NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

UNIVERSIDADE DE ÉVORA

ANALYTICAL APPROACHES FOR THE QUALITY AND FOOD SAFETY ASSESSMENT OF OLIVE OIL:

VOLATILE ORGANIC COMPOUNDS PROFILES, ANTIOXIDANTS AND PHTHALATES CONTENT MONITORING

FLÁVIA SOFIA SALGADO DE FREITAS

Master in Bioorganic Chemistry

DOCTORATE IN FOOD SCIENCES

NOVA University Lisbon and Évora University

January, 2025

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DOCTORATE IN FOOD SCIENCES

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Analytical Approaches for the Quality and Food Safety assessment of Olive Oil: Volatile Organic Compounds profiles, Antioxidants and Phthalates content monitoring

To my entire family, who have always been my safe harbor
and my greatest source of inspiration, love, and strength.
To you, all my gratitude.

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"There, where the olive tree gives up, is where the Mediterranean ends.
The tree of light is the nature and culture of the Mediterranean."
George Duhamel

PREFACE

This dissertation is submitted in partial fulfillment of the requirements for the Doctoral Degree in Food Sciences. It encompasses the results of my Ph.D. research conducted at the NOVA School of Science and Technology, NOVA University Lisbon, and the University of Évora.

Olive oil, one of the most iconic food products of the Mediterranean diet, has been widely studied due to its nutritional and organoleptic properties. However, issues related to its quality, safety, and sustainability, particularly contaminants and volatile compounds, remain critical areas of research. This dissertation addresses these challenges to advance knowledge about olive oil and improve its production and consumption, contributing to food quality and safety.

The dissertation is organized as follows:

1. General Introduction to Olive Oil

An overview of olive oil, highlighting its historical, economic, and nutritional importance, as well as the current challenges related to its production and quality.

2. Volatile Organic Compounds in Olive Oil: Organoleptic Perception and Shelf-Life

This chapter focuses on the volatile compounds in olive oil responsible for its sensory properties and shelf-life. The paper *"Early Identification of Olive Oil Defects throughout Shelf Life"* is one of the publications resulting from this research, addressing the early identification of defects throughout the olive oil shelf life.

3. Antioxidants: Nutraceutical Properties of Olive Oil

This chapter explores the antioxidant properties of olive oil, their health implications, and the development of methods to increase their concentration. The method developed reflects the innovative advancements in this field, aiming to enhance the nutraceutical qualities of olive oil.

4. Phthalates: Plasticizers in Olive Oil

The presence of phthalates in olive oil, due to the use of plastics during its production and packaging, is a significant issue for food safety. The paper *"A Critical Review of Analytical Methods for the Quantification of Phthalates Esters in Two Important European Food Products: Olive Oil and Wine"* discusses analytical methodologies for quantifying these contaminants. The article *"Analysis of Plasticizer Contamination Throughout Olive Oil Production"* presents the results of the analysis of plasticizer contamination throughout olive oil production.

I hereby affirm that, as the first author of the aforementioned manuscripts, I made a major contribution to the research and experimental work conducted, the interpretation of the results, and the preparation of these publications submitted during the Ph.D. project. The copyright of the publications has been transferred to the editors, and these articles are reproduced with the permission of the original publishers and subject to the copy restrictions imposed by them.

This work reflects the collective effort of several institutions and collaborators, to whom I am immensely grateful. Throughout this journey, I have sought not only to advance science but also to contribute to the improvement of olive oil quality and safety, ensuring that this essential food continues to play a vital role in healthy and sustainable diets.

ABSTRACT

In the plant kingdom, many fruits and seeds provide edible oils. However, olive oil, often referred to as "liquid gold," is the most important and widely used in the Mediterranean diet. Its nutritional value is considered to be twice that of cereals and ten times greater than that of wine. It is estimated that approximately 3 million tons of olive oil are consumed worldwide every year.

This doctoral thesis focused on the study of olive oil, addressing three main themes aimed at deepening the understanding of this food matrix, from its chemical composition to the factors affecting its quality and safety.

The first theme investigated the evolution of volatile organic compounds throughout the shelf life of olive oil, focusing on markers associated with positive attributes and sensory defects. A robust method was developed using solid-phase microextraction in headspace mode (HS-SPME), coupled with gas chromatography and mass spectrometry (GC/MS). This method allowed the identification of significant variations in the levels of volatile organic compounds (VOCs), particularly those derived from the lipoxygenase (LOX) pathway, over time. Principal component analysis (PCA) revealed a clear distinction between the volatile profiles of extra virgin olive oils and deteriorated oils. The relationship between *E*-2-hexenal and acetic acid proved to be a potential indicator for predicting the sensory disqualification of olive oil based on the evolution of volatile compounds during storage.

The second theme explored the potential of olive oil as a nutraceutical product, emphasizing the antioxidants hydroxytyrosol and tyrosol, both recognized for their health-promoting properties. A method was developed to efficiently extract these compounds from the by-products of olive oil production. These compounds were concentrated and added to the final olive oil, resulting in enriched olive oil and a concentrated antioxidant extract with functional applications.

The third theme focused on the presence of plasticizers in olive oil, aiming to identify and quantify phthalate esters. Through a critical review, various analytical methods used for quantifying phthalates in olive oils and wines were evaluated, discussing analytical challenges and mitigation strategies. Additionally, a study was conducted to monitor contamination by plasticizers, including 23 phthalates and 9 phthalate substitutes, throughout the olive oil production and storage process. Using liquid-liquid extraction with hexane/methanol and analysis by gas chromatography coupled with tandem mass spectrometry (GC-MS/MS), with detection limits ranging from 0.001 to 0.103 mg/kg, it was observed that plasticizer levels progressively increased as olive oil went through production stages. The main sources of contamination were identified, highlighting the importance of minimizing plastic use along the production line and during storage. Diisononyl phthalate (DINP) was the most prevalent compound, found at concentrations exceeding the migration limits established by the European Union in some olive oils.

The results presented in this thesis contribute to a deeper understanding of the quality, safety, and functional potential of olive oil, offering practical solutions to optimize its production and preservation, as well as promoting its valorization as a high-value-added food product.

Keywords: Olive Oil, Volatile Organic Compounds (VOCs), Sensory Quality, Shelf Life, Antioxidants, Hydroxytyrosol, Tyrosol, Plasticizers, Phthalates.

RESUMO

No reino vegetal, inúmeros frutos e sementes são fontes de óleos comestíveis. Entre eles, destaca-se o azeite, frequentemente chamado de "ouro líquido", que ocupa uma posição central na dieta mediterrânica. O seu valor nutritivo é estimado como sendo duas vezes superior ao dos cereais e dez vezes maior do que o do vinho. Estima-se que, anualmente, cerca de 3 milhões de toneladas de azeite sejam consumidas em todo o mundo.

A presente tese de doutoramento centrou-se no estudo do azeite, abordando três temáticas principais que visam aprofundar o conhecimento sobre esta matriz alimentar, desde a sua composição química até a fatores que afetam a sua qualidade e segurança.

No primeiro tema, foi investigada a evolução dos compostos orgânicos voláteis ao longo da vida útil do azeite, com foco nos marcadores associados aos atributos positivos e defeitos sensoriais. Foi desenvolvido um método robusto utilizando microextração em fase sólida em espaço de cabeça (HS-SPME) acoplada à cromatografia gasosa e espectrometria de massas (GC/MS), que permitiu identificar variações significativas nos níveis de compostos orgânicos voláteis (COVs), especialmente os derivados da via lipoxigenase (LOX), ao longo do tempo. A análise por componentes principais (PCA) revelou uma clara distinção entre os perfis voláteis de azeites virgem extra e de azeites deteriorados. A relação entre o *E*-2-hexenal e o ácido acético demonstrou ser um possível indicador para prever a desqualificação sensorial do azeite com base na evolução dos compostos voláteis durante o armazenamento.

O segundo tema explorou o potencial do azeite como um produto nutracêutico, com ênfase nos antioxidantes, hidroxitirosol e tirosol, ambos reconhecidos pelas suas propriedades benéficas para a saúde. Foi desenvolvido um método que permite a extração eficiente destes compostos a partir dos subprodutos da produção de azeite. Estes compostos foram concentrados e adicionados ao azeite final, resultando num azeite enriquecido e num extrato antioxidante concentrado com aplicações funcionais.

O terceiro tema focou-se na presença de plastificantes no azeite, com o objetivo de identificar e quantificar ésteres de ftalato. Através de uma revisão crítica avaliou-se diversos métodos analíticos aplicados na quantificação de ftalatos em azeites e vinhos, discutindo-se os desafios analíticos e as estratégias de mitigação. Adicionalmente, foi conduzido um estudo que acompanhou a contaminação por plastificantes, 23 ftalatos e 9 substitutos de ftalatos ao longo do processo de produção e armazenamento do azeite. Através de extração líquido-líquido com hexano/metanol e análise por cromatografia gasosa acoplada a espectrometria de massas (GC-MS/MS), com limite de deteção entre 0.001 to 0.103 mg/kg, observou-se que os níveis de plastificantes aumentavam progressivamente à medida que o azeite passava pelas etapas de produção. Foram identificadas as possíveis principais fontes de contaminação, destacando a importância de minimizar o uso de plásticos tanto ao longo da linha de produção quanto no armazenamento. O diisononil ftalato (DINP) foi o composto mais prevalente, sendo encontrado em concentrações superiores aos limites de migração estabelecidos pela União Europeia em alguns azeites.

Os resultados apresentados nesta tese contribuem para a compreensão aprofundada da qualidade, segurança e potencial funcional do azeite, oferecendo soluções práticas para otimizar a sua produção e conservação, além de promover a sua valorização como um alimento de elevado valor agregado.

Palavras chave: Azeite, Compostos Orgânicos Voláteis (COVs), Qualidade Sensorial, Vida Útil, Antioxidantes, Hidroxitirosol, Tirosol, Plastificantes, Ftalatos.

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ABBREVIATIONS

ANOVA	Analysis of variance
ATBC	Acetyltributyl citrate
BBP	Benzyl butyl phthalate
BMPP	Bis(4-methyl-2-pentyl) phthalate
BPA	Bisphenol A
CAS	Certificate of advanced studies
DAP	Diallyl phthalate
DBEP	Bis(2-butoxyethyl) phthalate
DBM	Dibutyl maleate
DBP	Dibutyl phthalate
DCHP	Dicyclohexyl phthalate
DEEP	Bis(2-ethoxyethyl) phthalate
DEHA	Bis(2-ethylhexyl) adipate
DEHP	Bis(2-ethylhexyl) phthalate
DEHS	Di(2-ethylhexyl) sebacate
DEHT	Di(2-ethylhexyl) terephthalate
DEP	Diethyl phthalate
DES	Diethyl sebacate

DHP	Diheptyl phthalate
DHXP	Dihexyl phthalate
DIBP	Diisobutyl phthalate
DIDP	Diisodecyl phthalate
DINCH	1,2-Cyclohexane dicarboxylic acid diisononyl ester
DINP	Diisononyl phthalate
DIPP	Diisopentyl phthalate
DiPrP	Diisopropyl phthalate
DMEP	Bis(2-methoxyethyl) phthalate
DMP	Dimethyl phthalate
DMTP	Dimethyl terephthalate
DNP	Dinonyl phthalate
DOI	Digital object identifier
DOP	Dioctyl phthalate
DPHP	Bis(2-propylheptyl) phthalate
DPhP	Diphenyl phthalate
DPP	Dipentyl phthalate
DPrP	Dipropyl phthalate
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
EEC	European Economic Community
EFSA	European Food Safety Authority
EU	European Union
eV	Electronvolt
EVOO	Extra virgin olive oil
FCM	Food contact materials
FID	Flame ionization detector

GC	Gas chromatography
GC x GC	Two-dimensional Gas Chromatography
GC-O	GC-olfactometry
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
HS-SPME	Headspace solid-phase microextraction
HTyr	Hydroxytyrosol
IOC	International Olive Council
IS	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LD_{50}	Median lethal dose
LDL	Low-density lipoprotein
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LRI	Linear retention index
LSD	Least significant difference
m/z	Mass-to-charge ratio
Md	Median of defects
MDGC	Multidimensional gas chromatography
Mf	Median of fruitiness
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NIST	National Institute of Standards and Technology

OMW	Olive mill wastewater
OO	Olive oil
PAEs	Phthalic acid esters
PCA	Principal component analysis
PET	Polyetylene terephthalate
PTV	Programmed temperature volatilization
QqQ	Triple Quadrupole
RPM	Revolutions per minute
SD	Standard deviation
SIM	Selected Ion Monitoring
SML	Specific migration limit
SNR	Signal-to-noise
TDI	Tolerable daily intake
TFDA	Taiwan Food and Drug Administration
TOFMS	Time-of-flight mass spectrometry
TOMT	Tris(2-ethylhexyl) trimellitate
Tyr	Tyrosol
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	Ultra-violet
VOCs	Volatile organic compounds
VOO	Virgin olive oil
WHO	World Health Organization

1

INTRODUCTION

Olive oil is one of humanity's oldest and most valued food products, renowned for its unique chemical composition, nutritional and organoleptic properties, and culinary versatility.

Derived from the fruit of the olive tree (*Olea europaea L.*), it is primarily obtained through mechanical methods, without the need for chemical treatments, which preserves its natural characteristics. Extra virgin olive oil, in particular, is the purest and most prized form of the oil, standing out for its low acidity and high concentration of bioactive compounds, such as polyphenols and oleic acid.

This food is not only an essential gastronomic ingredient but also a key component of cultural and historical practices that have shaped various societies.

1.1 Olive Oil and the Mediterranean Diet: A Millennial Heritage

The history of olive oil dates back more than 6,000 years, with records indicating that the olive tree was one of the first trees cultivated by humans. The earliest evidence of its use comes from the Eastern Mediterranean region, encompassing territories that today include the Middle East and Asia Minor. The Egyptians, for instance, used olive oil in religious rituals and as a cosmetic, while the ancient Greeks associated it with the gods, considering it a gift from the goddess Athena [1–5].

In Ancient Greece, olive oil was a symbol of status and a highly valued commodity, used in religious ceremonies, as fuel for lamps, and as a base for medicinal ointments [3,6]. During the Roman period, the production and trade of olive oil expanded significantly, driven by the introduction of more advanced agricultural techniques and the establishment of trade routes

connecting the Mediterranean to distant regions. The Romans also popularized its use in cooking, solidifying it as an essential food [7].

With the decline of the Roman Empire, olive oil production experienced a period of stagnation in some regions but remained vibrant in others, especially in areas influenced by Arab culture. During the Middle Ages, the Arabs contributed to the spread of cultivation and extraction techniques, ensuring the continuity of olive oil traditions. The Renaissance and the rise of European trade brought olive oil back into prominence, exporting it to new regions and integrating it into diverse cultural practices [8].

Thus, olive oil became a symbol of Mediterranean culture, representing not only a staple food but also an identity element for the region's communities. Globalization and advances in production technology have allowed olive oil to transcend Mediterranean borders, reaching international markets and gaining an increasingly broad audience[9].

Over the centuries, olive oil has solidified its position as a fundamental ingredient of the Mediterranean diet, recognized by UNESCO as Intangible Cultural Heritage of Humanity [10]. This diet, characterized by high consumption of fruits, vegetables, whole grains, fish, and olive oil as the primary fat source, is widely considered one of the healthiest in the world [11,12]. Scientific studies associate this diet with a significant reduction in the risk of cardiovascular diseases, type 2 diabetes, and certain types of cancer, as well as promoting longevity and quality of life [13–15]. These benefits are largely attributed to olive oil's chemical composition [16–18]. Oleic acid, the main fatty acid in olive oil, helps regulate cholesterol levels, while polyphenols have antioxidant and anti-inflammatory properties that protect against oxidative stress and cellular aging [19–22].

Beyond nutrition, the connection between olive oil and the Mediterranean diet represents a cultural and historical link rooted in traditional agricultural practices and the lifestyle of Mediterranean populations. Today, olive oil transcends borders, appreciated globally as a functional food that combines tradition, flavor, and health benefits [23–27].

1.2 Characterization of the Olive Oil Sector: Global and National Perspectives

The olive oil sector is one of the most emblematic in the food industry, representing centuries of tradition and innovation. Globally, production is heavily concentrated in the Mediterranean basin, led by countries like Spain, Italy, Portugal, and Greece, which together

dominate the world market, accounting for approximately 50% of global production (Figure 1) [28]. Recently, however, olive cultivation has expanded to non-traditional regions, such as Latin America, Australia, and the United States, driven by the growing appreciation of olive oil for its nutritional, functional, and sensory qualities [29,30].

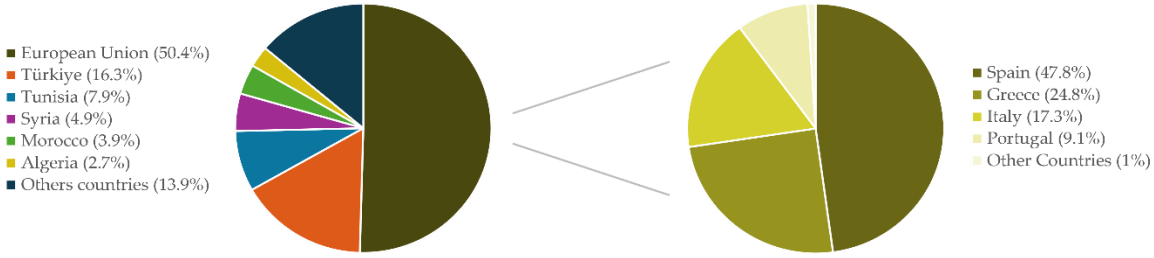

Figure 1. Olive oil production during the 2022/2023 season: contribution of countries to global production, including the European Union (right), and production exclusively within European Union countries (left) [28].

In Portugal, olive oil holds a prominent position in both food culture and the economy, being recognized as one of the world's leading producers. In 2022/2023, Portugal produced 1.375 million hectoliters of olive oil (approximately 126 thousand tons). And this amount is expected to increase, with projections for the 2024/2025 season indicating that Portugal is expected to produce 195 thousand tons of olive oil [28]. With a long tradition dating back to Roman times, the country combines traditional cultivation and extraction methods with technological innovations, resulting in high-quality olive oils known for their complex flavors and aromas [31].

The Alentejo region stands out as the main producer, contributing about 84% of national production, supported by favorable climatic conditions and the adoption of modern agricultural practices (Figure 2) [32]. This balance between tradition and modernity has significantly increased the productivity and quality of Portuguese olive oil, enhancing its prominence in international markets [33–35].

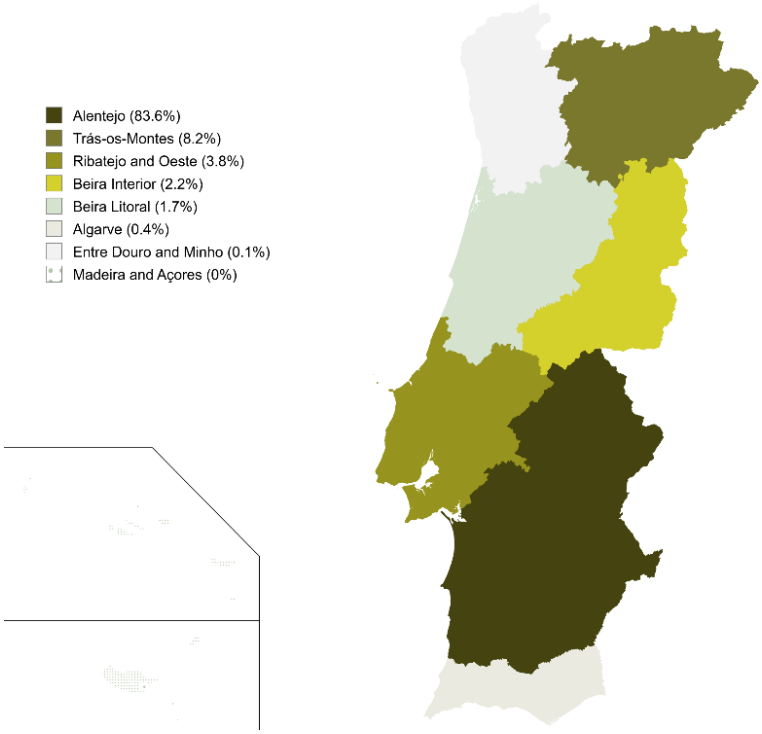

Figure 2. Olive oil production in Portugal by geographical location (agricultural region) in 2023 [32].

The valorization of national olive oil reflects not only its exceptional quality but also the growing interest in sustainable products with territorial identity. This context reinforces the importance of the sector for the Portuguese economy and consolidates Portugal's image as a producer of excellence on the global stage.

1.3 The Olive: Composition and Existing Varieties

The olive, a drupe fruit of the olive tree (*Olea europaea L.*), is essential for olive oil production and is widely consumed directly, either raw or after curing and fermentation processes that enhance its flavor and texture. This fruit has a unique chemical composition that makes it highly valued in gastronomy and the food industry. Structurally, the olive comprises three main layers: the epicarp, mesocarp, and endocarp. The epicarp, or skin, protects the fruit from external factors and is rich in phenolic compounds that determine its coloration, ranging from green to black, while also contributing antioxidant properties. The mesocarp, or pulp, makes up the majority of the fruit, serving as the primary source of lipids, especially monounsaturated fatty acids like oleic acid, as well as water, sugars, and dietary fibers, all essential for the yield and quality of olive oil. The endocarp, the hard layer surrounding the seed, can be ground during processing, marginally influencing the bioactive compound profile of the oil [36–39].

Detailed chemical composition highlights the importance of the olive. Water constitutes 50% to 75% of the fruit's weight, while lipids account for 15% to 30%, with a notable emphasis on the benefits of oleic acid. Carbohydrates represent 4% to 6%, present as simple sugars and fibers. Phenolic compounds provide bitter flavor and antioxidant properties, with their concentration varying according to ripeness. The fruit is also rich in vitamins and minerals, such as vitamin E, iron, calcium, and potassium, which promote bone, cardiovascular, and antioxidant health benefits [36–39].

The diversity of olive varieties is a striking aspect. It is estimated that there are hundreds of cultivars worldwide, each with specific characteristics of flavor, texture, color, and uses. In Portugal, for instance, there are around 30 native varieties, adapted to the climatic conditions of different regions. The most well-known varieties include Cobrançosa, Cordovil, Galega Vulgar, and Verdeal, each with distinct characteristics in terms of productivity, yield, and sensory profile [5,40,41].

For example, Cobrançosa, originating from Trás-os-Montes, is highly productive and resistant to diseases and adverse climatic conditions. Its oil is balanced, with bitter and pungent notes when the olives are harvested green, becoming sweeter and milder in mature harvests. Cordovil, typical of Alentejo, is widely used for both oil and table olives. Its oil has an intense fruity profile with prominent green notes and moderate bitterness [40,41].

Galega Vulgar is the most widespread variety in Portugal, accounting for about 80% of the national olive groves. Despite its moderate yield, its oil stands out for its mild and sweet flavor, with notes of dried fruits and ripe apple. Verdeal, cultivated in Alentejo and Trás-os-Montes, offers high productivity and yield, resulting in oils with a persistent fruity profile, marked bitterness, and pungency [40,41].

International varieties, such as Arbequina from Catalonia, also gain prominence. Known for its rusticity and adaptability, Arbequina allows for high planting densities. Its oil is fresh and fruity, ranging from spicy and green when harvested early to sweet and mild in late harvests [40].

Beyond genetic variety, factors such as climate, soil, cultivation techniques, and ripeness stage directly impact olive properties [42–50]. Ripeness, for example, influences both chemical composition and sensory profile: green fruits have higher phenolic compound concentrations and a more bitter flavor, while ripe fruits contain higher oil content and a milder flavor [47].

Understanding olive composition and diversity is essential to optimize the production of high-quality oils and direct consumption of the fruit. This approach is fundamental to

meeting the growing demands of the global market, which values nutritious, sensory-appealing, and sustainable products.

1.4 Olive Oil Production

The production of olive oil is a process that combines tradition and innovation, reflecting the diversity of olive varieties and their specific characteristics. Each stage, from harvesting to extraction, plays a crucial role in defining the quality and sensory profile of the oil. In Portugal, where olive cultivation is deeply rooted, production methods have evolved over centuries, integrating modern and sustainable practices that balance yield and quality. This focus extends not only to the oil itself but also to the utilization of its by-products, such as olive pomace, which has gained prominence for its beneficial properties and contributions to sustainability (Figure 3).

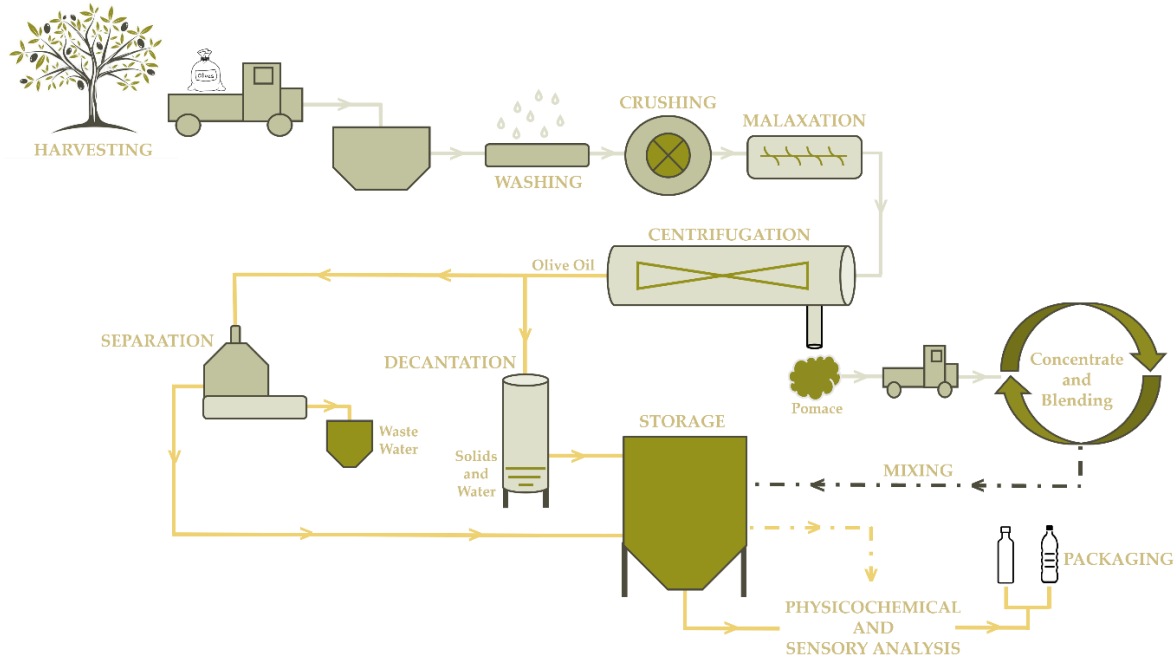

Figure 3. Representative diagram of an olive oil production line.

The harvesting of olives is the first and one of the most critical stages, as the timing of the harvest significantly influences the quality of the oil. High-quality olive oil is obtained from freshly harvested olives, free from pests and diseases, and picked at the optimal stage of ripeness. Greener olives produce oils with more bitterness and pungency, as well as being richer in polyphenols, while ripe olives yield milder, sweeter oils. Harvesting can be done manually, using poles and nets, or mechanically, with vibrating machines that shake the fruits off the trees, always prioritizing the preservation of the fruit's integrity [51–53].

After harvesting, the olives are quickly transported to the mill to avoid degradation processes, such as enzymatic and microbial fermentations, which can lead to defects like musty or winey aromas [54,55]. At the mill, the olives are carefully sorted to remove leaves, branches, and damaged fruits, ensuring that only high-quality olives are processed. The efficiency of this operation is crucial to prevent impurities from altering the organoleptic profile of the oil [2,56].

The next step is washing, where the olives are cleaned to remove impurities such as dust and pesticide residues before being crushed [57]. During the milling process, the olives are transformed into a homogeneous paste composed of pulp, pit, and skin, which is then subjected to malaxation. This process involves gently heating and slowly mixing the paste, allowing the oil droplets to coalesce, separating them from the solid and aqueous phases [58].

Malaxation must be carried out at controlled temperatures below 27°C (cold extraction) to classify the resulting oil as extra virgin and preserve its organoleptic and nutritional characteristics. Malaxation at higher temperatures can increase yield but compromises aromatic compounds and antioxidants [59,60]. After malaxation, the paste undergoes oil extraction, a critical stage where the oil is separated from the paste through centrifugation - an efficient method that has replaced traditional hydraulic pressing [56]. This process also produces olive pomace, a by-product consisting of the solid remnants after extraction, including residual pulp, pit fragments, and traces of oil and water [61].

Olive pomace, once discarded, has become a valuable resource in various sectors [62–64]. This by-product is rich in bioactive compounds, particularly antioxidants like hydroxytyrosol, one of the most potent polyphenols found in nature [65–67]. This compound exhibits anti-inflammatory, antimicrobial, and oxidative stress-protective properties and is extensively studied for its potential health benefits, including the prevention of cardiovascular and neurodegenerative diseases. Currently, hydroxytyrosol has numerous applications in the pharmaceutical, cosmetic, and food industries [68–72].

In addition to its beneficial properties, olive pomace plays an important role in promoting a circular economy model. Its applications include residual oil extraction, biomass production for energy generation, organic fertilizers, and even the development of functional ingredients for animal feed [73–77]. Recent studies explore the use of pomace for recovering high-value compounds, such as hydroxytyrosol, through green extraction technologies, reinforcing its environmental and economic relevance [66,78,79].

After extraction and the separation of olive pomace and wastewater, the oil still contains some impurities, such as residual water and solid particles, which can be removed during decantation and filtration stages. Decantation occurs by gravity, while filtration, often optional,

ensures that the oil is free of solid residues, improving its stability and appearance. Some producers choose to market unfiltered oils, valued for their rustic appearance and intense flavor. However, filtered oils tend to have greater stability and shelf life [80–82].

The olive oil is then stored in stainless steel tanks under controlled conditions, protected from light, oxygen, and temperature fluctuations, which can accelerate product degradation. Exposure to air and light can oxidize the oil, causing rancidity and loss of sensory and antioxidant properties [83–85].

Finally, the oil can be bottled in appropriate containers, such as dark glass bottles, cans, or opaque plastic packaging, which protect the contents from light and heat [85]. Labels provide information about the type of oil (extra virgin, virgin, etc.), origin, and often the sensory profile, helping consumers identify the product [86–88].

Quality control must be carried out at every stage of production, ensuring that the oil meets the rigorous criteria required to be classified as extra virgin or virgin. Physicochemical analyses, such as acidity and peroxide index tests, along with sensory evaluations by expert panels, ensure that the final oil complies with expected quality standards [89].

The olive oil production process, which combines traditional techniques with technological innovations, is fundamental for obtaining high-quality oils, respecting the characteristics of olive varieties, and meeting the demands of a consumer market that values fresh, authentic, and exceptionally flavorful products.

1.5 Chemical Composition of Olive Oil

The chemical composition of olive oil is highly variable, influenced by factors such as olive variety, fruit ripeness, environmental conditions, cultivation region, processing methods, and storage practices. Like other vegetable oils, olive oil comprises two main fractions: the saponifiable fraction, which constitutes the majority of its mass, and the unsaponifiable fraction, present in smaller amounts but with significant functional relevance [36,90].

The saponifiable fraction, accounting for approximately 97% to 99% of the total olive oil mass, is water-insoluble and predominantly composed of triglycerides, which determine the oil's physical and metabolic properties. This fraction also contains free fatty acids, which directly influence product acidity, along with small amounts of phospholipids, green pigments, and glucosides. The predominance of monounsaturated fatty acids, such as oleic acid, in this fraction is one of the factors responsible for the health benefits associated with olive oil consumption [91,92].

In contrast, the unsaponifiable fraction, representing around 1% to 3% of the composition, contains a wide variety of bioactive compounds, including hydrocarbons, sterols, waxes, triterpenic alcohols, carotenoids, tocopherols, polyphenols, and volatile substances. Despite its smaller proportion, this fraction plays a crucial role in olive oil quality, influencing its oxidative stability, organoleptic properties, and nutritional value. Additionally, compounds in the unsaponifiable fraction are often used as indicators in assessing olive oil quality and authenticity [91,92].

Olive oil quality is a priority for both producers and consumers [93]. To ensure the delivery of a premium product, commercial and food standards have been established to regulate the sector. These regulations aim to prevent fraud, eliminate adulteration, and ensure accurate labeling information, enhancing consumer trust [88,89].

In the commercial context, the chemical composition of olive oil plays a crucial role in its classification. Regulation (EU) No. 1308/2013 specifies three main categories for olive oils intended for human consumption [88]. These categories consider parameters such as sensory analyses conducted by specialized panels and acidity levels, ensuring that products meet global market demands and consumer expectations. Generally:

- Extra Virgin Olive Oil

  High-quality olive oil with good organoleptic characteristics, a score equal to or greater than 6.5 (on a scale of 0 to 10), no defects (median of defects [Md] = 0), a median fruitiness (Mf) greater than 0, and acidity less than or equal to 0.8% as oleic acid.
- Virgin Olive Oil

  Olive oil that may present slight defects in smell and taste, with an organoleptic score of at least 5.5 (on a scale of 0 to 10), a median of defects (Md) of 3.5 or less, a median fruitiness (Mf) greater than 0, and acidity not exceeding 2% as oleic acid.
- Lampante Olive Oil

  Virgin olive oil with abnormal organoleptic characteristics, a median of defects (Md) above 3.5, a median fruitiness (Mf) equal to 0, and acidity exceeding 2%. This oil is typically destined for refining or industrial transformation.

These categories establish a rigorous classification system designed to guarantee olive oil quality, provide greater transparency and trust to consumers, and protect product authenticity in the global market.

1.5.1 Saponifiable Fraction

The saponifiable fraction of olive oil is primarily composed of acylglycerols, with triacylglycerols being the most abundant, followed by smaller proportions of monoglycerides (less than 0.25%) and diglycerides (1.0% to 2.8%) [92] compounds result from the binding of fatty acids to glycerol and may vary due to processes such as incomplete biosynthesis or hydrolytic reactions. Although mono- and diglycerides are present in small quantities, they are relevant for quality assessment and detecting potential adulteration [94].

The predominant triacylglycerols in olive oil are presented in Table 1. These lipids, along with free fatty acids, play an essential role in defining the chemical properties of olive oil and indirectly influence its sensory characteristics through their degradation or interaction with other compounds [92,95].

Table 1. Composition of triacylglycerols in olive oil established by Regulation (EU) 2022/2104 [92].

Composition of triacylglycerols (%)	
Triolein (OOO)	40.0–59.0
Palmitodiolein (POO)	12.0–20.0
Linoleodiolein (LOO)	12.5–20.0
Palmitooleolinolein (PLO)	5.5–7.0
Stearodioleoyl (OOS)	3.0–7.0

The lipid composition of olive oil features a balance of saturated fatty acids (5% to 15%), monounsaturated fatty acids (55% to 85%), and polyunsaturated fatty acids (4% to 21%) [92]. This lipid profile contributes to its nutritional properties, with oleic acid being particularly notable for its high oxidative stability, making olive oil a healthy and functional choice[96,97]. Monounsaturated fatty acids, such as oleic acid, are fundamental to the cardiovascular benefits of olive oil consumption[13,15].

The main fatty acids in olive oil are listed in Table 2, including oleic acid (C18:1), linoleic acid (C18:2), palmitic acid (C16:0), stearic acid (C18:0), and linolenic acid (C18:3).

Table 2. Fatty acid composition of olive oil by International Olive Council [98].

Composition of fatty acids (% m/m methyl esters)	
Oleic (C18:1)	55.0–83.0
Palmitic (C16:0)	7.5–20.0

Linoleic (C18:2)	2.5–21.0
Stearic (C18:0)	0.5–5.0
Palmitoleic (C16:1)	0.3–3.5
Linolenic (C18:3)	⩽1.0
Arachidic (C20:0)	⩽0.60
Heptadecenoic (C17:1)	⩽0.6
Eicosenoic (C20:1)	⩽0.50
Heptadecanoic (C17:0)	⩽0.40
Myristic (C14:0)	⩽0.03
Behenic (C22:0)	⩽0.20
Lignoceric (C24:0)	⩽0.20

The composition of fatty acids is relatively constant qualitatively but varies quantitatively depending on factors such as olive variety, ripeness, climate, and technological processes [46,50,99]. For instance, warmer regions favor higher linoleic acid content, while cooler climates promote a higher proportion of oleic acid [100,101].

1.5.2 Unsaponifiable Fraction

Although the unsaponifiable fraction represents only about 2% of olive oil's total mass, it plays a crucial role in the product's nutritional, biological, and sensory value. This fraction comprises diverse compounds such as hydrocarbons, sterols, tocopherols, pigments, and phenolic compounds, which contribute to oxidative stability, organoleptic characteristics, and health benefits (Table 3) [17,91,92].

Table 3. Main unsaponifiable fractions of olive oil, their components, functions/importance, and approximate percentage of the total unsaponifiable matter [102,103].

Fraction	Components	Function/Importance	%
Hydrocarbons	Squalene	Antioxidant, precursor of bioactive compounds	30-50
Sterols	β -sitosterol, campesterol, stigmasterol	Cholesterol-lowering, authenticity markers for olive oil	15
Triterpenic Alcohols	Erythrodiol, uvaol	Anti-inflammatory and antioxidant properties	10
Higher Fatty Alcohols	C22 (behenic), C24 (lignoceric)	Quality and authenticity markers	n.s.
Carotenoid Pigments	β-carotene, lutein	Contribute to the color of olive oil, antioxidant properties	25-45

Tocopherols	α-tocopherol (Vitamin E)	Natural antioxidant, protects against lipid oxidation
Phenolic Antioxidants	Hydroxytyrosol, tyrosol, oleuropein	Protect against free radicals, provide health benefits
Volatile Compounds	Aldehydes, ketones, alcohols, esters (e.g., hexanal, *E*-2-hexenal)	Contribute to the characteristic aroma and flavor of olive oil
Others	Non-volatile phenolic compounds, altered-nucleus sterols (adulteration markers)	Indicators of olive oil purity and quality

Hydrocarbons, the main constituents of this fraction (30–50%), include saturated, unsaturated, linear, and branched compounds. These are by-products of the plant's natural metabolism, associated with fatty acid biosynthesis, and are found in higher concentrations in green olives compared to ripe ones [17,92,102]. Squalene stands out for its metabolic importance, serving as a precursor to sterols and triterpenic alcohols and participating in self-oxidation mechanisms that contribute to olive oil's oxidative stability [104].

Sterols constitute approximately 15% of the unsaponifiable fraction, with β-sitosterol being the predominant compound [92]. During olive maturation, sterol concentrations gradually decrease. Other sterols, such as stigmasterol and campesterol, are also present and play antioxidant roles essential to olive oil's chemical stability [17,104].

Phenolic compounds in olive oil play a key role in protecting against oxidative processes and enhancing sensory quality. These include phenolic alcohols (e.g., hydroxytyrosol, tyrosol, and hydroxytyrosol acetate), phenolic acids (e.g., vanillic, syringic, p-coumaric, and caffeic acids), flavonoids (e.g., luteolin and apigenin), lignans (e.g., pinoresinol and (+)-acetopinoresinol), and secoiridoids (e.g., oleuropein, oleacein, oleocanthal), wich are unique to the Oleaceae family. Furthermore, tocopherols, particularly α-tocopherol, are prominent phenolic antioxidants, preventing lipid oxidation reactions [105–107].

The concentration of phenolic compounds in olive oil is influenced by factors such as olive variety, ripeness, and processing and storage conditions. These compounds are recognized not only for their antioxidant contribution but also for antimutagenic properties and protective effects against cardiovascular diseases, cancer, and oxidative stress [47,105–109].

Olive oil also contains fat-soluble vitamins, such as vitamins A, D, K, and E, with α-tocopherol accounting for about 95% of total tocopherols. These vitamins play a vital role in

protecting polyunsaturated fatty acids from oxidation, indirectly influencing olive oil's aroma and stability [92,110,111].

The pigments present, such as chlorophylls (a and b), pheophytins (a and b), and carotenoids (e.g., lutein and β-carotene), not only give olive oil its color but also exhibit antioxidant activity in the absence of light. However, when exposed to light, they may act as pro-oxidants, impacting olive oil's oxidative stability. The concentration of these pigments varies depending on olive variety, ripeness, and environmental conditions [92,112–115].

The interaction between polyphenols, tocopherols, and carotenoids is fundamental to ensuring olive oil's oxidative stability while enhancing its antioxidant and anticancer properties. These compounds also play an essential role in maintaining organoleptic characteristics, such as bitterness and pungency, indicative of high product quality [116,117].

1.6 Selection and Quality of Olive Oil

High-quality olive oil is, essentially, a natural "juice" extracted from fresh and healthy fruits harvested at their optimal ripeness. It is crucial to avoid any treatment or handling that may alter the oil's chemical composition, both during the extraction process and throughout the storage period. The final quality reflects a synergy of various factors, including climatic conditions, soil characteristics, and the care applied during extraction and storage processes, highlighting the importance of an integrated and rigorous approach across the entire production chain.

Another important aspect is the differentiation among olive varieties and their impact on olive oil quality. Different cultivars produce oils with unique sensory profiles, encompassing variations in color, aroma, and flavor [118]. However, these organoleptic differences do not necessarily indicate variations in quality. The official classification of olive oil, as defined by the International Olive Council (IOC) and the European Economic Community (EEC), relies on technical criteria that recognize and value this diversity, allowing oils with distinct characteristics to be equally categorized as products of excellence.

The quality of olive oil results from a complex interaction between chemical, technological, sensory, and natural factors. Specific regulations, such as Regulation (EU) No 2022/2104, which complements Regulation (EU) No 1308/2013, along with the IOC guidelines, establish mandatory parameters for quality evaluation [88,89]. These include acidity level, peroxide value, ultra-violet (UV) spectrophotometric analysis, lipid profile, and sensory analysis, all of which ensure a trustworthy and high-quality product for consumers.

In addition to regulatory parameters, other attributes, such as polyphenol content, density, viscosity, and even visual aspects like color, play a fundamental role in the oil's stability and market acceptance. These factors influence the product's profile but primarily affect consumer perception and preference.

1.6.1 Physicochemical Characteristics

1.6.1.1 Acidity

Acidity in olive oil is a chemical parameter that reflects the level of free fatty acids present in the product. These acids are formed by the hydrolysis of triglycerides, a process triggered by the action of lipase enzymes when the olive tissue is damaged [119]. Such damage can result from factors like insect infestation, fungal diseases, delayed harvesting, improper storage, suboptimal or delayed extraction methods, and even contact between the oil and water after extraction [120–122].

Although acidity is often associated with olive oil quality, it does not directly affect flavor within regulated levels, as acidity is imperceptible to taste within the normal regulatory thresholds for consumer oils. In practice, superior-quality oils usually exhibit lower acidity, which correlates with healthy olives harvested at the optimal time and processed appropriately. Acidity is measured as the amount of oleic acid (in grams) per 100 grams of oil [9].

According to European regulations, olive oil is classified based on its acidity level as extra virgin olive oil if the free acidity is equal to or less than 0.8 grams of free oleic acid per 100 grams, as virgin olive oil if the free acidity is equal to or less than 2 grams of free oleic acid per 100 grams, and as lampante olive oil if the free acidity exceeds 2 grams of free oleic acid per 100 grams. The latter is unsuitable for direct consumption and requires refining [89].

Acidity, besides being an indicator of fruit quality and the production process, reflects the level of care taken with raw materials throughout the production chain. However, it is important to note that low acidity alone does not guarantee a complex or striking organoleptic profile; an oil may be technically flawless yet sensorially unremarkable. Conversely, oils with slightly higher acidity can exhibit intense and notable aroma and flavor characteristics.

1.6.1.2 Peroxide Value

The peroxide value is a key parameter for assessing the initial oxidation state of olive oil. It measures the presence of peroxides, compounds formed during the oxidation of oils and

fats through the action of oxygen. Even in small concentrations, these compounds can negatively impact the oil's flavor, contributing to undesirable attributes like rancidity [123,124].

This parameter is expressed in milliequivalents of active oxygen per kilogram of oil (meq O_2/kg) and is regulated by a maximum limit of 20 meq O_2/kg for virgin oils, as established by Regulation (EU) 2022/2104. Values exceeding this threshold indicate that the oil is unsuitable for human consumption [89].

A high peroxide value may indicate issues in handling the olives or paste during extraction or improper storage of the final oil. Moreover, monitoring this parameter provides insights into the degradation of natural antioxidants, such as tocopherols and polyphenols, which are critical for the stability and quality of the oil. Thus, determining the peroxide value is essential for assessing both the initial quality of the product and its expected durability during storage [123,124].

1.6.1.3 Ultraviolet Absorbance

The analysis of ultraviolet (UV) absorbance is a crucial tool for evaluating the quality and authenticity of olive oil. This method detects oxidized compounds, both primary and secondary, which may signal undesirable changes in the product. Measurements are conducted at specific wavelengths, with coefficients expressed as K_{232}, K_{270}, and ΔK.

Conjugated hydroperoxides, indicative of primary oxidation, show maximum absorption at 232 nm, while secondary oxidation products, such as aldehydes and ketones, absorb at other wavelengths. Conjugated dienes and trienes, associated with more advanced chemical changes, display maximum absorbance at 270 nm. According to regulations, the maximum limits for extra virgin oils are 2.5 for K_{232}, 0.22 for K_{270}, and 0.01 for ΔK. For virgin oils, the limits are 2.6, 0.25, and 0.01, respectively, with lower values indicating higher quality [89].

In addition to evaluating oxidation, UV spectrophotometry is often used to identify potential adulterations, as olive oil absorbs significantly less ultraviolet radiation at wavelengths between 208 and 210 nm compared to other vegetable oils. This method also provides valuable information about the oil's conservation state and any changes resulting from technological processing. Therefore, UV absorbance analysis is essential for ensuring the integrity and quality standards of the product [125,126].

1.6.1.4 Rancidity or Oxidative Stability

Oxidative stability is a critical parameter for evaluating the quality of oils and fats. This attribute is not solely dependent on the chemical composition or the quality of raw materials

but also reflects the conditions to which the product has been exposed during processing and storage until the time of analysis.

To determine this parameter, the induction period is used, measuring the time required for oxidation to begin. Samples are subjected to controlled conditions of accelerated oxidation, including elevated temperatures, increased oxygen exposure, and constant agitation, simulating extreme degradation scenarios. Stability is often assessed using the Rancimat equipment, which performs this analysis in a standardized and efficient manner, measuring the oil's resistance to oxidation.

The susceptibility of olive oil to oxidation is directly related to the degree of unsaturation of the fatty acids in its triglycerides. Higher unsaturation levels increase the propensity for oxidation. Oxidative stability is thus one of the most relevant quality indicators, particularly for oils used as ingredients in other products, as it directly influences manufacturing processes, sensory characteristics, and the shelf life of final products [95,124,127–130].

1.6.1.5 Sterols and Triterpenic Alcohols

Sterols constitute an important fraction of the unsaponifiable composition of olive oil and play a significant role in its characterization. Virgin olive oil contains various types of sterols with a specific composition, which allows for the identification of potential adulterations with other oils [131–134]. Additionally, phytosterols offer significant health benefits, helping to reduce plasma cholesterol levels and consequently preventing diseases such as arteriosclerosis and coronary conditions. The primary sterols found in virgin olive oil include β-sitosterol, Δ5-avenasterol, campesterol, stigmasterol, and Δ7-stigmastenol [17,104].

Refined olive oils and olive pomace oil exhibit total sterol values different from those found in virgin olive oils, where the minimum sterol content is 1000 mg/kg, as established by Regulation (EU) 2022/2104 [89].

Among triterpenic alcohols, compounds biosynthesized from fatty acids, the pentacyclic structures stand out, particularly two diols characteristic of the olive epicarp: erythrodiol and uvaol [134]. During the extraction process with solvents such as hexane, used in olive pomace oil, these compounds dissolve and are found in greater abundance in the skins and seeds of the olive than in its pulp. The maximum permissible content of these compounds in extra virgin and virgin olive oils is 4.5 g per 100 g of oil [89]. High levels of erythrodiol and uvaol may indicate the addition of olive pomace oil to virgin olive oil. Furthermore, an increase in these compounds in virgin olive oils can suggest practices such as a second extraction of the paste or the use of excessive pressures during processing [133–136].

1.6.1.6 Fatty Acids

The analysis of fatty acids in olive oil is essential for identifying non-characteristic compounds that may indicate adulterations [136–138]. Examples include myristic acid (C14:0, found in coconut oil), linolenic acid (C18:3, present in linseed oil), arachidic acid (C20:0, typical of peanut oil), eicosenoic acid (C20:1, common in rapeseed oil), behenic acid (C22:0, also in peanut oil), and lignoceric acid (C24:0, derived from peanut oil).

According to Regulation (EU) 2022/2104, in addition to established limits for the aforementioned acids, specific limits have been set for trans isomers of oleic acid, linoleic acid, and linolenic acid, collectively known as trans isomers. The sum of trans oleic isomers and the sum of trans linoleic and trans linolenic isomers must not exceed 0.05 g per 100 g of oil for extra virgin and virgin olive oils [89]. These compounds can be produced by illicit industrial processes that aim to disguise the addition of oils from other origins to olive oil. Such processes alter the composition of fatty acids, increasing the proportion of trans isomers. Thus, elevated levels of these isomers serve as reliable indicators of adulterations or fraud, such as the blending of virgin olive oils with refined oils.

Additionally, ethyl esters of fatty acids should not exceed 35 mg/kg for extra virgin olive oil [89]. This limit is important for ensuring the authenticity and purity of the product, as higher levels of ethyl esters could indicate the presence of refined oils or improper processing.

1.6.1.7 Fatty Acids in Position 2

The ratio of saturated to unsaturated fatty acids can be used to characterize the olive oil cultivar, as the fatty acid profile is largely influenced by the fruit's characteristics. Factors such as climate, irrigation practices, and fruit maturity also significantly impact the composition of fatty acids and triacylglycerols [139–141].

Additionally, the analysis of the fatty acid profile is useful for detecting the presence of esterified oils in olive oil. In natural olive oils, saturated fatty acids in triglycerides are predominantly attached to glycerol at positions 1 and 3, while position 2 is mostly occupied by unsaturated fatty acids. This specific distribution is related to the biosynthesis of triglycerides during oil formation in the fruit [141].

In contrast, the industrial synthesis of triacylglycerols (esterification) does not distinguish between saturated and unsaturated fatty acids, resulting in a higher proportion of saturated fatty acids at position 2. This difference can be used as an indicator of adulteration or the presence of non-natural oils in olive oil [142]. According to Regulation (EC) No. 1989/2003, the maximum permitted limit of these compounds in virgin olive oils is 1.5 g per 100 g of oil [143].

1.6.1.8 Waxes

Regulation (EU) 2022/2104 establishes guidelines for determining wax content in olive oil and defines concentration limits. For extra virgin and virgin olive oils, the wax content must not exceed 150 mg/kg [89]. Waxes are compounds naturally present in olives, consisting of esters of fatty acids and long-chain alcohols, distinct from glycerol. These compounds are primarily found in the epicarp of olives, and during the extraction process, some waxes are transferred to the oil.

When olive oil is adulterated with olive pomace oil, there is a significant increase in wax content. This is because the solvent used in olive pomace oil extraction, such as hexane, dissolves a greater quantity of waxes, which remain in the oil after the solvent evaporates. Consequently, wax content analysis is an effective tool for identifying the addition of olive pomace oil to olive oil, enabling the detection of potential fraud or adulteration [136,144].

1.6.1.9 Aliphatic Alcohols

The primary aliphatic alcohols present in olive oil include docosanol, tetracosanol, hexacosanol, and octacosanol [92]. These compounds are present in significantly higher concentrations in olive pomace oil compared to virgin olive oil . According to some studies, elevated levels of total aliphatic alcohols in certain olive oils are primarily attributed to their free (non-esterified) form. This phenomenon may result from adverse climatic conditions, such as prolonged drought periods [145].

1.6.1.10 Tocopherols

Tocopherols (such as α-tocopherol, a form of vitamin E) are important antioxidant components in olive oil. They are part of the unsaponifiable fraction of vegetable oils and fats, along with phytosterols [92]. Their multiple nutritional benefits are widely recognized and documented in the literature.

Tocopherols, associated with vitamin E properties, act as antioxidants, protecting body tissues from the harmful effects of free radicals generated during normal metabolism. Among tocopherols, α-tocopherol exhibits the highest biological activity. Although α-tocopherol is the most abundant and biologically relevant, analyzing other homologs is also significant. For instance, γ-tocopherol is believed to offer superior protection against harmful radicals, such as peroxynitrite, which causes damage to various cellular molecules, including DNA and proteins, due to its oxidative properties [17,103,146].

Thus, tocopherols are analyzed as indicators of olive oil quality and stability, being monitored to characterize the oils and verify their authenticity and freshness [94,133,147]. However, there are no specific regulatory limits for their presence.

1.6.1.11 Polyphenols

Phenolic compounds are secondary metabolites of plants, widely recognized for their structural diversity and broad phylogenetic distribution. In olives, four main classes of phenolic compounds stand out: phenolic acids, phenolic alcohols, flavonoids, and secoiridoids [92]. These compounds play essential roles in defining the sensory characteristics of olives and olive oils and in protecting against auto-oxidation and photo-oxidation processes [148,149].

The composition and concentration of phenolic compounds can vary significantly depending on the fruit's degree of maturity, influencing its quality both quantitatively and qualitatively [51]. Prominent phenolic compounds in olive oil include tyrosol, hydroxytyrosol, oleuropein, caffeic acid, and *p*-coumaric acid [92].

These compounds, in addition to playing a crucial role in the oxidative stability of olive oil due to their antioxidant properties, also contribute to various health benefits [16]. Although European legislation does not directly set limits for the levels of phenolic compounds in olive oil, their presence is indicative of quality. For instance, Regulation (EU) 432/2012 allows health claims for olive oils containing at least 5 mg of hydroxytyrosol and its derivatives, such as the oleuropein and tyrosol complex, per 20 g of olive oil, underscoring their importance in enhancing the product's value [150,151].

The polyphenolic compounds of olive oils, such as hydroxytyrosol and tyrosol, will be further explored in Section 3 of this dissertation.

1.6.2 Organoleptic Characteristics

The organoleptic evaluation is an indispensable procedure for determining the sensory quality of virgin olive oils and is one of the fundamental criteria for their classification as extra virgin, virgin, or lampante. This process complements physicochemical analyses, providing a comprehensive view of olive oil characteristics, particularly concerning sensory attributes such as fruitiness (green or ripe), bitterness, and pungency, as well as the identification of potential sensory defects [152–155].

Regulated by the European Union through Regulation (EU) 1348/2013, the organoleptic evaluation follows a standardized protocol to ensure the uniformity and reliability of results. This regulation establishes detailed criteria for the composition and functioning of tasting

panels, the method of sensory analysis, and the certification requirements for laboratories responsible for conducting these assessments [89,156].

The prescribed method involves sensory analysis conducted by trained tasters organized into officially recognized panels. Results are based on a sensory scale that evaluates the intensity of positive attributes, such as fruitiness, and potential defects, such as rancidity or mustiness, which compromise the product's quality. This system ensures that only oils meeting the required standards can be classified into superior categories.

Thus, organoleptic evaluation is a crucial tool for ensuring the authenticity, quality, and market value of olive oils while protecting consumers from fraud or inferior-quality products.

The organoleptic classification of olive oils will be further explored in Section 2 of this dissertation.

1.7 Packaging and Storage of Olive Oil: Impacts on Quality and Product Safety

After extraction and characterization, packaging and storage are fundamental steps in preserving the chemical, sensory, and nutritional properties of olive oil. The unsaponifiable fraction, phenolic compounds, tocopherols, and pigments, as previously discussed, play essential roles in olive oil stability and are sensitive to environmental factors such as light, oxygen, and temperature. Therefore, selecting appropriate packaging materials and storage conditions is crucial to maintaining product quality over time [157,158].

Olive oil is particularly vulnerable to lipid oxidation, which can lead to rancidity and the loss of beneficial compounds, reducing its nutritional and commercial value. This degradation is accelerated by exposure to light, high temperatures, and contact with oxygen. Additionally, pro-oxidant compounds, such as chlorophylls, can catalyze oxidation reactions when exposed to light, emphasizing the need for effective protective strategies during storage [124].

Packaging materials play a vital role in shielding olive oil from external factors. Dark or opaque glass bottles, coated metal containers, and polymeric packaging materials with oxygen barriers are widely used due to their ability to minimize exposure to light and oxygen. Studies have shown that inadequate packaging accelerates olive oil degradation, negatively affecting its aroma, flavor, and antioxidant properties [83–85,158,159].

Storage conditions are equally critical for preserving olive oil quality. Elevated temperatures increase the rate of oxidation reactions, while the presence of oxygen can trigger

autoxidation processes. For this reason, storage in cool, dark, and humidity-controlled environments is recommended to slow these reactions and maintain oxidative stability [158,159].

The effectiveness of packaging and storage strategies is directly linked to the preservation of bioactive compounds, such as phenolics and tocopherols, and the maintenance of olive oil's characteristic sensory profile. Recent research highlights that modern technologies, such as modified atmospheres and multifunctional packaging barriers, can offer superior protection and extend product shelf life [160,161].

Thus, studying the interaction between packaging type, storage conditions, and the chemical stability of olive oil is crucial to ensuring the delivery of a high-quality product to consumers, preserving both its sensory attributes and health benefits.

On the other hand, in the context of olive oil, prolonged contact with plastic packaging can lead to contamination by phthalates, which may compromise product quality and pose health risks [162–164].

The presence of phthalates in food is an increasing concern due to their potential migration from packaging materials. Phthalates are esters of phthalic acid widely used as plasticizers in polymers such as PVC, providing flexibility and durability to various products, including plastic food and beverage packaging. In the case of olive oil, its lipophilic composition makes it particularly susceptible to absorbing these compounds during storage, especially under conditions of heat, light, and prolonged contact [162,165].

This issue extends beyond product integrity to public health, as phthalates are classified as endocrine disruptors. These compounds have been linked to adverse effects on the reproductive system, hormonal imbalances, and potential impacts on child development [166–169].

International regulations, such as those imposed by the EFSA (European Food Safety Authority), establish limits for phthalates in food-contact materials, encouraging the use of safer alternatives such as glass or metal for olive oil storage [170,171].

However, beyond packaging, other elements throughout the olive oil production chain are potential sources of phthalate contamination, including harvesting nets, transport bags or containers, processing mats, O-rings, hoses, and seals.

Understanding the impact of phthalates on the quality and safety of olive oil is essential to improving production, packaging, and storage processes, ensuring a high-quality product while minimizing risks to consumer health.

The presence of plasticizers in olive oil will be explored in more detail in Section 4 of this dissertation.

2

VOLATILE ORGANIC COMPOUNDS

2.1 Sensory Analysis

Sensory analysis is an essential tool in evaluating food quality, being crucial for understanding the interactions between chemical stimuli and human senses, especially smell and taste (Figure 4). These two senses, known as chemical senses, play complementary roles in the perception of flavors and aromas [172–175].

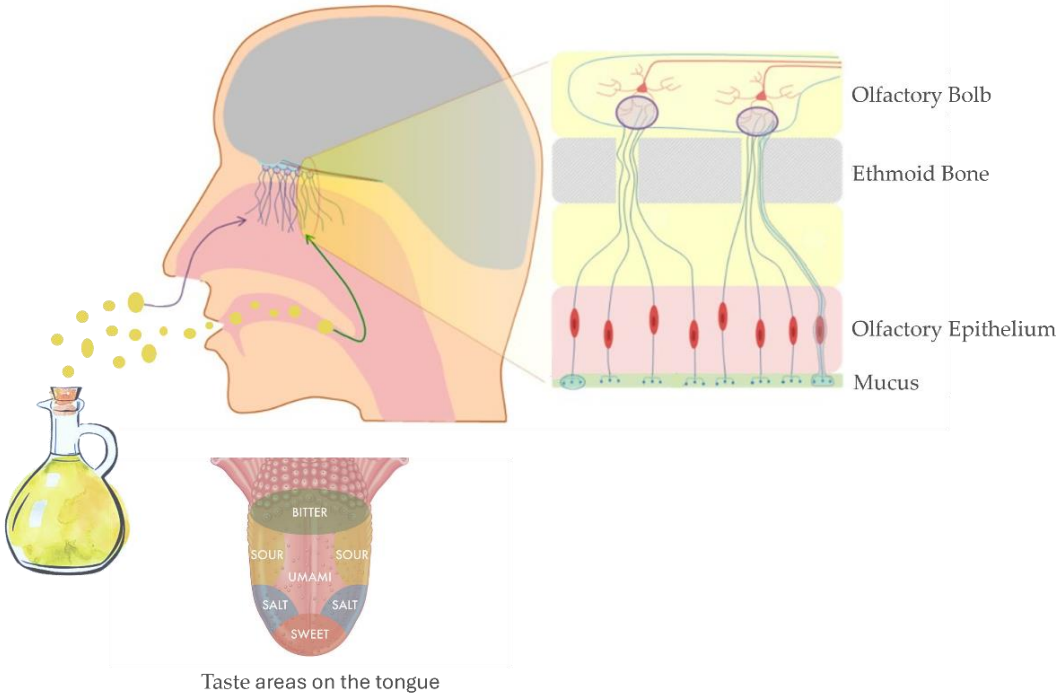

Figure 4. Illustration depicting the mechanisms of olfactory and taste perception, adapted [176].

Olfactory perception is triggered by odor molecules that, even in extremely low concentrations, can interact with receptors in the olfactory epithelium, located in the nasal cavity. For

a substance to be detected by smell, its molecules must have specific physicochemical characteristics, such as volatility, water solubility, or liposolubility. These factors allow the molecules to reach the sensory receptors, interacting with the cells of the olfactory epithelium through the mucus that covers it [177]. Substances like aldehydes, ketones, and polyphenols found in olive oil are responsible for stimulating sensory receptors and contributing to its organoleptic characteristics, such as aroma, flavor, and color [178].

Taste, on the other hand, occurs through the taste buds located on the tongue, which recognize compounds present in food and send signals to the brain. The brain, through integrative centers, combines information from smell and taste, allowing a complete and complex sensory experience [179].

2.2 Positive and Negative Attributes of Olive Oil

In sensory analysis of olive oil, these principles are applied to identify the intensity of positive and negative attributes of the product using trained taster panels. The process begins with olfactory evaluation, followed by tasting, during which taste and tactile impressions are observed. European Union regulations, such as Regulation (EU) 1348/2013, define specific guidelines for conducting these sensory tests, including scoring criteria and standardized tasting forms [156].

2.2.1 Positive Attributes

Positive attributes highlight desirable characteristics associated with high-quality olive oils, obtained from healthy and fresh fruit:

- **Fruity:** A set of olfactory sensations characteristic of oils from healthy olives, whether green or ripe, perceived through direct or retronasal pathways.
- **Bitter:** An elementary taste associated with oils made from green or early maturing olives, perceived by the caliciform taste buds on the back of the tongue.
- **Pungent:** A tactile sensation of sharpness, especially in the throat, typical of oils extracted at the beginning of the harvest, predominantly from green olives.

2.2.2 Negative Attributes

On the other hand, negative attributes indicate defects that compromise the quality of olive oil and may result from inadequate production, storage, or handling processes:

- **Fusty/muddy sediment:** A flavor characteristic of oils obtained from improperly stored olives, leading to anaerobic fermentation, or oils in prolonged contact with decanted matter.
- **Musty-humid-earthy:** A flavor resulting from contamination by molds, yeasts, or dirt due to improper storage of olives.
- **Winey-vinegary-acid-sour:** A flavor reminiscent of wine or vinegar, caused by aerobic fermentation, leading to the formation of compounds like acetic acid and ethanol.
- **Rancid:** A flavor resulting from advanced oxidation processes.
- **Frostbitten olives:** Characteristic of oils produced from olives frozen on the tree.
- **Heated or Burnt:** Resulting from excessive heating during thermal malaxation.
- **Hay-wood:** A flavor typical of oils from dried olives.
- **Rough:** A dense, pasty mouthfeel in older oils.
- **Greasy:** A flavor reminiscent of diesel or mineral oils.
- **Vegetable water:** A flavor acquired by prolonged contact with fermenting waters.
- **Brine:** A flavor associated with olives preserved in brine.
- **Metallic:** A flavor reminiscent of metals, resulting from prolonged contact with metal surfaces.
- **Esparto:** Characteristic of olives pressed in new esparto mats.
- **Grubby:** A flavor from olives infested with larvae of the olive fly (*Bactrocera oleae*).
- **Cucumber:** A flavor caused by prolonged hermetic storage, associated with the formation of compounds such as 2,6-nonadienal.

Thus, sensory evaluation uses human senses as measurement tools to detect and classify the aforementioned attributes. This process is fundamental for commercially classifying olive oil and its market value.

2.3 Volatile Organic Compounds

Volatile organic compounds (VOCs) are key elements in defining the aroma and flavor of olive oil, composing what is commonly referred to as *flavour* in English or *flaveur* in French. These terms encompass the combined sensations of odor and taste perceived in the mouth, which is particularly relevant in the context of olive oil, where these interactions define the product's sensory quality.

Approximately 150 volatile compounds have been identified in olive oil, belonging to various chemical classes such as hydrocarbons, alcohols, aldehydes, esters, phenols,

oxygenated terpenes, and furanoids. Most of these compounds are formed by the oxidative degradation of unsaturated fatty acids present in olive oil. This process occurs primarily through the action of lipoxygenase enzymes, which catalyze the formation of C6 aldehydes and alcohols, contributing green and fruity notes to the oil. Examples include hexanal and *E*-2-hexenal, which are considered quality markers due to their impact on aroma [155,180–182].

However, non-enzymatic chemical reactions, such as spontaneous lipid oxidation, can also generate VOCs. Compounds formed in this way, such as hexanal in high concentrations, are often associated with sensory defects like rancid odor. Furthermore, microbial activity under inadequate storage conditions can also contribute to the formation of undesirable compounds, resulting in unpleasant aromas [181–184].

In summary, the aromatic profile of olive oil arises from a combination of different pathways and metabolic processes, as illustrated in Figure 5.

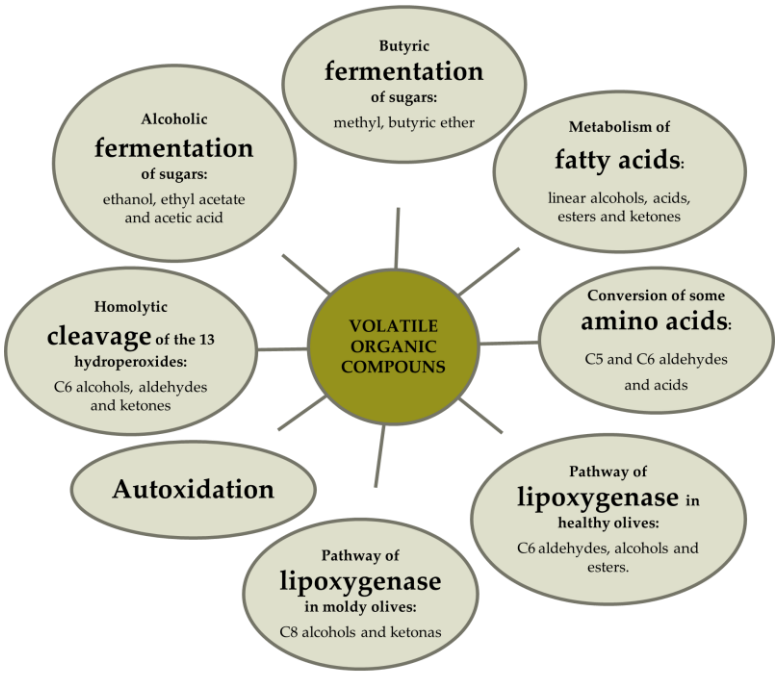

Figure 5. Main pathways involved in the formation of the volatile aroma profile of olive oil, adapted [154,182].

2.3.1 Sensory Characteristics and Contribution of VOCs

Volatile compounds have low molecular mass, generally below 300 Da, and volatilize easily at room temperature. The most common are C5 and C6 (with 5 and 6 carbon atoms in their structure), such as hexanal, *E*-2-hexenal, hexanol, isopentanol, 2-penten-1-ol, and pentanal, often associated with positive notes of freshness and fruitiness. However, the presence of compounds like hexanal in high concentrations can indicate advanced oxidation, resulting in undesirable aromas like rancidity. Other compounds, present at lower concentrations, are

also found, such as 1-octen-3-ol, *E*-2-octenal, nonanal, and limonene, which contribute to earthy, mushroom-like, green and citrus notes [54,92,116,124,182,185,186].

Although the concentration of these compounds in olive oil is relatively low, their contribution to the aroma is significant due to the high olfactory potency of many of them. Thus, a fundamental concept is that of odor activity, which relates the concentration of a compound to its detection threshold by smell. Odor activity is calculated by dividing the concentration of the compound in the olive oil by its odor threshold, which is the lowest concentration at which the compound can be detected by the human nose [187].

For example, *E*-2-hexenal at a concentration of 6670 µg/g has an odor activity of 16, while 1-penten-3-one at a very low concentration of 26 µg/g has a much higher odor activity of 36. This means that despite its lower concentration, 1-penten-3-one has a much stronger sensory impact due to its higher olfactory potency. Generally, the higher the odor activity, the more pronounced the aroma of the compound, meaning that a higher odor activity corresponds to a stronger perception of the scent [188].

Moreover, volatile compounds that are not directly detected by smell can play crucial roles as precursors or intermediates in the formation of other aromatic compounds. Thus, even substances present below the perception threshold can indirectly influence the sensory quality of olive oil.

The formation and composition of volatile compounds in olive oil depend on several factors related to the fruit's characteristics, processing methods, and storage conditions. Among the main factors, the following stand out:

- **Cultivar and Agronomic Conditions:** Different olive cultivars produce oils with distinct volatile profiles. This variation can be attributed to genetic differences that influence fatty acid composition and enzymatic activity. Additionally, environmental conditions such as climate, soil, and cultivation practices also play a significant role [189–192].
- **Fruit Maturity Stage:** The level of ripeness of the olives is a key determinant of the volatile composition of olive oil. During the climacteric period, there is an increase in the synthesis of aromatic compounds, driven by ethylene production and the activation of enzymes responsible for forming volatiles. Olives harvested at the optimal maturity stage tend to produce oils with more balanced sensory notes [49,118,193].
- **Processing Methods:** Processing stages, such as crushing the olives and thermal malaxation (heat-assisted kneading), play key roles in releasing enzymes that

catalyze the formation of VOCs. The time and temperature applied during these stages can significantly alter the aroma of the oil: higher temperatures or excessive times can lead to the degradation of desirable compounds and the formation of undesirable ones [194,195].

- **Storage and Conservation:** Improper storage of olives before processing can promote undesirable fermentations and the formation of compounds that compromise the quality of the oil. Furthermore, storing the final oil is also critical: exposure to oxygen, light, and high temperatures accelerates oxidation reactions, reducing both sensory and nutritional quality [159,183,186,196].

Thus, understanding the mechanisms leading to the formation of volatile compounds allows for the optimization of extraction and storage processes to maximize the quality of olive oil. By aligning chemical knowledge with industrial practice, it is possible not only to preserve but also to enhance the positive sensory attributes of olive oil, ensuring a high-quality product with added market value.

2.4 Factors of Deterioration

Lipid oxidation is one of the main factors compromising the quality and stability of olive oil, being a spontaneous and inevitable process that affects its sensory, nutritional characteristics, and shelf life. This phenomenon occurs due to the interaction of unsaturated fatty acids with reactive oxygen species, and is influenced by factors such as light, heat, the presence of oxygen and metals, as well as enzymatic processes. It can occur through auto-oxidation, photo-oxidation, and enzymatic oxidation [124,196,197].

Auto-oxidation is a chain process initiated by contact with oxygen, leading to the formation of hydroperoxides, which are the first products of oxidation. The degradation of hydroperoxides generates aldehydes and ketones, responsible for unpleasant odors and flavors, compromising the organoleptic and nutritional properties of olive oil [124].

In photo-oxidation, exposure to light, especially ultraviolet radiation, activates pigments like chlorophyll, triggering reactions that consume the available oxygen. This process is accelerated during storage in the presence of light, resulting in the formation of compounds that degrade both sensory and nutritional quality [124,198].

Enzymatic oxidation, catalyzed by enzymes like lipoxygenases, primarily occurs during processing or when the olive fruit is damaged (Figure 6) [124].

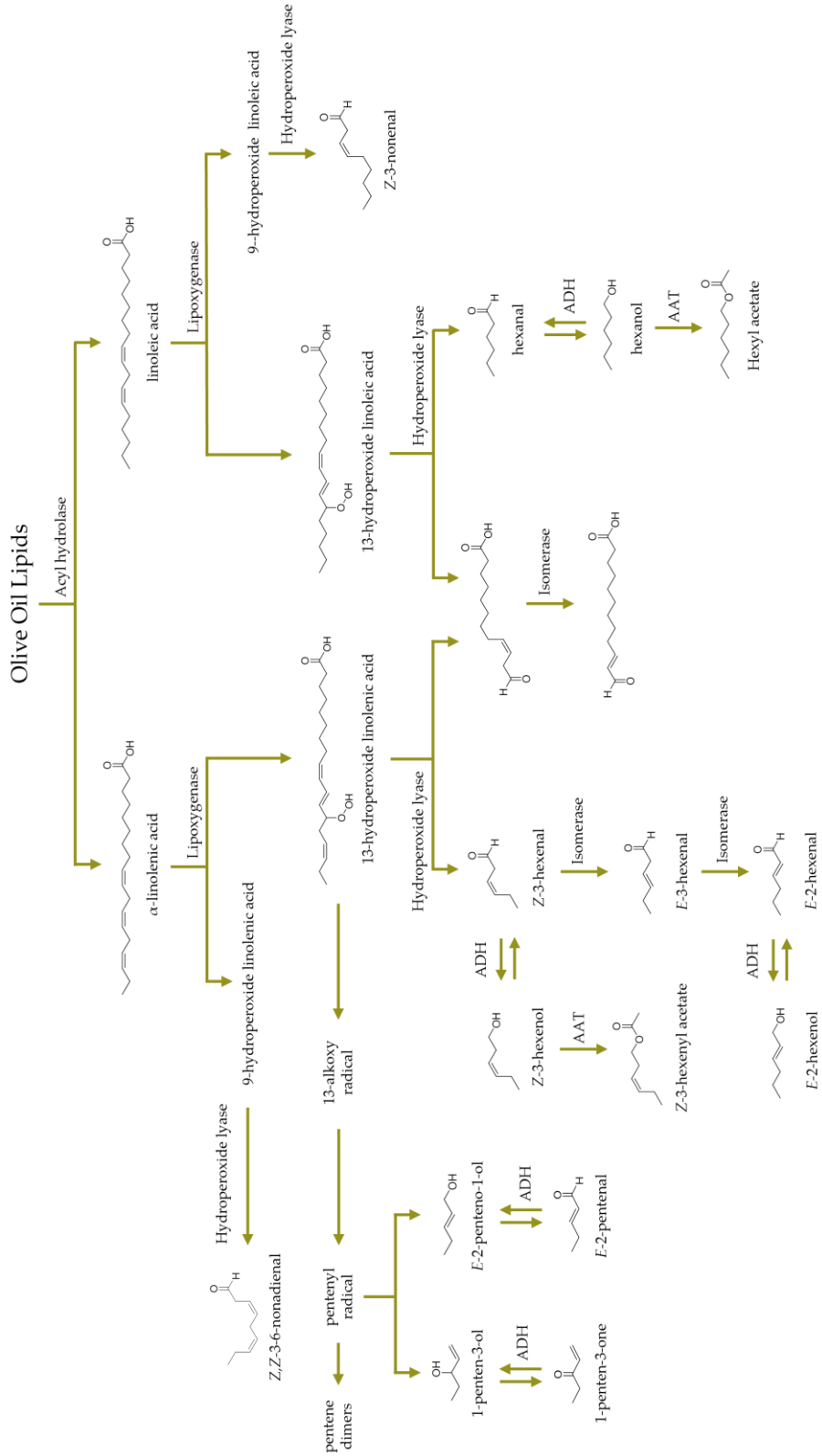

Figure 6. Scheme of the enzymatic oxidation process of linolic and linolenic acids - Lipoxygenase pathway. ADH: alcohol dehydrogenase; AAT: alcohol acyltransferase. Adapted [178,199,200].

This mechanism utilizes polyunsaturated fatty acids, such as linoleic and linolenic acids, to form volatile compounds. Lipoxygenase is responsible for producing volatile compounds like hexanal and *Z*-3-hexenal, derived from 13-hydroperoxide, and *Z*-3-nonenal and *Z,Z*-3,6-nonadienal, originating from 9-hydroperoxide. These C6 and C9 aldehydes contribute characteristic vegetal aromatic notes, such as "green," "herb," "cucumber," and "pear." Later, through enzymatic action, these aldehydes are transformed into short-chain alcohols and esters, contributing to the unique sensory profile of the olive oil [154,181,182,199,201,202].

From a biological perspective, the lipoxygenase pathway is a plant defense mechanism against stressors such as mechanical damage, extreme temperatures, and pathogen attacks. In high-quality olive oils, this pathway is responsible for the generation of volatile compounds, such as aldehydes, esters, alcohols, and ketones, which provide positive attributes like "green" and "fruity" aromas [203,204].

Other factors can also deteriorate olive oil. Heat (temperatures above 20°C) accelerates the formation of peroxides, while metals catalyze rancidity and can add metallic flavors to the oil. Prolonged exposure to oxygen intensifies oxidation, further compromising its quality.

Thus, the sensory profile of olive oil can be compromised, presenting sensory defects. The most common defects include "fusty", "mold", "winey-vinegary", and "rancid." The first three are typically associated with poor storage of olives before extraction, while rancidity results from oxidation or faulty storage of the oil [185].

After numerous studies, each of these defects has a more or less defined volatile profile. These sensory defects are responsible for the acceptability of olive oils, and when detected, the oil can be classified as lampante, meaning it is deemed unsuitable for direct consumption and is instead intended for refining or other industrial uses.

Oxidative processes and sensory defects compromise both the sensory attributes and the nutritional composition of olive oil, leading to the loss of essential fatty acids and fat-soluble vitamins. To minimize these impacts, it is essential to adopt measures such as good practices in harvesting and storing olives before extraction, storing in opaque and airtight containers to reduce exposure to light and oxygen, strictly controlling temperature during storage, and using natural antioxidants to delay the oxidation process.

Understanding and mitigating the factors that promote oxidation and sensory defects is essential for ensuring the quality of olive oil, guaranteeing consumer acceptance, and extending its shelf life.

2.5 Analysis of VOCs

The analysis of volatile organic compounds (VOCs) in extra virgin olive oil (EVOO) is crucial for quality control, as these compounds are responsible for the sensory characteristics of the product. However, VOC analysis in EVOO is not yet recognized or standardized by European Union regulations or the International Olive Council (IOC). The growing demand for fast and efficient methods has favored sensory approaches that provide immediate responses, such as "suitable/unsuitable" or "pleasant/unpleasant," at the expense of more detailed methods based on chemical identification [205].

The EVOO matrix presents significant analytical challenges due to the presence of hundreds of VOCs in concentrations ranging from trace levels to tens of milligrams per kilogram. These compounds belong to different chemical classes with varying polarities and volatilities, which increases the complexity of analysis and may lead to the loss of compounds during sample preparation [181,201].

The typical VOC analysis process in EVOO involves an initial sampling step to isolate and pre-concentrate the volatile compounds, followed by separation, identification, and quantification. The sampling step is considered the main bottleneck of the analytical procedure, with a direct impact on the quality of the results. Various methods have been developed over the past decades, allowing for the identification of hundreds of compounds [181]. Among these, headspace solid-phase microextraction (HS-SPME) combined with gas chromatography (GC) and mass spectrometry (MS) stands out as the most widely used approach for VOC analysis in EVOO [181,206,207].

HS-SPME is a solvent-free, fast, cost-effective technique that is easily adaptable to automation. Widely used in food analysis, this methodology is effective for pre-concentrating (semi)volatile compounds, acting as a bridge between static and dynamic headspace methods. The method is based on the adsorption of VOCs onto a fiber coating exposed to the free space of the sample, and its efficiency depends on the partitioning equilibrium between the oil matrix, the free space, and the fiber [181,189,207,208].

The distribution coefficients of VOCs between these phases determine the extraction efficiency, with analytical conditions adjusted according to the study's objective. For example, higher temperatures may intensify extraction but also induce the formation of unwanted artifacts. Although effective, HS-SPME has limitations, such as competition between compounds during adsorption onto the fiber, which can affect the accuracy of quantification. To minimize these effects, it is essential to select appropriate analytical conditions. The DVB/CAR/PDMS

fiber coating is widely recognized as the most efficient for VOCs in EVOO due to its high sensitivity and balanced absorption capacity [181,209,210].

Additionally, HS-SPME coupled with GC offers further advantages, such as low detection limits, operational simplicity, and feasibility in both manual and automated systems. These factors make the technique particularly suitable for analyzing complex matrices like EVOO [181].

Other analytical approaches include comprehensive two-dimensional gas chromatography (GC × GC), which combines two columns of different polarities, providing greater separation capacity and high sensitivity when coupled with mass spectrometry (GC × GC/MS). This technique allows for high-throughput screening and the creation of detailed profiles, organizing analyte patterns and homologous series logically on the two-dimensional chromatogram, which facilitates data interpretation [181,211–213].

Methods such as GC x GC-TOFMS, GC/MS, GC-FID, and GC-olfactometry (GC-O) are used in VOC analysis, each with specific advantages for different analytical objectives. However, the lack of uniformity in quantification methods complicates the comparison of results between studies, posing a challenge for harmonizing data and standardizing analyses [181].

2.6 Considerations and Objectives

Although advanced analytical techniques are widely employed, integrating fast, cost-effective methods that do not rely on trained sensory panels can offer a balance between efficiency and scientific accuracy. This hybrid approach could contribute significantly to standardizing VOC analyses in EVOO, optimizing quality control, and enhancing the product's competitiveness in the market.

Given the impact of volatile organic compounds (VOCs) on the sensory profile and quality of extra virgin olive oil (EVOO), the research developed as part of this thesis focused on the detailed analysis of these compounds, aiming to better understand the factors affecting their evolution and their relationship with sensory defects over shelf life.

The results were consolidated in the scientific article titled *"Early Identification of Olive Oil Defects throughout Shelf Life"* published in the journal Separations in 2024. This work presents the development and application of a robust analytical methodology, based on HS-SPME-GC/MS, for identifying VOCs associated with positive and negative attributes of EVOO. Furthermore, it proposes using the ratio between specific compounds as a predictive tool for the sensory declassification of olive oils.

In parallel, a study was conducted (data not shown in this dissertation) on the evolution of pheophytins and pyropheophytins, as chlorophylls are converted into pheophytins during the extraction and aging processes, which can subsequently transform into pyropheophytins. However, it was not possible to establish a direct correlation between the concentrations and percentages of the different pheophytins and either the sensory data or the chemical markers related to the shelf life of the oil. Pheophytins did not show a correlation with shelf life or sensory panel evaluations of EVOO, suggesting they may be not reliable markers for determining shelf life.

The full article follows, which forms a chapter of this thesis and reflects the contribution of this research to advancing scientific understanding in VOC analysis and quality control of extra virgin olive oil.

2.7 Article

"Early Identification of Olive Oil Defects throughout Shelf Life"

Flávia Freitas, Maria João Cabrita and Marco Gomes da Silva

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Article

Early Identification of Olive Oil Defects throughout Shelf Life

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Abstract: The unique aroma and flavor of extra virgin olive oil (EVOO) are generally associated with its volatile composition, which includes a variety of components responsible for positive attributes as well as sensory defects which result from chemical oxidation processes and the action of exogenous enzymes. In this study, a robust analytical method, headspace solid-phase microextraction combined with gas chromatography–mass spectrometry (HS-SPME-GC/MS), was developed to tentatively identify volatile organic compounds (VOCs) as markers of positive and negative attributes, correlating them with relative percentages to estimate the risk of disqualification during the shelf life of EVOO. Significant differences ($p < 0.05$) were identified in the levels of VOCs over time, mainly those derived from the lipoxygenase (LOX) pathway. Principal component analysis (PCA) was applied to process the experimental data. The ratio of *E*-2-hexenal to acetic acid allowed for the prediction of the disqualification of monovarietal EVOO by the sensory panel.

Keywords: extra virgin olive oil; VOCs; sensory panel; shelf life; defects

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1. Introduction

Olive oil (OO), derived from the olive tree (*Olea europaea* L.), is a fundamental element in the diet of Mediterranean countries and enjoys recognition as the most valued edible oil globally [1–3]. Its attractiveness stems mainly from the predominance of monounsaturated fatty acids, notably oleic acid, and the presence of minor compounds that contribute significantly to its high nutritional value [4,5].

Olive oil is highly prized for its characteristic flavor and pleasant aroma, mainly due to the wide variety and nature of various phenolic compounds and volatile organic compounds present in minor fractions [6].

Phenolic compounds in olive oil, such as phenolic acids and alcohols, lignans, flavones, and secoiridoids, have a significant influence not only on antioxidant activity but also on the ability to provide unique sensory descriptors. There is thus a positive correlation between the aroma and flavor of olive oil and its polyphenol content [7–9].

Other compounds of particular interest in influencing the flavor and aroma of olive oils are volatile organic compounds (VOCs). These compounds are produced by combinations of natural biochemical processes that mainly occur during olive maturation and oil extraction [10,11].

An example of a biochemical process is the lipoxygenase (LOX) biosynthetic pathway, responsible for the oxidation of polyunsaturated fatty acids, such as linoleic and linolenic acids, leading to the formation of C5–C6 VOCs, such as aldehydes, ketones, and alcohols, which contribute to the green and fruity aroma of olive oil [12–16].

However, volatile compounds can be related to both the positive attributes and sensory defects of olive oil [14,17,18]. While the pleasant characteristics of olive oil are predominantly influenced by endogenous plant enzymes through the LOX pathway, the presence of sensory defects is associated with chemical oxidation and the action of exogenous enzymes, often derived from microbial activity during storage. These defects are characterized by the low concentration or total absence of compounds from the LOX pathway, and the presence of monounsaturated aldehydes C7–C11, branched aldehydes of C5, and/or some C8 ketones [19–21].

The development of these VOCs and also phenolic compounds is mainly associated with the variety, quality, and ripeness of olives, pre- and post-harvest conditions, processing, and inadequate storage. The presence and quantity of these compounds can also be affected by other factors such as geographic origin, climate, and soil type [22–27].

Olive oil was the first food product for which a quality assessment by a certified and qualified sensory panel was legally required, as the sensory perception of aroma and taste plays a fundamental role in quality evaluation [28].

Positive sensory attributes include fruity, bitter, and pungent flavors. In addition to intensity, fruitiness can be classified as green or ripe. Bitterness is the characteristic bitter taste of olive oil, and pungency refers to the sensation of spiciness or burning in the throat [29].

Undesirable sensory attributes include defects such as rancid, vinegary, musty, metallic, and fusty flavors, among others [29]. When these defects are present, it is believed that inferior-quality olive oil is present, that there were problems in its production process, or that it has exceeded its shelf life [30–33].

The shelf life of olive oil can vary depending on the olive variety, production process, storage conditions, and the presence of deteriorating factors [34]. Olive oil has an extended shelf life compared to other vegetable oils due to its composition rich in natural antioxidants, such as polyphenols. Under ideal storage conditions, in a sealed bottle, away from excessive light and heat, extra virgin olive oil can be kept for about 12 to 18 months from the production date [35,36].

Thus, throughout this process, olive oil tasters are essential to provide valuable information about the quality, characteristics, and validity of olive oil, sensorially evaluating to obtain a classification within different categories, such as extra virgin (EVOO), virgin (VOO), or lampante, according to standards established by the International Olive Oil Council (IOOC) and European Union (EU) [37–39].

However, these sensory evaluations, which require specialized and trained individuals, may present some inconsistencies, as the result depends on the individual perception of each taster, leading to variations from one day to another, and even among the sensory panel itself [21,31,40,41]. In addition to sensory evaluation, other parameters of olive oil quality are regulated and should be considered to classify an oil, such as free acidity, peroxide value, UV absorbance, and ethyl esters of fatty acids [37–39].

Therefore, it is necessary to develop robust and reliable analytical methods that can support the evaluation performed by the sensory panel [42].

The good quality of EVOO is closely linked to its physicochemical and organoleptic characteristics, and consequently its volatile profile.

In recent years, solid-phase microextraction (SPME) has been widely used in the analysis of volatile organic compounds in olive oil, along with gas chromatography coupled with mass spectrometry (GC/MS) analysis [18,43]. SPME, besides being a simple, rapid, and low-cost method without the use of solvents, allows for the absorption and concentration of VOCs present in the matrix, facilitating their subsequent analysis and identification [44].

This study aimed to develop an HS-SPME-GC/MS methodology to determine the presence of VOCs as early markers for negative attributes such as rancidity, mustiness, and fustiness, establishing a correlation between compounds/concentrations/attributes in order to estimate the risk of disqualification during the shelf life of extra virgin olive oil.

2. Materials and Methods

2.1. Samples and Experimental Design

This study was divided into 3 major steps, represented graphically in Figure 1. The description of the samples is provided in Table 1.

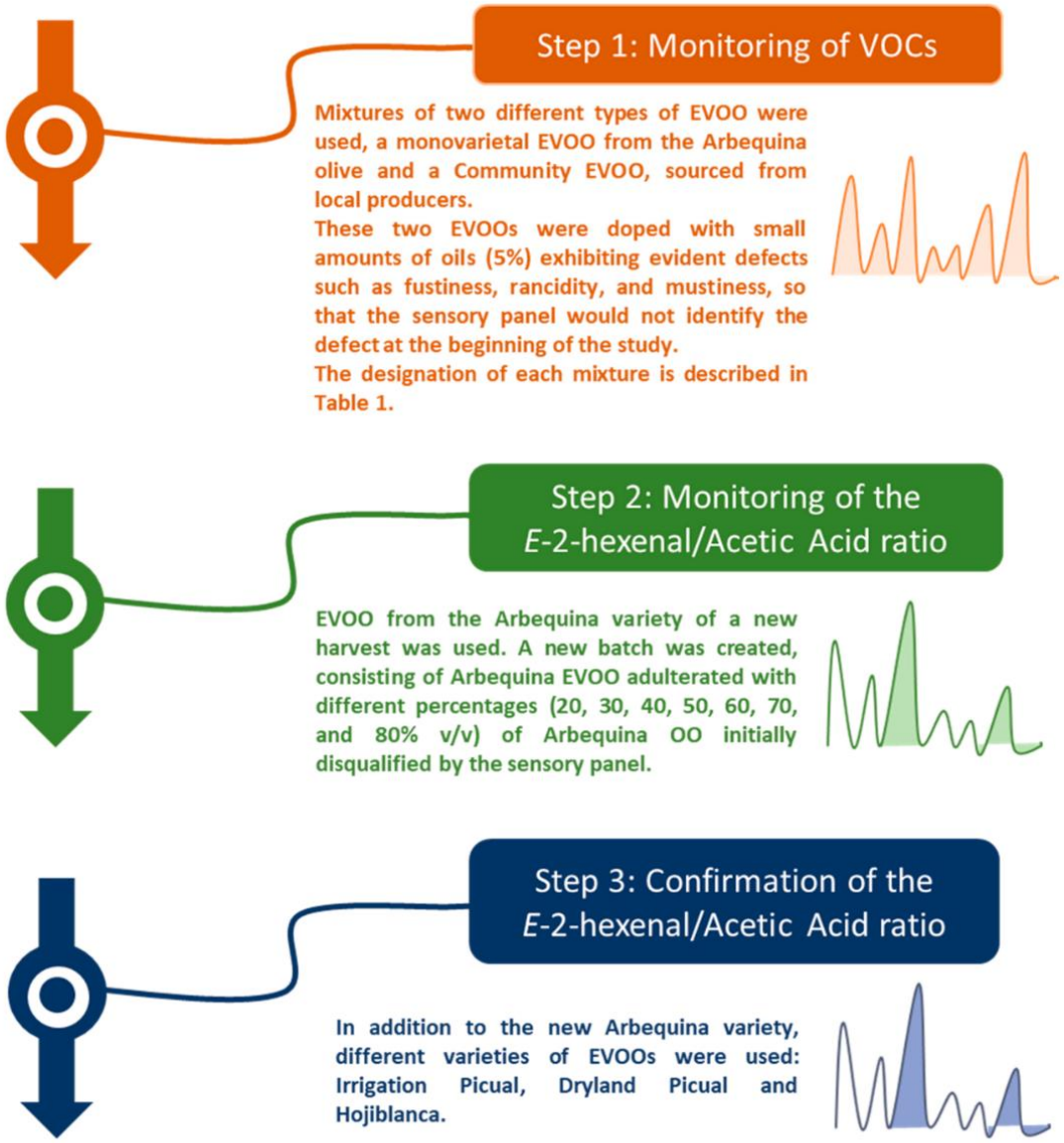

Figure 1. Graphical representation of the 3 main steps of the study.

Table 1. Description of the samples used in the study.

		Description of Samples
Monitoring of VOCs	Ar	Monovarietal EVOO from the Arbequina olives
	Ar musty	Arbequina EVOO doped with 5% (*v*/*v*) of disqualified olive oil with evident musty defect
	Ar rancid	Arbequina EVOO doped with 5% (*v*/*v*) of disqualified olive oil with evident rancidity defect
	Ar fusty	Arbequina EVOO doped with 5% (*v*/*v*) of disqualified olive oil with evident fusty defect
	Co	Community EVOO from local producers
	Co musty	Community EVOO doped with 5% (*v*/*v*) of disqualified olive oil with evident musty defect
	Co rancid	Community EVOO doped with 5% (*v*/*v*) of disqualified olive oil with evident rancidity defect
	Co fusty	Community EVOO doped with 5% (*v*/*v*) of disqualified olive oil with evident fusty defect
Monitoring of the *E*-2-hexenal/Acetic Acid	0% Def	Monovarietal EVOO from the Arbequina olives
	20% Def	Arbequina EVOO doped with 20% (*v*/*v*) of disqualified Arbequina olive oil (OO)
	30% Def	Arbequina EVOO doped with 30% (*v*/*v*) of disqualified Arbequina OO
	40% Def	Arbequina EVOO doped with 40% (*v*/*v*) of disqualified Arbequina OO
	50% Def	Arbequina EVOO doped with 50% (*v*/*v*) of disqualified Arbequina OO
	60% Def	Arbequina EVOO doped with 60% (*v*/*v*) of disqualified Arbequina OO
	70% Def	Arbequina EVOO doped with 70% (*v*/*v*) of disqualified Arbequina OO
	80% Def	Arbequina EVOO doped with 80% (*v*/*v*) of disqualified Arbequina OO
	100% Def	Disqualified Arbequina OO
Confirmation of the *E*-2-hexenal/Acetic Acid	PiR	Irrigation Picual EVOO
	Arb a	Arbequina EVOO
	PiD	Dryland Picual EVOO
	Arb b	Arbequina EVOO
	Hoj	Hojiblanca EVOO
	Hoj x	Disqualified Hojiblanca OO
	Arb x	Disqualified Arbequina OO
	PiD x	Disqualified Dryland Picual OO
	PiR x	Disqualified Irrigation Picual OO

2.2. Storage Conditions

All samples from different batches of the study, shown in Table 1, were stored in dark glass bottles and kept in a dry, dark place. Multiple bottles of the same sample type were stored so that throughout the study, new bottles were opened for analysis, thus replicating shelf storage conditions.

All samples were analyzed by HS-SPME-GC/MS in triplicate.

2.3. Physicochemical and Organoleptic Classification

The physicochemical and organoleptic classification was carried out following the criteria of Commission Regulation No. 1989/2003 of 6 November 2003, regarding the characteristics of olive oils and olive–pomace oils, as well as related analysis methods [38].

A sensory panel composed of a panel leader and eight selected and trained assessors was employed based on their ability to distinguish similar samples, following the IOOC manual on the selection, training, and monitoring of qualified virgin olive oil assessors [41]. All samples were analyzed by HS-SPME-GC/MS in triplicate.

2.4. Analytical Procedure

HS-SPME: 4 mL of each sample was subjected to Solid Phase Microextraction by Headspace (HS-SPME) with a 50/30 µm DVB/Carb/PDMS fiber of 1 cm in a 22 mL vial. The sample was equilibrated for 10 min at 50 °C and then extracted for 50 min at this temperature. Thermal desorption of analytes occurred by exposing the fiber to the GC injector at 260 °C for 3 min in splitless mode. Fiber blanks were periodically executed to verify the absence of contaminants and carryover.

GC/MS: A Bruker Scion TQ 456 GC-MS/MS (Bruker Corporation, Billerica, MA, USA) chromatograph equipped with a CTC-CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland) was used. Data were acquired with a Bruker MSWS 8.2 system

and analyzed with Bruker MS Data Review 8.0 software. Chromatographic separation was performed on a DB-WAX PLUS capillary column (60 m × 0.32 mm i.d., 1 µm film thickness (df)). The temperature program started at 40 °C, was held for 5 min, and was then ramped at 4 °C/min to 240 °C and held for 5 min. Helium was used as the carrier gas, with a constant flow rate of 1.7 mL/min. The transfer line of the MS and the source were set at 240 °C and 220 °C, respectively.

Mass spectra were compared using the NIST MS Search Program Version 2.0. For electron ionization (EI), the ionization energy was set at 70 eV, and spectra were recorded between 40 and 450 Da.

The fiber type was chosen according to various procedures described for olive oil that validate the extraction method [42,45–47]. Samples were prepared following validated procedures without the addition of NaCl.

The identification of VOCs was based on the analysis of their mass spectra by comparison with reference spectra provided by the NIST library. Additionally, identifications were confirmed by comparison of the linear retention indices (LRIs) relative to the homologous series of n-hydrocarbons (C8–C20), calculated by the formula proposed by Van den Dool and Kratz [48].

A relative semi-quantitative determination was made by comparing peak area intensities.

The software Minitab 19.2 (Minitab Inc., State College, PA, USA) was used for statistical data processing. Analysis of variance (ANOVA) was conducted, and significant differences ($p < 0.05$) between samples with and without added defects were highlighted by the post hoc Fisher's LSD test.

Principal component analysis (PCA) was used to characterize and classify the studied olive oils according to their volatile compounds and sensory panel classification. For this analysis, each peak was normalized to a percentage of the total chromatogram area. Calculation was performed with the Python programming language (Version 3.11.8) using the PCA class in the decomposition module of the Scikit-learn library (Version 1.4.2) [49].

3. Results and Discussion

3.1. Monitoring of VOCs

For the monitoring of VOCs, mixtures of two different types of EVOO were used: a monovarietal olive oil of the Arbequina olive variety and a Community olive oil sourced from local producers. The term "Community EVOO" is referred to when it is composed of one or more unknown varieties.

According to the sensory panel, the Community EVOO was described as a more bitter and pungent oil, whereas the Arbequina EVOO was milder and fruitier, characteristic of this variety (Figure 2). Additionally, there was a difference in color, with the Arbequina displaying a lighter golden-green hue, while the Community EVOO had a darker golden-green color.

The chromatographic profile between the two samples also showed significant differences, with the profile of the Arbequina being much more intense than that of the Community olive oil (Figure 3).

Throughout this study, both chromatographic and sensory analyses were consistently conducted during the same period. Figure 4 presents the samples analyzed chromatographically and sensorially over 14 months, with indications in red showing months in which the sensory panel confirmed the presence of defects and downgraded the EVOOs to VOOs (more than 50% of the panel), and in yellow when the samples resulted in defects for part of the panel but without agreement (less than 50%). Samples that continued to be considered "suitable" and classified as EVOOs by the sensory panel are marked in green.

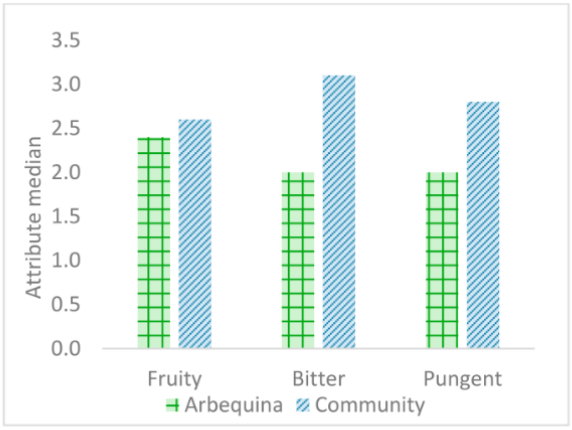

Figure 2. Sensory classification of Arbequina variety and Community EVOO at the beginning of the study.

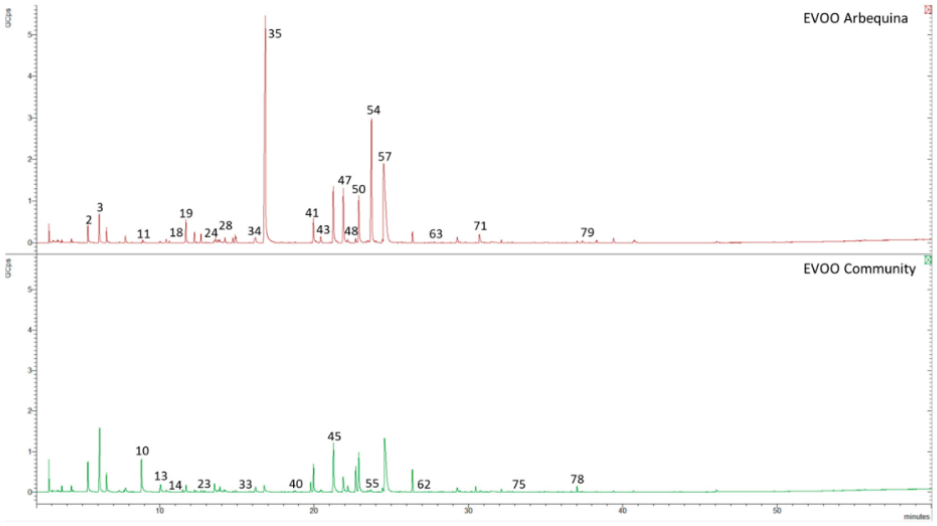

Figure 3. Chromatograms, on the same scale, of the Arbequina variety (top) versus Community (bottom). The compounds with significant differences over time are represented in the chromatograms. The peak numbers are identified in Table 2.

Despite the Community olive oil having a less rich and intense volatile profile, this EVOO took longer to be disqualified by the panel.

While the Community sample remained extra virgin throughout the 14 months, the Arbequina sample turned virgin before the 10-month mark of the study. This is likely due to it being a more bitter and pungent oil, positive attributes that may mask and overshadow some negative attributes. Samples to which olive oil with evident defects (5%) was added were downgraded 2 months earlier than their corresponding EVOO sample.

Olive oil is a highly complex matrix with a high concentration of volatile compounds with different physicochemical properties, such as volatility and polarity [50]. The HS-SPME-GC/MS technique used separated and tentatively identified around 80 volatile and semi-volatile compounds, highlighting the extreme complexity of olive oil aroma (Table 2).

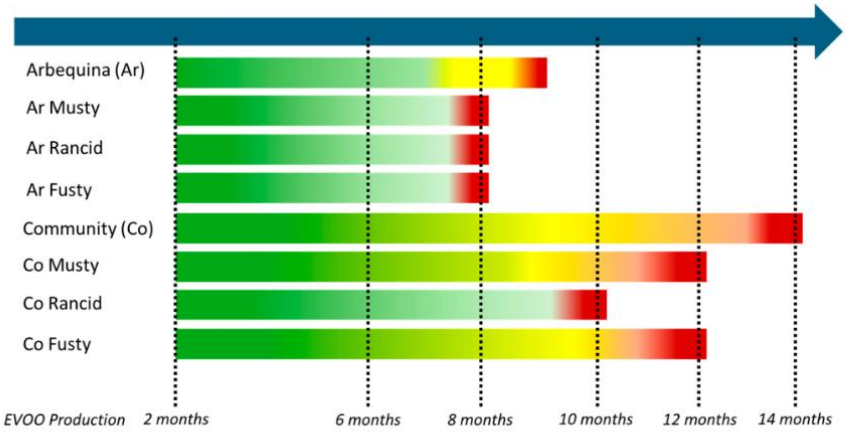

Figure 4. Samples analyzed with indication of when they are considered EVOO by the sensory panel (green), the moment the defect was detected (red) and the month in which part of the panel indicated the presence of the defect but without unanimity (yellow).

Table 2. Tentatively identified VOCs and their respective relative percentages of each compound calculated by the percent ratio of their peak area to the total chromatogram area in samples of Arbequina and Community. Compound nr. refers to the elution order.

Compound nr.	Compound [a]	LRI_{calc} [b]	LRI_{lit} [c]	% Arbequina	% Community
Aldehydes					
19	Hexanal	1067	1083	1.81 ± 0.04	1.12 ± 0.01
24	2-Pentenal (isomer)	1108		0.06 ± 0	0 ± 0
25	3-Hexenal (isomer)	1120		0 ± 0	0.25 ± 0.09
33	Heptanal	1175	1184	0.03 ± 0	0.06 ± 0
35	*E*-2-Hexenal	1192	1216	33.53 ± 0.13	2.08 ± 0.16
42	Octanal	1281	1289	0.01 ± 0	0.05 ± 0
44	2-Heptenal (isomer)	1299		0.05 ± 0	0.06 ± 0.01
51	2,4-Hexadienal (isomer)	1358		0 ± 0	0.04 ± 0.01
52	2,4-Hexadienal (isomer)	1358		0 ± 0	0 ± 0
56	Nonanal	1387	1391	0.21 ± 0.04	0.89 ± 0.11
60	*E,E*-2,4-Heptadienal	1427	1495	0 ± 0	0.1 ± 0.02
75	2-Decenal (isomer)	1620		0 ± 0	0.09 ± 0.01
Alcohols					
3	Isopropyl Alcohol	nc	927	2.49 ± 0.12	12.81 ± 0.47
23	2-Pentanol	1096	1119	0 ± 0	0.18 ± 0.01
28	1-Penten-3-ol (isomer)	1131		0.36 ± 0.01	0.11 ± 0
34	Isopentanol	1178	1209	0.8 ± 0.01	1.31 ± 0.05
36	Pentanol	1220	1250	0.09 ± 0	0.1 ± 0.02
43	2-Penten-1-ol (isomer)	1284		0.63 ± 0.01	0.39 ± 0.01
47	Hexanol	1321	1355	5.44 ± 0.06	3.48 ± 0.04
48	*E*-3-Hexenol	1329	1367	0.23 ± 0	1.14 ± 0.05
50	*Z*-3-Hexenol	1347	1382	4.88 ± 0.01	8.63 ± 0.15
54	*E*-2-Hexenol	1368	1405	12.49 ± 0.03	0.45 ± 0
55	*Z*-2-Hexenol	1377	1416	0.07 ± 0	0 ± 0
58	1-Octen-3-ol (isomer)	1399		0 ± 0	0 ± 0
59	Heptanol	1403	1453	0.05 ± 0	0 ± 0
62	2-Heptenol (isomer)	1450		0 ± 0	0 ± 0
64	Linalool	1480	1547	0 ± 0	0.04 ± 0
65	Octanol	1506	1557	0 ± 0	0 ± 0
80	Benzyl alcohol	1804	1870	0.26 ± 0	0.08 ± 0
81	Phenylethyl Alcohol	1841	1906	0.49 ± 0.01	0.22 ± 0.01
Carboxylic acids					

Table 2. *Cont.*

Compound nr.	Compound [a]	LRI_{calc} [b]	LRI_{lit} [c]	% Arbequina	% Community
57	Acetic acid	1392	1449	20.03 ± 0.27	24.21 ± 0.54
61	Formic acid	1445	1503	0 ± 0	0 ± 0
63	Propanoic acid	1479	1535	0.09 ± 0	0 ± 0
66	Isobutyric acid	1509	1570	0.02 ± 0	0 ± 0
71	Butanoic acid	1564	1625	1.08 ± 0.13	0 ± 0
76	Pentanoic acid	1671	1622	0.03 ± 0	0 ± 0
79	Hexanoic acid	1773	1846	0.15 ± 0	0.05 ± 0
83	2-Hexenoic acid (isomer)	1887		0.26 ± 0.01	0 ± 0
84	Octanoic acid	1979	2060	0 ± 0	0.05 ± 0
87	Nonanoic acid	2083	2171	0 ± 0	0 ± 0
Esters					
1	Methyl acetate	nc	810	0.18 ± 0	0.65 ± 0.02
2	Ethyl Acetate	nc	880	1.24 ± 0.03	5.26 ± 0.39
8	Ethyl isobutyrate	nc	961	0 ± 0	0.04 ± 0
9	Methyl butyrate	nc	982	0.03 ± 0	0.03 ± 0
14	Ethyl butyrate	1035	1035	0.32 ± 0	0.33 ± 0.03
17	Ethyl 2-methylbutyrate	1062	1051	0 ± 0	0.32 ± 0.02
18	Butyl acetate	1066	1074	0.02 ± 0	0.06 ± 0
20	Ethyl isovalerate	1074	1068	0 ± 0	0.04 ± 0.01
38	Ethyl hexanoate	1239	1233	0 ± 0	0.07 ± 0.01
41	Hexyl acetate	1268	1272	0 ± 0	0 ± 0
45	3-Hexenyl Acetate (isomer)	1304		5.58 ± 0.17	10.89 ± 0.47
72	Butyrolactone	1566	1632	0 ± 0	0.88 ± 0.75
73	Methyl benzoate	1576	1612	0.04 ± 0	0.03 ± 0
Hydrocarbons					
4	Unknown hydrocarbon	nc		0.95 ± 0.01	2.67 ± 0.22
12	Toluene	1022	1042	0.12 ± 0	0 ± 0
15	Ethyl octadiene (isomer)	1039		0.12 ± 0	0 ± 0
16	Ethyl octadiene (isomer)	1040		0 ± 0	0.04 ± 0.02
21	Ethyl octadiene (isomer)	1081		0.74 ± 0.02	0.32 ± 0.02
22	Ethyl octadiene (isomer)	1092		0.59 ± 0.02	0.25 ± 0.02
26	Unknown alkane	1122		0.13 ± 0.01	0.52 ± 0.06
27	*p*-xylene	1130	1138	0.03 ± 0	0.14 ± 0.04
29	Ethyl octadiene (isomer)	1142		0.3 ± 0.01	0.11 ± 0.01
30	Ethyl octadiene (isomer)	1147		0.79 ± 0.03	0.28 ± 0.01
31	*o*-Xylene	1169	1186	0.04 ± 0	0.11 ± 0.01
37	Styrene	1215	1261	0 ± 0	0 ± 0
49	Unknown alkene	1342		0.29 ± 0.02	4.23 ± 0.12
68	Hexadecane	1600	1600	0.06 ± 0	0.13 ± 0.04
74	Unknown alkane	1628		0.07 ± 0.01	0.14 ± 0.07
Ethers					
10	Hexyl methyl ether	nc	941	0 ± 0	6.13 ± 0.34
13	3-Hexen-1-ol, methyl ether	1025	980	0 ± 0	1.54 ± 0.15
53	Benzyl methyl ether	1363	1394	0 ± 0	0.32 ± 0.01
Terpenes					
40	*β*-Ocimene	1258	1250	0.56 ± 0.26	1.52 ± 0.17
69	Unknown sesquiterpene	1549		0 ± 0	0.14 ± 0.02
70	Unknown sesquiterpene	1558		0.05 ± 0	1.04 ± 0.08
77	Unknown sesquiterpene	1750		0 ± 0	0.13 ± 0
78	*α*-Farnesene	1762	1746	0.15 ± 0.01	0.95 ± 0
Ketones					

Table 2. *Cont.*

Compound nr.	Compound [a]	LRI_{calc} [b]	LRI_{lit} [c]	% Arbequina	% Community
6	Pentanone (isomer)	nc		0 ± 0	0.17 ± 0
7	Pentanone (isomer)	nc		0.72 ± 0	0.81 ± 0.08
11	1-Penten-3-one	nc	1019	0.27 ± 0.01	0 ± 0
32	2-Heptanone	1171	1182	0 ± 0	0 ± 0
46	Sulcatone	1316	1338	0 ± 0	0.03 ± 0
Others					
5	2-Ethylfuran	nc	950	0 ± 0	0 ± 0
39	Acetoin	1242	1284	0.07 ± 0	0.24 ± 0.02
67	Dimethyl Sulfoxide	1523	1573	0.6 ± 0.05	0.83 ± 0.24
82	Unknown	1886		0.12 ± 0.02	0.19 ± 0.01
85	Dimethyl salicylate	1995	2061	0 ± 0	0.06 ± 0.02
86	Phenol, 3-ethyl-	2083	2171	0.18 ± 0.05	0.35 ± 0.07

[a] Identification by NIST comparation; [b] linear retention indices calculated from C8 to C20 n-linear alkanes; [c] linear retention indices reported in NIST Chemistry WebBook for standard polar capillary column [51]; nc—not calculated because we had to take into account the solvent delay.

Within this vast group of compounds, we can predominantly find aldehydes, alkanes, alcohols, and ketones, among others, in different relative percentages for the two samples, as depicted in Figure 5.

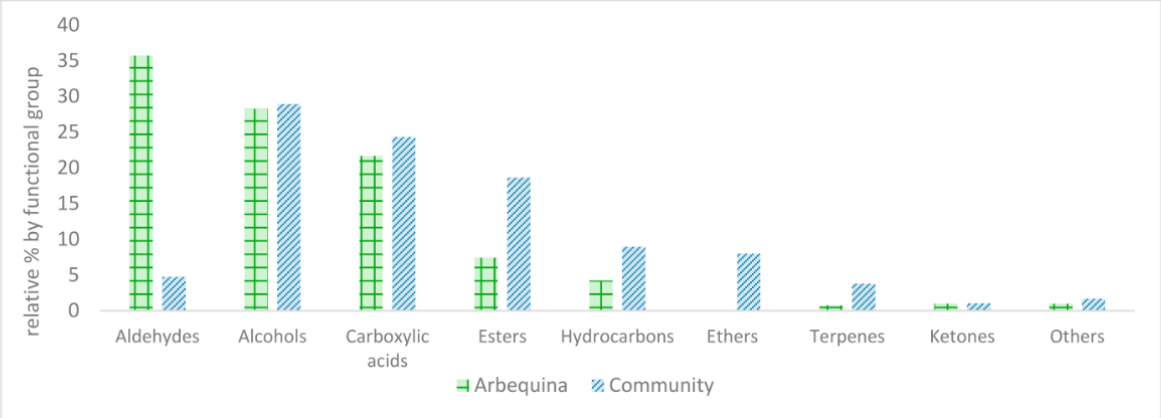

Figure 5. Graphical representation of the relative percentages for each functional group of Arbequina variety versus Community.

After a thorough analysis of all chromatograms of the samples, it was found that most of the VOCs were common to all samples of the two varieties with different defects, being present in different relative proportions. Therefore, it was necessary to identify which compounds contributed to the differentiation between these samples throughout the shelf life.

The majority of these compounds are related to the organoleptic properties of olive oil, having sensory characteristics that contribute to flavor sensations [10,52,53].

As observed in Table 2, it is the C6 alcohols and aldehydes that predominate. These compounds, along with their corresponding esters, are considered essential in the aromatic profile of EVOOs, both qualitatively and quantitatively [14]. They play a crucial role in expressing sweet and green notes, contributing significantly to the overall aroma [12].

Compounds such as hexanal (19), *Z*-3-hexenal (25), *E*-2-hexenal (35), hexanol (47), *Z*-3-hexenol (50), *E*-2-hexenol (54), hexyl acetate (41), and *Z*-3-hexenyl acetate (45) comprise the majority of the volatile fraction, representing about 60% of the total area for the Arbequina EVOO and 30% for the Community EVOO. For the Community olive oil, these compounds are found in very similar relative proportions to each other. However, in the

Arbequina variety, *E*-2-hexenal (35) stands out as a particularly prominent compound, representing about 30% of the total area of the chromatogram.

Therefore, the monovarietal Arbequina consists of approximately 36% of aldehydes versus 8% for the Community EVOO. Both EVOOs have approximately the same percentage of alcohols (28%). The Community EVOO exhibits a higher relative quantity of esters (19%) compared to the Arbequina variety (7%), mainly due to the greater presence of compounds such as ethyl acetate (2) and *Z*-3-hexenyl acetate (45).

According to the presented values, significant differences are observed, especially at the LOX pathway level for different cultivars, which leads to discrimination between the Arbequina EVOO and Community EVOO.

It is also worth considering that besides being sweet and fruity, these olive oils have bitter, pungent, and spicy attributes for the sensory panel, especially Community EVOO. These attributes are generally attributed to C5 compounds, such as 1-penten-3-one (11), which provides pungent sensations correlated with bitterness [54,55]. Despite C6 VOCs being in higher concentrations than C5, it does not necessarily mean they are the main contributors to the odor. For example, a concentration of 6770 μg/g of *E*-2-hexenal has an odor activity value corresponding to 16, while a concentration of 26 μg/g of 1-penten-3-one has a higher value of 36 [56].

In addition to these VOCs, these sensations are also attributed to phenolic compounds such as derivatives of oleuropein and ligstroside [57]. Therefore, all the VOCs found, whether major or minor, are responsible for the sensory notes and crucial in determining the quality of EVOO.

Even VOCs that are below their olfactory threshold and do not have a direct impact on aroma can play an important role in understanding the formation and degradation of volatile compounds that significantly contribute to aroma [12]. Additionally, these compounds can serve as useful quality markers. This fraction includes a variety of compounds such as carbonyl compounds, pentenols, hydrocarbons, ethers, and other minor compounds that are not the result of fatty acid transformations [58].

Some sesquiterpenes, such as α-farnesene (78) and ethyl octadiene isomers are present in both samples, whereas some ethers, such as hexyl methyl ether (10) and 3-hexen-1-ol methyl ether (13), are present only in the Community EVOO.

Another class of compounds with a notable presence in EVOOs and that do not derive from fatty acid transformation are carboxylic acids, mainly acetic acid (57) (about 20%). This compound has a natural origin and results from the fermentation process of sugars present in olives during maturation. It tends to increase over time due to continuous fermentation as well as the oxidation of the olive oil's fatty acids, giving rise to some organoleptic defects such as wine–vinegar flavor [21,33].

The evolution of VOCs in olive oil influences the organoleptic classification and, consequently, the classification of olive oil by the sensory panel. Several processes can alter the initially pleasant aroma and flavor, resulting in unpleasant sensory notes known as off flavors [53]. Current official olive oil regulations classify the most common off flavors into four groups: musty, musty–humid, wine–vinegar, and rancid [29].

The presence of a fusty flavor often indicates that the olives used in the oil production process were at an advanced stage of fermentation. Musty–humid flavor is typical of olive oils from olives stored in damp conditions for an extended period, leading to the development of various types of fungi. The wine–vinegar flavor arises due to high concentrations of acetic acid, ethyl acetate, and ethanol. Rancidity is a common sensory characteristic of all oils and fats that have undergone auto-oxidation due to prolonged exposure to air [21,32,33,59].

The first three defects result from improper storage of the fruits before olive oil processing, while the latter occurs during olive oil storage. These sensory defects become more pronounced over the olive oil's shelf life [33,60].

3.2. Evolution of VOCs over the Storage Time

In this study, Arbequina and Community EVOOs were both mixed with 5% of three different types of disqualified olive oil with distinct defects—musty, fusty, and rancid—and their evolution over 14 months was studied.

After the tentative identification of compounds (see Table 2), an analysis of variance (ANOVA) was conducted for the VOCs of the different varieties with and without disqualified olive oil added, comparing them over 14 months. This analysis revealed a set of 30 compounds that were significantly different among the samples ($p < 0.05$) responsible for the evolution and possible disqualification by the sensory panel (Table 3).

Table 3. Compounds with statistical differences ($p < 0.05$) determined by ANOVA.

Compound No.	Compound Name
2	Ethyl Acetate
3	Isopropyl Alcohol
10	Hexyl methyl ether
11	1-Penten-3-one
13	3-Hexen-1-ol, methyl ether
14	Ethyl butyrate
18	Butyl acetate
19	Hexanal
23	2-Pentanol
24	2-Pentenal
28	1-Penten-3-ol
33	Heptanal
34	Isopentanol
35	*E*-2-Hexenal
40	*β*-Ocimene
41	Hexyl acetate
43	2-Penten-1-ol
45	3-Hexenyl Acetate
47	Hexanol
48	*E*-3-Hexenol
50	*Z*-3-Hexenol
54	*E*-2-Hexenol
55	*Z*-2-Hexenol
57	Acetic acid
62	2-Heptenol
63	Propanoic acid
71	Butanoic acid
75	*Z*-2-Decenal
78	*α*-Farnesene
79	Hexanoic acid

The evolution of acetic acid (57) and VOCs derived from the LOX pathway that showed significant differences ($p < 0.05$) during the storage of the samples is depicted in Figure 6.

All compounds derived from LOX were affected by storage. Considering that fatty acid levels in olive oil decrease over time due to oxidation, it is important to evaluate the compounds formed by the main transformation pathway, the LOX pathway.

As observed in Figure 6, the relative percentage of compounds derived from α-linolenic acid was higher than that of compounds from linoleic acid for both samples, consistent with other published studies [61,62].

In the monovarietal OO Arbequina, the *E*-2-hexenal/*E*-2-hexenol pathway stands out, which is associated with the predominance of Z-3-hexenal isomerization. On the other hand, in the Community sample, the Z-3-hexenol/Z-3-hexenyl acetate pathway prevails, which may be related to a low level of isomerase and a high level of alcohol dehydrogenase (ADH) [63,64].

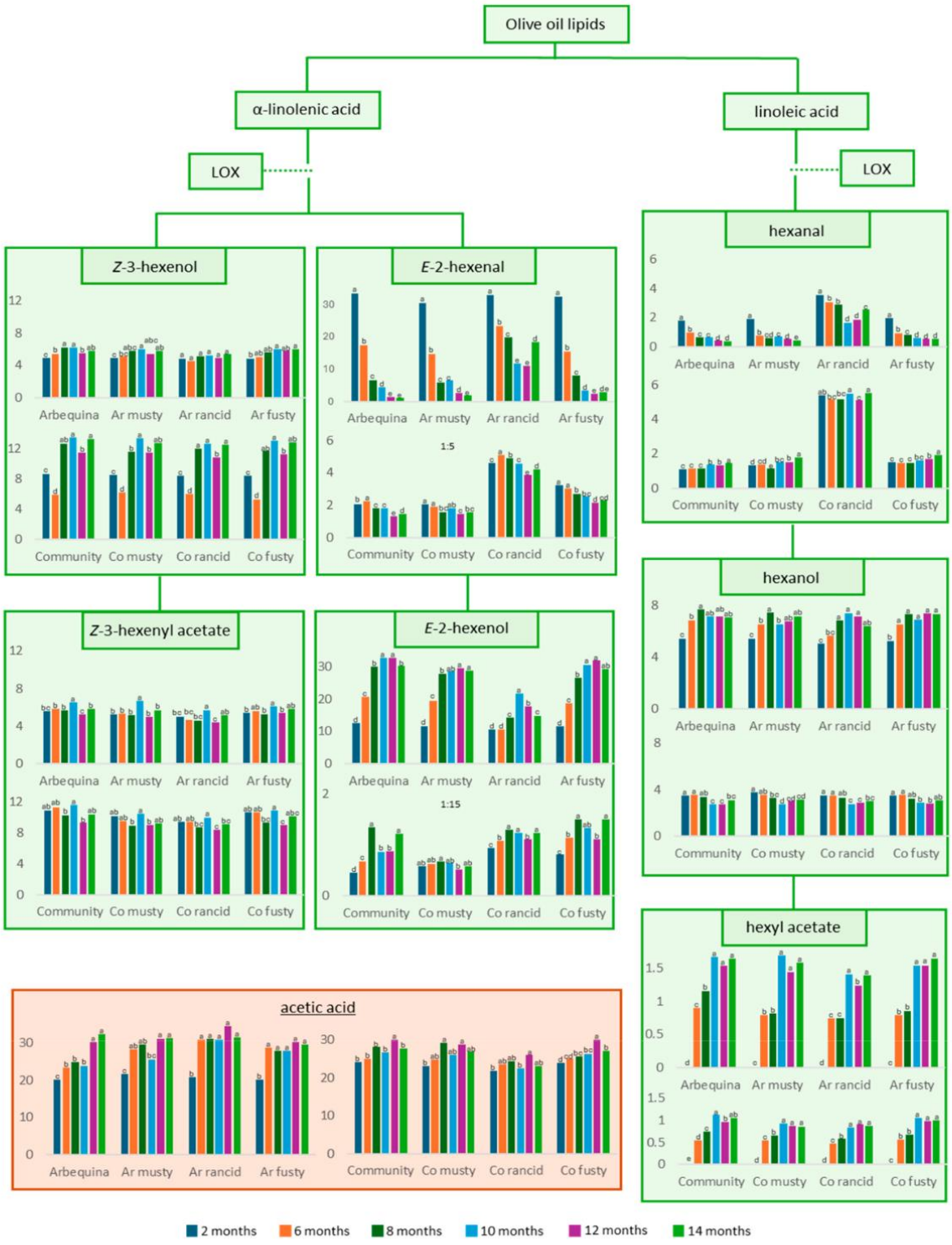

Figure 6. Evolution of compounds derived from the LOX pathway for the Arbequina and Community varieties with and without the addition of defects over 14 months. For each dataset, different letters above the bars indicate significant differences ($p < 0.05$) between time points.

Starting with compounds derived from linoleic acid, we observed an increasing trend in hexyl acetate resulting from the transformation of hexanal into hexanol.

On the other hand, compounds derived from α-linolenic acid, such as Z-3-hexen-1-ol and Z-3-hexenyl acetate, maintained a very similar relative percentage among themselves, both within the Arbequina and Community samples, making it difficult to discern a clear trend.

As for *E*-2-hexenal, one of the main VOCs originating from LOX and also a product of α-linolenic acid, it shows a clear decrease over time in both the Arbequina and Community samples. This is likely due to its conversion by ADH into *E*-2-hexenol, a compound that exhibits an increase over time for both samples.

The disqualification of EVOOs by the sensory panel is largely due to a decrease in *E*-2-hexenal, which is responsible for positive fruity and bitter notes [53,56]. With this decreasing trend, negative attributes become more pronounced, leading to the disqualification of the samples.

Another compound, not belonging to the LOX pathway but highlighted by its quantity in the olive oil, is acetic acid, which tends to increase over time.

With such an extensive universe of VOCs and numerous chemical reactions occurring simultaneously, including the LOX pathway, possible alcoholic and butyric fermentations of sugars, amino acid conversions, autooxidations, and homolytic cleavages of hydroperoxides, among others, principal component analysis (PCA) was applied in an attempt to differentiate between the Arbequina and Community samples classified as EVOO and the samples disqualified by the sensory panel (Figures 7 and 8).

PCA was performed using the means of the relative percentages of the 30 VOCs that showed statistically significant differences (Table 3) between the samples with and without the addition of disqualified olive oil and time.

For the Arbequina variety, in Figure 7, it can be observed that the first and second components (PC1 and PC2) explained about 99% of the total variance of the system. There is a clear separation of samples over time, primarily discriminated along PC1, with emphasis on the compounds *E*-2-hexenal, acetic acid, and *E*-2-hexenol. This suggests that acetic acid and *E*-2-hexenol are the compounds responsible for the samples disqualified (highlighted in red) by the sensory panel, while *E*-2-hexenal is the compound responsible for maintaining the samples as extra virgin.

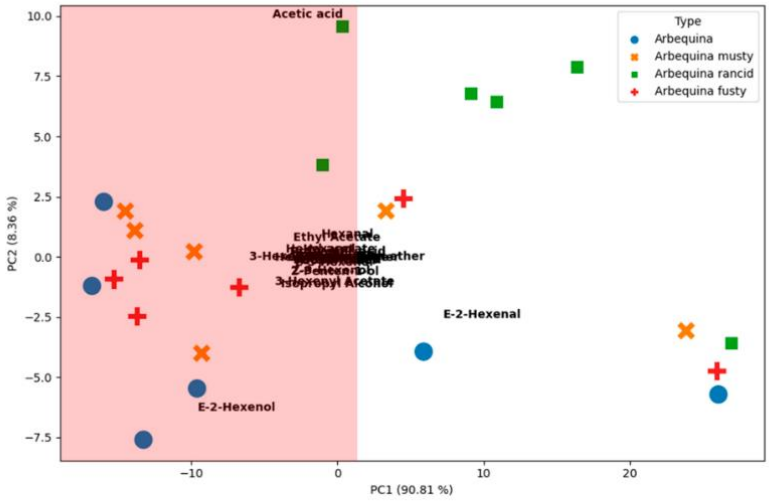

Figure 7. PCA biplot of VOCs selected by statistical analysis for the Arbequina olive oil, with and without added defects, over 14 months. In red shade are the samples disqualified by the sensory panel. The loadings (compounds) were scaled by a factor of 12.3 for legibility.

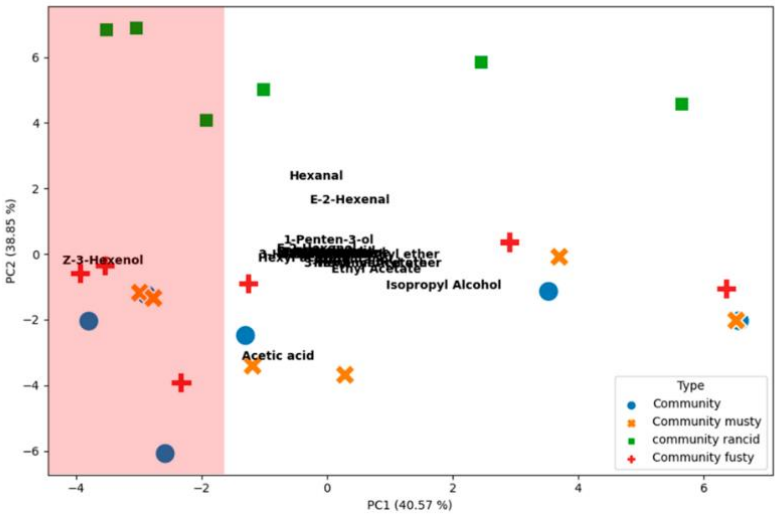

Figure 8. PCA biplot of VOCs selected by statistical analysis for the Community olive oil, with and without added defects, over 14 months. In red shade are the samples disqualified by the sensory panel. The loadings (compounds) were scaled by a factor of 4.5 for legibility.

For the Community variety, in Figure 8, PC1 and PC2 explained about 80% of the total variance of the system, and it is the compounds *E*-2-hexenal, hexanal, acetic acid, and *Z*-3-hexenol that distinguished the EVOO samples from the disqualified samples along PC1. Acetic acid and *Z*-3-hexenol are the compounds responsible for the disqualified samples (highlighted in red).

Given that *E*-2-hexenol is derived from *E*-2-hexenal, the predominant compound in Arbequina EVOO, it is normal for *E*-2-hexenol to increase over time as *E*-2-hexenal decreases. The same applies to *Z*-3-hexenol, which originates from the LOX pathway and is significantly present in Community EVOO.

Thus, considering that *E*-2-hexenal is one of the compounds influencing the PCAs of both samples and has been described as responsible for positive attributes, it was found to be a potential marker for the early detection of oxidation onset and future disqualification by the sensory panel when its relative percentage decreases in the olive oil. Conversely, the same reasoning applies to acetic acid, a compound responsible for negative attributes, which characterizes disqualified samples by increasing its relative percentage, as observed along PC1 for both Arbequina and Community varieties.

With this in mind, an attempt was made to establish a correlation between these two compounds to predict the level of oxidation, supporting the sensory panel. By predicting through a ratio between both compounds, it may be possible to anticipate when different olive oil varieties will become disqualified while remaining on the shelf throughout their shelf life.

3.3. Monitoring the Ratio of E-2-Hexenal to Acetic Acid

The ratio of *E*-2-hexenal to acetic acid was calculated for the new Arbequina variety with the addition of olive oil disqualified by the sensory panel and with evident defects, plotting the ratio over its shelf life, as shown in Figure 9.

As observed in Figure 9, as the percentage of disqualified EVOO added increases, the ratio of *E*-2-hexenal to acetic acid decreases. When the ratio value was equal to 3.2, the sensory panel unanimously disqualified the blends in which the ratio was below this value.

The ratio values for different EVOOs may vary slightly, as they depend heavily on the variety of olive oil used, as well as its composition in *E*-2-hexenal.

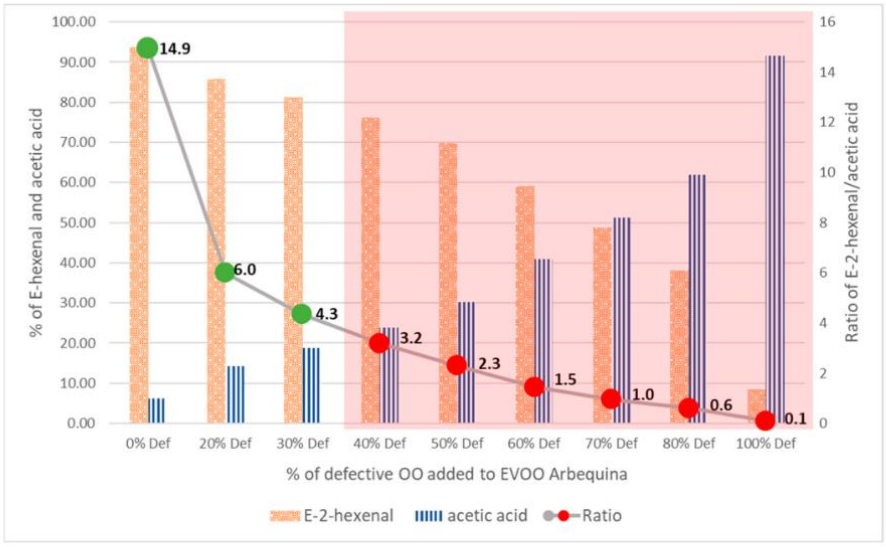

Figure 9. Variation in the *E*-2-hexenal/acetic acid ratio depending on the degree of mixing of EVOO Arbequina with declassified olive oil. Data on the values of the respective ratios are indicated. Mixtures that were disqualified by the sensory panel are red shaded.

3.4. Confirmation of the E-2-Hexenal/Acetic Acid Ratio

After estimating the ratio value for the sensory disqualification of the Arbequina monovariety, the same was applied to other varieties, as shown in Figure 10.

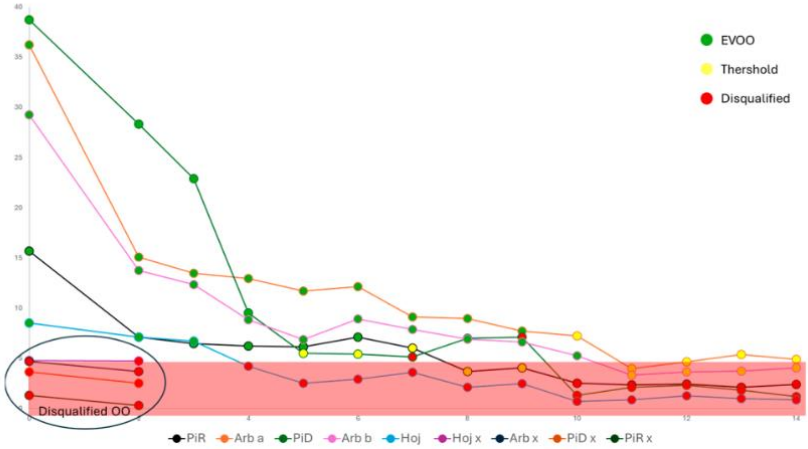

Figure 10. Variation in the *E*-2-hexenal/acetic acid ratio over time for different EVOO varieties. Samples disqualified by the sensory panel are represented by a red marker, and by a yellow marker when on the threshold of disqualification. The red shaded area represents the ratio below 5.

For the confirmation of the *E*-2-hexenal/acetic acid ratio deemed relevant for estimating EVOO shelf life, three different varieties of extra virgin olive oils (Irrigation Picual EVOO and Dryland EVOO, and two other Arbequina EVOOs) were used, as well as the same oils already disqualified by the panel, represented in Figure 5. The olive oils already disqualified by the panel showed a *E*-2-hexenal/acetic acid ratio value below 5 at the beginning of the study, so they were not further evaluated.

Although, as shown in Figure 9, the disqualification threshold for arbequina with mixed disqualified olive oil was 4.3, the later study that employed more varieties over

14 months showed a threshold of around 5, as can be seen in Figure 10. Thus, we were able to confirm this ratio of <5 for disqualified OO. Only Dryland Picual was disqualified by the sensory panel while still having a ratio slightly above 5.

However, it is necessary to always consider the variety of olive oil and the initial value of *E*-2-hexenal. Thus, one may consider that the higher the value of *E*-2-hexenal and the higher the ratio of *E*-2-hexenal to acetic acid, the greater the durability of the resulting mixture from a sensory standpoint.

It was not possible to establish a ratio value for the Community EVOO used in this study, as this EVOO had a very small amount of *E*-2-hexenal in its composition. The Community EVOO used came from local producers, and the variety or blend of varieties used is unknown. Therefore, it is necessary to find other compounds that can be correlated with each other for EVOOs which have a lower content of *E*-2-hexenal. Compounds derived from the LOX pathway, such as *Z*-3-hexenol and *Z*-3-hexenyl acetate may serve this purpose.

4. Conclusions

In this study, a sensory and analytical analysis over time was conducted on a monovarietal Arbequina EVOO and a Community EVOO with and without the addition of olive oil disqualified by the sensory panel due to evident defects such as mustiness, rancidity, and fustiness. A method using HS-SPME-GC/MS was established, allowing for the tentative identification of approximately 80 volatile organic compounds in these samples. Although the profiles of the two EVOOs were markedly different, the majority of compounds occurred in both oils.

The analysis of volatile profiles enabled the study of the impact of time and oxidation. Analysis of variance identified 30 compounds with significant differences between the respective samples and time, revealing that the evolution was primarily due to VOCs derived from the LOX pathway.

Through PCA, it was possible to differentiate the samples classified as EVOO from those disqualified by the sensory panel. The evolution of EVOOs over time was mainly attributed to compounds such as *E*-2-hexenal, *E*-2-hexenol, *Z*-3-hexenol, and acetic acid.

Compounds like *E*-2-hexenal and acetic acid were suggested as potential markers for early identification of the shelf life of an EVOO. A ratio between these two compounds was established and monitored over time for different olive oil varieties. The ratio of *E*-2-hexenal/acetic acid appears to be a good indicator of shelf life. Ratios lower than 5 indicate the possible disqualification of an monovarietal EVOO by the sensory panel.

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3

ANTIOXIDANTS

3.1 Olive Oil as a Nutraceutical Product

Since ancient times, products derived from the olive tree have been esteemed for their remarkable nutritional and therapeutic benefits. As early as 400 B.C., Hippocrates, regarded as the father of Western medicine, recommended the use of fresh olive juice to treat mental illnesses and applied poultices of crushed olives to heal ulcers. These products have traditionally been used to treat skin infections due to their emollient and healing properties, combat colds, herpes, and infections of the digestive and urinary tracts thanks to their antimicrobial properties, and address ulcers, stomach pains, and liver issues due to their anti-inflammatory effects [3,214]. Furthermore, their potent antioxidant and anti-inflammatory actions make them effective in preventing and treating chronic diseases such as cardiovascular conditions, cancer, and neurodegenerative disorders, where oxidative and inflammatory processes play a critical role [215–218].

The first investigation into the potential nutraceutical properties of olive oil was conducted by American biologist and physiologist Ancel Keys. In 1970, he introduced a comparative dietary study across seven countries (the United States, Italy, the Netherlands, Greece, Finland, Japan, and the former Yugoslavia), known as the "Seven Countries Study." This research provided compelling evidence that diets high in saturated fats increase the risk of coronary heart disease. The study found that the inhabitants of Crete had the lowest cardiovascular mortality rates, attributed to their predominant use of olive oil in cooking and seasoning,

contrasting with countries like Finland, where diets relied heavily on saturated fats such as butter and lard [219,220].

Subsequent, several studies suggest that these therapeutic properties of olive oil are largely attributed to its phenolic compounds [67,215,217,218,221–223].

3.2 Phenolic Compounds

Phenolic compounds are secondary metabolites synthesized by plants, chemically characterized by having an aromatic ring bonded to one or more hydroxyl groups. These compounds can also be defined based on their metabolic origin, as they are derived from the shikimate pathway, responsible for producing aromatic amino acids like phenylalanine and tyrosine, and from the metabolism of phenylpropanoids, which use phenylalanine as a precursor for synthesizing a wide range of phenolic compounds (Figure 7) [224]. These metabolites play essential roles in plants, including defense against predators and pathogens, protection against oxidative stress, and regulation of growth and developmental processes. The presence and diversity of phenolic compounds in plants reflect their importance in adapting to environmental conditions and interacting with their surroundings [222,225,226].

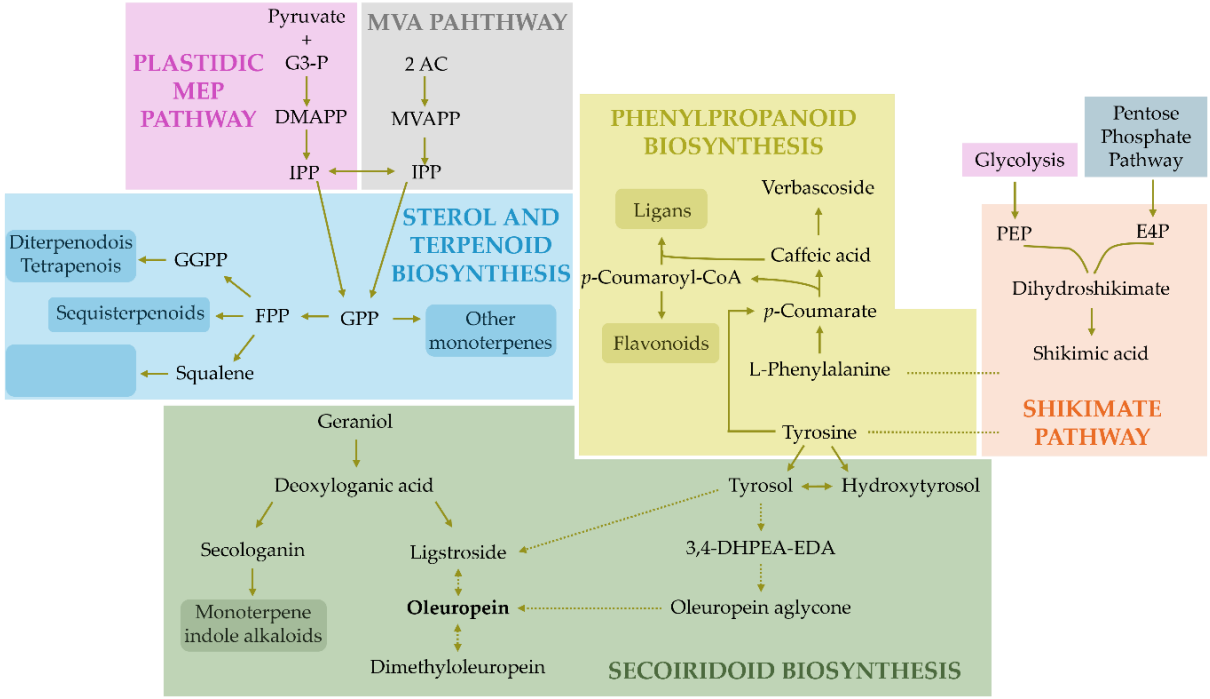

Figure 7. Schematic illustration depicting the biosynthetic pathways of phenolic compounds in olive fruits. G3-P: Glyceraldehyde 3-phosphate; DMAPP: Dimethylallyl diphosphate; IPP: Isopentenyl diphosphate; AC: Acetyl-CoA; MVAPP: Mevalonate diphosphate; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; GGPP: Geranylgeranyl

pyrophosphate; PEP: Phosphoenolpyruvate; E4P: Erythrose 4-phosphate; 3,4-DHPEA-EDA: Oleacein. Adapted [227,228]

Phenolic compounds can be classified into different groups based on the number of phenolic rings they possess and the molecular bonds present. Among the predominant phenolic compounds in olives are secoiridoids (such as oleuropein and ligstroside derivatives), phenolic alcohols (hydroxytyrosol - HTyr and tyrosol - Tyr), phenolic acids (*p*-coumaric acid and vanillic acid), flavonoids (luteolin and apigenin), and lignans (pinoresinol and acetoxypinoresinol) [225].

A study by Alagna *et al.* analyzed the metabolic and transcriptional profiles of phenolic compounds during olive maturation, suggesting the main biosynthetic pathways of these molecules [228]. These metabolic pathways are highly complex, and the production of phenolic compounds can vary significantly in response to environmental stimuli. In general, these pathways are interconnected, allowing their conversion during fruit maturation, processing, and storage. Typically, these metabolic pathways lead to the hydrolysis of precursor compounds, resulting in the formation of HTyr or Tyr [224,225,229].

During olive oil production, some phenolic compounds remain unchanged, while others undergo transformations due to chemical and enzymatic reactions. These processes largely depend on the nature of the compounds, the ripeness of the fruits, and processing conditions. Consequently, the profile and concentrations of phenolic compounds in olive oil vary widely [224,230].

Among the phenolic compounds found in olives, oleuropein, belonging to the hydroxytyrosol family, is the most abundant. This compound is linked to a sugar molecule (glucose) and reaches high concentrations during fruit development [231,232]. Ligstroside, its structural equivalent containing a tyrosol unit, is also significant. In virgin olive oil, the aglycones of oleuropein and ligstroside (compounds that have lost their sugar molecules) and their derivatives, mainly in oxidized forms, are present in higher quantities alongside their hydrolysis products, HTyr and Tyr [224].

During ripening, hydrolytic enzymatic activity reduces oleuropein and ligstroside concentrations while increasing hydrolysis products. Similar changes can occur during olive oil storage, resulting in higher concentrations of HTyr and Tyr due to hydrolysis of bound portions [224,229].

In virgin olive oil, phenols not only provide high oxidative stability, extending the product's shelf life and quality, but also directly influence its organoleptic properties, imparting characteristic flavors and aromas while contributing to nutritional benefits [108,224,233].

Unlike lipophilic phenols, found in various oils and fats, hydrophilic phenols are specific to virgin olive oil due to the unique characteristics of olives and the preservation of these compounds during minimal extraction processes. In refined oils, these molecules are significantly reduced during the refining process [234].

The importance of virgin olive oil's phenolic compounds for human health has garnered significant interest, particularly for their association with protective effects against cardiovascular and neurodegenerative diseases. These benefits are linked to the ability of phenols to act as antioxidants, neutralizing free radicals and preventing oxidative damage to cells [71,223,234,235].

3.2.1 Hydroxytyrosol and Tyrosol

Among the phenolic compounds in olive oil, HTyr and Tyr are the most widely recognized for their health benefits and play an essential role in virgin olive oil. These substances are highly valued for their antioxidant and anti-inflammatory properties, which not only contribute to olive oil's stability but also promote beneficial effects on human health [68,71,218,236].

Hydroxytyrosol is a chemical compound with the IUPAC name 4-(2-hydroxyethyl)-1,2-benzenediol. This alcohol is characteristically found in olives and other derivatives of the olive tree, either in free form or combined with various other natural compounds. Similarly, tyrosol or 4-(2-hydroxyethyl) phenol is another phenolic compound present in olives and their derivatives. It has a chemical structure similar to hydroxytyrosol but lacks one hydroxyl group (OH) on the aromatic ring. (Figure 8) [237].

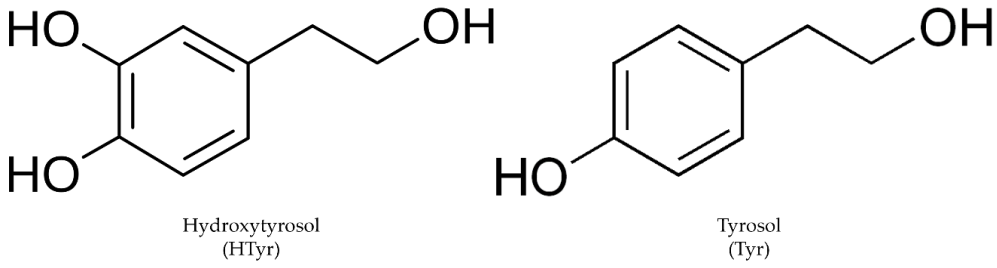

Figure 8. Chemical structures of hydroxytyrosol and tyrosol.

After ingestion, HTyr and Tyr are absorbed in the gastrointestinal tract and rapidly distributed throughout the body, being detected in urine, plasma, and low-density proteins (LDL) particles, which highlights their high bioavailability. This pharmacokinetic profile demonstrates that, despite being rapidly metabolized, these compounds can exert significant beneficial effects before excretion [236,238–241].

During digestion, precursor molecules such as oleuropein, oleuropein aglycone, oleacein, ligstroside, and oleocanthal are hydrolyzed, generating HTyr and Tyr as the main active metabolites. Their metabolism predominantly involves conjugation with sulfates and glucuronides, making them more water-soluble and facilitating renal excretion [236,238–241].

Several studies have reported their pharmacological properties [236,242]. HTyr stands out for its strong antioxidant potential, derived from the presence of two ortho-hydroxyl groups on the benzene ring, which confer a high capacity to neutralize reactive oxygen species (ROS). This antioxidant activity protects cellular components such as lipid membranes, proteins, and DNA against oxidative damage. Although less potent, Tyr also acts as an antioxidant due to the hydroxyl group present on its aromatic ring, complementing HTyr's action. Furthermore, both compounds exhibit significant anti-inflammatory effects, modulating intracellular signaling pathways that regulate the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α, and inhibiting the activity of the cyclooxygenase-2 (COX-2) enzyme. These characteristics are essential for preventing chronic diseases such as cardiovascular diseases, neurodegenerative disorders, type 2 diabetes, and cancer [236,238,243].

The consumption of HTyr and Tyr is strongly associated with the prevention of cardiovascular diseases, as they protect LDL particles from oxidation, a key process in the formation of atherosclerotic plaques. Additionally, their anti-inflammatory actions help reduce the risk of atherosclerosis [236,238,244].

In the context of neurodegenerative diseases, HTyr demonstrates the ability to cross the blood-brain barrier, protecting neurons from oxidative and inflammatory damage, making it promising for preventing conditions such as Alzheimer's and Parkinson's diseases [223,245]. Regarding type 2 diabetes, both compounds help modulate oxidative stress and inflammation while improving insulin sensitivity [246]. In the case of cancer, HTyr and Tyr contribute to suppressing tumor cell proliferation and reducing DNA damage caused by oxidizing agents [247,248].

Moreover, studies indicate that these phenols have an excellent safety profile, even at high doses, and are rapidly eliminated from the body, minimizing toxicity risks. The absence of bioaccumulation further reinforces their applicability in antioxidant therapies and the development of nutraceuticals. Including these compounds in the regular consumption of virgin olive oil, a key component of the Mediterranean diet, underscores their relevance to human health and their contribution to preventing various pathologies [236,249].

Based on extensive scientific evidence, the European Food Safety Authority (EFSA) has recognized the benefits of phenolic compounds derived from olives, including the fruit,

processing wastewater, olive oil, *Olea europaea L.* extracts, and leaves. These compounds, standardized by their hydroxytyrosol content and its derivatives (including the oleuropein complex), have been adequately characterized and associated with numerous positive health effects. These benefits include protecting LDL particles from oxidative damage, maintaining normal high-density lipoprotein (HDL) cholesterol levels in the blood, regulating blood pressure, anti-inflammatory properties, promoting upper respiratory tract health, supporting normal gastrointestinal function, and strengthening the body's defenses against external agents [150].

However, according to European Union Regulation 432/2012, for extra virgin olive oil to be marketed with nutritional or health claims related to these effects, it must contain at least 5 mg of hydroxytyrosol and its derivatives (such as the oleuropein complex and tyrosol) per 20 g of olive oil. Additionally, the legislation requires that the information provided to consumers highlights that the beneficial effects associated with consuming olive oil polyphenols are only obtained with a daily intake of 20 g of olive oil [151].

This requirement standardizes the minimum amount of these bioactive compounds and establishes clear guidelines for labeling, ensuring that the product offers sufficient concentrations to provide the claimed benefits. Thus, extra virgin olive oil meeting this criterion not only contributes to health promotion within the Mediterranean diet but also reinforces its status as a nutraceutical, enhancing its nutritional and functional value.

While olive oil phenols, such as hydroxytyrosol and tyrosol, are widely recognized for their health benefits, their concentrations in commercial oils are highly variable. Studies conducted on oils from different Portuguese olive varieties, indicated HTyr and Tyr concentrations (the sum of both) ranging from 2,027 mg to 10,973 mg per 20 g of oil (Table 4) [250].

Table 4. Concentration of Hydroxytyrosol (HTyr) and Tyrosol (Tyr) in Different Olive Oil Portuguese Cultivars. Vaues are expressed as mean ± standard deviation (SD) per 20 g of extra virgin olive oil (EVOO) [250].

Cultivar	HTyr + Tyr (mg/20 g EVOO)
Arbequina	2.027 ± 0.639
Picual	7.105 ± 2.413
Madural	2.255 ± 0.861
Cordovil de Serpa	4.847 ± 0.567
Cobrançosa	7.936 ± 3.767
Verdeal Alentejana	7.014 ± 1.664
Carrasquenha	1.787 ± 0.533
Blanqueta	10.973 ± 4.425
Galega Vulgar	3.520 ± 1.719

This variability reflects inherent differences in olive varieties, geographical origins, and factors such as processing and storage conditions [251,252].

It is estimated, however, that olive oil contains only about 1–2% of the total phenolic fraction available in the olive [253,254]. During processing, phenolic compounds are distributed among the aqueous, oily, and solid phases (pomace), with this distribution influenced by their specific solubilities [255].

While a small fraction is incorporated into the oily phase, about 53% of phenolic compounds are lost in wastewater, and approximately 45% remain in pomace, the solid fraction generated during processing. This composition highlights the limitations in the amount of phenolic compounds that can naturally transfer to olive oil, with concentrations ranging from 50 to 1000 μg/g in oil, depending on the olive variety and extraction conditions [255,256].

Since most phenols remain in the by-products, these materials represent rich and underutilized sources of bioactive compounds such as hydroxytyrosol, tyrosol, and other antioxidants. Studies indicate that pomace may contain hydroxytyrosol and tyrosol concentrations 10 to 100 times higher than those found in olive oil [257]. This has sparked significant interest in developing strategies for recovering, separating, purifying, and concentrating these compounds from pomace and other olive processing by-products, aiming to both enrich olive oil and create new products with high functional value.

In addition to the economic value added to the olive oil sector, valorizing pomace and wastewater promotes sustainability by transforming industrial waste into sources of functional ingredients with applications in the food, pharmaceutical, and cosmetic industries. These initiatives increase the market presence of phenolic compounds and reinforce the relevance of olive oil and its derivatives in promoting health, while contributing to developing nutraceutical products and innovative solutions for utilizing by-products.

3.3 Olive Pomace

As previously mentioned, olive pomace, generated during olive oil extraction, is one of the most significant by-products of this industry.

Currently, there are three main olive oil extraction systems: the classical system (presses) and continuous systems, which can operate in two or three phases (Figure 9) [258,259].

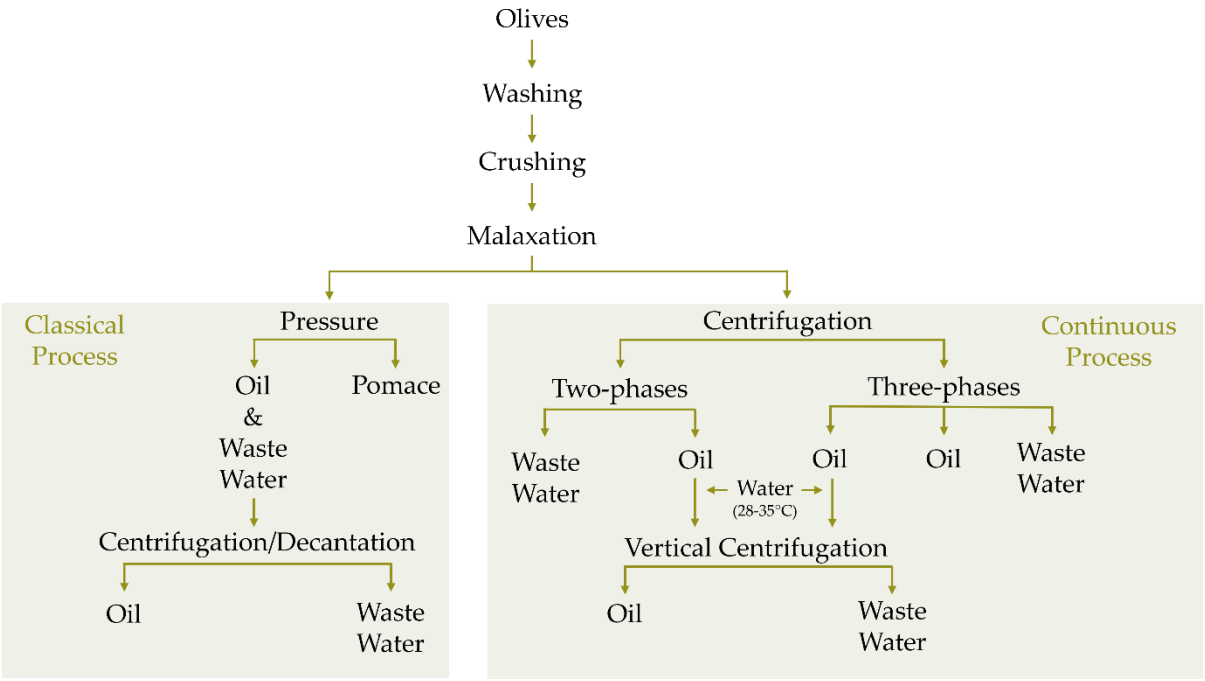

Figure 9. Classical and continuous process used for olive oil extraction [258,259].

The three-phase system, despite being technologically advanced, uses large amounts of water, resulting in higher volumes of wastewater and reduced phenolic content in the olive oil. On the other hand, the two-phase system was developed as an alternative to reduce water consumption and liquid waste volumes, such as olive mill wastewater. This system does not add water to the process but generates olive pomace with a high moisture content [258–260]. For two-phase systems, approximately 80 kg of wet pomace is produced for every 20 kg of olive oil [261].

In general, it is estimated that the production of pomace is approximately four times higher than that of olive oil. In Portugal, during the 2022/2023 season alone, the production of 126 thousand tons of olive oil resulted in approximately 500 thousand tons of olive pomace [28]. On a global scale, the olive oil industry is estimated to produce about 30 million m^3 of waste annually [262]. This significant waste generation, including olive mill wastewater and pomace, poses substantial environmental challenges, with pollution loads estimated to be 100 to 200 times higher than those of domestic sewage [263].

Olive pomace consists of water, solid residues from the olives, such as pulp, peel, and fragments of pits, along with a significant proportion of phenolic compounds. Olive mill wastewater, typically an acidic effluent containing various dissolved and suspended substances, consists of 83-94% water, 4-18% organic matter (including lipids, sugars, organic acids,

nitrogenous substances, polyalcohols, pectins, tannins, and polyphenols), and 0.4-2.5% mineral salts, primarily potassium, sodium, carbonates, and phosphates [258,264].

The improper disposal of these wastes in the environment leads to serious ecological consequences. Soil pollution caused by phenolic compounds and fatty acids inhibits plant growth. Water contamination resulting from discharge into water bodies can lead to eutrophication due to the high concentration of phosphorus and organic matter, promoting excessive algae growth and reducing the dissolved oxygen levels necessary for aquatic life. Additionally, waste degradation generates unpleasant odors, while phytotoxicity and low biodegradability, caused by the high presence of phenolic compounds, make treatment and proper reuse challenging [265,266].

Various approaches have been proposed to mitigate the environmental impacts of olive oil industry waste [258,267–269].

Physical processes are commonly used as a pretreatment step to remove suspended solids from olive mill wastewater. Thermal processes, effective for water removal and condensation of residues, have high operational costs. Advanced oxidation processes, while efficient in pollutant reduction, are similarly expensive. Physicochemical methods, such as neutralization, adsorption, and precipitation, offer more economical alternatives, though their effectiveness in reducing pollutant loads is limited. A more practical and economical solution for efficient removal is the use of evaporation ponds or storage reservoirs. These involve low investment costs and near-zero operational costs. Under favorable climatic conditions, the complete evaporation of olive mill wastewater reduces the by-products to solid residues, which then require appropriate management. When olive mill wastewater is used for soil irrigation, employing a storage reservoir can significantly lower initial investment costs, although neutralizing the effluent increases operational expenses [258,267–269].

Olive pomace is also used as a raw material for producing pomace oil, a process that has advanced significantly in recent years. After its generation, the pomace undergoes drying to reduce its moisture content, an essential step for subsequent stages. The dried pomace is then physically processed into a granular mass suitable for oil extraction. Hexane, a solvent with a high capacity for dissolving the oil present in pomace, is used for this extraction. The final product of this process is pomace oil, which is sent to refineries, while the extracted pomace, after oil removal, is generally used as fuel in factories or sold. It can also be used as animal feed or fertilizer, among other applications [61].

Despite the technical and financial challenges associated with these treatments, seeking solutions that are both sustainable and economically viable is essential for efficient waste management.

Furthermore, reusing olive pomace and olive mill wastewater as sources of phenolic compounds and other bioactives represents a strategic opportunity. This approach allows environmental liabilities to be transformed into high-value-added products, with promising applications in the food, pharmaceutical, and cosmetic industries.

3.3.1 Extraction of Phenolic Compounds from Olive Pomace

The extraction of phenolic compounds, such as hydroxytyrosol and tyrosol, from olive oil subproducts presents a technical challenge due to their low concentration in the oily phase and their greater solubility in water. However, various methods have been developed to perform this extraction efficiently.

3.3.1.1 Solvent Extraction Methods

Solvent extraction methods, such as liquid-liquid extraction (LLE) and solid-liquid extraction, are widely used to obtain antioxidants from plant matrices like olive pomace. Commonly used solvents include ethanol, methanol, acetonitrile, or mixtures of water with methanol or ethanol. The process involves mixing the pomace with the solvent, followed by agitation and settling, allowing phenolic compounds to migrate into the solvent phase. After phase separation, the phenolics can be recovered by solvent evaporation or other suitable methods, ensuring the efficient extraction of these high value bioactives [270–275].

A study by Suárez *et al.* investigated optimizing phenolic extraction from olive by-products. The researchers suggested accelerated solvent extraction as a more efficient alternative to solid-liquid extraction using methanol/water (80:20 v/v) under atmospheric pressure, often preceded by ultrasonic or thermal treatments to enhance phenol solubility [274]. However, due to the restrictions of European Regulation 2009/32 on methanol use in food products, ethanol, considered safer, is increasingly used [276]. Studies like those by Lafka *et al.* have shown ethanol to be among the most suitable solvents for recovering phenolic compounds from olive mill wastewater (OMW), confirming the efficacy of supercritical CO_2 as an alternative for extracting highly antioxidant phenolics [275].

Bouaziz *et al.* highlighted the successful use of an ethanol-water mixture (70:30 v/v) to extract polyphenols from olive leaves, showing the potential of food-grade solvents for applications in the food industry [277]. Ethyl acetate, widely studied, has proven effective in

recovering phenolics after aerobic or anaerobic digestion, achieving recovery rates above 90% [278,279]. Kalogerakis *et al.* investigated the application of different solvents, including ethyl acetate, diethyl ether, and a combination of chloroform and isopropanol, for the recovery of antioxidants from OMW. Among the solvents analyzed, ethyl acetate once again stood out for its high antioxidant recovery rates combined with lower environmental impacts. The analysis further indicated that optimizing the process could reduce environmental impacts by up to 29%, reinforcing the feasibility of this approach as a more sustainable alternative for extracting antioxidants from OMW [278]. Another "greener" alternative to conventional solvents for liquid-liquid extraction is the use of ionic liquids, with extraction efficiencies above 90% for HTyr and TYyr [280].

Despite its broad application, solvent extraction faces challenges such as the use of large solvent volumes, safety concerns, and environmental impacts. While olive pomace, being semisolid, requires solvent extraction, technical alternatives like membrane filtration are viable for recovering phenolics from liquid by-products such as OMW, thereby avoiding the use of solvents during the extraction and purification of phenolic compounds. [275,281,282]

3.3.1.2 Pressurized Liquid Extraction

Pressurized Liquid Extraction (PLE) has been employed to recover phenolic compounds from olive washing water. This process uses organic solvents at high temperatures and pressures and can combine static and dynamic conditions [271]. Acetonitrile, methanol, ethanol, ethyl acetate, and water are commonly used solvents. Methanol/water and ethanol/water mixtures are regarded as the most effective solvents for extracting phenolic compounds from OMW [270,274]. Compared to traditional extraction techniques, PLE offers advantages such as faster processing and reduced solvent volumes.

3.3.1.3 Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) has also been applied to optimize the extraction of phenolic compounds. This method uses microwave radiation to heat the material uniformly, facilitating the release of phenolic compounds into the solvent, resulting in time and solvent savings, in addition to improving process efficiency [283–285].

3.3.1.4 Ultrasound-Assisted Extraction

Ultrasound-assisted extraction (UAE) is an efficient and economically viable technique that does not require sophisticated equipment, making it an attractive option for small and

medium-sized industries. Ultrasound is used to enhance the efficiency of the process by facilitating cell rupture and promoting greater release of phenolic compounds. Among the main advantages of UAE are a significant reduction in extraction time and solvent consumption. The process is similar to liquid-liquid extraction (LLE), but it includes the application of ultrasonic waves to the olive oil, accelerating the transfer of phenolic compounds to the solvent [286–288].

A study by Jerman Klen *et al.* compared five extraction methods for phenolic compounds from OMW, including filtration, SPE, liquid-liquid extraction, and ultrasound-assisted extraction, with and without lyophilization. The findings revealed that UAE produced the highest yields in total and individual phenols, making it a promising alternative to conventional solvent-based methods [289].

3.3.1.5 Microorganism-Assisted Extraction

Microorganism-assisted extraction is an innovative approach where certain microorganisms are used to hydrolyze phenolic glycosides and release free phenolic compounds, such as hydroxytyrosol and tyrosol. This process can be performed using enzymes or fermentation, transforming complex phenols into simpler and more bioactive forms. Fungal enzymes, which are environmentally sustainable, are particularly effective in treating olive mill wastewater. Various enzymes, such as cellulase, pectinase, and hemicellulase, can be applied to hydrolyze the structural components of plant cell walls, increasing their permeability and thus allowing high yields in the extraction of phenolic compounds [290]. Fungal treatment provides excellent results in terms of yield, time efficiency, and process sustainability [291,292]. However, industrial-scale application is constrained due to the high cost of enzymes, and its reliance on environmental factors such as dissolved oxygen, temperature, and nutrient availability [292].

3.3.1.6 Supercritical Extraction

Supercritical extraction, especially with supercritical CO_2 combined with co-solvents like ethanol or methanol, has stood out as an efficient technique for extracting phenolic compounds. Supercritical CO_2 is an attractive alternative to traditional solvents, as operating above the critical point allows for better separation of bioactive compounds without the risks associated with flammable and toxic solvents [285,293]. Additionally, CO_2 is non-toxic, non-explosive, and widely recognized as safe, making it ideal for the food industry and other sectors requiring clean and safe processes. This method is particularly advantageous due to its environmental

sustainability and low toxicity, ensuring efficient extraction and minimizing oxidation phenomena that could compromise the final product's quality [294,295].

However, the main limitation of supercritical extraction lies in the high cost of the necessary equipment, as the process requires high pressures, making large-scale implementation more expensive. This high cost may outweigh the technical benefits of the technique, restricting its adoption in some industries, despite its clear advantages in terms of efficiency and sustainability [294,296].

3.3.1.7 Solid-Phase Extraction

Solid-phase extraction (SPE) is an efficient method for recovering phenolic compounds, in which the compounds are retained on adsorbent materials, such as modified silica or polymers, and subsequently eluted with appropriate solvents. This procedure is widely applied in the extraction of natural polyphenols, offering purer extracts, simplified protocols, shorter processing times, easier automation, and reduced costs compared to conventional methods [297–300]. Additionally, SPE can be effective in recovering more than 60% of the polyphenols from by-products like olive washing water, as shown in studies on the recovery of phenolic compounds from this source [299–301]. One example of successful industrial application was the use of reverse-phase solid-phase extraction (RP-SPE), enabling the recovery of roughly one gram of purified hydroxytyrosol per liter of OMW [299].

Although SPE is promising for small-scale extraction and analytical purposes, it has limitations when applied on a large scale, mainly due to the high cost of the process and potential solvent residue in the extract. This factor makes the method less viable for large industrial production volumes, although its benefits in terms of efficiency and operational simplicity continue to make SPE an attractive alternative in various scenarios [301].

3.3.1.8 Resin Adsorption Extraction

Resin adsorption extraction uses materials like polymeric resins to capture phenolic compounds directly from olive oil or by-products like olive mill wastewater and pomace. The process involves passing olive oil or extract through an adsorbent resin, where phenolic compounds adhere to the resin’s surface. After adsorption, the phenolic compounds can be eluted using appropriate solvents, such as ethanol [297,300–303].

Resin extraction offers significant advantages in terms of selectivity, allowing the recovery of specific phenols while providing high efficiency in recovering these compounds from olive oil by-products. This process is simple, effective, and relatively cheap, making it an

interesting alternative for industries seeking a more sustainable and cost-effective solution for extracting bioactive compounds [304].

The use of membranes for biophenol extraction from OMW is becoming increasingly popular due to its advantages over conventional methods. Membrane separation, which includes techniques such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), has gained attention for its benefits compared to solvent extraction [305].

The main advantage of this process is its high efficiency, which allows phenolic compounds to be separated based on their molecular weights, which is not possible with other methods. Membranes used, such as those for MF and UF, operate efficiently, requiring low energy consumption, with no need for additives, and providing easy scalability for industrial applications. These separation technologies are especially advantageous because they enable precise control over selectivity in recovering phenolic compounds from wastewater [305,306].

In practical application, several studies have demonstrated the effectiveness of using membranes for phenolic compound extraction from olive mill wastewater. Maurizio Servili *et al.* conducted an industrial application based on enzyme pre-treatment followed by a three-phase membrane system, resulting in a significant reduction in pollutant load and recovery of phenolic compounds [303]. Hamza *et al.* evaluated an ecologically friendly pilot-scale process combining enzymes such as β-glucosidase from Aspergillus niger with membrane filtration. This process was effective in recovering hydroxytyrosol, a valuable phenolic compound, free from chemicals. Microfiltration (MF) removed 72.12% of chemical oxygen demand (COD), and ultrafiltration (UF) increased hydroxytyrosol concentration to 7.2 g/L [307].

Additionally, Cassano *et al.* applied an integrated membrane system to recover low molecular weight phenolic compounds from olive oil wastewater, using a sequence of UF followed by NF. The reuse of olive mill water was an important feature of this study, highlighting the sustainability potential of the process [304]. On the other hand, D'Antuono *et al.* conducted a similar study, using membrane filtration to recover phenolic compounds from OMW from different olive cultivars. The process generated fractions with different phenol concentrations, with MF fractions containing 2.5–5.3 g/L phenols, while UF and NF fractions contained lower concentrations, ranging from 1.4–3.1 g/L and 0.4–1.6 g/L, respectively [308].

Garcia-Castello et al. also evaluated an integrated membrane system for the recovery, purification, and concentration of polyphenols from olive mill wastewater (OMW). The proposed system, which included microfiltration (MF), nanofiltration (NF), osmotic distillation (OD), and vacuum membrane distillation (VMD), successfully concentrated polyphenols in OMW. Microfiltration reduced TOC and suspended solids, recovering 78% of the initial polyphenol

content. Nanofiltration produced a polyphenol-rich solution, which was further concentrated by osmotic distillation, yielding approximately 0.5 g/L of polyphenols, primarily hydroxytyrosol [281].

Another relevant study by Zagklis *et al.* used reverse osmosis (RO) to concentrate phenolic compounds, followed by treatment with adsorption and desorption resins such as XAD4, XAD16, and XAD7HP, to obtain a concentration of 378 g/L in gallic acid equivalents from raw OMW with 2.64 g/L phenols. This process not only increased phenol concentration but also reduced the pollutant load, with the potential organic load (COD) being reduced by 97%. These applications demonstrate that the use of membrane systems, when combined with additional treatments such as adsorption, is an effective and promising approach for recovering phenolic compounds and other bioactive compounds from OMW [309].

These results reinforce the potential of membrane separation technologies as sustainable and efficient methods for treating and recovering valuable compounds from olive oil wastewater. The combined use of membrane filtration, enzymatic pre-treatments, and resin adsorption can not only increase yields of phenolic compounds but also reduce the environmental impact associated with the disposal of OMW, providing a promising alternative for reusing these by-products.

3.4 Considerations and Objectives

In summary, the use of by-products from the fruit and vegetable industry as sources of functional compounds has proven to be a promising area, with increasing interest in the application of these compounds in food. The use of phenolic extracts from by-products for the formulation of new food products with health benefits has stood out.

The recovery of these natural phenolic compounds is especially important due to their antioxidant properties, which help preserve quality and extend the shelf life of food products. This approach contributes to the creation of functional foods, transforming agro-industrial waste into valuable ingredients.

Several studies have investigated the extraction of these compounds from different parts of the olive, such as leaves, due to the higher efficiency in the process and the better yields obtained. While directly adding pure phenolic compounds like hydroxytyrosol is a viable option for food enrichment, this approach does not tackle the problem of olive industry waste and is more suited to laboratory research than large-scale industrial applications. The incorporation

of these compounds into food products has been explored in various applications, focusing on increasing the functional value of foods.

For example, the addition of phenolic extracts derived from olives to olive oils has proven to be an effective strategy to increase the antioxidant activity of these oils, which not only contributes to a significant extension of their shelf life but may also allow these oils to obtain a nutraceutical product label. As mentioned earlier, olive oil can contain between 2,027 mg to 10,973 mg of hydroxytyrosol and tyrosol per 20g of oil. However, they rarely reach the amount required by EFSA (5 mg per 20g of oil) to be officially recognized with health claims.

If it is possible to formulate an olive oil that meets this requirement, it could be marketed with nutritional and health claims recognized by EFSA. In this way, an olive oil formulated according to these criteria would not only follow EFSA guidelines but also meet the nutraceutical criteria, enhancing its nutritional, functional, and economic value while maintaining the extra-virgin oil classification by IOC.

For olive oil producers, the possibility of highlighting the specific health benefits of olive oil offers a strategic marketing advantage. The formulation and certification of the product add value, serving as an important differentiator and a powerful tool to promote olive oil, especially in the competitive olive oil market.

Thus, one of the main objectives of this dissertation was to extract phenolic compounds derived from olive pomace, in order to formulate an oil rich in hydroxytyrosol and tyrosol, 8 mg per 20g of oil, ensuring that it meets EFSA's guidelines until the end of the olive oil's shelf life (12-18 months). The process is summarized in Figure 10 and described in the document submitted with the patent application.

All analyses to monitor the levels of hydroxytyrosol and tyrosol in the concentrates obtained from olive pomace, as well as in the oils before and after the addition of the concentrates, were carried out according to the method previously described in the literature.

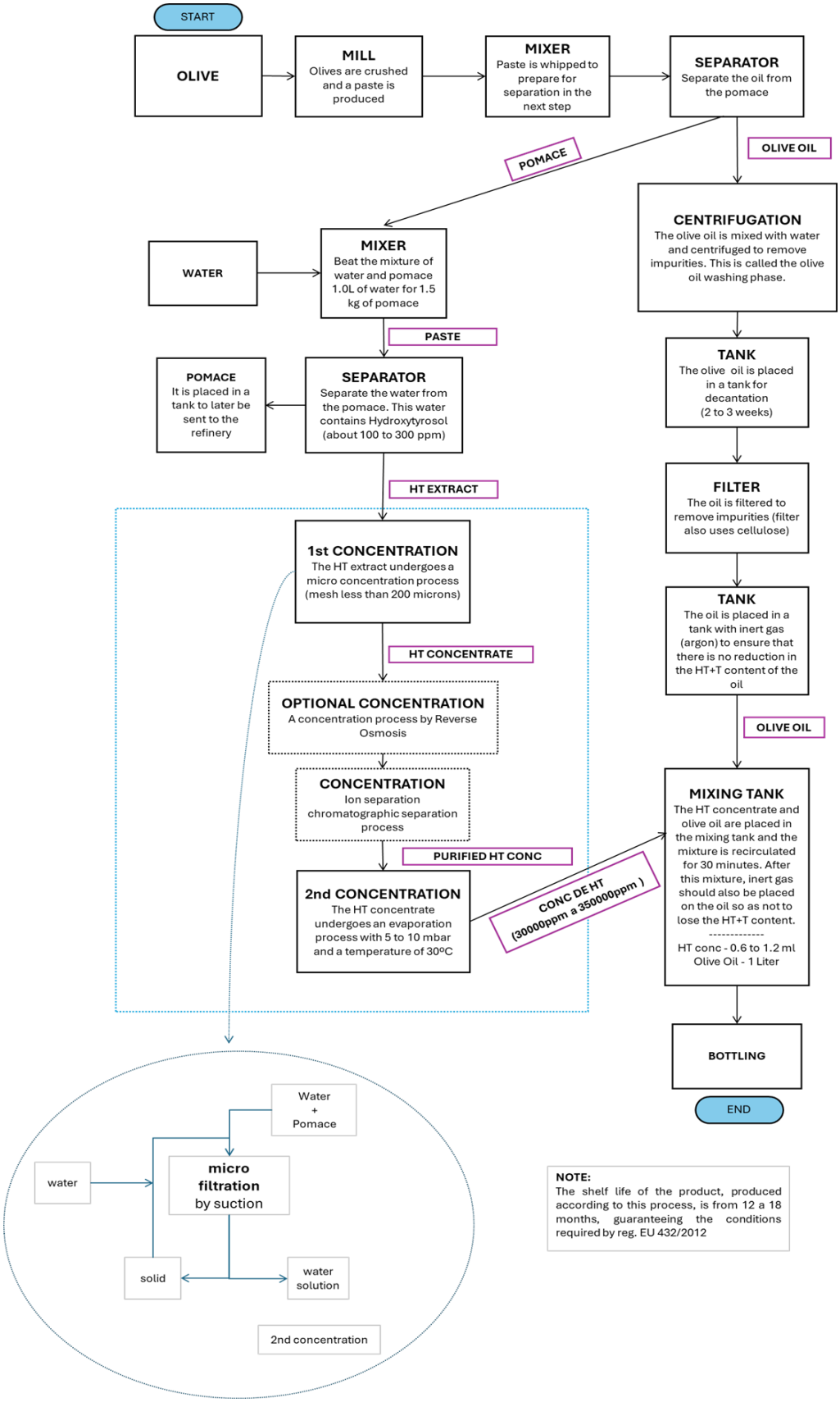

Figure 10. Scheme of the production process of olive oil with high antioxidant content.
HT: Antioxidants (Hydroxytyrosol and Tyrosol)

3.5 Patent

"Method for the Production of Olive Oil with a High Antioxidant Content and Antioxidant Concentrate"

Inventors: Luís Silva Pinto, Marco Gomes da Silva and Flávia Freitas

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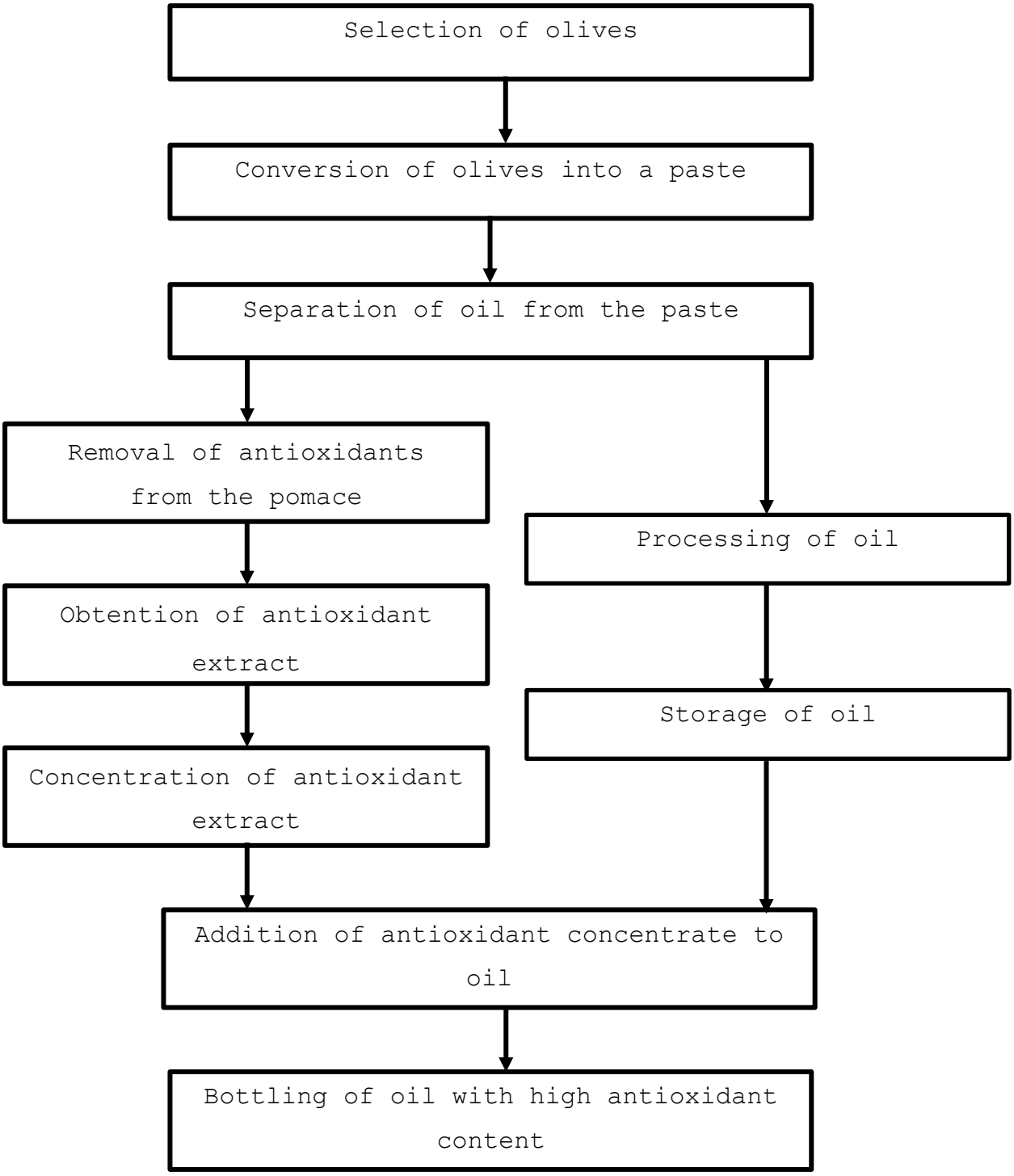
Selection of olives
Conversion of olives into a paste
Separation of oil from the paste
Removal of antioxidants from the pomace
Processing of oil
Obtention of antioxidant extract
Storage of oil
Concentration of antioxidant extract
Addition of antioxidant concentrate to oil
Bottling of oil with high antioxidant content

Figure 1

ABSTRACT

The present invention relates to a method of producing oil from olives, wherein the antioxidants of interest present in the olives can be practically used in full, without a substantial loss during the preparation of the oil, achieved by a first extraction of oil and a second extraction of the antioxidants present in the olives. The pomace, resulting from the grinding of the olives, is used to create a concentrate of antioxidants that is later added to, or mixed with, the oil to increase its antioxidant content. The preparation of the concentrate involves removing the antioxidants from the pomace by adding water to the pomace, forming a concentrated solution of antioxidants, this solution being evaporated under reduced pressure and at room temperature, so that there is no degradation of the antioxidants during the oil production method.

DESCRIPTION

METHOD FOR THE PRODUCTION OF OIL WITH A HIGH ANTIOXIDANT CONTENT AND ANTIOXIDANT CONCENTRATE

Technical Field of the Invention

The present invention falls within the field of agro-industrial production methods, more specifically the invention refers to a method of producing olive oil with a high antioxidant content from olives and an antioxidant concentrate.

Scope of Invention

In the Mediterranean tradition, olive oil is a widely used gastronomic component and therefore has a great economic importance in these countries. In addition to its taste, olive oil also has nutritious qualities that help the human body fight certain biochemical stresses, such as oxidative stress.

It is known that olives have a large number of antioxidants, both in number and value, namely oleuropein, tyrosol and hydroxytyrosol which, when ingested, help to mitigate the effects of oxidative species, toxic to humans.

However, although the olives possess these compounds, they often do not make it to the final product, *i.e.*, the olive oil, because during the olive oil production process, they are degraded, mainly due to the effects of high temperatures that degrade the antioxidant compounds, or because the process itself does not allow these compounds to reach the final product: olive oil. This happens because, after the olives are milled, there is a separation between the oil and the pomace (a by-product of semi-

solid or viscous consistency, formed by remains of pulp, olive skin or integument, crushed olive pit, water and olive oil), and a large percentage of the antioxidants, due to their hydrophilic character, remain in the pomace.

There are, however, mechanical, or physical methods that allow the extraction of antioxidants from the pomace, which are then returned to the oil in consequent processing.

That is precisely the goal of this invention, a method for extracting antioxidants from olives, obtaining a concentrate of antioxidants and then mixing them into olive oil.

State of the art

For the production of an olive oil that has higher levels of antioxidants in solution than those normally available in ready-to-eat olive oil, there are several solutions described in the state of the art.

The document EP1910257 describes a method of obtaining an antioxidant concentrate from olive pomace, the method comprising the extraction of antioxidants from olive pomace by means of biocompatible solvents, with the antioxidants being subsequently separated from the solvents by nanofiltration and reverse osmosis. This concentrate of antioxidants can later be added to olive oil.

The document WO2016087428 describes a method of fortifying cooking oils with hydroxytyrosol, wherein a concentrated solution with hydroxytyrosol is added to an edible oil, namely olive oil.

The document WO2018189730 describes an olive oil production method very similar to that of the present invention, a document that can be considered the closest state of the art. This method begins by grinding or crushing the olives, then separating the oil from the pomace by decantation. Thereafter, the olive oil is prepared by conventional means, while the pomace is used to create a concentrate of antioxidants. The water from the olives is removed from the pomace which has in its composition the antioxidants of the olive. This water is then concentrated by evaporation or reverse osmosis and is then added to the oil produced. At the end, the olive oil is decanted to remove excess water from the antioxidant concentrate.

Advantages of the Invention

The great advantage of this invention is that it allows to obtain a concentrate of antioxidants in quantities greater than those obtained by the methods referred to in the prior art documents. While in document WO 2018/189730 A1 the maximum amount of antioxidants obtained reached 56,000 ppm, the method of the present invention attained 300,000 ppm of total antioxidants. This advantage is achieved by changes made to the concentration step of the antioxidant concentrate, wherein an additional evaporation step is performed at a pressure of less than 10 mbar and at a temperature below 30 °C. This not only prevents the degradation of antioxidants at high temperatures, an event that occurs in the state of the art because evaporation occurs between 45°C and 75°C, but also allows to expend less energy during the concentration step.

Another major advantage of the olive oil production method of the present invention is based on the step of mixing the antioxidant concentrate into the oil. While in the state of the art

a large volume of the concentrate is mixed with the oil (6 mL per 1 L of oil), in the present invention a smaller quantity is mixed, equal to or less than 1.2 mL per 1 L of oil, this being the maximum amount of water dissolving in the oil because any higher amount that is placed in the oil separates from the oil, meaning that the final stage of decanting does not need to be carried out.

One of the major differences between the state of the art and the present invention lies in the stage commonly called "2nd extraction" or "2nd pass olive oil". This step, usually carried out for the sake of economic profitability, is carried out so that the olive oil that is still in the pomace can be used. In the present invention, this step is carried out so that the antioxidants that are present in the pomace can be extracted and used, allowing that in a subsequent step the antioxidants are mixed or joined to the oil.

Brief Description of the Drawings

These and other characteristics can be easily understood through the attached drawings, which should be considered as mere examples and not restrictive in any way to the scope of the invention. In the drawings, and for illustrative purposes, the measurements of some of the constituents may be exaggerated and not drawn to scale. The absolute dimensions and the relative dimensions do not correspond to the actual relations for the realization of the invention.

In a preferred embodiment:

Figure 1 shows a simplified diagram of the olive oil production method of the present invention.

Detailed Description of the Invention

By "antioxidants" it is meant the term known in the state of the art for compounds that inhibit the oxidation of other compounds. However, for the purposes of this invention, antioxidants are understood as antioxidants naturally present in olives, such as, in particular, but not limited to, the following compounds: oleuropein, hydroxytyrosol, tyrosol and other phenolic alcohols; phenolic acids, vitamin E and β-carotene; oleacein, oleocanthal and other secoiridoids; pinoresinol and other lignans; apigenin, luteolin and other flavones.

By "polyphenols" it is meant the term used in the state of the art to refer generically to phenolic alcohols and phenolic acids present in olives.

As will be clear to those skilled in the art, the application of the principles described here is not limited to the forms of implementation presented. Possible changes that may occur in the present invention, defined in number, remain within the scope of the present invention.

Methods to produce olive oil or antioxidant concentrates according to the principles described herein may comprise any number of the characteristics presented. Likewise, the principles described herein can be applied to any method of producing olive oil or antioxidant concentrates.

It will also be clear to those skilled in the art that, due to the nature of this invention, it can be applied to obtain a product with a high antioxidant content derived from any fruit, vegetable or seed, such as, but not limited to, olive oil, sunflower oil, corn oil, almond oil, safflower oil, palm oil,

soybean oil, and rapeseed oil, among others.

According to the Figures, the present invention refers to a method for the production of olive oil with a high antioxidant content and to a method for the production of an antioxidant concentrate.

The method of this invention begins with the choice of olives to be used in the method. Specifically, the olives must be of a variety that is known to be an olive presenting characteristics that allow the extraction of an amount of oil per olive that can be considered acceptable, as well as an antioxidant content that can be considered high.

By amount of oil per olive that can be considered acceptable, it is understood a volume of oil greater than 10% of the total weight of the olive. An antioxidant content that can be considered high means an antioxidant content of more than 100 mg/kg.

After the choice of the variety of the olives, where the state of ripeness of the olives of that variety has also been taken into account, the olives are harvested. In a preferred embodiment, the ripeness index calculated from the Jaén index is less than or equal to 2. Olive leaves and other macroscopic impurities are then separated from the olives, and the olives can be washed afterwards, preferably at room temperature.

Once the olives are selected, they are harvested, and the olive oil production method begins. The first step involves washing, followed by turning the olives into an olive paste. This transformation is carried out by crushing the olives, in order to break the vacuoles of the olives that contain the oil. This step can be carried out by any means known in the state of the art,

such as especially, but not exclusively, milling, beating, pressing/separating, malaxation, among others.

The crushing of the olives results in the production of a paste. This paste, in an embodiment, is then treated to promote a greater release of the oil from the olives, in order to optimize the efficiency of the separation or extraction step that occurs afterwards. The treatment of the paste includes any mechanical method suitable for this purpose, such as but not exclusively, beating the paste or mixing, among others.

In the following step of separation or extraction, the olive oil is separated or extracted from the above-mentioned olive paste. This separation or extraction can be carried out by any means of the state of the art suitable for the separation of liquids and solids, especially, but not exclusively, decantation, filtration or pressing, among others. From this step, olive oil and olive pomace are obtained.

The oil, after the separation or extraction step, is processed by any method known in the state of the art. In a preferred embodiment, olive oil is separated from impurities and water, especially, but not exclusively, by centrifugation, decantation, filtration, among others. The oil is then stored in a deposit whose atmosphere is mostly made up of, preferably, an inert gas, such as, especially, but not exclusively, helium, nitrogen, argon, among others.

Olive oil processing is always carried out at a temperature below 30 °C, in all possible steps, preferably in an inert atmosphere. A temperature below 30°C, along with an oxygen-poor atmosphere, prevents the degradation of any antioxidants that may be present in olive oil, also helping to maintain its nutritional and

organoleptic qualities. This guarantees the quality of the olive oil obtained, *i.e.,* the nutritional and organoleptic qualities are not neglected during the method of obtaining the oil. One of the main factors that influences the quality and characteristics of olive oil is the variety of the olive and its region.

The pomace, after the separation or extraction step, is processed in parallel and independently of the olive oil processing, to remove or extract as many antioxidants as possible. As an ordinary skilled in the art knows, the antioxidants present in olives are mostly hydrophilic. For this reason, most antioxidants are not naturally present in olive oil, but rather in pomace. To be able to take advantage of the antioxidants, and later add or mix them with the olive oil, it is necessary to extract them from the pomace.

To extract the antioxidants from the pomace coming from the olive mill, the step of extracting the antioxidant concentrate is carried out. This process begins with the separation or extraction of antioxidants from the pomace. This separation shall be carried out by any method known to the state of the art, especially but not exclusively, by pressing or beating, among others, preferably a combination of the two methods mentioned. In this step, water is mixed with the pomace, so that the antioxidants migrate to the water. The water and pomace are then separated. This water will hereinafter be referred to as antioxidant extract. The pomace is then reused by some method known in the technique, which is not part of the scope of the present invention. The antioxidant extract should preferably be centrifuged and decanted to separate impurities, namely fat residues.

The antioxidant extract contains between 100 to 400 ppm of hydroxytyrosol. Optionally, before concentrating the antioxidant

extract, the solution is centrifuged and decanted to remove any oil still present in the pomace and that has migrated to the antioxidant extract, which can then be mixed with the oil that is being processed in parallel. A decanting step can also be performed to remove impurities that are still present in the antioxidant extract.

For the concentration of the antioxidant extract, any appropriate step is carried out for the removal of water from a solution, such as, especially, but not exclusively, evaporation or reverse osmosis, among others. Reverse osmosis can be performed by any known method in the art, at a temperature below 30 °C. This first step of concentration is optional, and its implementation depends on the needs of the producer. This step, if it is performed, is called the first step of concentration. Prior to this concentration step, the extract is filtered by microfiltration.

After this optional step, a purification step may occur. The main objective of this step is to remove impurities from the pomace that may still be present in the antioxidant extract. Purification may be carried out by any appropriate method, such as, but not limited to, ion exchange chromatography. Within chromatography, the use of cationic resins is indicated, but not essential. The purification step is also optional and depends on the quality or condition of the antioxidant extract. By washing the cationic resins, hydroxytyrosol and tyrosol are eluted with water, allowing the extraction of the hydroxytyrosol and tyrosol that are still present.

After the purification step, the main step of the method of producing an antioxidant concentrate takes place. After the first step of concentration and/or purification, the antioxidant extract undergoes a second step of concentration, in which it is

concentrated by evaporation. This evaporation step is carried out by any means known in the art, however, it must be carried out at a temperature below 30 °C, preferably between 20 °C and 30 °C, more preferably between 26 °C and 30 °C. Evaporation should also be carried out at a pressure of less than 50 mbar, especially between 1 mbar and 15 mbar, preferably between 5 mbar and 10 mbar. Evaporation occurs over a period of more than 1 hour.

Unlike temperatures used in evaporation processes in other state-of-the-art arrangements, where the temperature reaches 75 °C, the evaporation step, like all other steps of the method of this invention, does not exceed 30 °C. This prevents the degradation of antioxidants, as these compounds are sensitive to high temperatures, reacting more easily with other compounds in these conditions, which accelerates degradation and volatilization. In this way, a high concentration of total antioxidants is maintained, increasing only by evaporation, with no degradation occurring. After the evaporation step is completed, a concentrate of antioxidants is obtained.

The antioxidant concentrate obtained has a concentration of hydroxytyrosol and tyrosol greater than 100,000 ppm, preferably greater than 200,000 ppm, especially between 250,000 ppm and 350,000 ppm.

After the second concentration step, the antioxidant concentrate is mixed with the olive oil that has been processed in parallel. Because the antioxidant concentrate has a very high content of antioxidants, an extremely low volume is mixed with the oil, preferably a volume equal to or less than 1.2 mL of antioxidant concentrate per 1 L of olive oil. The mixture is carried out by any means known in the state of the art, especially, but not

exclusively, by means of a mixer or a piston homogenizer, inert gas bubbles, pressing, among others, preferably by means of a circulator to allow the oil to circulate through pipes. This mixing step takes place for a period longer than 1 minute, preferably between 15 minutes and 1 hour for every 1000 L of olive oil, more specifically between 20 and 45 minutes, more specifically for 30 minutes for every 1000 L of olive oil. The mixture of the antioxidant concentrate with the olive oil is also carried out in an inert atmosphere, in order to prevent the quality of the antioxidant concentrate from degrading during the duration of the process. As will be clear, this mixing step can be carried out either with a continuous addition of the antioxidant concentrate to a continuous stream of oil, referred to in the technical area as continuous addition, or with the discrete addition of a certain volume of antioxidant concentrate to a discrete volume of olive oil, termed in the technical area as batch addition.

Although the antioxidant concentrate is hydrophilic, *i.e.*, considered immiscible with olive oil, a small amount of water is soluble in olive oil and vice versa. Specifically, it is known in the art that samples of extra virgin olive oil have an average amount of 1.2 mL of water per 1 L of olive oil. Therefore, the addition of the antioxidant concentrate to the oil in the referred volume allows not to mix excess water, which would have to be later decanted before the oil is packaged. In this way, this last step of decanting is avoided, as the oil is directly packaged after mixing the antioxidant concentrate. If packaging is not possible, olive oil with a high antioxidant content is stored in an inert atmosphere, and it is also packaged under the same atmospheric conditions.

The mixture of the antioxidant concentrate with the olive oil is carried out in such a way that the oil has a final concentration of hydroxytyrosol and tyrosol greater than 250 ppm, after 12 months shelf life, as required by European regulations for olive oil.

Results

The method of the present invention has been carried out and tested, and the composition of the antioxidant concentrate, and the final olive oil is compared with the equivalent products obtained by other methods known to the state of the art, especially by the method referred to in patent document WO2018189730.

By the method of the present invention, an antioxidant concentrate was obtained that was tested by any method known to the state of the art, namely, but not exclusively, by the standard HPLC method developed by the International Olive Council. However, only the concentration of hydroxytyrosol and tyrosol in the antioxidant concentrate was calculated, and a value of approximately 300,000 ppm of hydroxytyrosol and tyrosol was obtained.

The document WO2018189730 refers to a total antioxidant value of 56,000 ppm and 29,000 ppm, for two different olive varieties, of which 16,800 ppm and 9,800 ppm, respectively, correspond to hydroxytyrosol.

From these figures, it can be seen that the amount of antioxidants obtained by the method of the present invention is considerably higher than that obtained by the method referred to in document WO2018189730. While in documents of the state of the art it is mentioned to obtain a maximum of 56,000 ppm, with the

method of the present invention a value about 6 times higher was obtained for only hydroxytyrosol and tyrosol, not even considering the concentrations of the other antioxidants present in olives.

This difference is explained by the temperature conditions applied throughout the method, which does not exceed 30 °C throughout the method. Even more important for this difference is the second step of concentration, carried out by evaporation at a temperature below 30 °C, which prevents the degradation of the antioxidants in the antioxidant extract. In the example referred to in document WO2018189730, evaporation was carried out at 65 °C, which causes a considerable increase in antioxidants degradation.

The reduced pressure used in the example of the method of this invention (between 1 and 15 mbar) contributes not only to the reduction of the degradation of antioxidants, by creating anaerobic conditions important to maintain the quality of the antioxidants, but also contributes to the increase in the concentration of antioxidants since it is possible to eliminate a greater amount of water, therefore, less water is mixed with the oil eliminating the decanting method. In this way, the antioxidants remain in the oil so there are no undesirable deposits.

CLAIMS

1. Production process of olive oil with high antioxidant content and an antioxidante concentrate comprising the following steps:
   a) selection of olives;
   b) transformation of the olives into an olive paste;
   c) separation of the oil from the paste obtained in step b), thereby obtaining olive oil and olive pomace;
   d) extraction of antioxidants from pomace by mixing water with pomace; and
   e) at the same time, the olive oil is processed, followed by its storage in the warehouse whose atmosphere is mostly made up of an inert gas;
   f) optionally, concentration of the antioxidant extract obtained in step d) is carried out through the removal of water;
   g) optionally, the antioxidant extract obtained in step f) is purified;
   h) concentration of the antioxidant extract obtained in step g);
   i) mixing of the antioxidant concentrate obtained in step (h) with the olive oil that was previously stored;
   j) bottling of the olive oil;

   characterized in that:
   - steps (a) to (j) are carried out at a temperature below 30 °C;
   - in step (h) an additional evaporation step is introduced at a pressure of less than 50 mbar;
   - in step (i) a volume equal to or less than 1.2 mL of antioxidant concentrate per 1 L of olive oil is mixed.

2. Production process according to claim 1 characterized in

that the antioxidant concentrate obtained in step h) of claim 1 presents a concentration of hydroxytyrosol and tyrosol between 250.000 ppm and 350.000 ppm.

3. Production process according to any one of the preceding claims characterized in that the olive oil obtained has a final concentration of hydroxytyrosol and tyrosol greater than 250 ppm, after 12 months shelf life.

4. Production process according to any of the preceding claims, characterized in that the inert gas in step e) of claim 1 is, preferably, helium, nitrogen or argon.

5. Production process according to any of the preceding claims characterized in that the removal of water in step f) of claim 1 is accomplished through evaporation or reverse osmosis.

6. Production process according to any of the preceding claims characterized in that the purification in step g) of claim 1 is performed by chromatography.

7. Production process according to claim 6 characterized in that the chromatography is ion exchange chromatography.

8. Production process according to any of the preceding claims characterized in that the evaporation step in step h) of claim 1 is carried out between 20 °C and 30 °C.

9. Production process according to any of the preceding claims characterized in that the evaporation step in step h) of claim 1 is carried out between 1 mbar and 15 mbar.

10. Production process according to any of the preceding claims characterized in that the evaporation step in step h) of claim 1 is carried out for a period of time exceeding 1 hour.

11. Production process according to any of the preceding claims characterized in that the mixing step in step i) of claim 1 elapsing between 20 to 45 minutes.

12. Production process according to any of the foregoing claims characterized by the mixing step in step i) of claim 1 is carried out in an inert atmosphere.

4

PHTHALATES

4.1 Considerations and Objectives

Olive oil is celebrated for its numerous health benefits and cultural significance. However, like any widely produced food product, it faces challenges beyond quality and authenticity. A growing concern is the inadvertent introduction of contaminants, particularly plastic additives such as phthalates.

The increasing reliance on plastic materials in food production and storage has raised concerns about the migration of these substances into the final product. Ensuring the safety and quality of olive oil requires rigorous identification and mitigation of contaminants.

Understanding contamination sources and developing effective analytical methods are crucial for addressing these challenges.

This section introduces the topic, highlighting the importance of preserving the integrity of olive oil throughout its production and distribution chain to ensure a safe and high-quality product for consumers.

A review article *"A Critical Review of Analytical Methods for the Quantification of Phthalates Esters in Two Important European Food Products: Olive Oil and Wine"* was published, addressing analytical methods for determining phthalates in olive oil and wine. The article also summarized key information on the characteristics, toxicity, health effects, and regulations associated with these compounds. Techniques for extraction, purification, and quantification were also discussed in detail.

Although the review covered two food matrices—olive oil and wine—this thesis will focus exclusively on the olive oil matrix.

4.2 Article Review:

"A Critical Review of Analytical Methods for the Quantification of Phthalates Esters in Two Important European Food Products: Olive Oil and Wine"

Flávia Freitas, Maria João Cabrita and Marco Gomes da Silva

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Review

A Critical Review of Analytical Methods for the Quantification of Phthalates Esters in Two Important European Food Products: Olive Oil and Wine

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Abstract: Phthalic acid esters (PAEs) are a class of chemicals widely used as plasticizers. These compounds, considered toxic, do not bond to the polymeric matrix of plastic and can, therefore, migrate into the surrounding environment, posing a risk to human health. The primary source of human exposure is food, which can become contaminated during cultivation, production, and packaging. Therefore, it is imperative to control and regulate this exposure. This review covers the analytical methods used for their determination in two economically significant products: olive oil and wine. Additionally, it provides a summary and analysis of information regarding the characteristics, toxicity, effects on human health, and current regulations pertaining to PAEs in food. Various approaches for the extraction, purification, and quantification of these analytes are highlighted. Solvent and sorbent-based extraction techniques are reviewed, as are the chromatographic separation and other methods currently applied in the analysis of PAEs in wines and olive oils. The analysis of these contaminants is challenging due to the complexities of the matrices and the widespread presence of PAEs in analytical laboratories, demanding the implementation of appropriate strategies.

Keywords: phthalates esters; analytical methods; olive oil; wine

Citation: Freitas, F.; Cabrita, M.J.; da Silva, M.G. A Critical Review of Analytical Methods for the Quantification of Phthalates Esters in Two Important European Food Products: Olive Oil and Wine. *Molecules* **2023**, *28*, 7628. https://doi.org/10.3390/molecules28227628

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1. Phthalates Esters in Olive Oil and Wine

Olive oil and wine are daily staples in global consumption, and when enjoyed in moderation, they form the essential cornerstones of a wholesome Mediterranean diet. This is owing to the presence of macro and micronutrients endowed with antioxidant properties, including phenols and tocopherols [1]. It is estimated that around 3 million tons of olive oil and approximately 250 million hectoliters of wine are consumed worldwide each year [2,3].

The European Union (EU) is the world's primary producer, consumer, and exporter of olive oil. The primary member states engaged in the production and export of olive oil are Spain, Italy, Greece, and Portugal. Outside the EU, this role is taken on by Morocco, Tunisia, Turkey, and Syria. The EU produces about 70% of the world's olive oil and is responsible for 70% of global olive oil exports, with the United States, Brazil, and Japan being the main markets (Figure 1) [4].

When it comes to wine, the EU is responsible for approximately 60% of the world's production, with Italy, France, and Spain being the countries with the highest production. Together, these three countries account for about 60% of global exports. Beyond the EU, the United States, Australia, Chile, and Argentina are the countries with the highest global production and exports (Figure 1) [5].

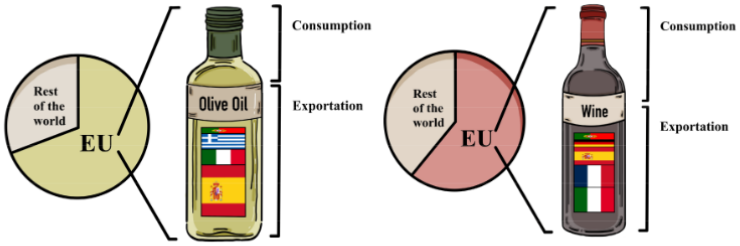


Figure 1. Data on the production, consumption, and export of olive oil and wine in the European Union.

Therefore, due to the significant consumption, production, and interest in these two products, the importance of studying and ensuring food control becomes evident, aiming for sustainability and a better quality of life.

Olive oil is directly obtained from olive fruits only through mechanical/physical processes that do not fundamentally alter oil composition. Wine is obtained from the fermentation of fresh grapes or juice, so its composition is also affected by biochemical processes.

In the past, most of the tools and equipment used in the production of these foods were produced from conventional materials, such as wood, metal, rock, ceramics, glass, and fabric, among others. However, over time, they have been replaced by large machinery that contains various plastics, also known as polymers, in their composition. Additionally, the packaging of these raw materials has been altered. While these products were once packaged in glass containers, it is now common to find them packaged in plastic materials [6–8].

Over the past few decades, the use of plastic materials has played a crucial role in the daily life of society [9]. Various types of polymers are used, such as polyethylene (PE), polyvinylidene chloride, polyvinyl chloride (PVC), polyethylene terephthalate (PET), ethylene vinyl alcohol (EVOH), polypropylene (PP), and polystyrene (PS), among others [10,11].

The choice of polymer will depend on various factors, such as cost-effectiveness, recyclability, and legal requirements. Its intended purpose also plays a role in the selection process. For example, whether it is for quick heating/reheating, frozen or ambient temperature storage, cooking in a bag or not, requiring heat stability, printability, durability, or several barrier properties (e.g., water, oxygen, carbon dioxide) [12,13].

Therefore, to make the applicability of these polymers more common in various uses and as versatile as possible, additives have been incorporated to enhance their properties. Phthalate esters or di-esters are among the most widely used additives by the polymer industries, and their high demand has increased to the point that, nowadays, approximately 6 million tons are produced annually [14]. Although these plasticizers have been extensively used for over 50 years, recent years have seen increasing research into the toxicity of various environmental pollutants, including phthalates esters. Moreover, several studies have reported potential health risks associated with these substances for human health [15].

Phthalic acid esters (PAEs) are colorless and odorless substances with low solubility in water, high solubility in lipid compounds, and present low volatility. They are capable of imparting a wide range of properties to materials, such as extreme rigidity or flexibility, opacity or transparency, coloration or translucency, and the ability to withstand high or low temperatures, among other characteristics. This wide range of property variation conferred by these plasticizers allows polymers to be used in different areas and applications, especially in industrial engineering (manufacturing rigid pipes and tubes) and the food industry (packaging and films for food packaging) [16,17].

Their structure consists of a benzene ring linked to two ester groups in the ortho position, resulting in two aliphatic chains (Figure 2).

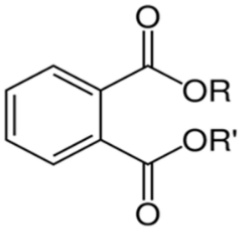


Figure 2. General chemical structure of phthalic acid esters. R and R′ denote linear and/or branched alkyl chains.

Depending on their substitution, this can generate more than 60 different types of PAEs with distinct properties (Table 1) [16].

Table 1. Physical–chemical properties and applications of various PAEs. Data from PubChem.

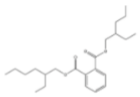
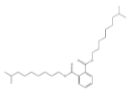
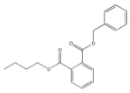
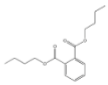
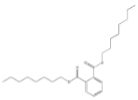
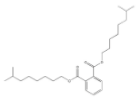
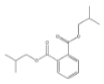
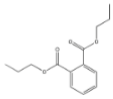
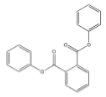
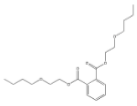
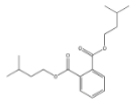
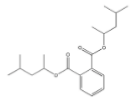
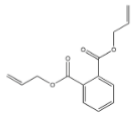
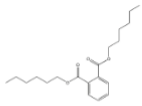
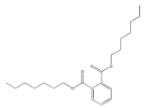
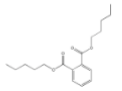
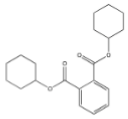
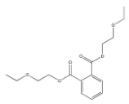
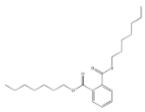
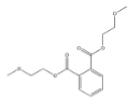
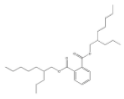
Name	Molecule	CAS	Molecular Structure	Molecular Weight (g/mol)	Density (g/cm^3)	Melting Point (°C)	Boiling Point (°C)	Solubility (mg/L in Water)	Applications
Bis(2-ethylhexyl) phthalate DEHP		117-81-7	$C_{24}H_{38}O_4$	390.6	0.981 (25 °C)	−50	384	0.27 (25 °C)	Used as a plasticizer; also used in pesticides (an inert ingredient), dielectric fluids, erasable inks, and vacuum pump oils;
Dimethyl phthalate DMP		131-11-3	$C_{10}H_{10}O_4$	194.2	1.194 (20 °C)	5.5	284	4 (25 °C)	Used as a plasticizer in solid rocket propellants, lacquers, plastics, safety glasses, rubber coating agents, molding powders, insect repellents, and pesticides.
Diisodecyl phthalate DIDP		26761-40-0	$C_{26}H_{46}O_4$	446.7	0.966 (20 °C)	−58	53	0.28 (25 °C)	Used as a plasticizer for polyvinyl chloride in calendered film, coated fabrics, building wire jackets, wire, and cable extrusion.
Benzyl butyl phthalate BBP		85-68-7	$C_{19}H_{20}O_4$	312.4	1.119 (25 °C)	−35	370	2.69 (25 °C)	Used as an organic intermediate and a plasticizer for PVC-based flooring products, polyvinyl acetate emulsion adhesives, polyvinyl and cellulose resins, vinyl foams, and other plastics.
Dibutyl phthalate DBP		84-74-2	$C_{16}H_{22}O_4$	278.3	1.049 (20 °C)	−35	340	11,2 (25 °C)	Used as a plasticizer to help make plastics soft and flexible; also used in shower curtains, raincoats, food wraps, bowls, car interiors, vinyl fabrics, and floor tiles.
Dioctyl phthalate DOP		117-84-0	$C_{24}H_{38}O_4$	390.4	0.978 (20 °C)	−25	220	0.022 (25 °C)	Used as a plasticizer in carpet backing, packaging films, medical tubing, blood storage bags, floor tile, wire, cables, adhesives, cosmetics, and pesticides.
Diisononyl phthalate DINP		28553-12-0	$C_{26}H_{42}O_4$	418.6	0.972 (20 °C)	−48	78	0.2 (20 °C)	Used to impart softness and flexibility to PVC products. Used in perfumes and cosmetics, vinyl swimming pools, plasticized vinyl seats, and clothing.
Diisobutyl phthalate DIBP		84-69-5	$C_{16}H_{22}O_4$	278.3	1.05 (15 °C)	−64	296	6.2 (25 °C)	Used as a plasticizer; used in paints, lacquers, and varnishes, in the paper and pulp industry, and to make boards, chemicals, polymers, adhesives, softeners, and viscosity adjusters.
Diethyl Phthalate DEP		84-66-2	$C_{12}H_{14}O_4$	222.2	1.12 (20 °C)	−41	295	1.08 (25 °C)	Used as a plasticizer, insect repellent, and solvent; as a solvent in cellulose acetate, fragrances, and cosmetics;
Dipropyl phthalate DPrP		131-16-8	$C_{14}H_{18}O_4$	250.3	1.07 (25 °C)	−31	317.5	108.1 (20 °C)	Used to make plasticizers and polymer additives. It is also used in chemical reagents and organic intermediates.

Table 1. *Cont.*

Name	Molecule	CAS	Molecular Structure	Molecular Weight (g/mol)	Density (g/cm^3)	Melting Point (°C)	Boiling Point (°C)	Solubility (mg/L in Water)	Applications
Diphenyl phthalate DPhP		84-62-8	$C_{20}H_{14}O_4$	318.3	1.28 (TNS)	75	402.5	0.082 (24 °C)	Used as a plasticizer in nitrocellulose lacquers.
Bis(2-butoxyethyl) phthalate DBEP		117-83-9	$C_{20}H_{30}O_6$	366.4	1.06 (20 °C)	−55	270	1.675 (25 °C)	Used as a plasticizer for resins, and as a softener and processing aid for chloroprene rubber, nitrile-butadiene rubber, and styrene-butadiene rubber.
Diisopentyl phthalate DIPP		605-50-5	$C_{14}H_{18}O_6$	306.4	1.02 (TNS)	<−25 °C	339	1.1 (20 °C)	Used as plasticizer of cellulose resin, polymethyl methacrylate, polystyrene, and chlorinated rubber.
Bis(4-methyl-2-pentyl) phthalate BMPP		84-63-9	$C_{20}H_{30}O_4$	334.4	0.995 (TNS)		341	<0.1%	Used as a plasticizer and found in cosmetics and baby skin care products.
Diallyl phthalate DAP		131-17-9	$C_{14}H_{14}O_4$	246.3	1.12 (20 °C)	−70	290	182 (25 °C)	Used to make insulators, potentiometers, and circuit boards in communication, computer, and aerospace systems, and a monomer in thermosetting plastics, a diluent in polyester spray systems, a dye carrier, and an impregnant for jewelry.
Dihexyl phthalate DHXP		84-75-3	$C_{20}H_{30}O_4$	334.4	1.01 (20 °C)	−59	350	0.05 (25 °C)	Used as a plasticizer; used to make plastisols for automobile parts and dip-molded products.
Diheptyl phthalate DHP		3648-21-3	$C_{24}H_{34}O_4$	362.5	1 (20 °C)	<−40	360	0.0018 (25 °C)	Used as a plasticizer for vinyl resins.
Dipentyl phthalate DPP		131-18-0	$C_{18}H_{26}O_4$	306.4	1.12 (20 °C)	<−55	342	0.8 (25 °C)	Used as plasticizers to soften polyvinyl chloride in shower curtains, vinyl upholstery, adhesives, floor tiles, food containers and wrappers, cleaning materials, and cosmetics.
Dicyclohexyl phthalate DCHP		84-61-7	$C_{20}H_{26}O_4$	330.4	1.383 (20 °C)	66	224	4.0 (24 °C)	Used as a plasticizer for nitrocellulose, ethyl cellulose, chlorinated rubber, polyvinyl acetate, polyvinyl chloride, and other polymers; And as a heat sealer for cellulose, in paper finishes, and to make printers ink water-resistant;
Bis(2-ethoxyethyl) phthalate DEEP		605-54-9	$C_{16}H_{22}O_6$	310.3	1.121 (20°C)	34	345	1946 (TNS)	Used as a plasticizer, an apoptosis inhibitor, and an androstane receptor agonist.
Dinonyl phthalate DNP		84-76-4	$C_{26}H_{42}O_4$	418.6	0.972 (20 °C)	−33.15	413	1.73×10^{-5} (25 °C)	Used in plastisols and coating pastes, as a low-volatility plasticizer for vinyl resins, as a stationary liquid phase in chromatography, and to make vinyl mixes resistant to heat and detergents;
Bis(2-methoxyethyl) phthalate DMEP		117-82-8	$C_{14}H_{18}O_6$	282.3	1.1596 (15 °C)	−45	340	8500 (25 °C)	Used in plastisols and coating pastes, as a plasticizer for vinyl resins, as a stationary liquid phase in chromatography, and to make vinyl mixes resistant to heat and detergents.
Bis(2-propylheptyl) phthalate DPHP		53306-54-0	$C_{28}H_{46}O_4$	446.7	0.964 (TNS)	−48	254	2×10^{-7} (25 °C)	Used as an adhesion/cohesion promoter, adhesives and sealant chemicals, intermediate, paint additives, and coating additives.

It is already known that the main sources of human exposure to PAEs are oral (through food, pacifiers, baby bottles), inhalation (air contaminated by building materials, accidental inhalation of soil, household dust, PVC-based medical devices), and dermal contact (creams, shampoo, soaps) with products containing these substances [17,18].

However, the most significant source of exposure is food intake, as it can absorb compounds that migrate from plastic packaging to the food matrix or become contaminated during the production process [19,20].

This phenomenon is due to the fact that phthalates do not chemically bind to the polymer matrix, leading to their easy migration over time through exposure, increased temperature, and mechanical stress, among other factors [12,17].

As a result, these compounds can migrate into food through the typically used manufacturing processes, packaging films, gloves used for food preparation, and storage containers. These compounds have also been found in the inks and adhesives of food packaging, as well as in coatings for kitchen utensils [19,21].

The risk of migration is more pronounced in nonpolar foods, such as olive oil, due to the lipophilic nature of phthalates. This is because phthalates have a specific affinity for fatty and nonpolar substances, which makes their migration easier.

1.1. Toxicity

Due to the lipophilic nature of PAEs, adsorption can occur through dermal and pulmonary tissues. However, the primary route of absorption occurs in the saliva or stomach after oral administration. In mammals, the metabolism of PAEs is rapid, and their distribution occurs uniformly throughout the body [22–24]. Phthalates esters undergo a biotransformation pathway that occurs in two stages, Figure 3.

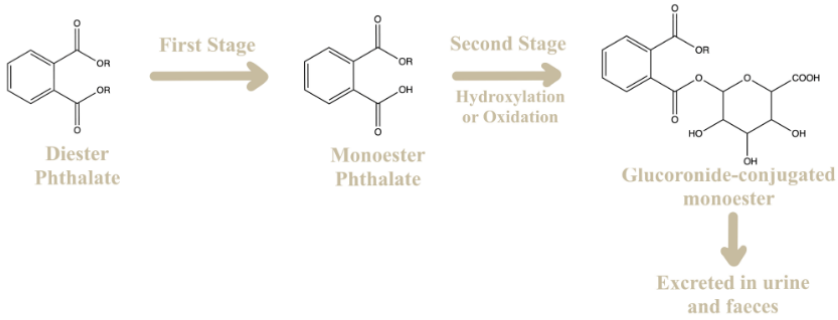


Figure 3. Metabolic pathway of phthalic acid esters.

In the first stage, lower molecular weight phthalates are hydrolyzed to form monoester phthalates through biotransformation catalyzed by lipases and esterases in the intestine and parenchyma. Typically, this initial metabolic step is associated with detoxification. However, in vitro and in vivo studies have shown that diester phthalates become more biologically active when they undergo hydrolysis and convert into monoester phthalates. On the other hand, higher molecular weight phthalates can be metabolized to form oxidative products. In the second stage, known as conjugation, both the hydrolyzed and oxidized monoesters can react with glucuronic acid catalyzed by the enzyme uridine 5′-diphospho-glucuronyl transferase. Glucuronidation facilitates excretion and can reduce the bioavailability of metabolites, minimizing their potential biological activity [25,26].

Relatively polar and short-chain phthalates (up to eight carbons), such as DMP and DEP, are rapidly hydrolyzed and have an elimination half-life in their free glucuronidated form of about 5–6 h. However, long-chain phthalates, like DEHP and DiNP, have a longer elimination half-life. Only 2–7% of DEHP is semi-eliminated from the human body in approximately 12 h [25,27].

1.2. Health Risks

Due to their unique physicochemical properties, certain phthalates and their metabolites have a severe toxic effect on human health, especially on the reproductive, endocrine, and respiratory systems (Figure 4). Several studies report that the accumulation of phthalates in the body disrupts growth and reproduction, as well as induces genotoxicity, neurotoxicity, and carcinogenicity [20,28–31].

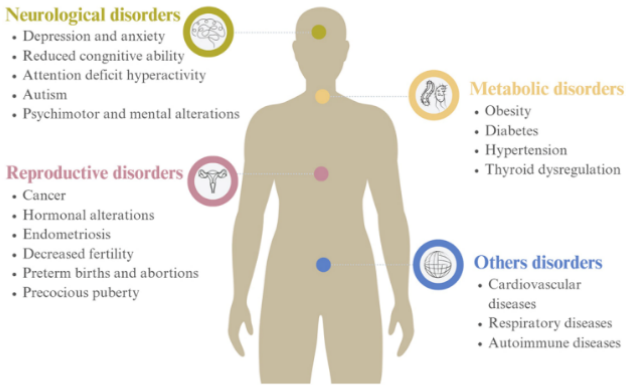


Figure 4. Negative health effects caused by PAEs in human health.

In general, phthalates demonstrate low acute toxicity in animals, with median lethal dose (LD_{50}) values ranging from 1 to 30 g/kg of body weight or above. In subchronic studies with rodents, phthalates induced dose-related adverse effects in the liver, kidneys, thyroid, and testicular tissue [17].

These studies have led several countries to intervene and regulate exposure to phthalates and other substances.

1.3. Regulation

Due to the increasing focus on consumer food safety in Europe, strict requirements for the use of food contact materials (FCMs) have been implemented, as outlined in Regulation (EC) No 1935/2004 [32]. This regulation emphasizes that any material or product intended to come into direct or indirect contact with food must not transfer chemical substances to food products in amounts that could pose a risk to human health or result in unacceptable changes in the composition of these foods or the deterioration of their organoleptic properties.

Furthermore, specific migration limits (SMLs) have been established for five permitted phthalates (DEHP, BBP, DBP, DINP, and DIDP) in FCMs, based on a toxicological assessment outlined in Annex I of Regulation (EU) 10/2011 [33]. In 2019, the European Food Safety Authority (EFSA) also defined a tolerable daily intake (TDI) of 50 µg/kg of body weight per day for DBP, BBP, DEHP, and DINP, and 150 µg/kg for DIDP [34].

It is worth noting that, although DIBP is not authorized as an additive for FCMs, it may be present in these materials in small quantities as an impurity or because of its use as an adjuvant in the manufacturing process of certain types of plastics [35].

In 2023, the EU reviewed these data and established new SMLs, implementing Regulation (EU) 2023/1442 [35], which amends Annex I of Regulation (EU) 10/2011 (Table 2).

This amendment came into effect on 1 August 2023. However, plastics in contact with food that were in compliance with the FCM regulation before the amendment's entry into force and were placed on the market before 1 August 2023 can remain on the market until their stocks are exhausted [35].

Table 2. Comparison between regulation 10/2011 and its amendment 2023/1442.

Substance	Regulation (EU) 2023/1442 Amending Annex I to Regulation (EU) 10/2011 *	Annex I to Regulation (EU) 10/2011	Only to Be Used as:
DBP	SML: 0.12 mg/kg Total SML group restriction no.32: 60 mg/kg Total SML group restriction no.36: 0.6 mg/kg	SML: 0.3 mg/kg Total SML group restriction no.32: 60 mg/kg	(a) Plasticizer in repeated use materials and articles contacting non-fatty foods; (b) Technical support agent in polyolefins in concentrations up to 0.05% (*w*/*w*) in the final product.
BBP	SML: 6.0 mg/kg Total SML group restriction no.32: 60 mg/kg Total SML group restriction no.36: 0.6 mg/kg	SML: 30 mg/kg Total SML group restriction no.32: 60 mg/kg	(a) Plasticizer in repeated use materials and articles; (b) Plasticizer in single-use materials and articles contacting non-fatty foods except for infant formula and follow-on formula; (c) Technical support agent in concentrations up to 0.1% (*w*/*w*) in the final product.
DEHP	SML: 0.6 mg/kg Total SML group restriction no.32: 60 mg/kg Total SML group restriction no.36: 0.6 mg/kg	SML: 1.5 mg/kg Total SML group restriction no.32: 60 mg/kg	(a) Plasticizer in repeated use materials and articles contacting non-fatty foods; (b) Technical support agent in concentrations up to 0.1% (*w*/*w*) in the final product.
DINP and DIDP	Total SML group restriction no.26: 1.8 mg/kg (sum of DINP and DIDP) Total SML group restriction no.32: 60 mg/kg Not to be used in combination with FCM substances DBP, BBP, DEHP, and DIBP.	Total SML: 9 mg/kg (sum of DINP and DIDP) Total SML group restriction no.32: 60 mg/kg	(a) Plasticizer in repeated use materials and articles; (b) Plasticizer in single-use materials and articles contacting non-fatty foods except for infant formula and follow-on formula; (c) technical support agent in concentrations up to 0.1% (*w*/*w*) in the final product.

* Group restriction no. 26 corresponds to the sum of DINP e DIDP; Group restriction no. 36 corresponds to the sum of DBP, DIBP, BBP, and DEHP expressed as DEHP equivalents using the following equation: DBP*5 + DIBP*4 + BBP*0,1 + DEHP*1; Group restriction no. 32 corresponds to the sum of DBP BBP DEHP DIBP and some plasticizing substances like adipates, sebacates, and terephthalates, among others. DIBP is not listed as an authorized substance; however, it may occur alongside other phthalates as a result of its use as a polymerization aid, and therefore, it is included in group restrictions.

Despite this, there is still no specific regulation for the permitted quantity of phthalates in food. Therefore, even though SMLs are controlled in packaging and other materials, it is necessary to identify the sources of migration of these plasticizers into food. If they are found to be contaminated, it indicates that they have come into contact with one or more materials that are not suitable during their production process.

In the case of wine and olive oil, several studies report that contamination with phthalates can occur both during the production and treatment of the fruit, as they are often used in harvesting nets, pipes, tanks, and other plastic materials, as well as during storage using synthetic corks and plastic containers [36,37].

Even the drinking water used for irrigation or for washing production materials can be contaminated with these plasticizers [38]. For example, the World Health Organization (WHO) recommends a maximum concentration of 8 µg/L for DEHP [39]. This compound, in particular, is a global issue and has become an omnipresent pollutant in the environment, particularly in food. This is the most commonly detected and/or quantified phthalate as it is used in the production of flexible plastics like PVC and PET, which are commonly used for producing caps and single-dose sachets [40,41].

In 2011, Taiwan reported the "largest episode of food contamination with plasticizers in human history," and various contaminated foods were found in the market [42]. This incident, along with new studies on the hazards of phthalates to human health, has made

this food safety issue a global concern. Therefore, it has become imperative to reconsider internationally accepted regulations to mitigate this problem in food products.

There are various regulations for phthalates in different parts of the world, and as a result, it is expected that there will be substantial variation in phthalate concentrations in foods depending on the region where they are produced. This makes phthalate contamination an increasingly cross-border food safety issue as the global market expands.

Due to the widespread use of plastics, it is impractical to completely eliminate the source of contamination. Since it is not possible to remove these products from the global market, more and more research is focusing on the removal of these plasticizers. Wang et al. reviewed the methods for removing PAEs from food [38]. For polar food matrices like drinking water and beverages, methods such as physical and chemical adsorption, microbial degradation, membrane filtration, and chemical oxidation, among others, are typically used. However, for non-polar food matrices, like vegetable oils, methods such as physical adsorption, steam distillation, molecular distillation, and solvent extraction are employed. Nevertheless, for the latter, research in this area is limited, primarily due to the significant susceptibility of vegetable oil quality to external conditions. It is important to note that regardless of the matrix, PEs can only be removed to a certain extent [38].

Finally, one believes it is more advantageous to review the materials used during the harvesting, production, and packaging of food matrices, incorporating strategies to prevent contamination by plasticizers.

Certainly, the quantity of phthalates entering the human body solely through the consumption of olive oil or wine is exceedingly minimal and might not reach levels capable of inducing toxicological effects. Nevertheless, when considering the cumulative exposure to all plasticizers found in various elements of dietary intake, the potential risks to human health should not be casually dismissed.

2. Identification and Quantification of Phthalate Esters in Olive Oil and Wine

It is well known that human exposure to foods containing PAEs is daily, leading to accumulation in the body and resulting in long-term harmful effects.

The development of analytical methods that allow the identification and quantification of these compounds at low concentration levels, especially in food matrices consumed in large quantities, is urgently needed.

Traditionally, the analysis of phthalates is commonly performed using either gas chromatography (GC) or liquid chromatography (LC), often followed by mass spectrometry (MS) detection. Other analytical techniques have also been used, such as ultraviolet spectrophotometry (UV), Raman spectroscopy, flow-injection chemiluminescence (FI-CL), and more recent methods employing enzyme-linked immunosorbent assay (ELISA) and Polymerase chain reaction (PCR), as shown in Table 3.

However, due to the complex nature of real matrices, direct injection into analytical systems is not advantageous, and therefore, prior sample preparation is required. This preparation depends on the physicochemical characteristics of the matrix, the target compounds, and the aimed concentration levels.

In addition to the complexity of the matrices in which they are present, phthalates are generally found at low concentrations, typically in the range of μg/L (ppb level) or less, often falling below the limit of detection (LOD) of instruments. It is almost strictly necessary to employ both extraction and cleaning/pre-concentration steps to maximize analyte recovery and minimize the presence of potential interferents [43–45].

As described in Table 3, different analytical pre-treatment approaches for the analysis of phthalates in olive oils and wines have been reported in the literature. These include liquid–liquid extraction (LLE), solid-phase microextraction (SPME), solid-phase extraction (SPE), molecularly imprinted solid-phase extraction (MISPE), magnetic solid-phase extraction (MSPE), dispersive liquid–liquid microextraction (DLLME), and quick, easy, cheap, effective, rugged, and safe (QuEChERS) methods, among others.

Due to their simplicity, customization, and automation capabilities, classical techniques, like LLE, with or without additional clean-up steps, are still preferred when it comes to phthalate esters extraction to isolate and concentrate target analytes. However, these methods have several limitations. They typically demand a substantial amount of time, intensive labor, and the use of significant volumes of potentially harmful.

Research and the advancement of alternative techniques, such as Solid-Phase Microextraction (SPME) and Liquid-Phase Microextraction (LPME), have demonstrated the capacity to mitigate certain limitations, all the while preserving elevated extraction efficiency and analyte enrichment levels.

Thus, given the worldwide importance of olive oil and wine in society and the growing interest in the analysis of food contaminants, a comprehensive effort has been made to address all articles published on the analysis of phthalate esters in olive oil and wine, excluding other vegetable oils and alcoholic beverages.

Table 3. Presents the analytical techniques reported in the literature for phthalate detection in the last 20 years, organized by matrix (wine and olive oil), phthalates esters analyzed, sample preparation, and analytical technique used. Limits of detection (LOD), quantification (LOQ), and Recoveries obtained in each study are represented, as well as the concentration of phthalates esters found in real samples. Studies on wine are represented in dark-shaded areas, and in light-shaded areas are the studies on olive oil.

PAEs	Sample Preparation	Analytical Technique Column	LOD	LOQ	Recovery %	Concentration of PAEs	R *
DMP, DEP, DIBP, DBP, BBP, DCHP, DEHP, DOP, DINP, DIDP	LLE Isohexane	GC/MS Agilent DB5- MS (30 m × 0.25 mm × 0.25 µm)	0.004–0.020 mg/L	0.01–0.05 mg/L	98–100	0.008–0.273 mg/kg DBP, BBP, DEHP	[46]
DEP	LLE 1,1,2-trichlorotrifluoroethane	GC/MS Varian VF-Xms column (29.3 m × 0.25 mm × 0.25 µm)	0.7 mg/L	2.6 mg/L	103.9–110.4	<LOD	[47]
DMP, DEP, DIPrP, DAP, DPrP, DIBP, DBP, DMEP, DIPP, BMPP, DEEP, DPP, DHXP, BBP, DBEP, DCHP, DEHP, DHP, DPhP, DNOP, DINP, DIDP, DNP	LLE Acetonitrile	LC/MS/MS Agilent Poroshell 120 EC-C18 (100 × 4.6 mm × 2.7 µm)	0.8–15 µg/kg	10–100 µg/kg	75.5–113.3	ns	[48]
DBP	LLE Hexane	FI–CL	0.03 pg/mL		96–103.3	0.09–0.22 µg/mL	[49]
DBP	LLE Hexane	icELISA	64.5 ng/mL		83.1–101.7	ns	[50]
DEP	LLE Hexane	GNP-rt-IPCR	1.06 pg/L		96.65–110.02	41.88 µg/kg	[51]
DMP	LLE Hexane	BA-rt-IPCR	1.98 pg/L		88.18–108.99	86.96–182.85 µg/L	[52]
DMP, DEP, DIBP, DBP, BBP, DEHP, DOP, DINP, DIDP	Filter 0.2 µm	HPLC/MS/MS Phenomenex 75 mm Synergi Hydro-RP (2 mm × 4 µm × 4 mm)	0.5–8.8 µg/L	1.6–26.6 µg/L	94.6–105.7	1.8–10.7 µg/L DIBP, DBP, BBP	[53]
DMP, DEP, DBP, BMEP, DPP	MA-LLME	GC/MS Teknokroma TRB-624 (30 m × 0.25 mm × 1.40 µm)	0.1–0.4 µg/L	0.3–1 µg/L		4.2–25 µg/L DBP, DPP, DEP	[54]
DMP, DEP, DBP, BBP, BBP, DEHP	USVADLLME	GC/MS Lab-made SE-54 (30 m × 250 µm × 0.25 µm)	0.022–0.1 µg/L	0.075–0.335 µg/L	85–100.5	11.5–312.4 pg/µL DBP, BBP, DEHP	[55]
DBP, BBP, DEP, DIOP	DLLME	GC-FID Lab-made SE-54 (15 m × 0.25 mm × 0.33 µm)	0.34–0.78 µg/L		70–120	1.2–5.8 µg/L BBP, DBP	[56]

Table 3. *Cont.*

PAEs	Sample Preparation	Analytical Technique Column	LOD	LOQ	Recovery %	Concentration of PAEs	R *
DBP, BBP, DCHP, DEHP, DOP	UA-DLLME-SFOD	GC-FID Agilent HP-5 (30 m × 0.250 mm × 0.25 µm)	0.64–2.82 µg/L	1.93–8.47 µg/L	75–98	ns	[57]
DIBP, DBP, BBP, DEHP	IL-DLLME [C8MIM] [PF6]	HPLC/DAD Waters Xterra C18 (15 cm × 4.6 mm × 5 µm)	1.5–2.2 ng/mL	5–7.3 ng/mL	91.6–10.6	0.018–0.122 µg/mL DIBP DBP	[58]
DMP, DEP, DBP, DEHP, BBP, DOP	HS-SPME PDMS	GC/MS Varian CP-WAX 52 CB (30 m × 0.32 mm × 0.25 µm)	16–35 ng/L		72–121	0.3–7.40 µg/L DMP, DEP, DBP, DEHP, BBP, DOP	[36]
DMP, DEP, DBP, DEHP, BBP, DOP	HS-SPME CW-DVB; PDMS-DVB with sodium chloride	GC/MS Varian CP-WAX 52 CB (30 m × 0.32 mm × 0.25 µm)	0.06–2.9 µg/L	0.1–4.2 µg/L	64–135	Total ranging from 7–12 ng/mL	[59]
DBP, BBP, BDE, DOP	HS-SPME PDMS-DVB with sodium chloride	GC/MS SGE HP-5 (60 m × 0.25 mm × 0.25 µm)	0.03–0.11 µg/L	0.09–0.36 µg/L	80.3–107.6	0.71–20.8 µg/L DBP, DOP	[60]
DEP, DBP, DEHP	DI-HF-SPME MWCNTs/SiO2 reinforced hollow fibre	GC/MS Thermo TR-5 MS (30 m × 0.25 mm × 0.25 µm)	0.006–0.03 ng/mL	0.02–0.1 ng/mL		<LOD	[61]
DBP	DI-SPME Graphene oxide	GC/MS HP-5 MS (30 m × 0.25 mm × 0.25 µm)	0.3 ng/L		98	<LOD	[62]
DMP, DEP, DBP, DIBP, BBP, DEHP	SPE Amberlite XAD-2	GC-FID Lab-made SE-54 (15 m × 0.25 mm × 0.24 µm)	1.21–2.51 pg/µL	2.42–5.03 pg/µL	94–103	4.9–12.3 pg/µLDBP, DEHP	[63]
DMP, DEP, DBP, BCEP, BBP, DEHP	SPE Carbograph 1	GC/MS Lab-made SE-54 (30 m × 250 µm × 0.23 µm)	0.2–14 ng/mL	0.5–25 ng/mL	78–105	0.1–23 ng/mL DBP, BBP, DEHP	[64]
DMP, DEP, DEHP, DIBP, DBP, BBP	SPE C18	GC/MS Restek RTX-5MS (30 m × 0.25 mm × 0.25 µm)	0.015–0.018 µg/mL	0.024–0.029 µg/mL	33–109	0.025–0.276 µg/mL DIBP, DBP, BBP, DEHP	[65]
DBP, DEHP, DEP	Filter; SPE 0.45 µm; C18	LC/DAD Poroshell 120 EC C18 (4.6 mm × 50 mm × 2.7 µm)	0.25–0.38 ng/mL	0.75–1.10 ng/mL		23.6–334 ng/mL DBP, DEHP, DEP	[66]
BBP, DEP, DBP, DMP	MIP-SPE	HPLC/MS Agilent ZORBAX Eclipse XDB-C8 (50 mm × 2.1 mm × 3.5 µm)	0.03–0.20 µg/L	0.09–0.68 µg/L	74–98	0.3–5 µg/L BBP, DEP, DBP, DMP	[67]
DBP, BBP, DEHP	QuEChERS	GC/MS J&W DB-5MS (30 m × 0.25 mm × 0.25 µm)	0.08–2.25 ng/mL		104–123	1.69–9.72 ng/mL DBP, BBP	[68]
DMP, DEP, DIBP, DBP, DHP, BBP, DCHP, DEHP, DNOP, DINP, DIDP	LLE Acetonitrile	GC/MS/MS Restek Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm)	0.004–0.130 mg/L	0.012–2.600 mg/L	90.1–108.2	0.03–7.52mg/kg DIBP, DBP, BBP, DEHP, DINP	[69]
DMP, DEP, DIBP, DBP, BBP, DHP, DEHP, DOP, DINP, DIDP	LLE Acetonitrile	GC/MS Supelco SPB-5MS (30 m × 0.25 mm × 0.25 mm)	0.003–1.2 mg/kg	0.010–4.0 mg/kg	93.5–99.4	0.060–6.249mg/kg DMP, DEP, DIBP, DBP, BBP, DEHP, DINP, DIDP	[70]
DMP, DEP, DBP, DEHP	LLE by means of the carbon nanotube Pseudophase Distilled water; MWCNTs	GC/MS Supelco SLB-5 ms (30 m × 0.25 mm × 0.25 µm)	25–50 µg/L		92–104	0.15–5.1 mg/L DMP, DEP, DBP, DEHP	[71]
DMP, DEP, DPP, DIBP, DBP, BBP, BMPP, DEHP, DOP	LLE Acetonitrile	GC/MS/MS Restek Rxi-5ms (30 m × 0.25 mm × 0.25 µm)	0.43–1.67 µg/L	1.48–5.75 µg/L	89–114	0.17 mg/kg DEHP	[72]

Table 3. *Cont.*

PAEs	Sample Preparation	Analytical Technique Column	LOD	LOQ	Recovery %	Concentration of PAEs	R *
DMP, DEP, DIBP, DBP, DMEP, BMPP, DEEP, DPP, DHXP, BBP, DBEP, DCHP, DEHP, DPhP, DOP	LLE hexane saturated acetonitrile and hexane	GC/MS/MS Agilent HP-5MS (30 m × 0.25 mm × 0.25 µm)	0.1–4.0 µg/kg		70.0–110.8	ns	[73]
DEHP, DBP, DIBP, DINP	Dilution Hexane	GC/MS Lab-made pre-column OV-1701-OH (0.5 m × 0.25 mm × 0.05 mm) in series w/2 lab-made columns OV-61-OH (2.5 m × 0.32 mm × 0.20 µm) OV-225-OH (15–20 m × 0.25 mm × 0.20 µm)	10 µg/kg - 1 mg/kg	40 µg/kg - 3 mg/kg	82–106	90–6480 µg/kg DEHP, DINP, DBP	[74]
DMP, DEP, DPP, DBP, BBP, DCHP, DEHP, DINP, DIDP	Dilution Hexane	GC×GC/MS/MS 1D Merck SLB-5 ms (10 m × 0.25 mm × 0.10 µm) 2D Merck SLB-35 ms (1.5 m × 0.10 mm × 0.10 µm)	0.02–0.63 mg/kg	0.06–2.10 mg/kg		0.22–8.0 mg/kgDPP, DEHP, DINP, DIDP	[75]
DMP, DEP, DPP, DBP, BBP, DCHP, DEHP, DINP, DIDP,	Dilution Hexane	GC/MS/MS Equity-5 (5 m × 0.53 mm × 0.53 µm)	0.004–0.341 mg/kg	0.013–1.136 mg/kg		0.018–55.9 mg/kgDEHP, DIDP DBP, DPP, DINP, DEP	[76]
DMP, DEP, DIPrP, DAP, DPrP, DIBP, DBP, DMEP, DIPP, BMPP, DEEP, DPP, DHXP, BBP, DBEP DCHP, DEHP, DHP, DPhP, DNOP, DINP, DIDP, DNP,	LLE Acetonitrile	LC/MS/MS Agilent Poroshell 120 EC-C18 (100 × 4.6 mm × 2.7 µm)	0.8–15 µg/kg	10–100 µg/kg	82.2–112.6	ns	[48]
BBP, DBP, DEHP, DEP, DIBP, DIDP, DINP, DMP, DHXP, DOP, DAP, DPP	LLE Hexane saturated acetonitrile	UHPLC/MS Thermo Accucore aQ C18 (2.6 µm × 2.1 × 100 mm)	0.02–0.35 mg/kg	0.07–1.17 mg/kg	79–109	0.3–256.2 mg/kg DEHP, DIDP, DINP, DMP, DNOP, BBP, DEP, DIBP	[77]
DMP, DEP, DAP, DPrP, DIBP, BBP, DBP, DCHP, DHXP, DEHP	LLE Acetonitrile	UHPLC/MS/MS Thermo Syncronis C18 (100 × 2.1 mm, 1.7 µm)	0.1–1 µg/kg	0.3–3.3 µg/kg	85.1–95.5	3.0–309 µg/kg DMP, DEP, DIBP, BBP, DBP, DEHP	[78]
PAEs hydrolyzed in Phthalic Acid	LPME Tributyl phosphate	HPLC/MS/MS GL Sciences Inertsil ODS-3 (250 mm × 4.6 mm × 5 m)	1 µmol/kg	1.3 µmol/kg	86–107	4.82 µmol/kg	[79]
DMP, DEP, DPP, DIBP, DBP, BBP, DCHP, DEHP, DINP, DIDP,	LLE and DI-SPME Acetonitrile; PDMS	GC/MS/MS Supelco SLB-5ms (10 m × 0.1 mm × 0.1 mm)		0.015–0.144 mg/kg		0.228–7.207 mg/kg DEP, DIBP, DBP, DEHP, DINP, DIDP,	[80]
DMP, DEP, DAP, DIBP, DBP, BBP, DCHP, DEHP	LLE and SPME Acetonitrile; MIL-88(Fe)/Go	GC-FID Agilent HP-5 (30 m × 0.32 mm × 0.25 µm)	0.5–2 ng/g	1.7–6.7 ng/g	83.1–104.1	<LOD	[81]
DPP, DBP, DEHP	HS-SPME G/PVC nanocomposite	GC-FID Varian CP-Sil 8 CB (30 m × 0.32 mm × 0.25 µm)	0.06–0.08 µg/L	0.2–0.3 µg/L	87–112	<LOD	[82]

Table 3. *Cont.*

PAEs	Sample Preparation	Analytical Technique Column	LOD	LOQ	Recovery %	Concentration of PAEs	R *
DMP, DEP, DIBP, DBP, DMEP, 1,2MPP, 1,3MPP, DEEP, DAP, DHP, BBP, BBEP, DCHP, DEHP, DOP, DNP	SPME DVB/CAR/PDMS	GC/MS/MS Phenomenex Zebron ZB-5ms (30 m × 0.25 mm × 0.25 m)	0.02–0.05 mg/kg			87–840 µg/kg DIBP, DBP, BBP, DEHP	[83]
DMP, DEP, DBP, BBP, DEHP	LLE and SPE Acetonitrile; PSA	GC/MS Thermo TG- 5MS column (30 m × 0.25 mm × 0.25 µm)	0.10–0.79 µg/kg	0.33–2.6 µg/kg	72,4–103	0.05–1.28 mg/kg DMP, DBP, BBP, DEHP	[84]
DBP, BBP, DEHP	LLE and SPE Acetonitrile; Florisil	GC/TOFMS Agilent DB-5MS column (30 m × 0.25 mm × 0.25 µm)	4.70–10 µg/kg	14.2–30.4 µg/kg	83.9–97.8	13.2–729 µg/kgDBP, DEHP	[85]
DMP, DEP, DBP, DIBP, DEHP, BBP, DINP, DIDP	LLE and SPE Acetonitrile and tetrahydrofuran; Alumina	GC/MS HP-5MS (30 m × 0.25 mm × 0.25 µm)	2–170 ng/mL	6–500 ng/g	62–110	<LOD	[86]
DMP, DEP, DIPrP, DPrP, DIBP, DBP, DMEP, DIPP, BMPP, DEEP, DPP, DHXP, BBP, DBEP, DCHP, DHP, DPhP, DEHP, DNOP, DNP	MAE-GPC-SPE C18	GC/MS/MS Agilent HP-5MS (30 m × 0.32 mm × 0.25 µm)	0.218–1.367 µg/kg	0.72–4.51 µg/kg	93.04–104.7	0.42–0.70 mg/kg DBP, DEHP	[87]
DMP, DEP, DPP, DBP, BBP, DOP	LLE and SPE Hexane; Florisil	GC/MS Santa Clara HP-5MS (30 m × 0.25 mm × 0.25 µm)	0.002–0.004 mg/L	0.006–0.012 mg/L	87–102	0.049–2.295 mg/L DMP, DEP, DBP, DPP, BBP, DOP	[88]
DMP, DEP, DIBP, DBP, BMPP, DEEP, DPP, DHP, BBP, DBEP, DCHP, DHP, DPhP, DOP, DNP	LLE and SPE (QuEChERS modified) Methanol; GCB and PSA	GC/MS/MS DB-5MS (30 m × 0,25 mm × 0,25 µm)	0.02–8 µg/kg	0.07–26.68 µg/kg	70.11–115.33	0.10–1.85 mg/kg DIBP, DHP	[89]
DMP, DEP, DPrP, DAP, DIBP, DBP, DPP, DHXP, BBP, DHP, DEHP, DPhP, DNP, DDP	LLE and dSPE Acetonitrile; Q-sep QuEChERS	GC/MS Shimadzu SHRXI-5MS (30 m × 0.25 mm × 0.25 µm)	1.4–7.5 µg/kg	4.8–25.1 µg/kg	60.9–101.3	14–6166 µg/kg DMP, DEP, DAP, DPP DIBP, DBP, DPP, DHXP, BBP, DEHP, DNP, DDP	[37]
DEP, DIBP, DBP, BBP, DEHP, DOP, DINP, DIDP	LLE and SPE Acetone: methanol; DSC-18	HPLC-MS/MS Phenomenex Kinetex C18 (50 mm × 2.1 mm × 5.0 µm)		5.5–110 µg/kg	42–100	0.014–4.7 mg/kg DIBP, DBP, BBP, DEHP, DOP, DINP, DIDP	[90]
DEP, DIBP, DBP, BBP	LLE and SPE (QuEChERS) Acetonitrile; PSA	HPLC/DAD	6–9 ng/g	18–29 ng/g		<LOD	[91]
DEP, DBP, BMPP, DEEP, DNPP, DHXP, BBP, DBEP, DCHP, DEHP, DNOP, DMP, DMEP, DPP, DINP, DIDP	SPE Florisil	LC-MS/MS Agilent ZORBAX SB-C18 (10 cm × 3.5 µm × 2.1mm)	0.5–25 µg/kg	1.4–65 µg/kg	50.94–140.83	ns	[92]
BBP, DEHP	LLE and SPE (QuEChERS) Acetonitrile; PSA	SFC-UV Thermo Acclaim 120 C18 (5 µm, 4.6 mm × 250 mm	0.09–0.12 µg/mL	0.30–0.39 µg/mL	80.3–106.4	<LOD	[93]

Table 3. *Cont.*

PAEs	Sample Preparation	Analytical Technique Column	LOD	LOQ	Recovery %	Concentration of PAEs	R *
DMP, DEP, DBP, BBP, DEHP, DOP	GPC Cyclohexane: dichloromethane	GC/MS/MS Varian Factor Four 5-ms (30 m × 0.25 mm × 0.25µm)	0.1–148 µg/kg	0.2–182 µg/kg		0.029–4.70 mg/kg DBP, BBP, DEHP	[41]
DBP, DEHP		Raman spectroscopy with SERS	At a concentration of 0.2 mg/kg, the peaks for both plasticizers were still clearly detectable			ns	[94]

* Reference: ns—not specified.

Out of these 52 studies, 90% use chromatographic analytical techniques, where 25% apply liquid chromatography and 67% apply gas chromatography. However, these techniques, besides having long analysis times and sometimes complex instrumentation, often do not provide all the information present in a sample. It may not be possible to separate and identify compounds in complex samples, especially when multiple analytes share the same retention time [95].

The identification and quantification of phthalates are also very challenging due to the issue of cross-contamination, which is a recurring problem in sample preparation, extraction/cleanup, and concentration, as well as in the chromatographic system. To address this problem, rigorous laboratory cleaning and handling procedures are typically applied, and internal standards, often isotopically labeled, are used to reduce matrix effects and correct potential variations during the analyses [44,45].

2.1. Sample Preparation

In recent years, there has been an increasing demand for new extraction techniques that can be automated and reduce both extraction times and the use of organic solvents. This aims to prevent environmental contamination in analytical laboratories and, most importantly, reduce the costs associated with sample preparation, contributing to greener analytical chemistry [96,97].

2.1.1. Liquid–Liquid Extraction

Liquid–liquid extraction (LLE), also known as solvent extraction, is one of the oldest and simplest extraction techniques and one of the most commonly used for the analysis of phthalate esters in food matrices. This technique is based on the separation of target analytes with different solubilities in two immiscible solvents. It is commonly used in aqueous samples to pre-concentrate and remove unwanted compounds from the matrix [98,99].

The choice of solvent, the volume used, and the affinity of the target compounds for the extraction solvent will determine the efficiency and duration of the technique. Generally, extraction efficiency increases with the use of larger volumes of extraction solvent; however, this will reduce the concentration of target analytes in the solution. To mitigate this problem, multiple extractions with smaller volumes are often performed [98,99].

However, the use of this simple method has significant disadvantages, such as its unsuitability for hydrophilic compounds, the formation of emulsions that hinder complete recovery of the extract, the recurring use of large amounts of organic solvents leading to significant hazardous waste disposal, and the difficulty of automating the entire process. Another drawback of this technique is that its selectivity is not as specific as some other methods, as it tends to extract undesired analytes from the matrix under study. But, this disadvantage can be an advantage for non-targeted analyses [98,99].

LLE is the most widely used method for the extraction of phthalates in both wines and olive oils. Several studies report LLE of phthalates in wine and olive oils using solvents such as acetonitrile, hexane, acetone, and methanol as extraction solvents (Table 3).

For a reliable and efficient method, several parameters should be optimized during implementation. Leitz et al. optimized an LLE method for the analysis of phthalates in wines, where they studied the best extraction solvent to use, the ratio of extraction solvent volume/sample volume, and the number of extraction repetitions [47]. After optimization, 1,1,2-trichlorotrifluoroethane was chosen as the best solvent, achieving recovery values between 103.9–110.4%. However, due to its contribution to ozone depletion, this solvent's production and use have been phased out under international agreements like the Montreal Protocol [100]. So today, following the principles of green chemistry, it would be necessary to use another solvent, such as hexane [47].

Dugo et al. used LLE with acetonitrile to extract phthalates from Italian olive oils, obtaining recoveries between 93.5 and 99.4%. In the study, it was observed that DEHP was present in higher concentrations in olive oil than allowed by the EU in food contact materials (1.5 mg/kg) [70].

However, as can be seen in Table 3, one considers that a simple LLE of olive oil, without clean-up steps and direct injection into the system, is a risk to the analytical instruments used. Conventional LLE should typically be used in conjunction with a clean-up step, such as SPE, using different phases like silica or Florisil. In the case of olive oils, clean-up steps are of utmost importance to remove co-extracted free fatty acids. Free fatty acids and phthalates have somewhat similar polarities, and when the extraction of phthalates is not well performed, fats can cause interference in chromatographic analysis or even system contamination.

Frankhauser-Noti sought to use a chromatographic methodology that would avoid these issues in the analytical system by separating the fatty matrix during injection with programmed temperature volatilization (PTV), forcing the compounds of interest to be transferred to the separation column while retaining the rest at the inlet [101].

Despite the good extraction efficiency of the LLE method, alternative extraction solutions based on the principles of green analytical chemistry are currently sought, including low volumes of organic solvents, simplicity, and speed.

2.1.2. Dispersive Liquid-Liquid Microextraction

In recent years, there has been significant attention given to liquid-phase microextraction techniques (LPME), particularly DLLME. It was first described in 2006 by Rezaee et al. and can be considered a miniaturized modification of conventional LLE as it uses only a few microliters of extractant [102]. When compared to the classical technique, it offers advantages of simplicity, speed, cost-effectiveness, user-friendliness, reduced utilization of organic solvents, high recovery, high enrichment factor, and compatibility with chromatographic techniques like LC and GC [103].

The basic concept of Dispersive Liquid–Liquid Microextraction (DLLME) revolves around the dispersion of an extraction solvent (typically a non-water-miscible chlorinated solvent) and a disperser solvent (which can mix with both water and the extraction solvent, often acetonitrile) within an aqueous solution. This creates a more extensive interaction zone between the aqueous phase and the extraction solvent [102].

In 2013, Cinelli et al. established an ultrasound and vortex-assisted DLLME method for the extraction of six phthalates in wine [55]. Zhu et al., 2014, extracted four phthalates from wine using a simpler and faster DLLME method, making it an operationally easier and quicker analysis method than Cinelli's [56]. LPME techniques help avoid the issue of large volumes of solvents used in classical liquid-liquid extraction but do not eliminate the use of toxic solvents, namely halogenated solvents, such as chloroform and carbon tetrachloride.

Therefore, new approaches to DLLME are regularly presented using ionic liquids as extractants. Zanjani et al. developed a new LPME method, known as solidification of organic drops (SFOD) assisted by ultrasound (UA-DLLME-SFOD). Using an extraction solvent with properties such as lower density than water, low toxicity, and a melting point close to room temperature, solidifies easily at low temperatures. In this technique, following the extraction process, the organic drop is solidified in an ice bath, collected using a spatula,

melted, and directed for analysis [104]. Following this, Perez et al. applied this technique to extract five phthalate esters in food simulants and liquid samples, including wine [57].

In 2013, another modification of traditional DLLME using ionic liquids (ILs) was addressed for the extraction of four phthalates in wine, known as ionic liquid dispersive liquid–liquid microextraction (IL-DLLME) [58]. Ionic liquids represent a new group of organic salts that maintain their liquid state at temperatures under 100 °C and possess unique physicochemical properties, such as minimal vapor pressures, strong thermal stability, and excellent solubility for both organic and inorganic substances. In addition to being non-toxic and non-volatile, ILs are also recyclable, making them considered green extraction solvents [105,106].

Xie et al. also applied the IL-DLLME technique for the extraction of four phthalates in edible oils [91]. However, for this matrix, a clean-up step before extraction was necessary. Despite several successful applications in aqueous matrices (water, urine, blood, etc.), DLLME lacks selectivity and encounters serious co-extractant interferences in oily matrices such as olive oil. Thus, Xie applied another technique called QuEChERS [91].

2.1.3. Solid-Phase Microextraction Extraction

Solid-Phase Microextraction (SPME) is an analytical technique that was invented and developed in the 1990s by Pawliszyn and associates to simplify the sample preparation procedure [107].

SPME is a rapid, simple, and effective approach for the adsorption/absorption and desorption of analytes, combining sampling, isolation, and enrichment in a single step without the need for solvents. It employs a needle, typically comprising fused silica, which is externally coated with a liquid polymer or solid sorbent material to extract analytes from a wide range of liquid or solid samples [107,108].

In the SPME technique, the property of the coating material is the most important key to enhancing its extraction efficiency since it relies on establishing the extraction equilibrium of analytes between the fiber coating and the sample based on the polarity of the target analytes. Depending on the fiber, there are two different processes for collecting volatile and non-volatile compounds: direct immersion of the fiber into the liquid sample (DI-SPME) or exposing the fiber to the headspace above the sample (HS-SPME) until equilibrium is reached [109,110].

After the required extraction time, the coated fiber containing the analytes of interest is introduced into a chromatographic system, and the analytes are desorbed. Nowadays, this technique can be automated with an autosampler in a chromatographic system, making the process of extracting, pre-concentrating, and transferring analytes to the chromatographic system an attractive and desirable method [111].

In addition to these advantages, since solvents are typically not used, SPME is considered a green technique with the significant benefit of no secondary contamination occurring during the sample pre-treatment step. Furthermore, a single fiber can be reused hundreds of times [112].

However, it has some limitations, such as the fragility of the fiber and the potential for analyte carryover during analysis if not fully desorbed during the previous injection [110,113].

Successful detection and quantification of phthalates in olive oil and wines using the SPME technique have already been reported. As phthalates are, in general, semi-volatile compounds, the HS-SPME method is preferred over DI-SPME to avoid interactions between the fiber and the sample matrix [114].

In the field of wines, Carrillo et al. compared different fiber coatings to select the most suitable one for phthalate analysis. The researchers investigated the impact of extraction temperature, salting-out effects, and sample volume. Their findings indicated that elevated temperatures promote better extraction results, the optimal sample volume decreases as the fiber's polarity increases, and the quantity of salt required increases with the fiber's polarity [59]. The authors also proposed the use of deuterated phthalates as internal

standards to correct potential errors during sample preparation, avoid matrix effects, and improve the reproducibility of the SPME extraction methodology [36].

In the realm of olive oils, Holadová et al. evaluated four different fiber types: polydimethylsiloxane (PDMS), polyacrylate (PA), carboxen/polydimethylsiloxane (CX/PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB), and tested various solvents as matrix modification agents to facilitate the transfer of some phthalates to the headspace. They also found that temperature and sample agitation are critical points during SPME extraction [115]. Barp et al. utilized the same SPME technique to identify and quantify phthalates in vegetable oils, studying only two different fibers and comparing direct immersion extraction with headspace extraction [80].

Rios et al. applied HS-SPME at high temperatures (250 °C) to analyze phthalates in olive oil [83]. The need to use high temperatures during sample incubation is due to the fact that some compounds, such as DNOP and DNP, have low volatility and do not easily transfer to the headspace like other compounds. However, the use of high temperatures presents challenges related to the durability of the fiber, as degradation can occur. Furthermore, the absorption/adsorption process from SPME is an exothermic process. Hence, high extraction temperatures tend to reduce the extraction efficiency. Moreover, constant monitoring and replacement of fibers when necessary are essential to maintain accurate results [83].

The main benefit of this extraction method is the absence of sample manipulation, thus avoiding potential contaminations from glassware, the environment, solvents, and samples. It is also a fast and cost-effective method compared to conventional cleaning processes, such as LLE and SPE.

2.1.4. Solid-Phase Extraction

Solid-phase extraction (SPE) was first introduced in the 1970s, and due to its effectiveness and versatility, it has become one of the most widely used extraction techniques for isolating, enriching, or cleaning analytes from various matrices [116,117].

In SPE, one or more analytes from a liquid sample are separated by extraction, partitioning, and/or adsorption onto a solid stationary phase. The wide variety of sorbents with different compositions and functional groups available allows for the separation of target analytes from the original matrix, as they have a greater affinity for the sorbent material than for the solvent used. After being retained on the sorbent material, analytes can be eluted and pre-concentrated using an appropriate solvent [118].

This technique allows for concentration factors of up to 500 times, which can be extremely useful for the targeted analysis of low-concentration compounds in real matrices, such as phthalates. As shown in Table 3, several methods using SPE for phthalate extraction in olive oil and wine have been developed. Currently, the most significant interest in scientific research has been in the development of new solid sorbents to achieve higher sensitivity and reliability [118].

In the extraction of six phthalates in wines, Russo et al. used the Carbograph 1 sorbent, which allowed for recoveries between 78% and 105% [64]. Later, Cinelli et al. from the same group used the Amberlite XAD-2 resin for the first time to extract the same phthalates from beverages with a wide alcohol range (10–40%) [63]. This group investigated both breakthrough curves to study the relationship and interactions between the phthalates, eluents, and adsorbents used, as well as the presence of NaCl to improve analyte recovery. XAD2 proved to be more efficient, enabling better recoveries (94–103%) and lower limits of quantification (LOQs) [63,64]

In the field of olive oils, SPE is commonly used after LLE as a clean-up step, using different phases such as PSA, C18, or Florisil (Table 3). However, it is considered a risk to use SPE cartridges because most of them are maunfactured from polyethylene or polypropylene, which can result in the release of phthalates into the adsorbent and potential cross-contamination of the real sample. It is advisable to use glass cartridges or extraction disks [119].

In addition to conventional SPE, other adaptations have been studied, such as the application of molecularly imprinted polymers (MIPs) as SPE sorbents [120]. MIPs are tailor-made polymeric materials designed for a specific analyte. Growing in popularity in the last decade due to advancements in their synthesis that allow for increased molecular recognition, MISPE has already been applied to the extraction of four phthalates in wine. Barciela-Alonso et al. prepared the MIP via precipitation polymerization using DBP phthalate as the template, and the SPE procedure coupled with HPLC/MS proved to be a precise and sensitive method, with recovery factors ranging from 74% to 98% in wines [67]. It was not found in any study describing the use of MIPSE in olive oil, probably because the triglycerides that comprise olive oil are too chemically similar to phthalate esters in order to allow target successful extraction.

Dispersive SPE (d-SPE) is commonly used for clean-up during phthalate extraction. This technique involves dispersive mixing sorbents so that they retain the target analytes present in the analytical solutions. Subsequently, after centrifugation and removal of the supernatant, the analytes are eluted with appropriate solvents. This method was applied as a clean-up step by Bi et al. after LLE extraction to analyze the presence of 15 phthalates in vegetable oil samples, where recoveries ranged from 60.9% to 101.3% for olive oils [37].

2.1.5. QuEChERS

To overcome some of the disadvantages of the traditional LLE method, either coupled or not with clean-up steps, the QuEChERS method emerged. QuEChERS, which stands for Quick, Easy, Cheap, Effective, Robust, and Safe, was first introduced by Anastassiades et al. in 2003. His group used QuEChERS to determine pesticides in fruits and vegetables [121].

This method, which is arguably the most successful development in the analysis of food contaminants in recent years, is a multi-step analytical procedure based on LLE with salting-out and d-SPE. There are five steps involved in the QuEChERS protocol. The procedure begins with the homogenization of the aqueous sample, followed by extraction with acetonitrile. Dehydration with MgSO4 or NaCl is performed to promote the separation of water from the organic solvent (salting-out effect), and then impurities are removed with a variety of sorbents (e.g., primary secondary amine, graphite carbon black, C18). After clean-up, the sample is analyzed using chromatographic techniques [121].

The rapid adoption of this simple and efficient method led to its adaptation for use with other matrices and analytes, including the determination of phthalates in food matrices, such as wine. Fasano et al. applied the QuEChERS method to extract three phthalate esters from wines packaged in laminated plastic-coated cardboard boxes (Tetra Pak). The most contaminated wine contained 9.72 μg/L of DBP [68].

In the case of olive oils, there were three articles mentioning the use of the QuEChERS method [89,91,93]. However, it appears that these authors simply followed a procedure involving LLE or UAE followed by d-SPE. They did not perform one of the main steps of the QuEChERS method: the salting-out extraction step, which promotes an equilibrium between the aqueous phase and the organic phase. This indiscriminate use of the term "QuEChERS," where LLE would be more appropriate, is problematic and unnecessary since it can lead to confusion between the two techniques. Nevertheless, several authors use the QuEChERS method for the determination of contaminants such as pesticides, followed by chromatographic analysis in olive oils [122–124].

2.1.6. Other Extraction/Clean-Up Methods

Although the most commonly used extraction procedures to extract and clean phthalate residues in wine and olive oil have already been mentioned in this review, other analytical approaches are also employed by some researcher teams.

For example, gel permeation chromatography (GPC), first used in the 1960's, is a powerful cleaning method that separates analytes based on molecular size, eluting larger molecules first, followed by smaller ones [125]. GPC is highly recommended for its effectiveness in removing fats and oils and is applicable to a wide range of analytes, such

as pesticides, polyaromatic hydrocarbons (PAHs), and phthalates, to clean extracts from complex samples, such as olive oil and wine [126]. However, this method has multiple disadvantages, such as the need for specialized equipment, which can be extremely expensive for some applications.

Some authors have reported the use of GPC as an additional cleaning step prior to analysis to remove interferences in wine and olive oil samples. For example, Cavaliere et al. used GPC as a cleaning step in a study aimed at determining the content of six phthalates in olive oil without the need for prior LLE or SPE cleaning after GPC [41].

On the other hand, Sun et al. used GPC coupled with Microwave-Assisted Extraction (MAE) and SPE to extract 20 phthalates from vegetable oil samples. The group sought an effective way to extract, clean, and concentrate analytes in the MAE–GPC–SPE method, overcoming lipid and pigment interference and increasing the sensitivity of their method [87]. However, the method involves very tedious and expensive steps, which probably prevents it from being used in routine analysis.

Microwave-assisted extraction is another extraction technique that, as the name suggests, uses microwave energy to heat the solvents in contact with the sample with the aim of transferring the analytes from the matrix into the solvent. It is suitable for routine analyses and allows for a significant reduction in time and solvent consumption, as well as enabling a high sample extraction throughput simultaneously [127].

2.2. Separation and Detection of Phthalates in Wine and Olive Oil

The extraction and cleaning procedures are generally the most critical and challenging aspects in the analysis of phthalates in foods, and both will influence the choice of analytical technique. The physicochemical characteristics of the target analytes and the required sensitivity also determine the suitable instrumental technique for separation, detection, and quantification.

However, establishing separation and detection techniques for phthalates in real samples is a challenge due to matrix interferences. Several traditional analysis techniques are used for the analysis of phthalates in olive oil and wine, but chromatography-based techniques are the most often employed: High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC), or more advanced approaches like GC/MS, GC/MS/MS, GC×GC/MS, UHPLC/MS/MS, and LC/MS, due to their sensitivity, separation, and identification capabilities. Other techniques, such as enzyme-linked immunosorbent assay (ELISA), Raman spectroscopy, IPCR, and FI-CL, have also been employed.

2.2.1. Gas Chromatography

It is undeniable that the most widely used analytical technique for the analysis of phthalates in olive oil and wine is gas chromatography, primarily coupled with mass spectrometry (GC/MS), given the thermal stability and volatile nature of phthalates.

GC is a separation and analysis technique for mixtures of volatile substances, equipped with an injector where the sample is vaporized, followed by a capillary column where the sample is carried by a mobile phase and separated according to volatility and/or polarity (depending on the nature of the stationary phase), and a detector [128].

Considering the trace presence of these contaminants in food matrices, the splitless injection mode is typically selected to achieve high sensitivity levels. However, their high boiling points make the analysis challenging, as they can decompose during injection. To prevent this and improve vaporization efficiency, the injector temperature is set to be similar to the boiling points of the PAEs [45,129].

One solution to avoid these two problems is the use of a programmed temperature vaporizer (PTV) injector, which mitigates discrimination in the injector, analyte decomposition, and increases the amount of sample injected into the column, thus achieving better sensitivity and lower limits of detection (LODs) [130]. Russo et al. used the PTV method for wine sample injection to determine six phthalates, obtaining LODs between 0.2–14 ng/mL [64].

Moreover, not only for aqueous samples, another significant advantage of using the PTV method is related to oily matrices, as this method can mitigate the problem of poor extract clean up, such as in olive oil, thus avoiding system contamination [101]. With PTV and the application of a backflush system, the sample pre-treatment can be reduced to a dilution, minimizing the risk of cross-contamination [74].

As seen in Table 3, the capillary columns used in GC are composed of fused silica, known for their high separation efficiency. The choice of column depends on the nature of the target analytes. Due to the nonpolar nature of phthalates, nonpolar fused silica capillary columns, such as those with 5% phenyl-95% dimethylpolysiloxane phases, are commonly used for separation.

The value of GC for the analysis of phthalates in complex matrices such as olive oil and wine is closely related to the availability of increasingly selective and sensitive mass detectors.

According to most of the research reported in Table 3, the analysis of phthalates is performed using GC coupled with Flame Ionization Detector (FID) or Mass Spectrometry (MS).

However, GC/MS is the most commonly reported technique due to its high sensitivity and specificity, allowing the detection of these contaminants at very low levels. For example, Cinelli et al. quantified six phthalates using GC-FID, which is a cost-effective, readily available, and easy-to-operate method. However, they used GC/MS with an ion trap mass analyzer for confirmation of peak identification [55].

Nowadays, there are various mass analyzers available, such as Single Quadrupole (Q), which works with Selected Ion Monitoring (SIM), and Triple Quadrupole (QqQ), which works with Multiple Reaction Monitoring (MRM). Both modes reduce the need for chromatographic separation and, to some extent, increase sensitivity. The latter mode has become more common due to its improved sensitivity, although some authors have preferred SIM mode, as both modes showed similar sensitivity [131].

As observed in Table 2, GC/MS has been used by several authors to determine phthalates in olive oil and wine, achieving LOQs in the range of μg/L or less.

From an analytical perspective, phthalates are molecules that present several challenges, from the care required throughout the experimental procedure to prevent cross-contamination to their identification and quantification. Since all phthalates are derived from phthalic acid, there is low specificity among them due to all mass spectra being dominated by the base peak m/z = 149, making it very difficult to separate them when they elute at the same retention time in the chromatogram.

Barp et al. determined 10 phthalates in olive oil, including DINP and DIDP, two phthalates regulated by EFSA. In this study, it was shown that DINP and DIDP partially coelute due to being composed of several structural isomers. Therefore, it was necessary to quantify them together as a sum, as suggested by regulations [80].

Coelutions and poor resolutions are commonly reported for these two phthalates, and when quantified, they often have higher limits of quantification than other phthalates that elute as a single peak. Additionally, in the same retention time as these two plasticizers, geranylgeraniol, a compound from the oily matrix, also elutes [80].

When it comes to chromatographic interferences in olive oil, squalene, present in quantities of 2000 to 4000 mg/kg, is considered a potential problem. To separate DEHP and DHP from the overloaded squalene, Fiselier et al. employed an analytical approach of thermal desorption of a diluted oil sample in the GC injector. In summary, the diluted oil is injected directly in splitless mode under desorption conditions for phthalates, with the oil layer retained on the liner wall. Subsequentially, a pre-column with a thin layer of a special material was used before the main analysis in chromatography. At the end of each analysis, a technique called "backflushing" was employed to push out and remove heavy compounds that tend to stay in the precolumn. This was performed through a specific exit designed for this purpose [132].

With that said, it is safe to say that the analysis of phthalates is a significant challenge, and therefore, more powerful separation techniques have been suggested.

In the last decade, one of the major trends in gas chromatography has been the combination of independent techniques to enhance the resolving power. The development and application of multidimensional gas chromatography (MDGC), particularly two-dimensional gas chromatography (GC×GC) coupled with mass spectrometry, has been reported.

GC×GC consists of two orthogonal mechanisms based on the use of two capillary columns coated with different phases, which separate sample constituents in a single analysis. The two columns are connected in series by a modulator interface, allowing small portions (a few seconds) of the first dimension (1D) to elute and be cryo-focused onto the second column (2D). Compounds coeluting in 1D undergo further separation in 2D, often resolving coelutions [133].

Therefore, there is great potential for separation, which makes GC×GC/MS have numerous advantages over conventional one-dimensional GC, such as higher peak capacity, improved resolution of thousands of peaks, acquisition of unique structured chromatograms and mass spectra with high sensitivity, and the ability to reduce matrix-related interferences [134]. Thus, it allows the deconvolution of spectra from coeluted peaks. This technique has already been successfully used for various complex matrices, such as food and environmental samples [135,136].

Arena et al. developed a direct method for the analysis of four phthalates in vegetable oils without any sample preparation, using cryogenic modulation GC×GC coupled to a triple quadrupole. With this analytical technique, it was possible to quantify the four phthalates, including the problematic DINP and DIDP pair, where cases of coelution were spectrally resolved [75].

However, this latter technique is considerably more expensive in terms of both purchase and operation, as well as being more complex.

In summary, without a doubt, GC/MS is a superior technique that measures the mass-to-charge ratio of ions produced in the sample. It is the interface of the technique, typically electron ionization (EI)—a strong ionization method—that is responsible for the extensive fragmentation of molecules like phthalates. As a result, highly reproducible mass spectra of each molecule are obtained using the standard ionization energy of 70 eV, regardless of the chosen chromatographic conditions. Therefore, it is possible to identify compounds by comparing them with thousands of spectra available in standard database libraries.

2.2.2. Liquid Chromatography

As mentioned earlier, GC serves as the primary separation method for the analysis of phthalate esters (PAEs). Nonetheless, liquid chromatography (LC) emerges as a dependable substitute for GC, particularly when assessing isomeric blends like DINP and DIDP, offering enhanced selectivity [137].

In this regard, HPLC has been the most commonly used modality, although ultra-high-performance liquid chromatography (UHPLC) has also been applied (Table 3). In LC, the PAEs are injected and dissolved in a mobile phase, passing through a stationary phase where they are separated and subsequently detected [138].

To achieve adequate chromatographic separation and improve the sensitivity of the method, it is crucial to choose appropriate mobile and stationary phases [138]. Gradient elution is generally applied due to differences in the physicochemical properties of PAEs, and mixtures of ACN/water and MeOH/water have been the most common mobile phases for proper separation of these analytes. Regarding the stationary phases, the approach generally involves using a reverse-phase system, and C18 has by far been the most applied stationary phase for the separation of PAEs in wines and olive oils due to the non-polar nature of these compounds. Shorter-chain columns, like C8, have also been selected.

As for detectors, diode-array (DAD) has sometimes been used for the analysis of phthalates in olive oil and wine, but its identification capability is unsatisfactory when

compared to mass spectrometric detectors. With the significant advancement of the latter, MS/MS has become the most robust approach for the analysis of these analytes, as they are present in very complex matrices with various interferents.

Electrospray ionization (ESI) has been the most commonly reported, and MS analyzers, such as single quadrupole, triple quadrupole, high-resolution time-of-flight, and q-Orbitrap have also been applied, achieving LODs at the μg/L or μg/kg level in all studies.

Hayasaka et al. successfully applied a simple, practical, and robust HPLC/MS/MS method without sample extraction or enrichment for the analysis of nine phthalates in wine, which prevents or significantly reduces the effect of contamination by leaching from laboratory materials. The group used a retention column placed upstream of the injection valve to retain contaminants in the system, avoiding coelutions. Additionally, they used various internal standards, in this case, deuterium-labeled phthalate esters, to avoid quantification issues (matrix effects), obtaining LODs between 1.6–26.6 μg/L [53].

Vavrous et al. applied the same chromatographic technique as the previous group for the determination of eight phthalates in edible oils, including olive oil. The group performed LLE followed by SPE to remove the major matrix components, attempting to minimize sample handling to avoid cross-contamination. They also equipped their analytical system with a contamination trap, as the previous group did, achieving similar LODs of 5.5–110 μg/kg [90].

These studies had the advantage over GC of achieving lower LODs for phthalate isomeric mixtures, such as DINP and DIDP. As mentioned earlier, these compounds are one of the major challenges when it comes to phthalate monitoring and were confirmed to be as common as DEHP, the most abundant phthalate in real matrices. Therefore, these mixtures become promising targets for future efforts in the application of this chromatographic technique.

Frequently, one often handles LC and GC as competing techniques; however, if one considers exploring and combining both techniques (LC–GC), one can obtain the most of both. Thus, one can employ LC to isolate compounds, given its high sampling capacity, and subsequently direct the eluate to GC analysis, where compounds are separated with higher resolution and sensibility, which can be improved by means of mass spectrometry (MS) [139,140].

This technique is typically applied to complex matrices, and the use of LC helps to eliminate or reduce the time-consuming sample preparation step, minimizing the need for manipulation and thereby reducing the risk of compound loss and cross-contamination, which is crucial in the analysis of phthalates.

LC–GC is frequently employed for the analysis of various analytes in both wines and olive oils [141,142]. However, concerning the analysis of phthalates using LC–GC, one can only find applications to water samples [143,144]. To date, to the best of our knowledge, no studies have been found that apply it to the analysis of phthalates in olive oil and wine. Therefore, it would be meaningful to explore this possibility.

2.2.3. Other Analytical Techniques

Although the chromatographic methods mentioned above are suitable and accurate for the analysis of phthalates in olive oils and wines, they typically require laborious, time-consuming, and costly procedures.

Therefore, there is a continuous search for alternative methods that are simple, fast, and cost-effective, preferably allowing for improved LODs and LOQs.

Biochemical tests that apply enzymes or antibodies as identification components have been receiving increasing attention due to their high specificity and sensitivity. Examples include the Enzyme-Linked Immunosorbent Assay (ELISA) and Immuno Polymerase Chain Reaction (iPCR).

The chemiluminescence-based ELISA (icELISA) method was applied for the detection of DBP in wine, achieving an LOD of 64.5 ng/mL and recoveries between 83.5–101.7%. When compared to GC/MS, it obtained a correlation of 0.928 in detecting a real sample [50].

On the other hand, the real-time immuno-PCR (rt–IPCR) method was applied for the detection of DMP, and the results were consistent with those obtained by GC/MS [52].

However, despite their precision and reliability, these methods are highly specific and limit the number of phthalates that can be analyzed at once since they are designed for a single specific target molecule.

Table 3 also shows other methods, such as flow-injection chemiluminescence (FI-CL), Supercritical Fluid Chromatography with Ultraviolet (SFC-UV) detection, and Surface-Enhanced Raman Spectroscopy (SERS) technology combined with chemometrics have also been used to determine phthalates in olive oil and wine. Colorimetric methods are generally more basic and user-friendly, and when combined with nanomaterials, they can provide highly sensitive results due to their selectivity [145].

2.3. The Major Challenge in the Laboratory Analysis of Phthalates

Due to the widespread use of products containing phthalates, these contaminants have become omnipresent everywhere, including analytical laboratories. The low cost of plastic materials has led to their use in various laboratory applications, making them a considerable problem during sampling, sample preparation, extraction, and instrumental analysis. Besides causing contamination issues with blanks, they increase the risk of cross-contamination, leading to background signals that complicate the analysis of real samples.

Nonetheless, it is not solely plastic materials, such as pipette tips or storage containers, that have the potential to contaminate the sample. Various other laboratory components, including solvents, chemical sorbents, water, glassware, and even ambient air and dust within the laboratory, can harbor phthalates [146–149]. DBP and DEHP were the most frequently found contaminants. For example, Fankhauser-Noti et al. detected laboratory air at concentrations of 3 and 2.4 $\mu g/m^3$. It was even estimated that a 1.5 mL autosampler vial contains 10 ng of DBP and 4 ng of DEHP [146]. Phthalates were also found in small amounts in Milli-Q water [148]. In fact, even in high-purity organic solvents used for the extraction and analysis of PAEs in foods, phthalates were found at levels of up to mg/L [147]. The authors illustrated that the primary concern in phthalate analysis does not lie in the analysis process itself but rather in the susceptibility to contamination at various stages of the analytical procedure. Such contamination can potentially result in false positives or overestimation of results.

To avoid such contaminations, different strategies have been adopted by analysts. It is recommended that the analysis of PAEs is conducted in a separate area of the laboratory, preferably one with air filters, and that plastic materials in all procedures be replaced with glass, Teflon, PTFE, aluminum, or stainless steel [150,151]. However, it is known that this is not sufficient, and other measures are still recommended. Starting with glass materials, which should be washed with solvents and heated to 400 °C for several hours [146]. Glass materials that cannot be cleaned by heating should be washed with pure solvents taken from containers to which aluminum oxide (oxidizing agents) has been added [152]. The materials required for analysis, such as sample vials, should, whenever possible, be stored in desiccators containing aluminum oxide and/or covered with aluminum foil [153]. Alternatively, they can be stored in suitable glass or PTFE containers to prevent the adsorption of PAEs from the air.

Checking for the absence of PAEs in gloves and pipette tips is crucial. Caps of vials, extraction cartridges, syringes, filters, and septa should also be checked for the presence of PAEs before the start of the analytical procedure [146].

It is also recommended to avoid personal care products by the analysts. Creams, perfumes, and lotions may contain significant amounts of PAEs.

Finally, to track all possible contamination routes, it is essential to perform analytical blanks for each stage of the analytical procedure simultaneously with the set of samples analyzed, preferably in triplicate [154]. Blanks are expected to be free of PAEs to ensure that no contamination occurs during the procedure. Additionally, if high contamination levels are expected, increasing the number of blanks is recommended [150].

In addition to blanks, another recommended measure in the quantification of phthalates is the use of internal or external standards [154]. These measures are advisable because of the multiple stages involved between sampling and the ultimate analysis. These stages encompass extraction, purification, pre-concentration, transfer, and storage, where target analytes can be lost, such as more volatile phthalates. Thus, the use of internal standards allows for the correction of both the potential loss of target analytes throughout the procedure, as well as variations in the injected volume, detector response, and matrix effects, ensuring greater precision in the analysis.

Therefore, a well-selected internal standard, for example, isotopic ISs, along with blank analysis, is crucial to ensure accuracy and precision in quantifications.

3. Conclusions and Future Perspectives

Phthalates, known to migrate from polymers into food, require strict measures and regulations. First and foremost, the careful selection of materials for both industrial machinery and packaging during food production is essential to reduce the potential risk of migration. Furthermore, specific limits for phthalates in food should be related to already regulated migration limits in food packaging. Currently, only five phthalates (DBP, BBP, DEHP, DINP, and DIDP) are regulated, even though studies indicate the presence of other phthalates in food, some exceeding regulated limits. With the 2023 update to Regulation (EU) 10/2011, specific migration limits were lowered, raising concerns regarding these plasticizers.

The chemical properties of phthalates pose analytical challenges in sample preparation, identification, and quantification. Additionally, detection limits are very low, and cross-contamination is a concerning factor.

Advances in sample preparation techniques, such as SPME and DLLME, align with the principles of "Green Analytical Chemistry," offering simpler, faster methods with reduced solvent usage. However, no technique is considered suitable for a complete analysis of phthalates in complex matrices like olive oil and wine.

The analysis of phthalates in olive oil and wine primarily relies on conventional methods, such as GC and LC coupled with various detectors, with mass spectrometry being the primary choice due to its exceptional capabilities for identification and quantification. Emerging techniques, like GC×GC/MS and LC-GC/MS, show significant potential to enhance phthalate analysis.

It is important to note that, as certain phthalates face restrictions and increased scrutiny from the scientific community, alternative compounds are emerging, such as terephthalates, trimellitates, adipates, and sebacates. However, the migration of these compounds into food and their impact on human health remains uncertain. It is imperative to subject these alternatives to epidemiological studies to assess their effects on health and explore potential analytical methods for future controls.

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4.3 Analysis of Phthalates

4.3.1 Cross-Contamination in the Laboratory: Preventive Measures

The review article discussed various techniques for the extraction of phthalates in olive oils. However, a significant and recurring issue with these methods is cross-contamination in the laboratory, which directly impacts the reliability of the analytical results. To achieve accurate analysis of these contaminants, it is crucial to thoroughly evaluate all materials and solvents used during the stages of analytical preparation, extraction, and injection into the equipment. Phthalates and other plasticizers are ubiquitous in laboratory environments, posing a high risk of contaminating samples and reagents.

One of the initial studies conducted in this chapter focused on testing common laboratory materials, such as volumetric flask stoppers, laboratory spray nozzles, vial septa, vials and their caps, pipette tips, syringe filters, test tubes, among others. The results revealed that all analyzed materials contained plasticizers. This finding underscores the pervasive presence of plasticizers and highlights the need for stringent precautions to prevent their unintentional introduction during analytical procedures. Therefore, controlling cross-contamination is an essential step in ensuring the accuracy and reliability of the quantification of these compounds in olive oils.

To address this issue, several preventive measures were implemented throughout the study:

- A glass surface was placed on the laboratory bench to prevent contact with potentially contaminated surfaces;
- Glassware was used whenever possible;
- All caps of volumetric flasks were replaced with glass caps and caps of the vials used were made of phthalate-free materials;
- For the collection of real samples, glass containers with bamboo lids were used.
- Glassware and other lab materials were meticulously washed, rinsed, and stored at 100°C before use;
- Phthalate-free chemical solvents were selected and analyzed daily for the presence of plasticizers;
- The chromatographic system was routinely checked for plasticizers by performing three blank injections at the start, during, and at the end of analyses.

These measures collectively ensured that cross-contamination was minimized, thereby enhancing the reliability of the results obtained during the quantification of phthalates in olive oils.

4.3.2 Preliminary Study of Production Line Materials

As highlighted in the review article, the sources of olive oil contamination by plasticizers extend far beyond the containers used for storage or commercial packaging. The production line itself constitutes a significant source of contamination, involving materials such as olive harvesting nets, plastic buckets and bags for temporary olive storage, conveyor belts, hoses in production lines, and storage containers or tanks in processing facilities, among others.

In our laboratory, some of these materials were subjected to preliminary analyses for eight phthalates by GC-TOFMS, and phthalates were detected in almost all of them (Table 5). This finding highlights the pervasive nature of contamination throughout the production process.

Table 5. Preliminary results of the analysis of eight phthalates in hoses, O-rings, nets, and slabs using GC-TOFMS. The limit of quantification for the preliminary method for all phthalates was 0.060 mg/kg, except for DIDP and DINP, which was 0.600 mg/kg. *Phthalates are present in very high concentrations, exceeding the quantification range. DMP: dimethyl phthalate; DIBP: diisobutyl phthalate; DBP: dibutyl phthalate; BBP: benzyl butyl phthalate; DEHP: bis(2-ethylhexyl) phthalate; DOP: dioctyl phthalate; DINP: diisononyl phthalate; DIDP: diisodecyl phthalate.

Production Material	DMP	DIBP	DBP	BBP	DEHP	DOP	DINP	DIDP
	<LOQ	<LOQ	<LOQ	<LOQ	**0,064**	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	**0,598**	<LOQ	**1,001**	<LOQ
	*	*	*	*	*	*	*	*
	<LOQ	<LOQ	<LOQ	<LOQ	**0,150**	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	**0,410**	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	**0,071**	<LOQ	<LOQ	<LOQ

	<LOQ	<LOQ	<LOQ	<LOQ	**0,170**	<LOQ	**0,480**	<LOQ
	<LOQ	**0,251**	**0,292**	**0,061**	**2,045**	*	*	*

To better understand the critical contamination points along the olive oil production line, samples were collected from three production lines located in different regions of Portugal—North, Central, and South. This study culminated in the publication of the article titled *"Analysis of Plasticizers Contamination Throughout Olive Oil Production"* which explored contamination across these three production lines in detail. A total of 23 phthalates and 9 phthalate substitutes were analyzed, providing valuable insights into the extent and sources of contamination.

4.4 Article

"Analysis of Plasticizers Contamination Throughout Olive Oil Production"

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Analysis of Plasticizer Contamination Throughout Olive Oil Production

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Abstract: This study monitored the contamination of 32 plasticizers in olive oil throughout the production and storage process. Samples were collected at different stages of production from three olive oil production lines in distinct regions of Portugal and analyzed for 23 phthalates and 9 phthalates substitutes to identify contamination sources. The developed analytical method employed liquid–liquid extraction with hexane/methanol (1:4, *v*/*v*), followed by centrifugation, extract removal, and freezing as a clean-up step. Analysis was conducted using gas chromatography tandem mass spectrometry (GC-MS/MS), with detection limits ranging from 0.001 to 0.103 mg/kg. The results revealed that plasticizer concentrations progressively increased at each stage of the production process, although unprocessed olives also contained contaminants. Di-isononyl phthalate (DINP) was the most prevalent compound, but all phthalates regulated by the European Union for food contact materials were detected, as well as some unregulated plasticizers. In a few packaged olive oils, DINP concentrations exceeded the specific migration limits established by European regulations. Samples stored in glass and plastic bottles showed no significant differences in plasticizer concentrations after six months of storage. However, higher concentrations were observed in plastic-packaged samples after 18 months of storage. Our findings indicate that the primary source of plasticizer contamination in olive oil originates from the production process itself, except for prolonged storage in plastic bottles, which should be avoided.

Keywords: olive oil; plasticizers; phthalates; contamination; production line; analysis

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1. Introduction

Olive oil, rich in antioxidants such as phenols and tocopherols, is essential in the Mediterranean diet and widely consumed worldwide, with a consumption of approximately three million tons per year [1,2]. Its processing occurs mechanically and/or physically, ideally without altering its chemical composition.

Initially, the materials used in the production and storage of olive oil included wood, glass, metal, and clay, but industrial evolution led to the use of polymers, such as polyethylene and polypropylene, especially in packaging, due to their advantages in cost, recyclability, and durability [3–7].

The versatility of polymers led to the development of additives to enhance their properties, such as phthalates, which are used to make the material more flexible or rigid, depending on the need. These plasticizers are widely used in the food and engineering

industries in products ranging from food packaging to pipes, tubes, and mats [8–11]. Despite their benefits for polymer durability and functionality, phthalates have low solubility in water and high solubility in lipid matrices, such as olive oil, which may result in their migration into food products [9,10].

Several studies have indicated that these additives may pose health risks, leading to extensive research into the toxicity of phthalates [12–22]. As a result, several countries have intervened and regulated exposure to these plasticizers.

In Europe, food safety concerns have led to the imposition of strict requirements for food contact materials (FCMs), as specified in Regulation (EC) No. 1935/2004 [23]. This regulation prohibits materials from transferring substances to food in quantities that could harm human health or adversely affect its organoleptic properties. Specific migration limits (SMLs) have been defined for five permitted phthalates (DEHP, BBP, DBP, DINP, and DIDP, see Table 1) in Annex I of Regulation (EU) No. 10/2011, based on toxicological assessments [24]. Additionally, in 2019, the European Food Safety Authority (EFSA) established tolerable daily intake for four of these phthalates, ranging from 50 µg/kg for DBP, BBP, DEHP, and DINP to 150 µg/kg for DIDP [25].

Later, in 2023, Regulation (EU) 2023/1442 updated the migration limits to strengthen consumer protection further (Table 1) [26].

Table 1. Phthalates permitted in food contact materials by regulation (EU) 2023/1442, their SML and intended uses.

Substance	Regulation (EU) 2023/1442 Amending Annex I to Regulation (EU) 10/2011	Only to Be Used as:
Dibutyl Phthalate (DBP)	SML: 0.12 mg/kg Total SML group restriction no. 32: 60 mg/kg Total SML group restriction no. 36: 0.6 mg/kg	(a) Plasticizer in repeated use materials and articles contacting non-fatty foods; (b) Technical support agent in polyolefins in concentrations up to 0.05% (*w*/*w*) in the final product.
Benzyl Butyl Phthalate (BBP)	SML: 6.0 mg/kg Total SML group restriction no. 32: 60 mg/kg Total SML group restriction no. 36: 0.6 mg/kg	(a) Plasticizer in repeated use materials and articles; (b) Plasticizer in single-use materials and articles contacting non-fatty foods except for infant formula and follow-on formula; (c) Technical support agent in concentrations up to 0.1% (*w*/*w*) in the final product.
Di(2-Ethylhexyl) Phthalate (DEHP)	SML: 0.6 mg/kg Total SML group restriction no. 32: 60 mg/kg Total SML group restriction no. 36: 0.6 mg/kg	(a) Plasticizer in repeated use materials and articles contacting non-fatty foods; (b) Technical support agent in concentrations up to 0.1% (*w*/*w*) in the final product.
Di-isononyl Phthalate and Di-isodecyl Phthalate (DINP and DIDP)	Total SML group restriction no. 26: 1.8 mg/kg (sum of DINP and DIDP) Total SML group restriction no. 32: 60 mg/kg Not to be used in combination with FCM substances DBP, BBP, DEHP, and DIBP.	(a) Plasticizer in repeated use materials and articles; (b) Plasticizer in single-use materials and articles contacting non-fatty foods except for infant formula and follow-on formula; (c) technical support agent in concentrations up to 0.1% (*w*/*w*) in the final product.

Group restriction no. 26 corresponds to the sum of DINP e DIDP; Group restriction no. 36 corresponds to the sum of DBP, DIBP, BBP, and DEHP expressed as DEHP equivalents using the following equation: DBP × 5 + DIBP × 4 + BBP × 0.1 + DEHP × 1; Group restriction no. 32 corresponds to the sum of DBP BBP DEHP DIBP and some plasticizing substances like adipates, sebacates, and terephthalates, among others. DIBP is not listed as an authorized substance; however, it may occur alongside other phthalates as a result of its use as a polymerization aid, and therefore, it is included in group restrictions.

Currently, there are no specific regulations defining the permissible levels of phthalates in food. Therefore, even though migration limits are monitored in packaging, it is essential

to identify the sources of these plasticizers migrating into food. Detecting contamination may indicate that the food has come into contact with unsuitable materials during the production process.

For olive oil, research indicates that phthalates may be introduced both during the production process and during the treatment of the olives. This is because these compounds are commonly found in materials such as harvesting nets, pipes, tanks, and various other plastic components. [27,28]. Additionally, storage in synthetic corks and plastic containers can contribute to this contamination. Even the drinking water used for irrigation or washing production materials may contain these plasticizers [29]. Thus, controlling and studying the use of polymers and additives in the olive oil production chain is essential to ensure food safety and consumer health.

Lastly, it is important to note that due to the restriction on the use of certain phthalates and the ongoing pressure from the scientific community, alternative compounds to these plasticizers are emerging, such as terephthalates, trimellitates, adipates, and sebacates, among others [30–34]. Some of these are authorized for use in the manufacture of plastic materials intended to come into contact with food and are included in the Restriction Group No. 32 of Regulation (EU) 2023/1442 (Table 1) [26]. However, the migration of these substances into food and their implications for human health are still not well understood. This highlights the need for toxicological studies to examine their impact on human health, as well as further analytical exploration for potential future regulatory controls [33,35–37].

Therefore, there is an urgent need to develop analytical methods that allow the identification and quantification of phthalates and phthalate substitutes at low concentrations throughout the olive oil production line [28,38].

The analysis of plasticizers in olive oil presents challenges due to the low concentrations of these compounds and the interference of the lipophilic matrix, which requires methods with adequate clean-up/separation and low detection limits [39–41]. It is essential to avoid contamination during laboratory handling, given the omnipresence of plasticizers in plastic materials. To ensure data reliability, stringent control measures are necessary [42–45].

Due to the complexity of the matrix, samples require prior preparation before analysis, typically by gas chromatography (GC) or liquid chromatography (LC), with mass spectrometry (MS) as the detection method. Other techniques, such as UV spectrophotometry, Raman spectroscopy, and chemiluminescence, are also employed [38].

To optimize the recovery of plasticizers and minimize interferences, different extraction and pre-treatment approaches are recommended, such as liquid–liquid extraction (LLE), solid-phase microextraction (SPME), solid-phase extraction (SPE), and the QuEChERS method, among others. These methods enable more accurate quantification of plasticizers in complex food matrices [38].

The objective of this study was to investigate contamination by 23 phthalates and 7 phthalate substitutes in olive oil throughout the production process and in packaged olive oil to ascertain where plasticizer contamination was originating. To this end, several samples were taken at different stages of the production process from three olive oil production lines. Plasticizer determination was achieved using a simple, fast, and reliable analytical method that combines LLE extraction followed by freezing. This method was employed for efficient extraction and clean-up, with reduced solvent consumption, minimizing cross-contamination from laboratory materials in order to lower background contamination levels in the analytical procedure. Separation and detection were carried out by GC-MS/MS, without the need for pre-concentration steps.

2. Results and Discussion

2.1. Analytical Method Development

For sample analysis, we intended to develop a robust method that reached low detection limits whilst also minimizing the amount of oil in the final extract. Furthermore, the entire method should be performed without recourse to plastic material in an attempt

to avoid contamination. Several methods have already been developed for the analysis of phthalates in olive oil [38]. Most methods employ either a liquid–liquid extraction, often with acetonitrile and followed by some type of clean-up such as dispersive solid-phase microextraction (d-SPE), or they simply dilute the olive oil with hexane and inject it ("dilute-and-shoot"). Although liquid–liquid extraction with acetonitrile has shown adequate results, the need for extensive clean-up presents an extra step and a possible source of contamination (plastic Eppendorf tubes commonly used in d-SPE are a problem, for example). Furthermore, acetonitrile has a relatively high boiling point and thus is not amiable to pre-concentration techniques such as solvent-drying or programmed-temperature volatilization, especially when analyzing low boiling point phthalates. Simple "dilute-and-shoot" with hexane is an incredibly simple technique that greatly reduces the possibility of laboratory contamination but often requires specialized GC equipment such as pre-columns and frequent inlet liner changes due to the introduction of waxes and other low-volatility components of olive oil.

The original method that was adapted used an extraction with pentane/acetone, followed by centrifugation and removal of the supernatant. After experimentation with different solvents, it was found that hexane/methanol (1:4 v/v) was a better mixture, both because pentane was too volatile for quantitative work and methanol provided a better phase separation. The hexane reduced the extraction solvent's polarity since only methanol was found not to adequately extract most plasticizers. The original method used 3 mL of extraction solvent twice for 0.5 g of olive oil, but we found 2 mL to be ideal, as 1 mL was operationally difficult to remove after centrifugation, and 3 mL had a lower concentration factor. After injection of an extract into a GC-FID with a high-temperature DB-5MS column, it was found that many high-boiling point compounds had been extracted; thus, a simple clean-up method was devised by freezing the samples overnight and then removing the liquid phase. In most frozen samples, a significant amount of solid precipitate was found.

Matrix-induced response enhancement for olive-oil extracts was quite significant. For some compounds, calculated plasticizer concentrations of a spiked blank using a calibration in pure hexane/methanol (1:4 v/v) were over twice the actual spiked concentration. Thus, calibrations were performed by spiking the blank oil and then performing the extraction. Simple matrix-matched calibration (by extracting the blank and spiking just before injection) would have adequately corrected for matrix effects but not for recovery. Since excellent repeatability was obtained for extraction triplicates of every spiked concentration (the highest being 9.6% RSD for DMP at 343 ng/g, and commonly between 1–5% RSD), this calibration method proved successful and more accurate. Sunflower oil was used as a surrogate matrix instead of olive oil because, during initial testing, it was found that all olive oils analyzed, including an olive oil analytical standard, contained significant amounts of plasticizers. The sunflower oil in question was selected because it was the only oil among several tested that showed no appreciable contamination by the evaluated plasticizers. Moreover, it mimics the oily nature of olive oil as well as most of its constituents, although in distinct proportions. Indeed, sunflower oil is similar to olive oil in terms of lipid composition and physical characteristics. Since calibration without some form of matrix-matching would significantly compromise the method, the choice of sunflower oil was, therefore, the best alternative available to ensure accuracy in method calibration and validation.

2.2. Olive Oil Production and Plasticizer Contamination

Olive oil is present as small droplets within the vacuoles of mesocarp cells in olive fruits. It is also found, in smaller proportions, within the colloidal system of the cell cytoplasm and, in even smaller amounts, in the epicarp and the endosperm [46].

To obtain olive oil, several individual steps are required. Figure 1 shows a simplified diagram of olive oil production, as well as the different intermediate products obtained, from olives to packaged olive oil.

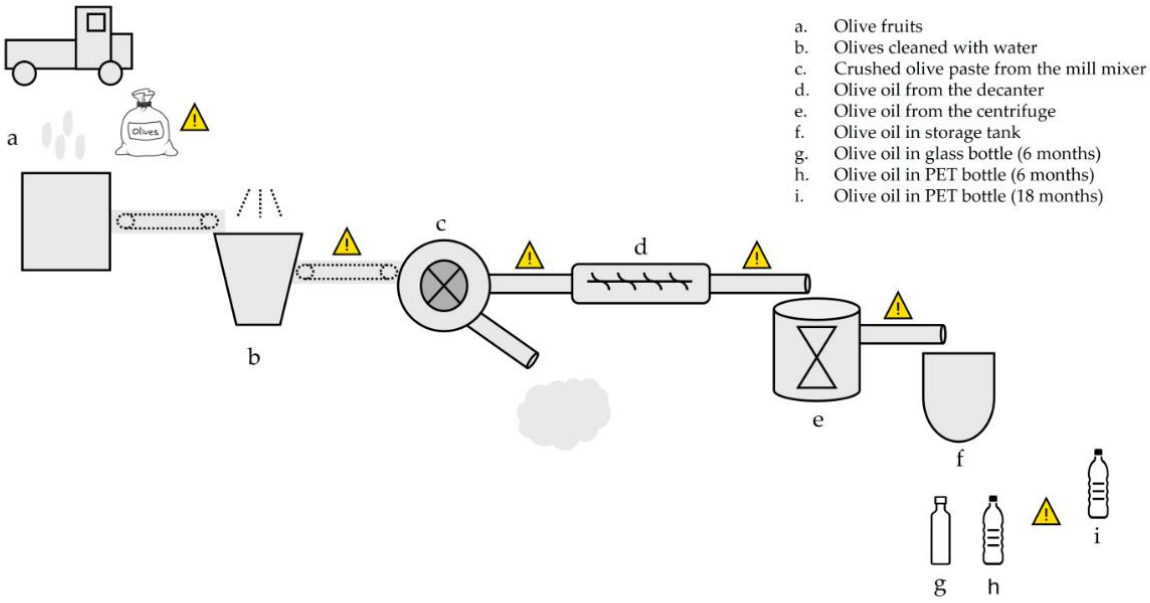

Figure 1. Diagram of olive oil production. The letters represent the samples taken from each step of the production process. Samples g and h were analyzed 6 months after bottling, whereas sample i was analyzed 18 months after bottling. Olive oil production follows from sample a to f. The warning symbol represents critical points of contamination during olive oil production, such as plastic bags, conveyor belts, tubes and hoses, and storage containers.

After harvesting, the olives are transported in baskets, containers, or plastic bags (sample a). Once they arrive at the mill, leaves, branches, and other foreign materials must be removed, followed by a thorough washing with clean water to eliminate impurities that could damage the equipment or compromise the final product's quality (sample b). For example, the presence of leaves can impart a bitter taste to the oil.

Next, the olives are transported via conveyor belts to the next stage: crushing and malaxation (sample c). This process, carried out in the mill, aims to crush and rupture the pulp cells to release the oil stored in the vacuoles. After crushing, the resulting paste undergoes malaxation, a slow and continuous mixing process that promotes the coalescence of oil droplets, thereby increasing extraction efficiency.

After malaxation, the olive paste mainly consists of oil, small fragments of olive pits, water, and cellular residues. The next step is separating the oil from other by-products. This involves extracting the liquid phase (the mixed oil or "olive must") from the solid phase (pomace). The oil is then transferred through hoses to a decanter, where suspended particles are removed, allowing for an initial purification (sample d). Finally, the oil undergoes a final separation in a centrifuge to remove water residues and any other unwanted substances, resulting in a product ready for consumption or storage (sample e).

After processing, the olive oil is typically stored in stainless steel tanks (sample f), which protect the product from oxidation and preserve its organoleptic characteristics. Subsequently, the oil is packaged in glass bottles (sample g), which provide excellent protection against light and external contaminants, or in polyethylene terephthalate (PET) bottles (sample h), a lighter and more economical alternative often used for oils intended for quick consumption. These containers are then prepared for commercialization, ensuring the product reaches consumers with its quality intact.

In the past, most of the utensils and equipment used in olive oil production were made from conventional materials such as stone, ceramic, fabric, glass, and wood. Nowadays, these materials have largely been replaced by large machines that incorporate various

types of plastics in their composition. Examples include plastic bags and/or rigid baskets used in olive harvesting, conveyor belt bases, hoses for transporting the product along the production line, tanks, sealing rings (O-rings), unions, and sealing plugs, among others. Even in storage, stainless steel tanks are commonly used, but plastic tanks are also employed. Likewise, in the final packaging stage, plastic bottles are often used instead of glass.

Thus, the contamination of olive oil with plasticizers added to plastics can occur at various stages of the production chain, with packaging contact being, in most cases, only the final stage. This means that packaging may not necessarily be the primary source of plasticizers found in olive oil.

The contamination occurs because plasticizers do not chemically bind to the polymer matrix, which allows them to migrate over time due to factors such as exposure, increased temperature, and mechanical stress, among others [47].

To determine the sources of plasticizer contamination, samples from different stages across three olive oil production lines in Portugal, as well as samples of packaged and stored olive oil (Figure 1), were analyzed using the previously described analytical method.

The quantities of all plasticizers analyzed and detected in the samples are reported in Table S1 of the Supplementary Material. Table 2 summarizes the data specifically for the concentrations of DIBP, DBP, BBP, DEHP, and the sum of DINP and DIDP, selected based on the European Commission Regulation (2023/1442). Additionally, the table includes the total sum of all plasticizers involved in the study, as defined under Restriction Group No. 32 of the same regulation, which establishes a specific migration limit of 60 mg/kg for the combined levels of DBP, BBP, DEHP, DIBP, and other plasticizing substances. For plasticizers found under the quantification limit (<LOQ), this value was added to the sum to obtain a "worst-case scenario".

Table 2. Concentration (mg/kg) of phthalates regulated by Regulation (EU) 2023/1442 and the sum of the 32 plasticizers studied in samples collected from the three production lines, expressed as average ± standard deviation. For the sums of analytes, the standard deviation was calculated by the square root of the sum of variances. Bold and underlined values indicate those exceeding the specific migration limits defined. n. a.—not analyzed.

	Samples of Production Line	DIBP	DBP	BBP	DEHP	Sum of DINP and DIDP	Sum of 32 Plasticizers
NORTH	a. Olive fruits in plastic bag	0.011 ± 0.004	<LOD	<LOD	<LOD	<LOD	1.987 ± 0.571
	b. Olives cleaned with water	0.008 ± 0.001	<LOD	<LOD	<LOD	<LOD	0.971 ± 0.133
	c. Crushed olive paste from the mill mixer	n. a.					
	d. Olive oil from the decanter	<LOD	<LOQ	<LOQ	<LOD	<LOD	1.253 ± 0.100
	e. Olive oil from the centrifuge	0.007 ± 0.002	<LOQ	<LOQ	<LOD	<LOD	1.277 ± 0.066
	f. Olive oil in storage tank	<LOD	<LOD	<LOQ	<LOD	0.103 ± 0.061	1.522 ± 0.071
	g. Olive oil in glass bottle (6 months)	<LOD	<LOD	<LOQ	<LOD	1.278 ± 0.161	8.277 ± 0.752
	h. Olive oil in PET bottle (6 months)	0.019 ± 0.008	<LOQ	<LOQ	<LOD	**3.527 ± 0.214**	11.946 ± 1.028
	i. Olive oil in PET bottle (18 months)	0.028 ± 0.009	**0.127 ± 0.007**	0.006 ± 0.001	0.454 ± 0.013	**6.000 ± 0.203**	14.751 ± 0.506
CENTRE	a. Olive fruits in plastic bag	n. a.					
	b. Olives cleaned with water	0.019 ± 0.010	0.073 ± 0.004	<LOD	0.076 ± 0.007	0.146 ± 0.056	5.377 ± 0.289
	c. Crushed olive paste from the mill mixer	0.052 ± 0.010	0.085 ± 0.004	<LOD	<LOD	0.431 ± 0.145	6.372 ± 0.359
	d. Olive oil from the decanter	0.061 ± 0.007	0.083 ± 0.012	<LOD	<LOD	0.318 ± 0.065	7.615 ± 0.086
	e. Olive oil from the centrifuge	0.079 ± 0.013	0.082 ± 0.007	<LOD	<LOD	0.625 ± 0.309	7.780 ± 0.888
	f. Olive oil in storage tank	0.093 ± 0.008	0.092 ± 0.002	<LOQ	<LOD	1.211 ± 0.172	9.821 ± 0.331
	g. Olive oil in glass bottle (6 months)	n. a.					
	h. Olive oil in PET bottle (6 months)	0.072 ± 0.007	0.096 ± 0.009	<LOQ	<LOD	**3.528 ± 0.323**	16.224 ± 0.807
	i. Olive oil in PET bottle (18 months)	n. a.					
SOUTH	a. Olive fruits in plastic bag	n. a.					
	b. Olives cleaned with water	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c. Crushed olive paste from the mill mixer	0.013 ± 0.006	<LOQ	<LOD	<LOD	<LOQ	4.385 ± 0.330
	d. Olive oil from the decanter	0.010 ± 0.008	<LOQ	<LOD	<LOD	0.103 ± 0.023	6.285 ± 0.271
	e. Olive oil from the centrifuge	0.010 ± 0.002	<LOD	<LOD	<LOD	0.156 ± 0.015	6.429 ± 0.212
	f. Olive oil in storage tank	<LOQ	<LOD	<LOD	<LOD	0.264 ± 0.064	6.621 ± 0.200
	g. Olive oil in glass bottle (6 months)	<LOQ	<LOQ	<LOD	0.037 ± 0.003	**4.396 ± 0.156**	5.590 ± 0.215
	h. Olive oil in PET bottle (6 months)	<LOQ	<LOQ	<LOD	0.078 ± 0.009	**5.112 ± 0.228**	14.595 ± 0.262
	i. Olive oil in PET bottle (18 months)	<LOD	0.038 ± 0.002	<LOD	0.095 ± 0.001	**9.393 ± 0.580**	24.991 ± 0.786

It is important to emphasize that this regulation pertains only to migration limits for materials in contact with food and does not define the allowable limits of these substances in the food itself.

All regulated phthalates were detected at least once along the production lines. BBP was only found in two production lines and only in a single sample from northern Portugal above LOQ. DBP, on the other hand, was primarily detected in the Central production line. But it was in the North line that the concentration of DBP in olive oil packaged in PET for 18 months slightly exceeded the specific migration limit established by European regulations. DEHP, however, was almost always below the LOD across all production lines. DIBP was detected in all lines but always at concentrations below 0.093 mg/kg. DINP and DIDP stood out as the main contributors to the increase in plasticizers along the production lines, with concentrations exceeding the specific migration limits established by Regulation (EU) 2023/1442 for the sum of these two phthalates (1.8 mg/kg). DINP, known for replacing DEHP in many industrial applications, was the most abundant compound overall (see Table S1 in the Supplementary Material) [48].

Similar results were reported by Nanni et al., who investigated 172 samples of vegetable oils marketed in Italy, including olive oil. In their study, DINP was also identified as the plasticizer present at the highest levels in olive oils, with an average concentration of 1.7 mg/kg in extra virgin olive oils and 2.9 mg/kg in regular olive oils [49]. Likewise, Pereira et al. detected DINP in European olive oil samples, reporting it as one of the phthalates with the highest concentrations. Their study found an average DINP concentration of 1.5 mg/kg across samples, with a maximum value of 6.29 mg/kg [50]. Similarly, Arena et al. observed comparable results, with DINP concentrations ranging from 2.4 to 7.60 mg/kg in extra virgin olive oils [51].

Regarding the sum of the 32 plasticizers analyzed, no sample exceeded the specific migration limits established by Regulation (EU) for Restriction Group No. 32, which stipulates that the sum of DBP, BBP, DEHP, DIBP, and other plasticizing substances such as adipates, sebacates, and terephthalates, among others, must not exceed 60 mg/kg.

In addition to the regulated phthalates, other phthalates and plasticizers contributed significantly to the total values observed, particularly in packaged olive oils. Among these, the most notable were DMEP, with concentrations ranging from 0.969 to 4.342 mg/kg; DPP, between 0.005 and 9.818 mg/kg; DEHT, with values between 0.153 and 8.538 mg/kg (see Table S1 in the Supplementary Material).

Although these compounds are not specifically regulated in the European Union's table of SMLs for phthalates, they represent a significant contribution to the total load of plasticizers detected. DMEP is widely used as a solvent and plasticizer, particularly in paints and resins. Despite its relatively low toxicity compared to other phthalates, its presence in food warrants attention due to potential migration from contact materials [52]. DPP, though less studied, is used in industrial applications and exhibits low volatility, which may favor its accumulation [53]. Meanwhile, DEHT is often employed as an alternative to more toxic phthalates and is considered a low-risk plasticizer in food-related applications [54]. However, recent studies suggest that even plasticizers deemed safe may pose potential long-term risks due to cumulative exposure [55].

Additionally, it is crucial to note that the total plasticizer values reported in this study reflect only the 32 compounds analyzed, while hundreds of plasticizers are currently available in the market, whose presence and impact on food remain underexplored.

These findings highlight the importance of monitoring not only the regulated plasticizers but also widely used substitutes, ensuring a comprehensive assessment of the human health risks associated with their presence in food.

The three olive oil production lines from northern, central, and southern Portugal exhibited distinct patterns regarding the presence of plasticizers, making it difficult to identify the main sources of contamination. In the Northern production line, olives were analyzed before cleaning, and it was observed that those transported in reusable plastic bags (Figure 2) were already contaminated upon arrival, albeit at low levels. After the olives

were cleaned with water, a reduction in these contaminants was observed, suggesting that washing partially removes plasticizer particles originating from the bags.

(**a**) (**b**)

Figure 2. Plastic bags used for olive transport in the northern production line. Photo (**a**) shows bags filled with olives and photo (**b**) shows empty bags and containers used to transport the olives.

In the Central line, the levels of plasticizer contamination in the olives were higher from the start than in the Northern and Southern lines. Interestingly, in the Southern line, no plasticizers were quantified in the unprocessed olives. Nevertheless, contamination accumulated throughout the production process, similar to what was observed in the Central line. On the other hand, the Northern line appeared to contribute the least to olive oil contamination up to the storage stage.

Overall, a progressive increase in plasticizer contamination was observed along the production lines (Figure 3).

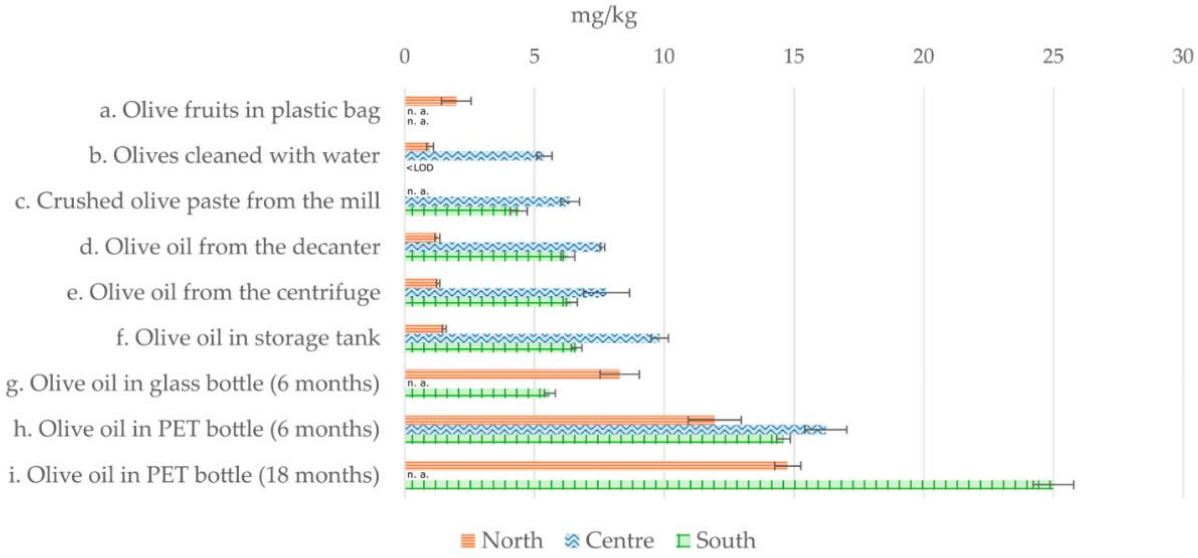

Figure 3. Graphical representation of the sum of the 32 plasticizers analyzed across the three production lines. n. a.—not analyzed.

This increase may be associated with the equipment, tools, and containers used during production. However, the most significant increment in contamination was observed during storage and packaging. During sample collection in all production lines, it was noted that the crushed olive paste and olive oil were transported mainly through stainless steel tubes between the mill mixer, the decanter, and the centrifuge, as well as between the centrifuge and the storage tanks (which were made of either stainless steel or rigid plastic).

However, during the bottling process (in glass or plastic bottles), the use of plastic hoses to transport the olive oil was identified (Figure 4).

(**a**) (**b**)

Figure 4. Production line equipped with stainless steel tubes versus plastic hoses. Photo (**a**) shows steel tubing, whereas (**b**) shows plastic hoses.

This detail suggests that the primary contamination source is not directly related to the material of bottles but rather to the hoses used during transportation to the packaging stage.

Another relevant observation was made when comparing olive oil packaged in plastic bottles and stored for 6 months versus 18 months in the Northern and Southern lines. A considerable increase in plasticizer concentrations was observed over time, likely due to prolonged contact between the olive oil and the packaging, resulting in greater migration of plasticizer compounds. The Southern line showed a more significant increase, suggesting differences in the PET composition used across the production lines. These results emphasize the importance of carefully selecting the appropriate packaging material to minimize contamination during storage, as well as not prioritizing plastic bottles for long-term storage.

2.3. Off-the-Shelf Olive Oil

In the previously investigated production lines, only two of them used glass bottles as packaging material in addition to PET. Although an increase in plasticizer concentrations was observed in both lines during storage, their behaviors differed. In the production line in the North, the difference in plasticizer concentrations between olive oil packaged in glass and olive oil packaged in PET was smaller compared to the production line in the South. This observation highlighted the need for a more detailed analysis of the impact of different packaging materials, considering their potential influence on plasticizer contamination levels.

In order to test these findings, samples of olive oil from the same brand, packaged in glass bottles and PET containers, were purchased from a local supermarket. Sample identification and the results of the analyzed plasticizers are presented in Table 3.

Table 3. Concentration (mg/kg) of phthalates regulated by Regulation (EU) 2023/1442 and the sum of the 32 plasticizers studied in olive oils purchased from local supermarket. Expressed as average ± standard deviation. For the sums of analytes, the standard deviation was calculated by the square root of the sum of variances. Bold and underlined values indicate those exceeding the specific migration limits defined.

		DIBP	DBP	BBP	DEHP	Sum of DINP and DIDP	Sum of 32 Plasticizers
Olive Oil 1	GLASS	0.014 ± 0.009	<LOD	<LOD	<LOD	1.598 ± 0.145	2.688 ± 0.146
	PET	<LOQ	<LOD	<LOD	<LOD	**1.807 ± 0.199**	4.109 ± 0.204
Olive Oil 2	GLASS	<LOQ	0.020 ± 0.005	<LOD	<LOD	0.395 ± 0.076	1.168 ± 0.116
	PET	0.018 ± 0.005	<LOQ	<LOD	<LOD	0.716 ± 0.112	1.516 ± 0.117
Olive Oil 3	GLASS	<LOD	<LOD	<LOD	<LOD	**3.310 ± 0.053**	5.763 ± 0.122
	PET	<LOQ	<LOD	<LOQ	<LOD	**3.245 ± 0.050**	5.847 ± 0.131
Olive Oil 4	GLASS	<LOD	<LOD	<LOQ	**0.656 ± 0.006**	1.244 ± 0.086	3.299 ± 0.093
Olive Oil 5	GLASS	0.02 ± 0.002	<LOD	<LOD	<LOD	**5.976 ± 0.389**	7.145 ± 0.391
Olive Oil 6	CAN	<LOD	<LOD	<LOD	0.082 ± 0.013	<LOD	0.882 ± 0.056

A slight increase in plasticizer levels was observed in the samples packaged in PET compared to those in glass. Among the three analyzed samples, plasticizer levels varied significantly, with olive oil two exhibiting the lowest plasticizer concentrations compared to the other two olive oils. Consistent with the results observed in the production lines, the sum of DINP and DIDP represented the main contribution to plasticizer contamination, with concentrations exceeding the specific migration limits established by Regulation (EU) 2023/1442.

Additionally, three other oils were analyzed, two packaged in glass and one in a metal can, which showed plasticizer levels within different ranges from the previously analyzed samples. The oil packaged in a metal can exhibited the lowest plasticizer concentrations among all the samples studied, but it was also the only one not produced or packaged in Portugal.

Despite the differences observed between oils packaged in glass and PET, these were minor and consistent with findings from other published studies [47,49,50,56]. For instance, Bi et al. studied edible oils, including olive oil, in the United States and found no significant differences in plasticizer concentrations among glass, plastic, and metal packaging, leading the authors to conclude that packaging is not the primary source of contamination [27]. Similarly, a European study also concluded that the presence of phthalates in olive oil is not necessarily associated with plastic packaging after comparing various packaging materials [50].

These results reinforce the hypothesis that the main source of plasticizer contamination may not be exclusively related to the type of packaging but rather to the widespread use of plastic materials throughout the production process. Additionally, the impact of environmental factors on food contamination with plasticizers should also be considered, an aspect that warrants further investigation.

3. Experimental Section

3.1. Chemicals

Thirty-two plasticizers were analyzed: dimethyl phthalate (DMP), diethyl phthalate (DEP), diallyl phthalate (DAP), dipropyl phthalate (DPrP), diisobutyl phthalate (DIBP), dibutyl phthalate (DBP), bis(2-methoxyethyl) phthalate (DMEP), diisopentyl phthalate (DIPP), bisphenol A (BPA), benzyl butyl phthalate (BBP), dihexyl phthalate (DHXP), dicyclohexyl phthalate (DCHP), diphenyl phthalate (DPhP), bis(2-ethylhexyl) phthalate (DEHP), di-n-heptyl phthalate (DHP), dioctyl phthalate (DOP), di(2-ethylhexyl) terephtha-

late (DEHT), diisononyl phthalate (DINP), and diisodecyl phthalate (DIDP), which were acquired from Sigma-Aldrich (Steinheim, Germany). Additionally, dimethyl terephthalate (DMTP), dibutyl maleate (DBM), diisopropyl phthalate (DiPrP), diethyl sebacate (DES), bis(4-methyl-2-pentyl) phthalate (BMPP), bis(2-ethoxyethyl) phthalate (DEEP), dipentyl phthalate (DPP), acetyltributyl citrate (ATBC), bis(2-ethylhexyl) adipate (DEHA), bis(2-n-butoxyethyl) phthalate (DBEP), di(2-ethylhexyl) sebacate (DEHS), dinonyl phthalate (DNP), tris(2-ethylhexyl) trimellitate (TOMT), and the internal standard Benzyl Butyl Phthalate-d4 were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetone, hexane, and methanol of GC-MS grade were obtained from Carlo Erba (Emmendingen, Germany).

Stock solutions for each plasticizer were prepared in acetone at 500 µg/mL and stored at 4 °C for at most one month.

As certified olive oil without plasticizers was not commercially available, an organic virgin sunflower oil with no detectable plasticizers was used for method validation. This oil was previously tested for plasticizer presence and content to ensure it could be considered suitable for matrix effect simulation. When residual contamination was detected in blank injections, it was subtracted from the sample results.

All solvents used for sample preparation were analyzed daily for the presence of plasticizers. Only glassware lab material was used, which was carefully washed, rinsed, and stored at 100 °C before use.

Additionally, the chromatographic system was checked daily for plasticizers by performing three blank injections at the start, during, and at the end of analyses.

3.2. Sampling

Samples of olives, olive paste, and olive oil at different steps of the production process were collected from three olive oil production lines located in different regions of Portugal (North, Center, and South). These were stored in glass containers with non-plastic lids, namely bamboo and glass, and frozen until analysis. Olive oil at the end of each production line was collected in both glass and PET containers and stored at room temperature to simulate normal shelf conditions. These containers were supplied by each production line.

Olive samples were processed into olive oil in the laboratory, firstly using an IKA-Werke A 10 stainless-steel grinder (Staufen, Germany). The paste was then transferred to glass culture tubes, heated to around 50 °C, and centrifuged at 3000 RPMs until sufficient oil was separated. This was removed and immediately weighed for analysis.

Off-the-shelf extra virgin olive oils, in both glass and plastic bottles, were purchased from a local supermarket.

3.3. Sample Analysis and Method Validation

The analytical method was adapted from a protocol TFDAA0008.02 established by the Taiwan Food and Drug Administration (TFDA) for testing phthalate plasticizers in foods. The method was optimized based on the extraction solvents used, the amount of solvent used, and the speed and timing of the vortex mix and centrifugation. Additionally, a freezing step was added to the procedure.

A 500 mg oil sample was weighed into a 15 mL glass test tube, and then 100 µL of internal standard (IS) (2.5 mg/L) was added and vortexed for 30 s. Then, 0.3 mL of hexane and 2 mL of hexane/methanol (1:4, v/v) were added, vortexed again for 1 min, and centrifuged at 2000 RPMs for 2 min. A 1.2 mL aliquot of the supernatant was carefully removed, after which another 2 mL of hexane/methanol (1:4, v/v) was added to the tube, vortexed 1 min, and centrifuged as above. A total of 2 mL of the supernatant was removed and added to the previous one for a total volume of 3.2 mL. This extract was then frozen at −24 °C overnight.

With the sample still frozen, an aliquot of the liquid phase was quickly transferred to a glass vial, allowed to reach room temperature, and injected into the chromatographic system. For the blanks, the entire procedure was performed with unspiked sunflower oil. Three replicates were performed for each sample.

Standard calibration solutions were prepared by spiking the sunflower oil at 0.001–16 mg/kg for all plasticizers. When linearity was not observed throughout the calibration range, two different regression curves were constructed.

Limits of detection (LOD) and quantification (LOQ) were determined considering that the lowest calibration concentration for each compound with a signal-to-noise ratio greater than 3 was the experimental LOD, and greater than 10 was the LOQ.

3.4. Chromatographic Conditions for GC-MS/MS

Analyses were performed on a Bruker (Bremen, Germany) GC 456 coupled with a Bruker Scion TQ (Triple Quadrupole) system equipped with a CTC (Zwingen, Switzerland) CombiPAL autosampler. Data acquisition was managed using Bruker MSWS 8.2 software, and analysis was conducted with Bruker MS Data Review 8.0. Chromatographic separation was achieved with a ZB-5MS Plus capillary column (20 m × 0.18 mm ID, 0.18 µm film thickness) supplied by Phenomenex (Torrance, CA, USA). The oven temperature program started at 50 °C, held for 1 min, increased at 20 °C/min to 140 °C, then 4 °C/min to 240 °C, followed by 10 °C/min to 280 °C, and finally 20 °C/min to 310 °C, where it was held for 9 min.

High-purity helium (99.9999%) was used as the carrier gas at a constant flow rate of 0.7 mL/min, with an injection volume of 1 µL. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, using argon as the collision gas at 2.4 mTorr. The transfer line was maintained at 300 °C, and the ion source at 270 °C. A solvent delay of 7 min was applied.

The MRM transitions, associated with selected precursor and product ion pairs for each analyte, are listed in Table 4. Quadrupoles operated at unit resolution, and ion ratios between the quantifier and qualifier ions were required to be within ± 30% of the average standard injections for positive identification [57]. Determination coefficients (R^2) obtained for all compounds were between 0.958 and 0.998.

Table 4. MRM parameters for the analysis of the 32 plasticizers, as well as detection and quantification limits (LOD and LOQ, respectively).

Plasticizers	CAS	Quantifier Transition (eV)	Qualifier Transition (eV)	LOD (mg/kg)	LOQ (mg/kg)
DMP	131-11-3	163 > 77 (14)	163 > 92 (28)	0.002	0.007
DMTP	120-61-6	163 > 75 (30)	163 > 103 (18)	0.005	0.018
DBM	105-76-0	117 > 99 (10)	117 > 71 (16)	0.001	0.004
DEP	84-66-2	149 > 65 (22)	149 > 121 (14)	0.005	0.018
DiPrP	605-45-8	149 > 65 (24)	149 > 121 (16)	0.001	0.004
DAP	131-17-9	149 > 65 (22)	149 > 121 (14)	0.013	0.043
DPrp	131-16-8	149 > 65 (24)	149 > 121 (14)	0.005	0.018
DES	110-40-7	171 > 55 (23)	171 > 97 (12)	0.031	0.103
DIBP	84-69-5	149 > 65 (24)	149 > 121 (16)	0.002	0.007
DBP	84-74-2	149 > 65 (24)	149 > 121 (16)	0.005	0.018
DMEP	117-82-8	149 > 65 (24)	149 > 121 (16)	0.103	0.343
BMPP	84-63-9	149 > 65 (24)	251 > 149 (15)	0.005	0.018
DIPP	605-50-5	149 > 65 (24)	237 > 149 (12)	0.005	0.018
DEEP	605-54-9	149 > 65 (22)	149 > 121 (14)	0.005	0.018
DPP	131-18-0	149 > 65 (24)	149 > 121 (16)	0.001	0.004

Table 4. *Cont.*

Plasticizers	CAS	Quantifier Transition (eV)	Qualifier Transition (eV)	LOD (mg/kg)	LOQ (mg/kg)
BPA	80-05-7	231 > 91 (28)	119 > 91 (14)	0.031	0.103
ATBC	77-90-7	129 > 69 (18)	185 > 69 (24)	0.001	0.004
BBP	85-68-7	149 > 65 (24)	238 > 149 (18)	0.001	0.004
DHXP	84-75-3	149 > 65 (24)	251 > 149 (14)	0.001	0.004
DEHA	103-23-1	129 > 55 (16)	129 > 111 (17)	0.005	0.018
DBEP	117-83-9	149 > 65 (22)	149 > 121 (14)	0.031	0.103
DCHP	84-61-7	149 > 65 (24)	167 > 149 (10)	0.005	0.018
DPhP	84-62-8	225 > 77 (22)	225 > 51 (50)	0.001	0.004
DEHP	117-81-7	149 > 65 (20)	279 > 149 (18)	0.005	0.018
DHP	3648-21-3	149 > 65 (24)	265 > 149 (15)	0.009	0.030
DOP	117-84-0	149 > 65 (24)	149 > 121 (16)	0.001	0.004
DEHT	6422-86-2	149 > 65 (19)	167 > 79 (14)	0.005	0.018
DEHS	122-62-3	185 > 69 (16)	203 > 121 (14)	0.005	0.018
DNP	84-76-4	149 > 65 (24)	149 > 121 (16)	0.005	0.018
DINP	28553-12-0	293 > 149 (5)	293 > 71 (5)	0.031	0.103
DIDP	26761-40-0	307 > 149 (5)	307 > 71 (5)	0.103	0.343
TOMT	3319-31-1	305 > 193 (20)	193 > 81 (26)	0.005	0.018

4. Conclusions

This study investigated the sources of plasticizer contamination in olive oil. The optimized analytical method used for quantification demonstrated adequate performance in terms of detection limits and excellent repeatability while requiring relatively small solvent volumes and ensuring effective sample clean-up. Analyses conducted throughout the production line revealed a progressive increase in plasticizer concentrations, having identified olive harvesting and industrial processes as predominant contamination sources, along with storage, particularly in PET packaging, over long periods.

Initial contamination in olives may have been influenced by factors such as the use of plastic nets and bags, as well as by the metabolism of the olive tree, which facilitates the absorption of compounds from soil, water, and air.

Among the compounds analyzed, DINP was the most frequent, with an average concentration of 3.387 mg/kg and a maximum value of 9.393 mg/kg in oils stored in both glass and PET. These results indicate that some stored oils exceeded the specific migration limits established by European regulations (1.8 mg/kg). The significant presence of DINP, as opposed to plasticizers like DEHP, reflects the gradual replacement of the latter in industrial applications and highlights the growing prevalence of DINP in construction materials, industrial machinery, and ecosystems.

Although the total concentration of plasticizers analyzed did not exceed the limit set by European regulations (60 mg/kg), it should be noted that this study covered only 32 compounds, whereas many other plasticizers are currently in use.

Given the importance of olive oil as a widely consumed food product, it is essential to precisely identify contamination sources and implement effective mitigation strategies. Replacing plastic materials with safer alternatives, such as stainless steel or adopting phthalate-free plastics, are fundamental measures. However, it is equally crucial to monitor and evaluate these materials, as even those labeled as phthalate-free may release contami-

nants over time due to mechanical stress and temperature. Additionally, new phthalate replacement plasticizers must be monitored and toxicologically tested.

Accurate diagnostics and the implementation of mitigation strategies will significantly reduce plasticizer contamination, ensuring greater consumer safety and preserving the quality of this essential food product.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/molecules29246013/s1, Table S1: Concentration (mg/kg) of the 32 plasticizers studied in all collected samples, expressed as average ± standard deviation. Bold and underlined values indicate those exceeding the specific migration limits defined.

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4.4.1 Article Supplementary Material

		DMP	DMTP	DBM	DEP	DIPrP	DAP	DPrP
North	a.	<LOQ	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD
	b.	<LOD	<LOD	<LOD	0.019 ± 0.001	<LOD	<LOD	<LOD
	c.	not analyzed						
	d.	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ
	e.	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOQ	<LOQ
	f.	<LOD	<LOD	<LOD	0.026 ± 0.010	<LOD	<LOD	<LOQ
	g.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	h.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	i.	0.013 ± 0.001	<LOD	<LOD	<LOD	0.004 ± 0.001	<LOD	<LOD
Centre	a.	not analyzed						
	b.	<LOQ	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOQ
	c.	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOQ
	d.	<LOQ	<LOD	<LOD	<LOD	<LOQ	0.045 ± 0.001	0.018 ± 0.001
	e.	0.007 ± 0.001	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ
	f.	0.009 ± 0.001	<LOD	<LOD	<LOD	<LOQ	0.098 ± 0.004	0.066 ± 0.001
	g.	not analyzed						
	h.	0.007 ± 0.001	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ
	i.	not analyzed						
South	a.	not analyzed						
	b.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c.	<LOD	<LOD	<LOD	0.023 ± 0.005	<LOD	<LOQ	<LOD
	d.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD
	e.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	f.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	g.	<LOQ	<LOD	<LOD	0.041 ± 0.014	<LOD	<LOD	<LOD
	h.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	i.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 1	Glass	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOQ	<LOD
	PET	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 2	Glass	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD
	PET	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD
Olive Oil 3	Glass	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD
	PET	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD
Olive Oil 4	Glass	<LOD	<LOQ	<LOD	<LOQ	<LOD	<LOQ	<LOD
Olive Oil 5	Glass	<LOD	<LOQ	<LOD	0.020 ± 0.010	<LOD	0.059 ± 0.003	<LOD
Olive Oil 6	Can	<LOD	<LOD	<LOD	0.029 ± 0.005	<LOD	<LOD	<LOD

Table S1: Concentration (mg/kg) of the 32 plasticizers studied in all collected samples, expressed as average ± standard deviation. Bold and underlined values indicate those exceeding the specific migration limits defined.

		DES	DIBP	DBP	DMEP	BMPP	DIPP
North	a.	<LOD	0.011 ± 0.004	<LOD	<LOD	<LOD	<LOD
	b.	<LOD	0.008 ± 0.001	<LOD	<LOD	<LOD	<LOQ
	c.	not analyzed					
	d.	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD
	e.	<LOD	0.007 ± 0.002	<LOQ	<LOD	<LOD	<LOD
	f.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	g.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	h.	<LOD	0.019 ± 0.008	<LOQ	0.969 ± 0.107	0.023 ± 0.001	0.032 ± 0.001
	i.	<LOD	0.028 ± 0.009	0.127 ± 0.007	4.342 ± 0.448	0.029 ± 0.001	0.043 ± 0.000
Centre	a.	not analyzed					
	b.	<LOD	0.019 ± 0.010	0.073 ± 0.004	<LOD	<LOD	<LOD
	c.	<LOD	0.052 ± 0.010	0.085 ± 0.004	<LOD	<LOD	<LOD
	d.	<LOD	0.061 ± 0.007	0.083 ± 0.012	<LOD	<LOD	<LOQ
	e.	<LOD	0.079 ± 0.013	0.082 ± 0.007	<LOD	<LOQ	<LOQ
	f.	<LOD	0.093 ± 0.008	0.092 ± 0.002	<LOD	<LOQ	<LOQ
	g.	not analyzed					
	h.	<LOD	0.072 ± 0.007	0.096 ± 0.009	1.686 ± 0.643	0.035 ± 0.001	0.050 ± 0.003
	i.	not analyzed					
South	a.	not analyzed					
	b.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c.	<LOD	0.013 ± 0.006	<LOQ	<LOD	<LOD	<LOD
	d.	<LOD	0.010 ± 0.008	<LOQ	<LOD	<LOD	<LOD
	e.	<LOD	0.010 ± 0.002	<LOD	<LOD	<LOD	<LOD
	f.	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
	g.	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD
	h.	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD
	i.	<LOD	<LOD	0.038 ± 0.002	<LOD	<LOD	<LOD
Olive Oil 1	Glass	<LOD	0.014 ± 0.009	<LOD	<LOD	<LOD	<LOD
	PET	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
Olive Oil 2	Glass	<LOD	<LOQ	0.020 ± 0.005	<LOD	<LOD	<LOD
	PET	<LOD	0.018 ± 0.005	<LOQ	<LOD	<LOD	<LOD
Olive Oil 3	Glass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	PET	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD
Olive Oil 4	Glass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 5	Glass	<LOD	0.020 ± 0.002	<LOD	<LOD	<LOD	<LOD
Olive Oil 6	Can	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ

Table S1 (Cont.): Concentration (mg/kg) of the 32 plasticizers studied in all collected samples, expressed as average ± standard deviation. Bold and underlined values indicate those exceeding the specific migration limits defined.

		DEEP	DPP	BPA	ATBC	BBP	DHXP
North	a.	0.096 ± 0.027	<LOD	<LOD	<LOD	<LOD	<LOD
	b.	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD
	c.	not analyzed					
	d.	<LOD	<LOD	<LOD	0.007 ± 0.000	<LOQ	<LOD
	e.	<LOD	<LOD	<LOD	0.008 ± 0.001	<LOQ	<LOQ
	f.	<LOD	<LOD	<LOD	0.011 ± 0.001	<LOQ	<LOQ
	g.	<LOD	5.747 ± 0.734	<LOD	0.008 ± 0.001	<LOQ	<LOQ
	h.	<LOD	5.998 ± 1.000	<LOD	0.009 ± 0.002	<LOQ	<LOQ
	i.	0.019 ± 0.004	<LOD	<LOD	0.015 ± 0.000	0.006 ± 0.001	<LOD
Centre	a.	not analyzed					
	b.	0.024 ± 0.000	4.325 ± 0.282	<LOD	0.014 ± 0.000	<LOD	<LOD
	c.	<LOD	5.204 ± 0.327	<LOD	0.019 ± 0.002	<LOD	<LOD
	d.	<LOQ	6.422 ± 0.020	<LOD	0.016 ± 0.001	<LOD	<LOD
	e.	<LOD	6.213 ± 0.830	<LOD	0.015 ± 0.001	<LOD	<LOQ
	f.	<LOD	6.801 ± 0.279	<LOD	0.016 ± 0.001	<LOQ	<LOQ
	g.	not analyzed					
	h.	<LOD	9.818 ± 0.363	<LOD	0.018 ± 0.003	<LOQ	<LOQ
	i.	not analyzed					
South	a.	not analyzed					
	b.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c.	<LOD	3.389 ± 0.297	<LOD	0.013 ± 0.001	<LOD	<LOD
	d.	<LOD	5.435 ± 0.267	<LOD	<LOD	<LOD	<LOD
	e.	<LOQ	5.716 ± 0.210	<LOD	<LOQ	<LOD	<LOD
	f.	<LOD	5.494 ± 0.179	<LOD	0.015 ± 0.001	<LOD	<LOD
	g.	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD
	h.	0.043 ± 0.005	5.921 ± 0.087	<LOD	0.015 ± 0.001	<LOD	<LOD
	i.	<LOQ	6.243 ± 0.440	<LOD	0.029 ± 0.002	<LOD	<LOD
Olive Oil 1	Glass	0.024 ± 0.001	<LOD	<LOD	0.005 ± 0.000	<LOD	<LOD
	PET	<LOQ	1.125 ± 0.040	<LOD	0.008 ± 0.000	<LOD	<LOD
Olive Oil 2	Glass	<LOD	0.005 ± 0.081	<LOD	0.005 ± 0.000	<LOD	<LOD
	PET	<LOD	<LOD	<LOD	0.007 ± 0.000	<LOD	<LOD
Olive Oil 3	Glass	<LOQ	0.656 ± 0.107	<LOD	0.016 ± 0.001	<LOD	<LOD
	PET	<LOD	<LOD	<LOD	0.016 ± 0.000	<LOQ	<LOD
Olive Oil 4	Glass	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ
Olive Oil 5	Glass	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD
Olive Oil 6	Can	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD

Table S1 (Cont.): Concentration (mg/kg) of the 32 plasticizers studied in all collected samples, expressed as average ± standard deviation. Bold and underlined values indicate those exceeding the specific migration limits defined.

		DEHA	DBEP	DCHP	DPhP	DEHP	DHP	DOP
	a.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	b.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c.				not analyzed			
	d.	0.135 ± 0.004	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
North	**e.**	0.124 ± 0.009	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	f.	0.161 ± 0.011	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	g.	0.181 ± 0.003	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	h.	0.188 ± 0.007	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	i.	0.245 ± 0.015	<LOD	<LOD	<LOD	0.454 ± 0.013	0.468 ± 0.016	<LOD
	a.				not analyzed			
	b.	<LOD	<LOD	<LOD	<LOD	0.076 ± 0.007	0.044 ± 0.007	<LOD
	c.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	d.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Centre	**e.**	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD
	f.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	g.				not analyzed			
	h.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	i.				not analyzed			
	a.				not analyzed			
	b.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c.	0.022 ± 0.004	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	d.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
South	**e.**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	f.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	g.	0.068 ± 0.002	<LOD	<LOD	<LOD	0.037 ± 0.003	<LOD	<LOD
	h.	0.095 ± 0.003	<LOD	<LOD	<LOD	0.078 ± 0.009	0.051 ± 0.009	<LOD
	i.	0.161 ± 0.008	<LOD	<LOD	<LOD	0.095 ± 0.001	<LOQ	<LOD
	Glass	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 1	**PET**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Glass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 2	**PET**	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Glass	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 3	**PET**	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 4	**Glass**	<LOD	<LOD	<LOD	<LOD	0.656 ± 0.006	0.692 ± 0.006	<LOD
Olive Oil 5	**Glass**	0.034 ± 0.014	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Olive Oil 6	**Can**	<LOD	<LOD	<LOD	<LOD	0.082 ± 0.013	0.051 ± 0.014	<LOD

Table S1 (Cont.): Concentration (mg/kg) of the 32 plasticizers studied in all collected samples, expressed as average ± standard deviation. Bold and underlined values indicate those exceeding the specific migration limits defined.

		DEHT	DEHS	DNP	DINP	DIDP	TOMT
North	a.	1.760 ± 0.543	0.035 ± 0.011	<LOQ	<LOD	<LOD	0.027 ± 0.004
	b.	0.736 ± 0.132	<LOQ	0.034 ± 0.001	<LOD	<LOD	0.024 ± 0.001
	c.	not analyzed					
	d.	0.858 ± 0.041	<LOQ	0.041 ± 0.002	<LOD	<LOD	0.040 ± 0.001
	e.	0.878 ± 0.054	<LOQ	0.045 ± 0.002	<LOD	<LOD	0.042 ± 0.001
	f.	0.975 ± 0.028	<LOQ	0.059 ± 0.002	0.103 ± 0.061	<LOD	0.041 ± 0.002
	g.	0.850 ± 0.017	<LOQ	0.042 ± 0.001	1.278 ± 0.161	<LOD	0.032 ± 0.001
	h.	0.860 ± 0.023	<LOQ	0.107 ± 0.003	3.527 ± 0.214	<LOD	0.050 ± 0.003
	i.	2.686 ± 0.099	<LOQ	0.192 ± 0.007	6.000 ± 0.203	<LOD	0.051 ± 0.000
Centre	a.	not analyzed					
	b.	0.206 ± 0.021	<LOQ	0.033 ± 0.002	0.146 ± 0.056	<LOD	<LOQ
	c.	0.153 ± 0.004	<LOQ	0.029 ± 0.004	0.431 ± 0.145	<LOD	<LOQ
	d.	0.185 ± 0.005	<LOQ	0.035 ± 0.004	0.318 ± 0.065	<LOD	<LOQ
	e.	0.190 ± 0.009	<LOQ	0.037 ± 0.003	0.625 ± 0.309	<LOD	<LOQ
	f.	0.767 ± 0.006	<LOQ	0.059 ± 0.003	1.211 ± 0.172	<LOD	<LOQ
	g.	not analyzed					
	h.	0.315 ± 0.005	<LOQ	0.109 ± 0.008	3.528 ± 0.323	<LOD	<LOQ
	i.	not analyzed					
South	a.	not analyzed					
	b.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	c.	0.505 ± 0.012	<LOQ	<LOQ	<LOQ	<LOD	<LOQ
	d.	0.317 ± 0.038	<LOQ	<LOQ	0.103 ± 0.023	<LOD	<LOQ
	e.	<LOQ	<LOQ	<LOQ	0.156 ± 0.015	<LOD	<LOQ
	f.	0.436 ± 0.062	<LOQ	<LOQ	0.264 ± 0.064	<LOD	<LOQ
	g.	0.520 ± 0.011	<LOQ	0.024 ± 0.003	2.768 ± 0.132	1.627 ± 0.083	<LOQ
	h.	2.859 ± 0.091	<LOQ	<LOQ	4.595 ± 0.222	0.518 ± 0.053	<LOQ
	i.	8.538 ± 0.292	<LOQ	0.043 ± 0.006	6.513 ± 0.579	2.880 ± 0.046	<LOQ
Olive Oil 1	Glass	0.631 ± 0.007	<LOQ	<LOQ	1.598 ± 0.145	<LOD	<LOQ
	PET	0.730 ± 0.012	<LOQ	0.027 ± 0.001	1.807 ± 0.199	<LOD	<LOQ
Olive Oil 2	Glass	0.326 ± 0.012	<LOQ	<LOQ	0.395 ± 0.076	<LOD	<LOQ
	PET	0.378 ± 0.019	0.018 ± 0.003	0.024 ± 0.001	0.716 ± 0.112	<LOD	<LOQ
Olive Oil 3	Glass	1.337 ± 0.019	<LOQ	<LOQ	3.310 ± 0.053	<LOD	<LOQ
	PET	2.048 ± 0.074	<LOQ	0.019 ± 0.003	3.245 ± 0.050	<LOD	<LOQ
Olive Oil 4	Glass	<LOD	0.100 ± 0.003	0.026 ± 0.001	1.244 ± 0.086	<LOD	<LOQ
Olive Oil 5	Glass	0.504 ± 0.012	<LOQ	0.036 ± 0.002	5.976 ± 0.389	<LOD	<LOQ
Olive Oil 6	Can	<LOD	<LOQ	0.124 ± 0.004	<LOD	<LOD	<LOQ

Table S1 (Cont.): Concentration (mg/kg) of the 32 plasticizers studied in all collected samples, expressed as average ± standard deviation. Bold and underlined values indicate those exceeding the specific migration limits define.

4.5 Future Perspectives: GC x GC

Phthalates are predominantly analyzed using chromatographic techniques, as their detection and quantification require high specificity and sensitivity. However, several analytical challenges arise due to their structural and chemical characteristics. Phthalates are derivatives of phthalic acid, resulting in a high degree of similarity among them. This similarity is particularly evident in their mass spectral fragmentation patterns, where almost all phthalates exhibit a dominant ion with an m/z of 149. This creates significant difficulty in differentiating between phthalates, especially when they co-elute during chromatography (Figure 11).

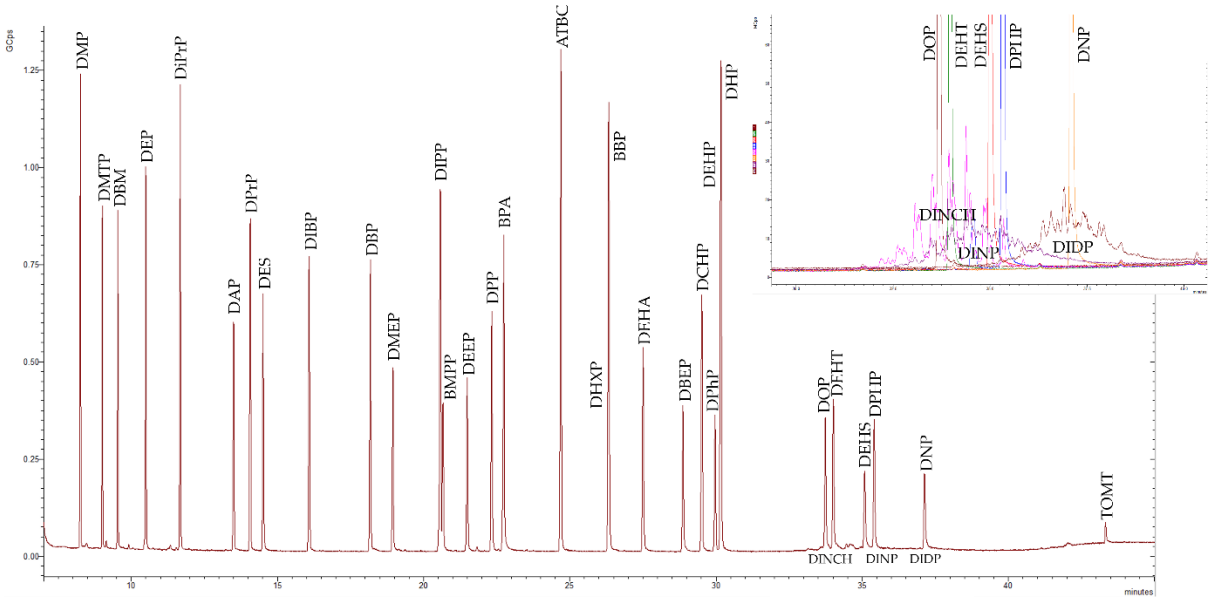

Figure 11. Chromatogram obtained by GC/MS for 34 plasticizers, including DINP and DIDP, using a Bruker Scion TQ 456 GC-MS/MS (Bruker Corporation, Billerica, MA, USA) chromatograph. Chromatographic separation was performed on a ZB-5MS Plus capillary column (20 m × 0.18 mm ID, 0.18 µm film thickness). The temperature program started at 50 °C, held for 1 min, increased at 20 °C/min to 140 °C, then 4 °C/min to 240 °C, followed by 10 °C/min to 280 °C, and finally 20 °C/min to 310 °C, where it was held for 9 min. Helium was used as the carrier gas at a constant flow rate of 0.7 mL/min. The MS transfer line and source were set at 300 °C and 270 °C, respectively. A solvent delay of 7 min was applied.

Among the phthalates, diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP) represent some of the most challenging analytes. These compounds not only lack unique ions with sufficient signal intensity to distinguish them but also elute as broad, overlapping peaks due to their composition of multiple skeletal isomers. Their partial co-elution exacerbates the difficulty of identification and quantification, leading to substantially higher limits of detection

(LOD) and quantification (LOQ) compared to other phthalates that elute as single, well-defined peaks.

To address these challenges, multidimensional gas chromatography (GC), particularly comprehensive two-dimensional gas chromatography (GC × GC), has been explored as a viable alternative to classical one-dimensional GC (1D-GC). GC × GC offers enhanced separation capacity through the coupling of two columns with differing selectivity, enabling improved resolution of complex mixtures [310–313].

Traditionally, cryogenic modulation has been employed in GC × GC systems to maximize resolution. Cryogenic modulators trap and refocus analytes between the first and second dimensions, resulting in narrow peak widths and high chromatographic efficiency. This approach significantly improves the signal-to-noise ratio (S/N) for a given sample mass, allowing for the use of detectors such as flame ionization detectors (FID) with enhanced sensitivity. However, cryogenic modulation is associated with high operational costs, both in terms of instrument acquisition and maintenance. Additionally, the sharp peaks generated by this technique necessitate mass spectrometers with extremely high scanning speeds, which can limit the use of simpler quadrupole mass analyzers in full-scan mode. Despite these limitations, targeted analyses using methods like selected ion monitoring (SIM) or multiple reaction monitoring (MRM) on triple quadrupole systems can mitigate these challenges [314,315].

An alternative to cryogenic modulation is flow modulation, which offers comparable chromatographic resolution under optimal conditions but at a significantly reduced operational cost. Flow-modulated GC × GC systems eliminate the need for cryogenic cooling, simplifying operation and maintenance. However, these systems face challenges related to the handling of larger eluate volumes from the second dimension, which are typically an order of magnitude greater than those from traditional GC or cryogenic GC × GC systems. To address this, a majority of the eluate is vented, and only a small fraction is directed to the mass spectrometer [314–317].

Figure 12 exemplifies the performance of a flow-modulated GC × GC system, highlighting its potential to resolve plasticizers co-elutions in the future, with adequate optimization. While the resolution achieved is similar to that of cryogenic modulation, flow modulation offers the advantage of reduced costs, making it more suitable for routine analysis. Moreover, coupling flow-modulated GC × GC with triple quadrupole mass spectrometers can potentially resolve co-elutions and maintain adequate sensitivity. Such systems are well-suited for targeted analyses where cost efficiency and reliable quantification are critical.

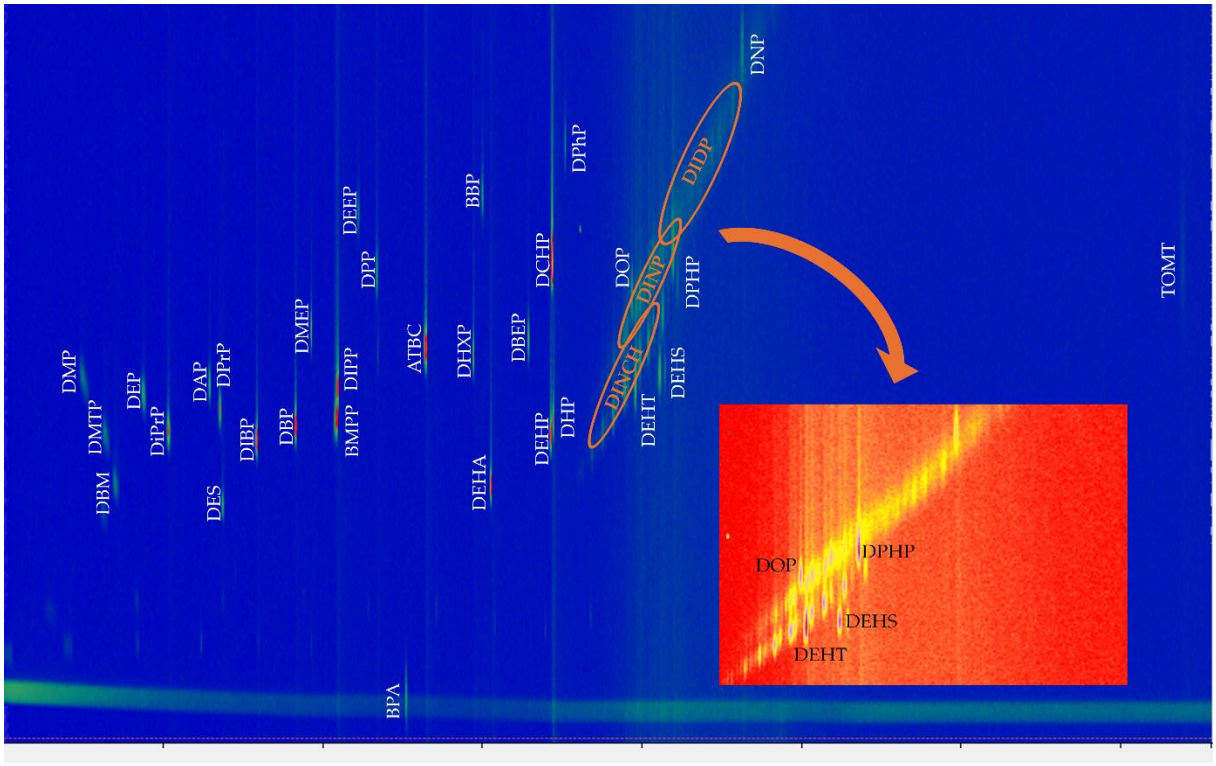

Figure 12. Test chromatogram obtained by flow-modulated comprehensive GC × GC-TOFMS for 34 plasticizers, including DINP and DIDP, using a Agilent 8890GC System (Shanghai, China) with a BenchTOF-Select detector (Markes International, Bridgend, UK). Chromatographic separation was performed with the INSIGHT™ flow modulator (SepSolve Analytical), equipped with a loop with 50 µL, a BPX5 column (20 m length × 0.18 mm i.d. and 0.18 μ m film thickness) as the first dimension (1D), and a BPX50 column (5 m length × 0.25 mm i.d. and 0.1 μ m film thickness) as the second dimension (2D). The modulation period (PM) used was 5s. The temperature program started at 120 °C, was held for 3 minutes, and was then ramped at 4 °C/min per minute to 225 °C and held for 5 minutes. It was further ramped at 4 degrees Celsius per minute to 250 °C and held for 20 minutes, followed by a final ramp at 4 °C/min to 280 °C, which was held for 40 minutes. Helium was used as carrier gas with a flow of 0.5 mL/min in the first column and 20 mL/min in the second column. The MS transfer line and source temperatures were set at 270 °C.

The analytical challenges posed by phthalates, particularly DINP and DIDP, underscore the need for advanced chromatographic techniques. Comprehensive GC × GC, whether cryogenically or flow-modulated, represents a powerful tool for overcoming these challenges. While cryogenic modulation provides unparalleled resolution, its high costs limit widespread adoption. Conversely, flow-modulated GC × GC strikes a balance between resolution and affordability, offering a practical solution for routine analysis. Theoretically, a flow-modulated GC x GC coupled to a triple-quadrupole might be able to resolve all co-elutions and still provide good sensitivity. Future advancements in detector technology and modulation strategies may further enhance the applicability and efficiency of GC × GC systems for the analysis of phthalates and other complex mixtures.

5

Conclusion

This work focused on the study of olive oil, a food matrix of high chemical complexity and significant nutritional, cultural, and economic importance. Throughout the thesis, three main themes were addressed, contributing to a deeper understanding of the chemical composition, organoleptic characteristics, nutraceutical potential, and safety of this product.

To achieve the proposed objectives, a multidisciplinary approach was employed, integrating various advanced analytical techniques and sample preparation methods. These methodologies enabled a detailed characterization of different olive oil components, including volatile organic compounds, antioxidants, and plasticizers, addressing essential aspects for monitoring and improving its quality.

The study on volatile organic compounds throughout olive oil's shelf life provided important tools for predicting sensory disqualification, particularly through the ratio between *E*-2-hexenal and acetic acid, improving the evaluation of the shelf life of extra virgin olive oils. The analysis of antioxidants led to the development of a methodology that maximizes the extraction of hydroxytyrosol (HTyr) and tyrosol (Tyr), enhancing olive oil as a functional food with nutraceutical applications, which resulted in an international patent. Additionally, the investigation of plasticizers identified the main sources of contamination during production and storage processes, providing practical strategies for mitigating these contaminants and ensuring compliance with international regulations.

This thesis thus represents a significant contribution to the improvement of olive oil quality monitoring and control, encompassing its organoleptic characteristics, functional properties, and the evaluation and mitigation of contaminants. The results reinforce olive oil's value

as a high-added-value food while ensuring its safety and integrity, benefiting both producers and consumers.

6

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A

SCIENTIFIC DISSEMINATION

A.1 Published Work

Patent developed in collaboration with AZAL (Azeites do Alentejo) while a PhD student at Resolution Lab

- "Method for the production of olive oil with a high antioxidant content and antioxidant concentrate". Inventors: Luís Silva Pinto, Marco Gomes da Silva and Flávia Freitas. WO 2024/095132; PCT/IB2023/0609223 (2024)

Papers associated to the present dissertation

- Flávia Freitas; João Brinco; Maria João Cabrita; Marco Gomes da Silva. "Analysis of Plasticizer Contamination Throughout Olive Oil Production". *Molecules* (2024): https://doi.org/10.3390/molecules29246013

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Early Identification of Olive Oil Defects throughout Shelf Life". *Separations* (2024): https://doi.org/10.3390/separations11060167

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "A Critical Review of Analytical Methods for the Quantification of Phthalates Esters in Two Important European Food Products: Olive Oil and Wine". *Molecules* (2023): https://doi.org/10.3390/molecules28227628

Papers produced as a member of Resolution Lab during PhD

(https://resolution-lab.com)

- Vitor Rosa; Helena Laronha; Clara S. B. Gomes; Cristina M. Cordas; João Brinco; Flávia Freitas; Marco D. R. Gomes da Silva; Teresa Avilés. "Aerobic oxidation of benzylic alcohols catalysed by new(aryl-BIAN)copper(I) complexes: Their synthesis and structural characterization". *Applied Organometallic Chemistry* (2023): https://doi.org/10.1002/aoc.7193

- Inês M. Ferreira; Flávia Freitas; Sara Pinheiro; Maria Filipa Mourão; Luís Ferreira Guido; Marco Gomes da Silva. "Impact of temperature during beer storage on beer chemical profile". *LWT* (2022): https://doi.org/10.1016/j.lwt.2021.112688

- Nuno Martins; Nicasio T. Jiménez-Morillo; Flávia Freitas; Raquel Garcia; Marco Gomes da Silva; Maria João Cabrita. " Revisiting 3D van Krevelen diagrams as a tool for the visualization of volatile profile of varietal olive oils from Alentejo region, Portugal". *Talanta* (2020): https://doi.org/10.1016/j.talanta.2019.120276

A.2 Conference Contributions

Oral Communications

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Analytical techniques for assessing olive oil quality and shelf life". *13th National Chromatography Meeting (13ENC)*. Faculty of Pharmacy, University of Lisbon (Lisbon, Portugal), December 17-19, 2023

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Phthalates, a danger in food: a new analytical approach for risk assessment". *IV PhD Students Meeting in Environment and Agriculture.* University of Évora (Évora, Portugal), November 11-12, 2019

Poster Communications associated to the present dissertation

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Volatile Organic Compounds in Extra Virgin Olive Oil: Sensory Quality and Shelf Life Assessment". *28th International Symposium on Separation Sciences*. Campus University of Messina (Messina, Italy), September 22-25, 2024

- Matilde Afonso Costa; Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "New insights into the analysis of phthalate esters". *13th National Chromatography Meeting (13ENC)*. Faculty of Pharmacy, University of Lisbon (Lisbon, Portugal), December 17-19, 2023

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Phthalates - Preventing Future Health Risks: Exploring GC/MS tools for monitoring phthalates in food matrices". *Science Meeting 2023*. Campus of Santiago, University of Aveiro (Aveiro, Portugal), July 5-7, 2023

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "New insides into the analysis of phthalates esters: The problem of contamination in the laboratory". *12th National Chromatography Meeting (12ENC)*. University of Aveiro (Aveiro, Portugal), December 6-8, 2022

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Phthalates: Preventing Future Health Risks - Exploring GC/MS tools for monitoring phthalates in food matrices". *XV Food Chemistry Meeting*. University of Madeira (Madeira, Portugal), September 5-8, 2021

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Preventing Future Health Risks: monitorization of very low levels of some phthalates in food matrices by GC/TOFMS". *3rd International Caparica Conference on Pollutant Toxic Ions and Molecules*. Caparica (Almada, Portugal), November 4-7, 2019

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Phthalates, Preventing Future Health Risks: exploring GC/MS tools for monitoring phthalates in food matrices". *11th National Chromatography Meeting (11ENC)*. Caparica (Almada, Portugal), December 9-11, 2019

Poster Communications produced while Resolution member

- Sara Pinheiro; Flávia Freitas; Francisco Campos; Paulo Lopes; Miguel Cabral; Marco Gomes da Silva. "From Cork Stoppers to Sparkling Wine – The impact of different cork stoppers in the aroma profile". *12th National Chromatography Meeting (12ENC)*. University of Aveiro (Aveiro, Portugal), December 6-8, 2022

- Sara Pinheiro; Flávia Freitas; Francisco Campos; Paulo Lopes; Miguel Cabral; Marco Gomes da Silva. From Cork Stoppers to Champagne – Influence of different bottling in the aroma profile". *XV Food Chemistry Meeting.* University of Madeira (Madeira, Portugal), September 5-8, 2021

- Cátia Magro; Davide Mendes; Flávia Freitas; Marco Gomes da Silva; Alexandra B. Ribeiro; Eduardo P. Mateus. " Development of target methods for triclosan monitorization in effluents at trace levels using gas chromatography-triple quadrupole mass spectrometry and electronic tongues". *11th National Chromatography Meeting (11ENC).* Caparica (Almada, Portugal), December 9-11, 2019

Oral Communications - Abstracts

- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Analytical techniques for assessing olive oil quality and shelf life". *13th National Chromatography Meeting (13ENC)*. Faculty of Pharmacy, University of Lisbon (Lisbon, Portugal), December 17-19, 2023

Abstract: Olive oil is a vegetable oil extracted from olives without the use of solvents or chemicals. It is a cornerstone of the Mediterranean diet due to its health benefits. Its distinctive flavour and aroma result from volatile organic compounds (VOCs), the presence and quantity of which vary due to olive variety, ripeness, processing, and storage.1,2

VOCs are produced through natural biochemical processes, including the lipoxygenase (LOX) pathway, contributing to the green and fruity flavour of olive oil. However, sensory defects can arise from chemical oxidation and the action of exogenous enzymes, often stemming from microbial activity.3

Olive oil is the only food product legally required to undergo quality evaluation by a certified sensory panel. This evaluation considers positive attributes such as fruity, bitter, and pungent flavours, as well as negative attributes like rancidity and mustiness. The shelf life of olive oil ranges from 18 to 24 months, thanks to natural antioxidants such as polyphenols.4,5

Robust analytical methods, such as solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC/MS), are essential to support sensory evaluation. This study aimed to develop an HS-SPME-GC/MS methodology to identify VOCs as markers of both positive and negative attributes, correlating them with concentrations to estimate the risk of disqualification during the olive oil's shelf life.

Acknowledgements: This work was supported by National Funds through FCT - Foundation for Science and Technology under the Ph.D. Grant [2020.08089.BD]; Associate Laboratory for Green Chemistry - LAQV which is financed by national funds from FCT/MCTES [UIDB/QUI/50006/2020 and UIDP/50006/2020] and MED – Mediterranean Institute for Agriculture, Environment and Development funded by National Funds through FCT - Foundation for Science and Technology under the Project [UIDB/05183/2020]. This research was also anchored by the RESOLUTION LAB, an infrastructure at NOVA School of Science and Technology.

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- Flávia Freitas; Maria João Cabrita; Marco Gomes da Silva. "Phthalates, a danger in food: a new analytical approach for risk assessment". *IV PhD Students Meeting in Environment and Agriculture.* University of Évora (Évora, Portugal), November 11-12, 2019

Abstract: Phthalate esters (PE's), better known as phthalates, are a group of chemical compounds widely used since 1960 as plasticizing agents in order to impart flexibility, durability and longevity to plastics.[1]
Given their unique physicochemical properties, some phthalates and their metabolites have a severe toxic effect on human health, primarily in the reproductive, endocrine and respiratory systems.[2,3]
Several studies have led the EU and the USA, among other countries, to intervene and regulate exposure to phthalates.[4] Exposure to PE's is daily, causing an accumulation in the body, leading to long-term harmful effects. The control must be rigorous with very low levels of detection (ppb or lower), so it is important to define methodologies that respond to this need. Traditionally, the analysis of PEs is performed using 1D gas chromatography techniques. In the future, this project will apply classical and alternative 2D analytical methodologies (GC x GC and/or MD-GC) in order to obtain better separation, detection and sensitivity for PEs in complex food matrices, wine and olive oil.
Up to this moment, nine phthalates have been quantified in Portuguese olive oil and different materials used in it's production, such as hoses. Liquid extraction with hexane/Methanol was performed, and chromatographic analysis was carried on a LECO GC/TOFMS with an apolar capillary column. The limit of detection ranged from 0.2 to 1 ppm (mg/kg) for all analytes. Further matrices are under study, namely wine.

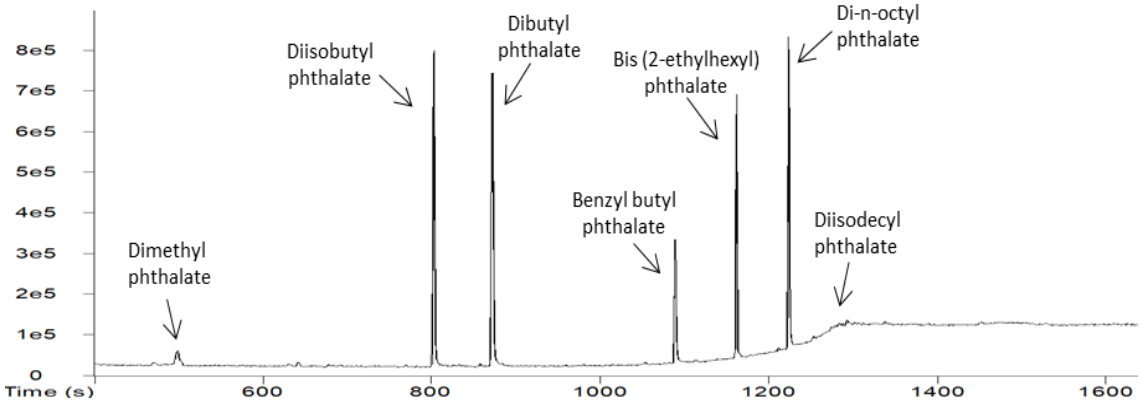

Figure 1: Extracted ion chromatogram showing m/z 149, displaying seven phthalates at a concentration of 60 ng/mL

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Acknowledgements: This work was supported by the Associate Laboratory for Green Chemistry- LAQV which is financed by national funds from FCT/MCTES (UID/QUI/50006/2019), and ICAAM funding by FCT - Foundation for Science and Technology under the Projects UID/AGR/00115/2019.

POSTERS COMMUNICATIONS - FIGURES

VOLATILE ORGANIC COMPOUNDS IN EXTRA VIRGIN OLIVE OIL
SENSORY QUALITY AND SHELF-LIFE ASSESSMENT

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INTRODUCTION

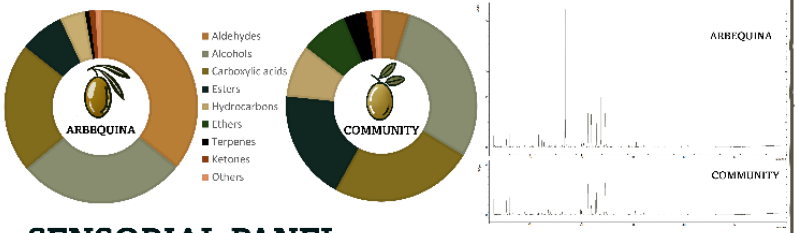
Olive oil, derived from *Olea europaea* L., is a fundamental element of the Mediterranean diet and globally valued for its quality. Its appeal mainly comes from the high content of monounsaturated fatty acids, such as oleic acid, and minor compounds that enhance its nutritional and sensory value, including phenolic compounds and volatile organic compounds (VOCs). Phenolics, like phenolic acids and flavones, contribute to antioxidant activity and the unique sensory profile, such as fruity aroma. The lipoxygenase pathway (LOX) produces VOCs that influence flavor, but unwanted VOCs can indicate sensory defects, such as rancidity, caused by oxidation. Processing and storage conditions affect these compounds. Techniques like SPME-GC/MS allow for the detection of VOCs, serving as early markers of defects, helping to improve the assessment of olive oil quality, especially for extra virgin olive oil (EVOO).[1]

OBJECTIVES

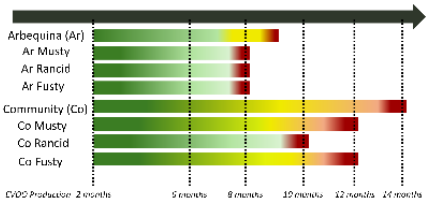
- Development of an HS-SPME-GC/MS analytical method to identify VOCs in EVOO as markers of sensory attributes.
- Correlate these markers with the risk of EVOO disqualification throughout its shelf life.
- Monitor VOC levels, especially those from the LOX pathway, over time.
- Demonstrate that the developed method can be an effective tool to predict EVOO disqualification and assist in quality control and assurance

EXPERIMENTAL

Mixtures of monovarietal Arbequina EVOO and Community EVOO were doped with 5% defective oils (musty, rancid, fusty) to prevent early defect detection by the sensory panel. Chromatographic and sensory analyses were consistently performed over 14 months until disqualification by the sensory panel.



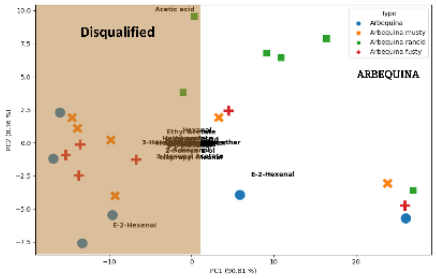
SENSORIAL PANEL

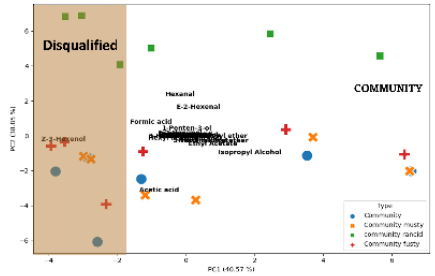


Around 80 VOCs were identified, highlighting the complexity of aroma, with differing relative percentages between the two EVOOs.

The Arbequina EVOO showed a more intense chromatographic profile compared to the Community EVOO, yet the Community, despite its less intense volatile profile, remained extra virgin for a longer period before being disqualified.

RESULTS

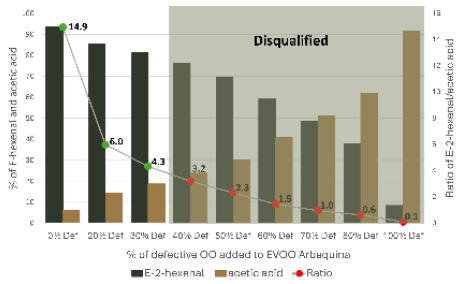




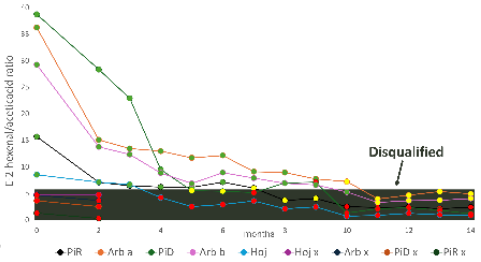
After statistical analysis, significant differences were found in compounds from the LOX pathway. Principal component analysis (PCA) analyzed the relative percentages of 30 VOCs, considering disqualified olive oil and time.

The evolution of EVOOs over time was mainly attributed to compounds such as *E*-2-hexenal, *E*-2-hexenol, *Z*-3-hexenol, and acetic acid.

***E*-2-hexenal**, linked to positive attributes, and **acetic acid**, linked to negative ones, were identified as potential markers for oxidation onset and disqualification. Their ratio could predict oxidation levels, aiding the sensory panel in assessing olive oil shelf life.



The ratio of ***E*-2-hexenal** to **acetic acid** was calculated for Arbequina EVOO with added disqualified oil, showing that as disqualified EVOO increased, the ratio decreased. At a ratio of 3.2, all blends were disqualified. This threshold was then applied to other varieties, EVOO, and disqualified oils for consistency.



CONCLUSION

This study conducted sensory and analytical analysis over time on monovarietal Arbequina EVOO and Community EVOO, with and without the addition of disqualified olive oil due to sensory defects. Around 80 VOCs were identified. The profiles of the oils evolved over time, primarily due to VOCs from the LOX pathway, with 30 VOCs showing significant differences. PCA differentiated EVOO samples from disqualified ones. *E*-2-hexenal and acetic acid were proposed as shelf-life markers, with a ratio below 5 indicating potential disqualification of monovarietal EVOO.

Reference: [1]Freitas F., Cabrita MJ., Gomes da Silva M., (2024). Early Identification of Olive Oil Defects throughout Shelf Life, Separations, 11(6), 167

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New insights into the analysis of phthalate esters

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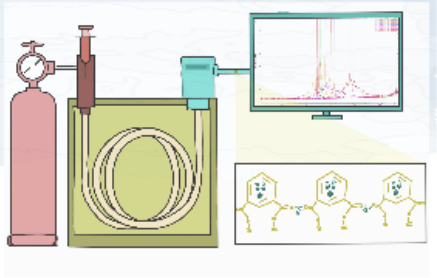
[2] MED, Mediterranean Institute for Agriculture, Environment and Development; CHANGE – Global Change and Sustainability Institute, Departamento de Fitotecnia, Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, 7006-554 Évora, Portugal



1. ABSTRACT

Phthalates are commonly used as plasticizers in polymer manufacturing due to their versatile properties. However, their propensity to migrate into the environment raises concerns for human health, being associated with complications in the reproductive, endocrine, and respiratory systems. This has led to increasing regulations aiming to reduce their usage.

In this study, a gas chromatography method coupled with a triple quadrupole (GC/MS/MS) was developed to identify and quantify 34 plasticizers in food matrices. Despite laboratory cross contamination challenges, the method was tested, but some coelutions persisted due to the presence of the m/z 149 ion, common to all phthalates. To enhance the separation and resolution of challenging peaks, two-dimensional gas chromatography (GCxGC) was employed.

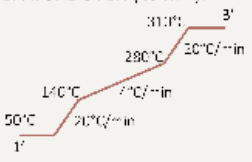
2. RESULTS WITH GC/MS/MS

Chromatographic Method

Split less injection
Flow: Helium; 0,7 mL/min

Phenomenex ZB-5ms
(20m x 0.18 mm i.d., 0,18µm df)
Multiple Reaction Monitoring
Bruker Scion 456 TQ

TEMPERATURE OVEN (45 min):



	Compound		Compound
2	Dimethyl phthalate	4	Benzyl butyl phthalate
29	Dimethyl terephthalate	16	Dihexyl phthalate
30	Dibutyl maleate	24	Bis(2-ethylhexyl) adipate
9	Diethyl phthalate	12	Bis(2-n-butoxyethyl) phthalate
10	Diisopropyl phthalate	19	Dicyclohexyl phthalate
15	Diallyl phthalate	11	Diphenyl phthalate
18	Dipropyl phthalate	1	Bis (2-ethylhexyl) phthalate
27	Diethyl sebacate	17	Di-n-octyl phthalate
8	Diisobutyl phthalate	6	Dioctyl phthalate
5	Dibutyl phthalate	31	Di(2-ethylhexyl) terephthalate
23	Bis(2-methoxyethyl) phthalate	26	di(2-ethylhexyl) sebacate
14	Bis(4-methyl-2-pentyl) phthalate	34	Di(2-propylheptyl) phthalate
13	Diisopentyl phthalate	22	Dinonyl phthalate
21	Bis(2-ethoxyethyl) phthalate	7	Diisononyl phthalate
20	Dipentyl phthalate	3	Didecyl phthalate
25	Bisphenol A	33	Tris(2-ethylhexyl) trimellitate
28	Acetyltributyl citrate	32	1,2 Cyclohexane dicarboxylic acid diisononyl ester

Table 1. Plasticizers identification.

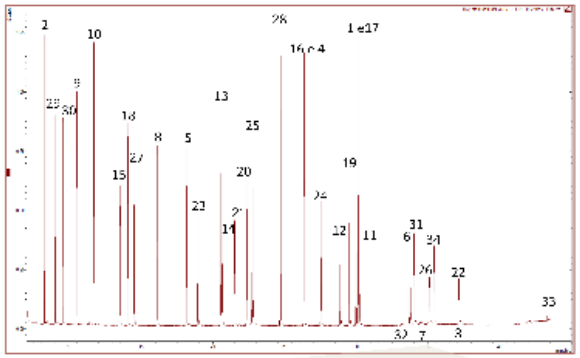


Image 1. Chromatogram of 34 plasticizers.

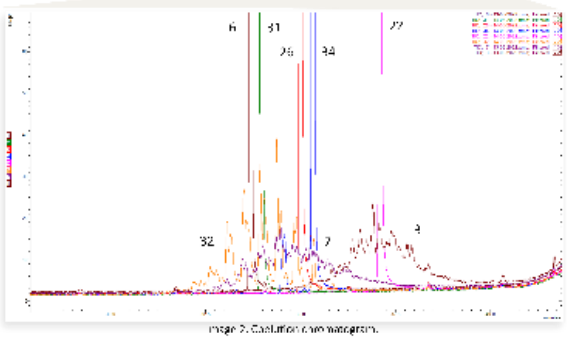


Image 2. Coelution chromatogram.

3. RESULTS WITH GCXGC

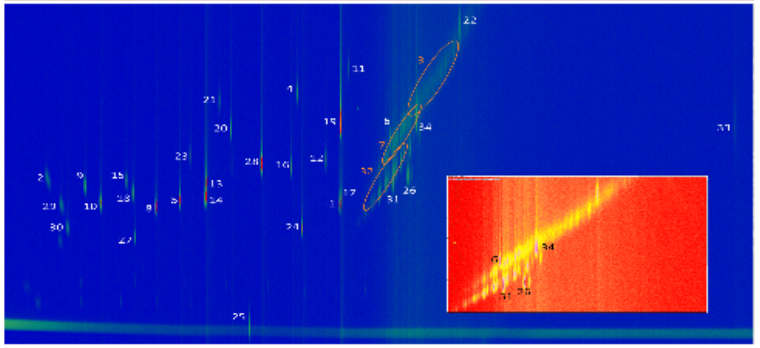


Image 3. Two-dimensional chromatogram of 34 plasticizers.

4. CONCLUSION

The most common methods used to analyze phthalates relies one dimensional gas chromatography (1D), however not all compounds can be separated by using this technique. The experimental testes made with a 2D multidimensional technique (GCxGC) has shown some promising results to increase the separation of the hardest phthalates to separate.

Acknowledgements: We would like to thank the Fundação para a Ciência e Tecnologia for financial support and for the PhD Scholarship 2020.08089.BD. This research was anchored by the RESOLUTION LAB, an infrastructure at NOVA School of Science and Technology.

Funding: Associate Laboratory for Green Chemistry – LAQV which is financed by national funds from FCT/MCTES (UIDB/50006/2020 and UIDP/50006/2020), and MED funding by FCT – Foundation for Science and Technology under the Projects UIDB/05183/2020.

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New insides into the analysis of phthalates esters

The problem of contamination in the laboratory

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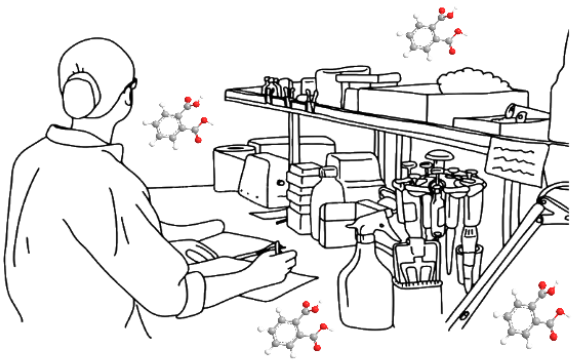
[2] MED, Mediterranean Institute for Agriculture, Environment and Development; CHANGE – Global Change and Sustainability Institute, Departamento de Fitotecnia, Escola de Ciências e Tecnologia, Universidade de Évora, Pólo da Mitra, 7006-554 Évora, Portugal

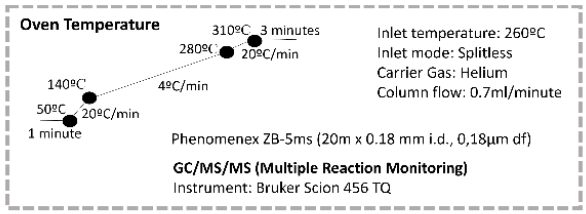
INTRODUCTION

Phthalate esters (PE'S), better known as phthalates, are a group of chemical compounds widely used as plasticizing agents in order to impart flexibility, durability and longevity to plastics.[1]
Given their unique physicochemical properties, some phthalates and their metabolites have a severe toxic effect on human health.[2]
The most relevant source of exposure to these contaminants is food intake.[3]
Nowadays other compounds are used but their effects on human health are unknown. So, it is important that there is regulation and control of these contaminants. Analytical methods are needed to control the contamination of these plasticizers at very low levels of detection.
Traditionally, the analysis of PEs is performed using 1D gas chromatography techniques, e.g. GC/MS and GC/MS/MS.
However, these have shown several problems both in identification and quantification, mainly due to co-elutions between different PE`s, and also with compounds in the matrix. Another major problem in identifying these compounds in real matrices is their ubiquity around us, including in the analytical chemistry laboratory.
In this work, 25 phthalates and 7 phthalate substitutes were separated using a non polar column and detected using a triple quadrupole in multiple reaction monitoring mode, taking into account all possible contaminations during the analyte preparation, extraction and injection process.

How many TIMES have you found PHTHALATES in your samples?



EXPERIMENTAL



RESULTS

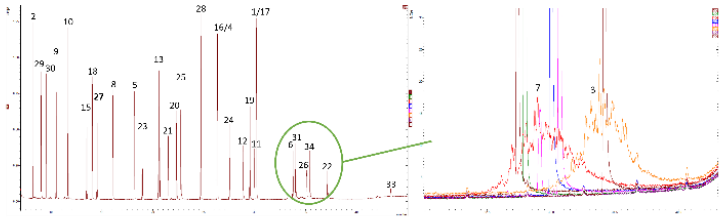


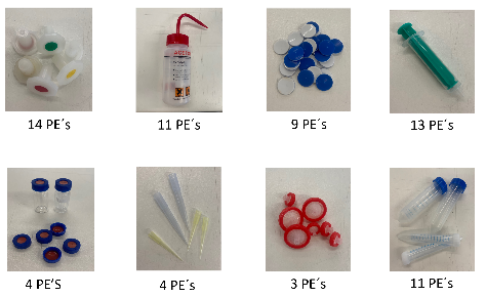
Figure 1. Full scan chromatogram for all compounds

GC/MS/MS MRM

	Compound	Quantifier ion	Qualifier ion
2	Dimethyl phthalate	163-77 (24)	194-63 (10)
29	Dimethyl terephthalate	163-103 (19)	194-163 (12)
30	Dibutyl maleate	117-99 (9)	155-99 (10)
9	Diethyl phthalate	149-65 (23)	177-149 (12)
10	Diisopropyl phthalate	149-65 (23)	167-149 (11)
15	Diallyl phthalate	149-65 (23)	189-41 (10)
18	Dipropyl phthalate	149-65(23)	191-149 (10)
27	Diethyl sebacate	171-55 (11)	213-121 (22)
8	Diisobutyl phthalate	149-65 (23)	167-149 (11)
5	Dibutyl phthalate	149-65 (23)	205-149 (10)
23	Bis(2-methoxyethyl) phthalate	149-65 (23)	207-59 (9)
13	Diisopentyl phthalate	149-65 (23)	167-149 (11)
21	Bis(2-ethoxyethyl) phthalate	149-65 (23)	176-149 (12)
20	Dipentyl phthalate	149-65 (23)	219-149 (9)
25	Bisphenol A	213-91 (28)	228-213 (14)
28	Acetyltributylcitrate	185-69 (25)	213-139 (14)
4	Benzyl butyl phthalate	149-65 (23)	206-149 (12)
16	dihexyl phthalate	149-65 (23)	251-149 (13)
24	Bis(2-ethylhexyl) adipate	129-55 (16)	147-129 (7)
12	bis(2-n-butoxyethyl) phthalate	149-65 (23)	176-149 (11)
19	Dicyclohexyl phthalate	149-65 (23)	167-149 (11)
11	diphenyl phthalate	149-65 (23)	225-77 (27)
1	Bis (2-ethylhexyl) phthalate	149-65 (23)	279-149 (77)
17	Di-n-heptyl phthalate	149-65 (23)	265-149 (12)
6	Dioctyl phthalate	149-65 (23)	167-149 (11)
31	Di(2-ethylhexyl) terephthalate	149-65 (23)	167-79 (14)
26	di(2-ethylhexyl) sebacate	185-69 (23)	185-55 (17)
34	Di(2-propylheptyl) phthalate	149-65 (23)	307-149 (19)
22	Dinonyl phthalate	149-65 (23)	293-149 (14)
7	Diisononyl phthalate	149-65 (23)	293-149 (14)
3	Diisodecyl phthalate	149-65 (23)	307-149 (19)
33	Tris(2-ethylhexyl)trimellitate	305-193 (20)	193-81 (28)

Table 1. MS/MS parameters for PE´s and PE's substitutes. Quantifier and qualifier ion pair, m/z (collision energy, eV)

How many phthalates have been identified in these materials?



CONCLUSION

Several PEs were detected in various laboratory materials.
Traditionally, the analysis of PE's is perfomed using one dimensional gas chromatography (1D), however not all compounds can be separated by using this technique.
Thus, it is intended to apply 2D multidimensional techniques (GCxGC, MD-GC and LC-GC) to increase the separation, detection and sensitivity of the chromatographic system.

ADVICES

- ✓ Using glass materials, such as glass micropipettes
- ✓ Avoid closing volumetric flasks with teflon caps
- ✓ Don´t inject from the same vial more than once, as over time the solution becomes contaminated with phthalates from the cap.
- ✓ Wash all materials with solvent and place in oven before preparing solutions
- ✓ Pay attention to washing detergents and solvents

References
[1] Gómez-Hens, A.; M.P. Aguilar-Caballos, M.P. (2003). Social and economic interest in the control of phthalic acid esters. Trends in Analytical Chemistry, Vol. 22, No. 11, 847-857.
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PHTHALATES

Preventing Future Health Risks

Exploring GC/MS tools for monitoring phthalates in food matrices

Flávia Freitas[1*], Maria João Cabrita[2], Marco Gomes da Silva[1]

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INTRODUCTION

Phthalate esters (PE's), better known as phthalates, are a group of chemical compounds widely used as plasticizing agents in order to impart flexibility, durability and longevity to plastics.[1]

Given their unique physicochemical properties, some phthalates and their metabolites have a severe toxic effect on human health, primarily in the reproductive, endocrine and respiratory systems.[2]

The most relevant source of exposure to these contaminants is food intake.[3]

PEs are not only used in food packaging but also in equipment materials for processing, handling, transport and storage of products. Thus, product contamination does not arise solely because of packaging, which is the last step of production, but also during the manufacturing process.[4]

Since olive oil is one important product for our national industry and anticipating that PEs control will become more strict, requiring very low levels of detection, it is important to define methodologies that respond to this need.

RESULTS

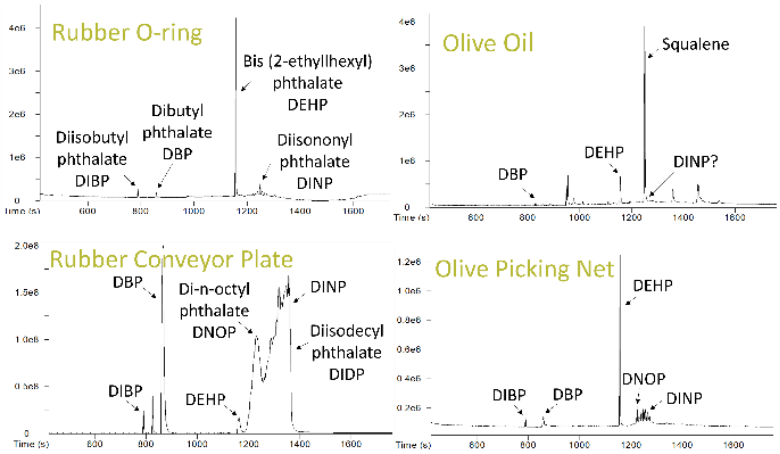


Figure 1. Extracted ion chromatograms showing m/z 149 (characteristic of PEs) from the extract of different materials used in olive oil production, and of olive oil itself

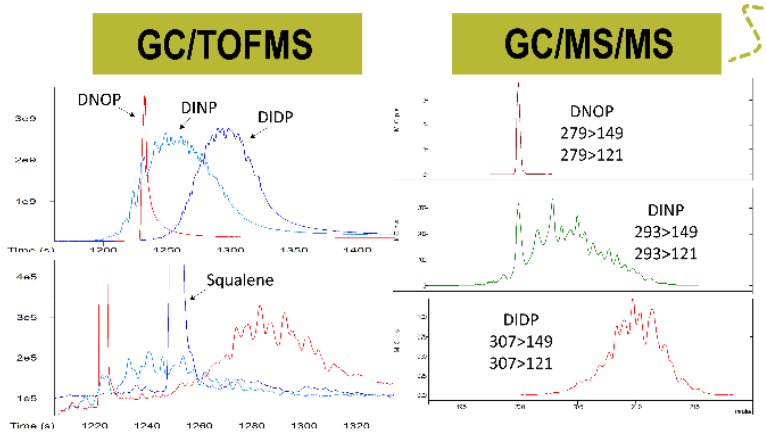
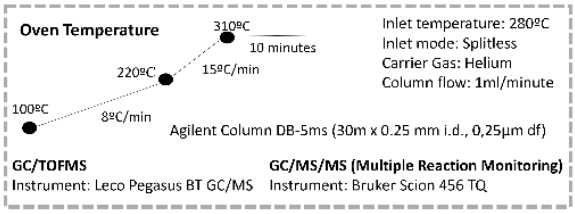
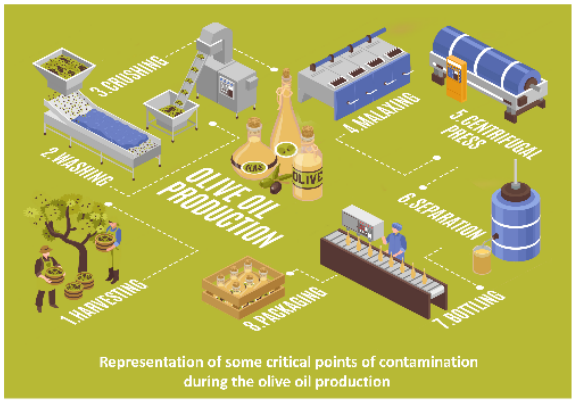


Figure 2. Extracted ion chromatogram showing m/z 149 in GC/TOFMS and MRM chromatograms from the same run, displaying the three different analytes

Did you KNOW that your FOOD may contain PHTHALATES?

EXPERIMENTAL





DINP and DIDP, are found as a mixture of skeletal isomers, resulting in many peaks, leading to co-elution, regardless of oven program.
The most abundant m/z for each of these compounds is the same (149), therefore they can not be identified or quantified by full scan MS

CONCLUSION

Several PEs were detected in various matrices from production materials, packaging and the final product.

Traditionally, the analysis of PE's is perfomed using one dimensional gas chromatography (1D), however not all compounds can be separated by using this technique. For example, in olive oil squalene may be coeluting with DINP, and DNOP, DINP and DIDP also co-elute.

In MS/MS, we used the molecular ion for each compound as precursor, and were able to separate the compounds in the same chromatographic run, except for DINP because it still had DNOP contamination.

In the future, it is intended to apply 2D multidimensional techniques (GCxGC, MD-GC and LC-GC), to increase the separation, detection and sensitivity of the chromatographic system.

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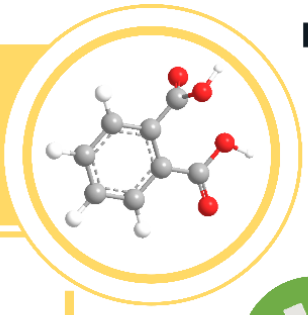
PHTHALATES

Preventing Future Health Risks



Exploring GC/MS tools for monitoring phthalates in food matrices

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Representation of some critical points of contamination during the olive oil production

Introduction

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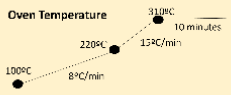
Given their unique physicochemical properties, some phthalates and their metabolites have a severe toxic effect on human health, primarily in the reproductive, endocrine and respiratory systems.[2]

The most relevant source of exposure to these contaminants is food intake.[3]

PEs are not only used in food packaging but also in equipment materials for processing, handling, transport and storage of products. Thus, product contamination does not arise solely because of packaging, which is the last step of production, but also during the manufacturing process.[4]

Since olive oil is one important product for our national industry and anticipating that PEs control will become more strict, requiring very low levels of detection, it is important to define methodologies that respond to this need.

Experimental



Agilent Column DB 5ms (30m x 0.25 mm i.d., 0,25µm df)
Inlet temperature: 280ºC
Inlet mode: Splitless
Carrier Gas: Helium
Column flow: 1ml/minute

GC/TOFMS
Instrument: Leco Pegasus BT GC/MS

GC/MS/MS (Multiple Reaction Monitoring)
Instrument: Bruker Scion 456 TQ

But there are some problems...

X DINP and DIDP, are found as a mixture of skeletal isomers, resulting in many peaks, leading to co-elution, regardless of oven program.

X The most abundant m/z for each of these compounds is the same (149), therefore they can not be identified or quantified by full scan MS.

Results

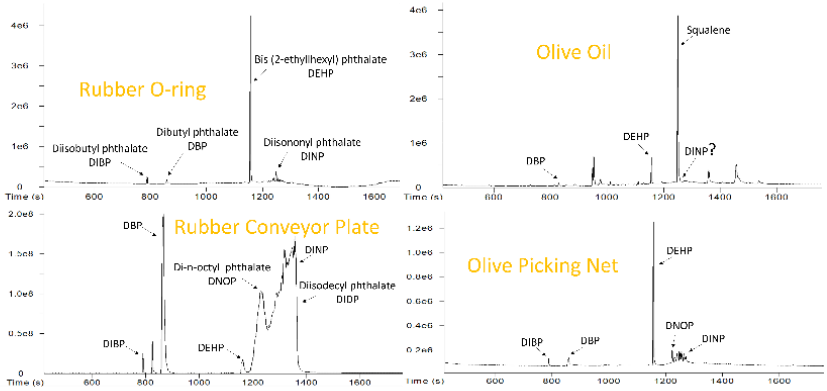
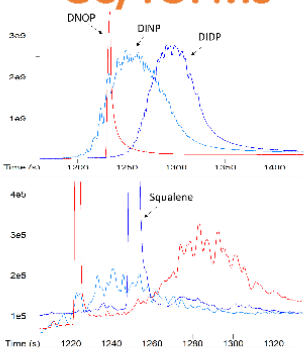


Figure 1. Extracted ion chromatograms showing m/z 149 (characteristic of PEs) from the extract of different materials used in olive oil production, and of olive oil itself

GC/TOFMS



GC/MS/MS

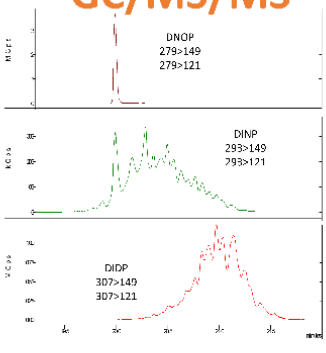


Figure 2. Extracted ion chromatogram showing m/z 149 in GC/TOFMS and MRM chromatograms from the same run, displaying the three different analytes

Conclusion

- Several PEs were detected in various matrices from production materials, packaging and the final product.
- Traditionally, the analysis of PE's is perfomed using one dimensional gas chromatography (1D), however not all compounds can be separated by using this technique. For example, in olive oil squalene may be coeluting with DINP, and DNOP, DINP and DIDP also co-elute.
- In MS/MS, we used the molecular ion for each compound as precursor, and were able to separate the compounds in the same chromatographic run, except for DINP because it still had DNOP contamination.
- In the future, it is intended to apply 2D multidimensional techniques (GCxGC, MD-GC and LC-GC), to increase the separation, detection and sensitivity of the chromatographic system.

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Preventing Future Health Risks

Monitorization of very low levels of some phthalates in food matrices by GC/TOFMS

Flávia Freitas[1*], Maria João Cabrita[2], Marco Gomes da Silva[1]

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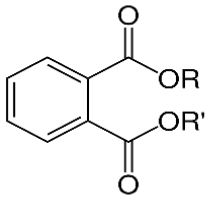

Figure 1. General chemical structure of phthalate ester

Introduction

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Given their unique physicochemical properties, some phthalates and their metabolites have a severe toxic effect on human health, primarily in the reproductive, endocrine and respiratory systems.[2]

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Since olive oil is one important product for our national industry and anticipating that PEs control will become more strict, requiring very low levels of detection, it is important to define methodologies that respond to this need.

Experimental

Olive Oil Extraction Method

0,5 g olive oil + 0,5 ml hexane + 3 ml hexane:metanol 1:4 → vortex → 24 hours rest → organic phase → GC/TOFMS

Production Material Extraction Method

Piece of material + hexane → 24 hours rest → GC/TOFMS

Cromatographic Method

Instrument: Leco Pegasus BT GC/MS
Agilent Column HP-5MS (30m x 0.25 mm i.d., 0,25µm df)

Inlet temperature: 280ºC
Inlet mode: Splitless
Column flow: 1ml/minute

Oven Temperature
310ºC — 10 minutes
220ºC — 15ºC/min
100ºC — 8ºC/min

Results

Figure 2. Extracted ion chromatograms showing m/z 149 (characteristic of PEs) from the extract of different materials used in olive oil production, and of olive oil itself.
⚠ Representation of some critical points of contamination during the olive oil production

Conclusions

- Several PEs were detected in various matrices from production materials, packaging and the final product. The limit of detection is from 0.2 to 1 ppm (mg/kg) for all analytes, and we wish to improve the technique in order to lower these values.
- Traditionally, the analysis of PE's is perfomed using one dimensional gas chromatography (1D), however not all compounds can be separated by using this technique. For example, in olive oil squalene may be coeluting with DINP.
- In the future, it is intended to apply 2D multidimensional techniques (GCxGC e MD-GC), to increase the separation, detection and sensitivity of the chromatographic system.
- This technique may be applied in several different matrices, both aqueous and oily.

References

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2025

FLÁVIA SOFIA SALGADO
DE FREITAS

ANALYTICAL APPROACHES FOR THE QUALITY AND FOOD SAFETY ASSESS-MENT OF OLIVE OIL