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Optimization of Polyphenol Extraction from Olive Leaves using Ohmic Heating (Emerging Processing) and Particle Size Reduction (Pre-processing)

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I further dedicate this work to the present and future food scientists whose discoveries play a major role in promoting green valorization and circular bio-economy. Help us build a zero-waste future for sustainable agricultural/food system.

DECLARATION

I hereby declare that this thesis has been written solely by myself and has not been submitted for any previous degree. I confirm the originality of the research work and that there is no plagiarism or any form of inappropriate use of information or falsification of results in any part of the thesis.

I further declare that I have fully acknowledged the ethical principles of the University of Minho defined in the Code of Ethical Conduct for the research integrity.

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ABSTRACT

Optimization of Polyphenol Extraction from Olive Leaves using Ohmic Heating (Emerging Processing) and Particle Size Reduction (Pre-processing)

Olive leaves are among the most inexhaustible biomass residues generated not only from agricultural/pruning activities, but also from industrial processing of olive oil (namely, olive mill leaves). These products contain a substantial amount of polyphenols, such as secoiridoids, with great antioxidant potentials but they are currently under-utilized; mostly have found low to moderate added-value applications. The present research project, after identification of the knowledge gaps related to the sustainable recovery of polyphenols from olive leaves, performed investigations based on two separate principal approaches: (i) possibilities for sustainable re-utilization of olive leaves through the extraction processes, and (ii) efficient extractability of polyphenols from olive leaves through pre-processing system and possibilities for sustainable processes in olive oil industry.

The first part of the project – olive leaf extracts, obtained by ohmic heating (*OH*), were examined, compared to the conventional heating (*Conven*) and solvent extraction (*Control*). Among the response variables examined, special attentions were placed on the content of polar phenols (total and target constituents) and antioxidant capacity (at the initial point of the extraction and throughout the stability assessments). The findings from the initial point of the extracts support the role of ohmic heating as a value-added processing means, over conventional heating system, having being effective in the rise of total phenolic content (TPC), antiradical activities, and, above all, target polyphenol content (including oleuropein and verbascoside) $p < 0.05$. The findings from the stability study, justify the role of ohmic heating in the stability of leaf extracts because substantial levels of oleuropein and total phenols remained in the extracts, after being stored/heated (over times) and pH modified. The data may provide useful information for future investigations on the development of prototyping of an ohmic heater to potentially deliver an ideal mechanical tool for the enterprises to sustainably valorize olive leaf residues.

The second part of the project– dealing with the valorization of major polyphenols of dry olive leaves through the pre-processing system, the influence of different particle size fractions of leaf powders, after their incorporations during crushing olives (pitted and unpitted) before malaxation (30 and 60 min), on the phenolic content of the resulting leaf-added olive oils was primarily assessed. Firstly, the investigation was made on the original point of the extracted oils (as the preliminary study). Secondarily, the assessments were made throughout the storage conditions. The data from the initial point of the extracted oils give grounds for the role of size reduction of the added olive leaves in the enhancement of polyphenols and antiradical activities of the resulting oils. Importantly, the use of 0.3 mm leaf powders during crushing of pitted olives, prior to a 30-min malaxation exerted significant effects on: (i) the increase in TPC, oleuropein, and verbascoside and antioxidant capacity, and (ii) the decrease in peroxide value and free acidity of the resultant virgin olive oil. The data from the stability of the leaf-enriched olive oils support the significant role of 0.3 mm leaf powders present in the oil (30-min malaxation), especially in terms of oleuropein as considerable amounts still remained over the course of exposure to oxygen and light. Overall, as a potential solution, the findings show an efficient/inexpensive re-use of olive leaf powders (with appropriate mesh size) for the enhancement of polyphenol content of olive oil, particularly when olives are pitted and the malaxation duration is shortened.

Keywords: Ohmic heating; olive leaves; particle size; polyphenols, sustainable reutilization.

RESUMO

Otimização da Extração de Polifenóis de Folhas de Oliveira usando Aquecimento Ohmico (Processamento Emergente) e Redução do Tamanho de Partículas (Pré-processamento)

As folhas de oliveira estão entre os resíduos de biomassa mais abundantes gerados não só pelas atividades agrícolas/podas, mas também pelo processamento industrial do azeite (nomeadamente, folhas de lagar). Estes produtos contêm uma quantidade substancial de polifenóis, como os secoiridoides, com grande potencial antioxidante, mas atualmente são subutilizados sendo aplicados em soluções de valor agregado baixo a moderado. O presente projeto de investigação, após identificação das lacunas de conhecimento relacionadas com a recuperação sustentável de polifenóis de folhas de oliveira, desenvolveu investigação baseada em duas abordagens principais distintas: (i) possibilidade de reutilização sustentável de folhas de oliveira através dos processos de extração, e (ii) extração eficiente de polifenóis de folhas de oliveira através de sistema de pré-processamento e possibilidade de aplicação de processos sustentáveis na indústria do azeite.

A primeira parte do projeto – extratos de folhas de oliveira, obtidos por aquecimento óhmico (*OH*), foram examinados, comparados com aquecimento convencional (*Conven*) e extração com solvente (*Controlo*). Entre as variáveis de resposta examinadas, foi dada especial atenção ao conteúdo de fenóis polares (constituintes totais e alvo) e à capacidade antioxidante (no ponto inicial da extração e ao longo das avaliações de estabilidade). Os resultados obtidos a partir do valor inicial dos extratos apoiam o papel do aquecimento óhmico como meio de processamento de valor agregado, em relação ao sistema de aquecimento convencional, tendo sido eficaz no aumento do conteúdo fenólico total (TPC), atividades antirradicalar e, acima de tudo, conteúdo de polifenóis alvo (incluindo oleuropeína e verbascosídeo) $p <$ 0,05. Os resultados do estudo de estabilidade justificam o papel do aquecimento óhmico na estabilidade dos extratos de folhas porque níveis substanciais de oleuropeína e fenóis totais permaneceram nos extratos, após serem armazenados/aquecidos (ao longo do tempo) e com pH modificado. Os dados podem fornecer informações úteis para futuras investigações sobre o desenvolvimento de um protótipo de um aquecedor óhmico como ferramenta mecânica ideal para as empresas valorizarem de forma sustentável os resíduos de folhas de oliveira.

A segunda parte do projeto – tratando da valorização dos principais polifenóis das folhas secas de oliveira através do sistema de pré-processamento, a influência de frações do pó das folhas com diferente granulometria, após a sua incorporação no azeite durante o esmagamento das azeitonas (sem caroço e com caroço) antes da malaxação (30 e 60 min), no conteúdo fenólico dos azeites adicionados de folhas resultantes foi avaliada. Primeiramente, foi avaliada o efeito imediatamente após extração e, de seguida, ao longo das condições de armazenamento. Os dados obtidos imediatamente após extração apoiam o papel fundamental da redução do tamanho dos pós de folhas de oliveira adicionados no aumento dos polifenóis e das atividades antirradicalares dos óleos resultantes. É importante ressaltar que o uso de folhas em pó de 0,3 mm durante o esmagamento de azeitonas sem caroço, antes de uma malaxação de 30 minutos, exerceu efeitos significativos sobre: (i) o aumento de TPC, oleuropeína e verbascosídeo e capacidade antioxidante, e (ii) a diminuição de valor de peróxido e acidez livre do azeite virgem resultante. Os dados da estabilidade dos azeites enriquecidos com folhas apoiam o papel significativo dos pós de folhas de 0,3 mm presentes no azeite (malaxação de 30 minutos), particularmente em termos de oleuropeína, uma vez que ainda restavam quantidades consideráveis após exposição ao oxigénio e à luz. tempo. No geral, como uma solução potencial, os resultados mostram uma reutilização eficiente/barata de pós de folhas (com tamanho de partícula apropriado) para aumentar o teor de polifenóis no azeite com adição de folhas, especialmente quando as azeitonas são descaroçadas e a duração da malaxação é encurtada.

Palavras-chave: Aquecimento ôhmico; folhas de Oliveira; polifenóis; reutilização sustentável; tamanho da partícula.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degrees centigrade
µL	Microliter
µm	Micrometer
µmol	Micromole
3,4-DHPEA-EA	3,4-dixydroxyphenylethanol elenolic acid (oleuropein aglycone)
5-LOX	5-lipoxygenase enzyme
ABTS	2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists Society
BHT	Butylated hydroxytoluene
CE	Catechin equivalents
cm	Centimeter
CO_2	Carbon dioxide gas
Conven	Conventional heating
COX-2	Cyclooxygenase-2 enzyme
DPPH	2,2-diphenyl-1-picrylhydrazyl
d.w.	Dry weight
EC50	Half maximal effective concentration
EtOH	Ethanol
EVOO	Extra virgin olive oil
FFA	Free fatty acids
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
h	Hours
HPLC	High-performance liquid chromatography
HVED	High-voltage electric discharges
IC50	Half-maximal inhibitory concentration
in vitro	In glass (outside the living organism)
Kg	Kilogram
kHz	Kilohertz

LC–MS/MS	Liquid chromatography with tandem mass spectrometry
M	Molarity
m/s	Meter per second
M^2/s	Meter squared per second
MAE	Microwave assisted extraction
mEq. O_2	Milliequivalents of active oxygen
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimole
N	Normality
nm	Nanometer
NMR	Nuclear magnetic resonance spectroscopy
–OH	Hydroxyl group
OH	Ohmic heating
OMWW	Olive mill wastewater
p	Probability
PEF	Pulsed electric field
pH	Potential of hydrogen
p-HPEA-EA	p-hydroxyphenyl-ethanol linked to elenolic acid (Ligstroside aglycone)
PLE	Pressurized liquid extraction
PV	Peroxide value
QE	Quercetin equivalents
rpm	Rotation per minute
SD	Standard deviation
SFE	Supercritical fluid extraction
SPSS	Statistical Package for the Social Sciences
TCT	Total condensed tannins
TE	Trolox Equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content

TPC	Total phenolic content
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
Trolox	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UAE	Ultrasound assisted extraction
UHPLC	Ultra-high-performance liquid chromatography
UV-vis	Ultraviolet–visible
v/v	Volume per volume
VOO	Virgin olive oil
W/O	Water in oil
w/w	Weight in weight
Yr	Year

SCIENTIFIC OUTPUT

Published in peer-reviewed journals:

- ✓ Safarzadeh Markhali, F., Teixeira, J.A. and Rocha, C.M., 2020. Olive tree leaves—A source of valuable active compounds. *Processes*, *8*(9), 1177. https://doi.org/10.3390/pr8091177

- ✓ Safarzadeh Markhali, F., 2021. Roles of drying, size reduction, and blanching in sustainable extraction of phenolics from olive leaves. *Processes*, *9*(9), 1662. https://doi.org/10.3390/pr9091662

- ✓ Safarzadeh Markhali, F., 2021. Effect of processing on phenolic composition of olive oil products and olive mill by-products and possibilities for enhancement of sustainable processes, *Processes*, *9*(6), 953. https://doi.org/10.3390/pr9060953

- ✓ Safarzadeh Markhali, F., Teixeira, J.A. and Rocha, C.M., 2022. Effect of ohmic heating on the extraction yield, polyphenol content and antioxidant activity of olive mill leaves. *Clean Technologies*, *4*(2), 512–528. https://doi.org/10.3390/cleantechnol4020031

- ✓ Safarzadeh Markhali, F., and Teixeira, J.A., 2023. Comparative study of different particle sizes of added olive leaves for the content of target polyphenols in virgin olive oil. *Sustainable Food Technology*, *1*(6), 896–905. https://doi.org/10.1039/D3FB00108C

Submitted to peer-review publication:

- ✓ Safarzadeh Markhali, F., and Teixeira, J.A., 2023. Stability of Target Polyphenols of Leaf-Added Virgin Olive Oil under Different Storage Conditions over Time. *Sustainable Food Technology* (under review)

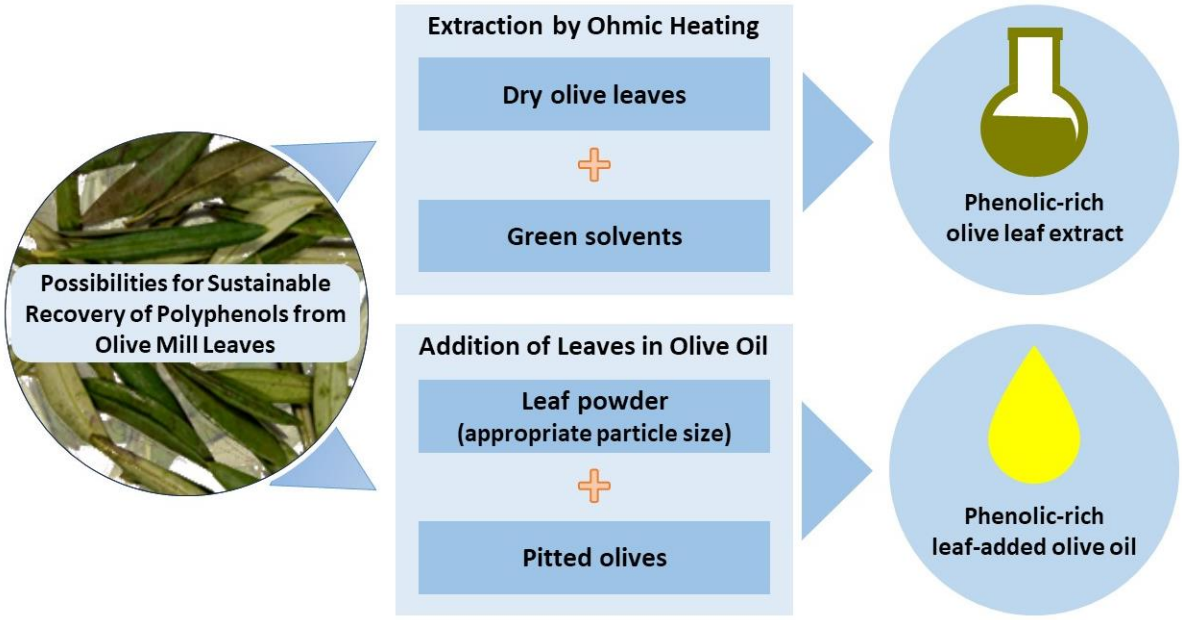
- ✓ Safarzadeh Markhali, F., and Teixeira, J.A., 2023. Extractability of Oleuropein, Hydroxytyrosol, Tyrosol, Verbascoside, and Flavonoid-Derivatives from Olive Leaves using Ohmic Heating (A Green Process for Value Addition). *Sustainable Food Technology* (under review)

THESIS SUMMARY

RESEARCH BACKGROUND, JUSTIFICATION, AND OBJECTIVES

Research background and justification – Olive mill leaves account for a substantial amount of plant biomass residues generated from olive oil industry. They are a source of important antioxidant polyphenols with distinctive molecular properties, in particular, secoiridoids which are uniquely found in Oleaceae plants (olive family). Such valuable ingredients remain potent/little affected because they are removed at the preliminary stage of the oil processing (leaves are removed before milling process). The growing awareness of such benefits has prompted a rising interest in the investigation of efficient recovery of polyphenols from olive leaf residues using a broad range of mechanical techniques. However, while many scientific attempts have been made, due to the challenges involved, olive leaf residues are currently underutilized. Therefore, the potential solutions for their sustainable valorizations are yet to be explored. In this regard, through an in-depth review of literature, the present doctoral research project performed investigations on two novel strategies that may be considered as a partial contribution to filling the current knowledge gaps. Importantly, the extractability of target polar phenols (including oleuropein) from olive leaves was investigated based on the following separate approaches: (i) through the extraction process, using ohmic heating as an emerging approach, compared to the conventional heating, to assess the target analytes in the resulting olive leaf extracts, and (ii) through pre-processing (particle size reduction) of dry leaves (without having them solvent extracted), with subsequent addition of size fractions of leaf powders to the extraction process of olive oil to assess the target analytes in the resulting leaf-added oils.

Thesis Graphical Abstract



Research objectives – dealing with the possibilities for sustainable recovery of polyphenols from olive mill leaves the overall objectives were: (i) green extraction using ohmic heating with potential favorable phenolic recovery in the leaf extracts, and (ii) size reduction of leaves with potential favorable phenolic enhancement in the leaf-added olive oils. The specific objectives of this research are listed below:

- To evaluate the effect of ohmic heating, compared to the conventional method, on the extraction yield, total and target polyphenols, and *in vitro* antioxidant capacity of olive mill leaves, thorough a range of solvent ratios (aqueous ethanol) at different temperatures.
- To determine the stability of olive leaf extracts under various storage conditions over time, thermal conditions over time, and pH conditions.
- To evaluate the effect of particle size of dry ground olive leaves on the phenolic content of olive oil when added to the crushed olives in advance of malaxation of olive paste.
- To examine the storage stability of virgin olive oils enriched with olive leaf powders, under light and oxygen exposures over time.

Thesis outline – This thesis covers a total of nine chapters as outlined below:

PART I

Chapter 1 – An overview based on the key endogenous bioactive compounds in olive tree (*Olea europaea* L.) leaves is provided in this chapter. Importantly, the following aspects are discussed: (i) bioactive compounds in olive leaves, (ii) current/proposed technological means for the extraction of antioxidant ingredients including polyphenols, (iii) importance of stability of olive leaf extracts to their bioactivities, (iv) potential industrial applications of the leaf extracts, and (v) future perspectives and challenges associated with sustainable re-utilization of olive leaves through maximum isolation of desired bio-molecules.

Chapter 2 – Dealing with the extractability of polyphenols from olive mill leaves through the mechanical processes, ohmic heating (*OH*), as an emerging approach, was applied using a range of extraction temperatures (45–75 °C) and solvent ratios (40–80% ethanol). The resulting leaf extracts were assessed, compared to those obtained by conventional heating (*Conven*) and *Control* (solvent extraction without heating) in terms of the extraction yield, total phenolic content (TPC), and antioxidant activities of olive mill leaves. The data from this model study demonestrate that, compared to the conventional system, the ohmic heating was significantly effective in the enhanced recovery of polyphenols, and, correspondingly, higher antioxidant capacity of the leaf extracts $p < 0.05$.

Chapter 3 – The outcome from the previous chapter (Chapter 2) served as a preliminary study for the current chapter that followed up the same extraction designs to further evaluate (i) concentrations of individual target polar phenols (oleuropein, verbascoside, hydroxytyrosol, tyrosol, luteolin 7-O-glucoside, apigenin 7-O-glucoside, and rutin) via chromatographic analysis using UHPLC, (ii) total flavonoid content (TFC), total condensed tannins (TCT), as well as repeated measurement of TPC to confirm the data, and to compare the trends of the variations across all extraction groups, to the corresponding values of TFC and TCT, and (ii) *in vitro* antioxidant capacity, using three standard analytical methods. Overall The data from this study support the significant potential of ohmic heating for improved recovery of the chief biophenols, particularly oleuropein, from olive mill leaves.

Chapter 4 – To gain a good knowledge of keepability of the polyphenols of the leaf extracts, this chapter further extends previous scheme (Chapter 3) to monitor the changes of the major polar phenols in olive leaf extracts under different conditions/time points as follows: (i) storage temperatures (4 °C, –20 °C, and 25 °C) over time, (ii) thermal trials (70–110 °C) over time, and (iii) pH solutions (3–9). The

main idea was to assess the stability of ohmic extracts, compared to the conventional groups, under the given surrounding conditions, for each dependent variable of the study (particularly concentrations of target polar phenols and antioxidant capacity). Overall the data justify the role of ohmic heating as a preferred extraction method in the stability of extracts because, compared to the conventional groups, appreciably significant proportions of polyphenols (particularly oleuropein) and antiradical capacity remained after the *OH* extracts were stored and heated (over time), and exposed to various pH conditions.

PART II

Chapter 5 – As a novel concept, this chapter provides an insight into the importance of preprocessing operations, particularly particle size reduction (throughout grinding and sieving) and drying of olive leaves that commonly employed at the initial stage of olive leaf exploitations. This study highlights that operation parameters/methods involved in preprocessing system are equally important as those associated with the processing/extraction system; a viewpoint that has been overshadowed in the literature. Additionally, it emphasizes that there is a need to optimize the preprocessing parameters particularly when the selective recovery of bio-compounds from olive leaves is of interest. Further, the study points out that it is highly crucial to determine whether/not the desired polyphenols are largely liberated from the leaf matrix during preprocessing before/without being subjected to the extraction process.

Chapter 6 – The current chapter further provides an insight into possible solutions for sustainable processing system in olive oil industry, wherein underlines the key factors responsible for efficient technologies that potentially assist in (i) intensification of recovery of antioxidant polyphenols in olive oil, and (ii) green extraction of phenolic constituents from olive mill by-products (including olive leaves). The information gained in present chapter and previous chapter (**Chapter 5**) further bring to light a new solution that prompted the performance of an experiment (see **Chapter 7**) for potential enrichment of olive oil with dry olive leaves with a range of particle size fractions.

Chapter 7 – Having gained a good knowledge from previous works (**Chapter 6** & **7)**, a novel study was formulated for the research investigation performed in the current chapter. The experiment deals primarily with the current knowledge gaps based on: (i) the crucial role of preprocessing of olive leaves in the diffusion and liberation of bio-phenols from the plant tissue, and (ii) the need to compensate the loss of polyphenols (specially oleuropein and verbascoside) in the extracted olive oil. In this model study, a range of particle size fractions of the added dry olive leaves are compared for their effects on the quality of the resulting leaf-added oils in respect of: (i) total and target polar phenols (oleuropein, verbascoside,

hydroxytyrosol, tyrosol, luteolin, and apigenin), (ii) antioxidant activities, and (iii) physicochemical quality – free fatty acids (FFA), peroxide value (PV), oil extractability (extraction yield), and pigments (chlorophylls and carotenoids). The results demonstrate that depending on the size reduction, significant proportions of target polyphenols are released from the leaf matrix (during crushing of pitted olives) and transferred to the crushed paste before a short-term malaxation. Overall the data highlights that the reduction of the leaf particles to an appropriate sieve size potentially offers additional benefit for delivering an affordable means to valorize dry ground olive leaves for the enhancement of total & target polyphenols in olive oil.

Chapter 8 – In this chapter, the results of the previous experiment (**Chapter 7**) served as the preliminary experiment that was referred to as the partial benchmark in this experiment, in respect of independent variables – where the effects of the factors of previous work (Chapter 7) on the selected response variables were statistically significant, they were included in this chapter for the investigation of the stability of olive oils (containing optimum particle size of leaf powders) under various storage conditions over the course of six-month period. The storage situations of olive oil samples were as follows: (i) storage of oils without exposure to light and oxygen, (ii) storage of oils with light exposure only, and (iii) storage of oils with oxygen exposure only. All samples were stored at laboratory room temperature (20 ± 5 °C). Overall, the data justify the significant role of the appropriate particle size of added leaves in the oils as, in effect, a significant amount of oleuropein remained after the oils were exposed to the severe storage conditions (oxygen and light) over time.

PART III

Chapter 9 – The thesis ends with the most important conclusions drawn on the combined data presented in previous chapters (**1– 8**). Based on the given conclusions, a list of suggestions for the future relevant studies are also provided in this chapter.

Part I

Possibilities for Sustainable Re-Utilization of Olive Leaves through the Extraction Processes

CHAPTER 1

OLIVE TREE LEAVES—A SOURCE OF VALUABLE ACTIVE COMPOUNDS

This work, cited below, is published by MDPI under the special issue of: "Sustainable Development of Waste towards Green Growth":

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processes

MDPI

Review

Olive Tree Leaves—A Source of Valuable Active Compounds

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Abstract – The agricultural and processing activities of olive crops generate a substantial amount of food by-products, particularly olive leaves, which are mostly underexploited, representing a significant threat to the environment. Olive leaves are endowed with endogenous bioactive compounds. Their beneficial/health-promoting potential, together with environmental protection and circular economy, merit their exploitation to recover and reuse natural components that are potentially safer alternatives to synthetic counterparts. These biomass residues have great potential for extended industrial applications in food/dietary systems but have had limited commercial uses so far. In this regard, many researchers have endeavored to determine a green/sustainable means to replace the conventional/inefficient methods currently used. This is not an easy task as a sustainable bio-processing approach entails careful designing to maximize the liberation of compounds with minimum use of *(i)* processing time, (*ii)* toxic solvent *(iii)* fossil fuel energy, and (iv) overall cost. Thus, it is necessary to device viable strategies to *(i)* optimize the extraction of valuable biomolecules from olive leaves and enable their conversion into high added-value products, and (ii) minimize generation of agro-industrial waste streams. This review provides an insight to the principal bioactive components naturally present in olive leaves, and an overview of the existing/proposed methods associated with their analysis, extraction, applications, and stability.

Keywords: olive leaves; by-products; bioactive compounds; polyphenols; extraction; sustainability.

1.1. Introduction

Olive tree (*Olea europaea* L.), a member of the Oleaceae (Gilani & Khan, 2010), is a drought-tolerant plant essentially native to the Mediterranean climate (Mannina & Segre, 2010). It is universally planted in tropical and subtropical regions (Ray et al. 2015), predominantly in Greece, Italy, Spain, Australia, Portugal, France, Cyprus, Israel, Jordan, USA, Morocco, Turkey, and Tunisia (Caballero et al. 2003). The Mediterranean region, representing above 90% of the cultivated area, is estimated to be above 11 million hectares in 2017 (Zipori et al. 2020).

The leaves of the olive tree are within easy reach either from the olive orchard or from the residues remained after agricultural (Rahmanian et al. 2015) and industrial by-products (Contreras et al. 2020). The olive mill leaves constitute a relatively sizable portion, in the range of 4–7% (Contreras et al. 2020), up to 10% of overall weight of processing olives (Abaza et al. 2015) and account for almost 5% of overall yield from olive oil by-products (Lama-Muñoz et al. 2020). During the horticultural system, a significant proportion of leaf residue is also generated. In the course of pruning, depending on variations in geography, horticultural routine, and tree lifetime, the amount of leaf by-products roughly accounts for 25% of total weight of pruned residue (Romero-García et al. 2014).

The increment of these residues represents a major problem through its adverse effect on environmental sustainability, as a large proportion of leftovers is underexploited and/or inadequately disposed of, e.g., through incineration (Talhaoui et al. 2015). There is an increased awareness that this underutilized biomass could be regarded as a valuable/health-promoting resource, if properly exploited, with great market potential in the food and dietary system. The principal benefits of olive leaves (Contreras 2020) are attributed to their distinctive molecular and biological properties (Rahmanian et al. 2015) which confer protective activities of (i) antioxidation (Bulotta et al. 2011), (ii) anti-inflammation (Cavaca et al. 2020), (iii) anti-hypertension (Gilani & Khan, 2010), (iv) anti-arrhythmia (Benavente-García et al. 2000), and (v) inhibiting low-density lipoprotein receptors (Jemai et al. 2008). Indeed the exertion of bioactivity stems from the action of intrinsic phytochemicals, particularly polyphenols (Balasundram et al. 2006), present in olive leaves (Erbay & Icier, 2010). In this respect, recovery of bioactive ingredients from this residual biomass and their conversion into value-added ingredients in the food and dietary system, helps promote a sustainable processing method to provide bio-based/nutritional products with a minimum/zero waste stream.

At present, commercial applications of olive leaves are mostly limited to (i) folk medicine, to protect body against chronic conditions such as cardiovascular disease and diabetes (Contreras et al. 2020; Acar-Tek & Ağagündüz, 2020; Tsimidou & Papoti, 2010), and (ii) animal feed (Rahmanian et al. 2015).

Research studies have now focused to determine broader applications of olive leaves across various industrial sectors including foods, modern medicines, and pharmaceuticals (Tsimidou & Papoti, 2010; Souilem et al. 2017). Although much effort has been made to extend the use of leaves from traditional to modern/industrial applications, the sustainable recovery of natural biomolecules comes with several challenges, which requires better performance to ensure (i) optimum extraction that is less time-consuming/economical, (ii) the stability of biomass and leave extracts, (iii) the safety and non-toxicity of outputs and inputs, (iv) the bioavailability of product, and (v) up-scalability of the processing system. This review provides an outline of the principal endogenous bioactive compounds in olive leaves, their characteristics, with respect to their biological activities and industrial uses, and discusses the existing processing approaches/proposals for the recovery of these compounds.

1.2. Bioactive compounds in olive leaves

Bioactive compounds are the naturally occurring ingredients derived from a variety of food crops (Hamzalıoğlu & Gökmen, 2016). They are grouped as either essential or non-essential components (Bernhoft, 2010). Unlike essential nutrients that are necessary for the human biological system—as the body is unable to synthesize them and their deficiency may bring about biological disorders—non-essential compounds are those which are not necessary for biological functions and their insufficient/lack of intake has no influence on physiological disorders (Ortega & Campos, 2019). However, the presence of non-essential groups in food crops, predominantly phytochemicals (Ortega & Campos, 2019), are appreciably prized for their auxiliary physiological effects in promoting health to a further extent than those exerted by essential nutrients present in foods (Hamzalıoğlu & Gökmen, 2016; Bernhoft, 2010; Šaponjac et al. 2016). Of all the beneficial effects, antioxidative, anti-atherosclerotic, antimicrobial, and anti-inflammatory functions prevail in most bioactive compounds (Hamzalıoğlu & Gökmen, 2016). The processing methodologies involved in food functionalization and nutraceutical supplements substantially determine the extent of (i) bio-accessibility, that is the liberation of bio-compounds from the cell walls of the food before being absorbed in the blood cells (ii) bioavailability, namely, the availability of active compounds for physiological activity/metabolism upon absorption, and (iii) bioactivity (Galanakis, 2017).

1.2.1. Polyphenolic compounds

Polyphenols, a group of heterogenous compounds, refer to naturally occurring secondary metabolites largely found in plant species (Moreno & Peinado, 2012). Their molecular structure is based on a linkage between hydroxyl groups (polar phase) and one aromatic ring (Souto et al. 2019). The magnitude of antioxidation of phenolics (Petti & Scully, 2009) partly relies on the location and

arrangement of hydroxyl groups (Belitz et al. 2009). It also depends on their presence/proportion in the food, and their molecular formation in the matrix, i.e., whether they are in free or bound state (Oniszczuk et al. 2019). Research has demonstrated that the aglycone portion displays higher biological activity than the glycoside portion (aglycones bound to glycones) (Oniszczuk et al. 2019). The free radical scavenging activity exerted by phenolics, can limit the initiation phase, and postpone the propagation phase of lipid oxidation, with the potential of minimizing the production of volatile components from hydroperoxide degradation (Shahidi and Ambigaipalan 2015). Polyphenols, together with displaying physiological activities including antioxidation, are organoleptically responsible for the flavor and color of the foods. On this account, they are considered beneficial to promoting health and food quality (Balasundram et al. 2006; Belitz et al. 2009).

Phenolics relatively have good stability upon stomach digestion but the level of bio-accessibility may vary depending on numerous decisive factors, such as (i) plant origin and physical structure, (ii) chemical composition of phenolics and their reaction with other active components released from the cell walls of the ingested foods, and (iii) processing methods/conditions (Wojtunik-Kulesza et al. 2020). The processing parameters include (i) heat treatment, and (ii) drying when a medium/low temperature is used (around 40–50 °C) which may adversely affect phenolic contents and bioactivity, that is attributed to the fact that the survived enzymes (that were not deactivated at low temperature) can bring about oxidative and/or hydrolytic reactions, and hence the degradation of phenolic constituents (Oniszczuk et al. 2020). Heat treatment may be advantageous (through exerting solubility and liberation of phenolics from the food matrix, and/or developing disintegration of the cellular structure (Oniszczuk et al. 2020). It can also be unfavorable, giving rise to degradation of the components that are unable to withstand thermal conditions (Wojtunik-Kulesza et al. 2020).

Olive leaves are abundant in a range of known phenolic groups that are broadly clustered into (i) secoiridoids (including oleuropein and oleuropein-aglycone), (ii) flavonoids (such as rutin and luteolin-7-glucoside), and (iii) simple phenols (such as hydroxytyrosol and tyrosol). Of these, secoiridoids are characteristically present in the Oleaceae family that well applies to *Olea europaea* L. (Segura-Carretero et al. 2010), have notably gained attention owing to their distinctive potential for biological activity (Huang et al. 2019). Iridoids, usually with glycosidic structure in plants, derive mainly from monoterpenes (Huang et al. 2019). The ring-breakage of cyclopentane gives rise to the formation of secoiridoids (Mander & Liu, 2010; Rodriguez et al., 1998) that are typically attached to glycosides and distinguished by the presence of elenolic acid linkage within their atomic arrangements (Segura-Carretero et al. 2010).

Among the most typical phenolics in olive leaves include hydroxytyrosol, tyrosol, rutin, and oleuropein (Rahmanian et al. 2015; Benavente-García et al. 2000). Indeed, oleuropein and hydroxytyrosol are detectably prominent in olive leaves. Oleuropein, a key component of secoiridoids in olive leaves (Erbay & Icier, 2010) is characterized by an ester linkage of elenolic acid glucoside and hydroxytyrosol (Segura-Carretero et al. 2010; Nediani et al. 2019). Because of its unique secoiridoid structure, it is organoleptically characterized by a strong bitterness (Acar-Tek & Ağagündüz, 2020). The concentration of oleuropein in olive leaves has been reported to be in the range of 6 to 9%, dry basis (Romani et al. 2017). Bouaziz & Sayadi (2005), in their research on evaluating antioxidant ability of olive leaves from Tunisian cultivar, observed that oleuropein (6.8 g/100 g fresh leaves) was the principal phenolic constituent in the leaves of olive trees.

The protective attributes of oleuropein are reflected typically by their inhibiting effects against (i) oxidation (Benavente-García et al. 2000), (ii) microbial disorders (Benavente-García et al. 2000), (iii) inflammation (Visioli et al. 1998), and (iv) platelet aggregation (Benavente-García et al. 2000). In addition, oleuropein is found to be effectively capable of re-building the tissue damage, caused by cisplatin in stomach and lung organs (Geyikoglu et al. 2017). It is noteworthy that the phenolic concentration in olive drupes is comparatively as great as those in olive leaves (Rahmanian et al. 2015), however the proportion adversely decreases during the maturation and processing steps. For example, significant depletion of oleuropein, during processing, is highly likely, particularly through enzymatic reactions (Paiva-Martins & Pinto, 2008) and de-bittering operations (Zoidou et al. 2017). The degradation of oleuropein in olive processing further merits exploitation of olive leaves, as oleuropein is appreciably retained/unaffected in olive by-products. In principle, there are numerous decisive factors (**Table 1.1**), acting dependently or independently, that markedly determine the proportion, structural feature, and bioavailability of polyphenols in olive leaves.

Oleuropein aglycon, is liberated in the course of oleuropein de-glycosylation (Xu et al. 2018). Research demonstrated that oleuropein aglycon has a great contribution to developing bitterness/astringency (Siliani et al. 2006). The health benefits associated with this compound include its ability to (i) curtail neurodegeneration, mainly through suppression of Aβ1–42 toxicity (Leri et al. 2019), (ii) decrease low-density lipoprotein cholesterol (Rahmanian et al. 2015), and (iii) promote oxidative stability of lipids (Jemai et al. 2008).

Olive leaves, ubiquitous with flavonoids, are further valued for dietary applications. Flavonoids exert a favorable protection against carcinogenic, cardiovascular (Babu & Liu, 2009), and microbial (Havsteen, 2002) diseases. Typical examples of flavonoids in olive leaves are rutin, luteolin, and luteolin-7-glucoside

(Rahmanian et al. 2015; Benavente-García et al. 2000). Total flavonoids in olive leaves, on a dry-basis, using different extraction techniques were: (i) through maceration, 54.92 mg/g (with 80% ethanol) and 34.50 mg/g (with distilled water), and (ii) through sonication, 21.15 mg/g (with 80% ethanol), and 36.40 mg/g, with distilled water as the solvent (Ghomari et al. 2019).

Hydroxytyrosol, largely found in olive leaves, is among the main simple phenolic alcohol (Tamasi et al. 2016). The formation of hydroxytyrosol, derives from the hydrolysis of oleuropein, and their numbers grow through (i) stage of maturity, (ii) processing line (Rahmanian et al. 2015), and (iii) metabolism of oleuropein upon intake of oleuropein-based foods (Erbay & Icier, 2010). Hydroxytyrosol generally offers dietary health benefits in terms of antioxidation, anti-atherosclerosis, anti-carcinogenic and anti-inflammation (Martínez et al. 2018). The antioxidation potency of hydroxytyrosol is attributable to its ability to inhibit free radicals, mainly through donating hydrogen atoms, typically from hydroxyl groups, that initiates the formation of low-energy linkage with minimum reactivity arising from delocalization in phenoxyl radical (Rahmanian et al. 2015). The hydroxytyrosol content of olive leaves, using high-performance liquid chromatography (HPLC) method, has been reported to be around 2.28 mg per 100 g leaf extract (Fki et al. 2020).

Tyrosol, a group of phenolic alcohol, is usually present in a trace amount in olive leaves (Tamasi et al. 2016). Due to its chemical stability, it is less prone to be degraded by auto-oxidation (Karković Marković et al. 2019). Although the antioxidative potency of tyrosol is relatively low (compared to those of other prominent phenolics such as flavonoids), it remains stable/unaffected during the process of oxidation (Karković Marković et al. 2019). The concentration of tyrosol in olive leaves, by maceration using ethanol and water solvent extraction, is approximately 0.0007 mg/g leaves, dry basis (Ghomari et al. 2019).

Table 1. 1 Typical factors affecting concentration and functional properties of olive leaf phenolic compounds.

Harvest stage	Determinant	Reference(s)
Pre-harvest	Geographical origin	Bilgin,& Şahin (2013)
	Maturity stage	Tsimidou & Papoti (2010)
	Climate	Wang et al. (2019)
	Cultivar	Bilgin,& Şahin (2013)
	Tree/leaf lifetime	Ranalli et al. (2006)
Post-harvest	Storage (time/temperature)	Tsimidou & Papoti (2010)
	Harvest time/period	Di Donna et al. (2010)
	Extraction technique	Bilgin,& Şahin (2013); Tsimidou & Papoti (2010)

1.2.2. Triterpenoids

Triterpenoids, namely triterpenes (Howes, 2018), are the secondary metabolites abundantly found in the waxes/outer coating of leaves (Jäger et al. 2009; Stiti &Hartmann, 2012). They are chemically characterized by having six isoprene groups, typically made up of thirty carbons (Du et al. 2014). The occurrence of triterpenoids stems from the production of their precursor, squalene, through the ring formation of their lipidic molecules, farnesyl pyrophosphates (Du et al. 2014). Triterpenoids are chemically structured either in a free or glycosidic form (attached to carbohydrate molecules) (Du et al. 2014). They appreciably exert protection against oxidative stress (Sánchez-Quesada et al. 2013), carcinogenic, microbial, fungal, viral (Rascon-Valenzuela et al. 2017; Martín-García et al 2019), and hypertensive activities (Hsu & Yen, 2014).

Olive leaves contain considerable amount of pentacyclic triterpenoids, with oleanolic acid, the prevailing component ranging from 3.0 to 3.5% (Guinda et al. 2010), and uvaol in the range of 0.07 to 0.11% (Guinda et al. 2015). Concentrations may vary depending on harvesting/sampling time and cultivar type (Tsimidou & Papoti, 2010). Research demonstrated that triterpenes-rich olive leaves, with the presence of oleanolic acid and uvaol, exhibit suppression against diabetes and inflammatory diseases (Canabarro et al. 2019). Other research, by Tsoumani et al. (2016), confirmed that olive leave extracts, following hexane extraction that yielded oleanolic acid (around 56.6 mg/g) and uvaol (around 37.8 mg/kg), can display protection against platelet aggregation.

1.2.3. Tocopherols

Tocopherols, the naturally occurring antioxidants, are grouped in fat soluble compounds, typically with vitamin E characteristics (Duncan & Chang, 2012). They are indicatively intrinsic to plants and other living organisms with photosynthetic activity (Duncan & Chang, 2012; Mokrosnop, 2014). The presence of tocopherols in most lipid-based foods such as seeds and vegetable oils is a paramount, mainly because of the protective action exerted by tocopherols to inhibit activities of reactive oxygen species such as peroxides (Mokrosnop, 2014) which in turn assists in the prevention of lipid peroxidation in the food (Colville, 2017). The competitive reaction of tocopherol with peroxy free radicals brings about limitation of free radical reaction with fatty acids of foods, and hence reducing the process of lipids autocatalysis/autoxidation (Tena et al. 2019). The functionality of tocopherols has a great reliance on i) occurrence and structural isomers (namely, alpha, beta, gamma, and delta), and ii) their proportions and availability (Tena et al. 2019). Research on optimization of tocopherol recovery, though supercritical CO_2 extraction process, demonstrated that tocopherol proportion/concentration accounts for 10.10 mg/ 100 g leaves, and the isolated/recovered tocopherol accounts for 6.94 mg per 100 g leaves (De Lucas et al.

2002). Concentration of alpha-tocopherols in olive leaves, is reportedly around 284.6 μg/g (dry basis) (Botsoglou et al. 2012), 82.37 μg/g (dry basis) of Neb Jmel cultivar, and 10.12 μg/g (dry basis) of Oueslati cultivar (Tarchoune et al. 2019).

1.2.4. Pigments

Pigments of plants are mainly responsible for the color, light reactions (photosynthesis), ripening, and maturity of the plant components (Sudhakar et al. 2016). Chlorophylls and carotenoids, the pigments prevalent in plants, are valued for their color attributes, and functional properties (Schoefs, 2003) Carotenoids, the hydrophobic (fat-soluble) compounds, signify yellow/reddish orange color of the cell membranes (Aadil et al. 2019; Garcia-Vaquero & Rajauria, 2018). In addition to photosynthesizing functions, the physiological activities of carotenoids are highlighted by their antioxidative effects, acting as free radical scavengers (Aadil et al. 2019; Domonkos et al. 2013), and carcino-preventive agents (Aadil et al. 2019). In the research of Tarchoune et al. (2019), carotenoid concentration in olive leaves ranged from 26.90 to 44.33 μg/g (dry basis) in Neb Jmel and Oueslati cultivars, respectively.

Chlorophylls, the fat-soluble (Solymosi et al. 2015) green pigments, appreciably contribute to photosynthesis in plants (Roca et al. 2016) through enabling absorbance and transformation of light to chemical energy, and development of adenosine triphosphate (Casida, 2009). They find applications in food industry as food additives/colorants (Indrasti et al. 2018). Chlorophyll-content foods confer a range of health protections that include disabling inflammation (Indrasti et al. 2018), oxidation and mutagenicity (Yilmaz & Gökmen 2016). There are broadly two forms of chlorophyll pigments (a and b) that are distinguished by their chemical structure (Aramrueang et al. 2019) and biological functions. Chlorophyll *a* is the chief pigment in photosynthesizing system, while chlorophyll *b* is the secondary (accessory) component that facilitates greater light absorption and energy conversion (Trees et al. 2000). The presence and proportion of chlorophyll notably determines the color attributes of olive leaves (Bahloul et al. 2014). Concentration of chlorophyll in Tunisian olive leaves (Oueslati cultivars) has been reported to be around 829.29 μg/g dry basis (Tarchoune et al. 2019). Proportion of total chlorophyll in leaves may vary largely in various cultivars (Bahloul et al. 2014). Moreover, the retention of chlorophyll and carotenoids potentially increase with the increase of leaves lifetime (Brahmi et al. 2012).

1.3. Technological aspects for extraction of bioactive compounds from olive leaves

The extraction process, The extraction process, that is the separation of desired ingredients, requires careful selection of methodology. This is because unlike synthetic antioxidants, a great challenge is involved for recovery of natural ingredients (Pokorný & Korczak, 2001). Although synthetic antioxidants

confer efficiency and stability, by reason of being potentially hazardous, e.g., developing carcinogenicity and toxicity, the use of natural antioxidants is viewed as a safer alternative (Duh & Yen, 1997; Hayes et al. 2011). The extraction system should be ideally designed to effectively satisfy processing, economics, and environmental sustainability. The main features associated with the sustainable extraction of functional ingredients from olive leaves are highlighted in **Figure 1.1**.

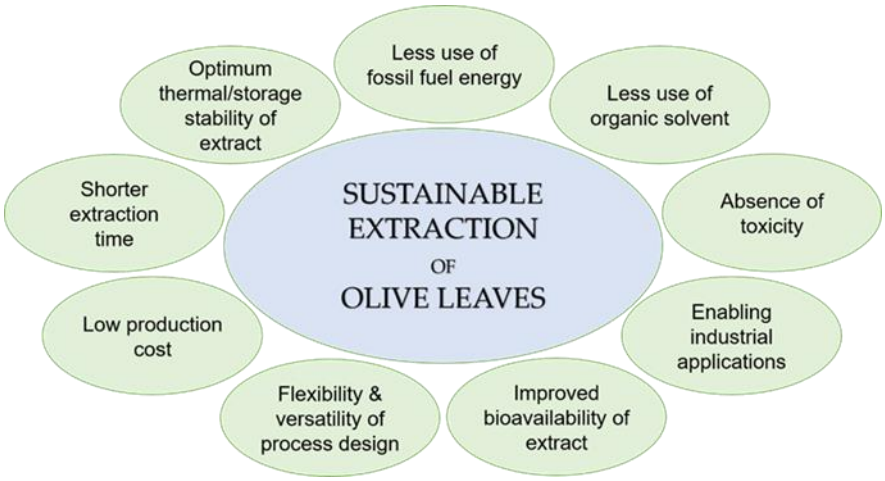

Figure 1. 1 Factors involved in the sustainable extraction of active compounds from olive leaves.

The processing parameters, including sample preparation/handling, extraction time/temperature, and solvent type/ratio, are highly influential in the level of extractability and remaining functionality of the final product (Brglez Mojzeret al. 2016). The choice of methodology depends on the intended final application/product that should be formulated suitably to be applicable to the desired extract composition and purity. For instance, the processing design may effectively enable the exertion of de-glycosylation and/or hydrolysis of oleuropein to hydroxytyrosol, that is appreciably prized for its biological activity and functionality (Rahmanian et al. 2015). However, if oleuropein is the desired final product, the extraction system should be optimally devised to ensure recovery of target pure/intact molecules with minimum degradation/chemical reaction. In this regard, the accuracy of the processing system control ("from farm to final product") is of great importance given the high susceptibility of the compounds to be extracted, particularly when aiming at more demanding applications, including those at pharmaceutical and medicinal sectors.

1.3.1. Stabilization of raw biomass to promote physicochemical quality of olive leaves

Due to the high sensitivity of the bioactive compounds of olive leaves to environmental stresses, post-harvest treatments for biomass stabilization may be crucial to allow maximum recovery of bioactivity. The trend of research studies on extraction systems has moved partly towards optimization and efficiency

of processing components and operational parameters associated with extraction system. These include drying treatments such as freeze drying and air drying (Dai & Mumper, 2010). Drying operation plays a major part in the resultant quantity and quality of the desired extract (Brglez Mojzer et al. 2016). The research on optimization of drying performance has progressed to some extent. Drying approaches such as (i) tray drying based on Response Surface Methodology (Erbay & Icier, 2009a), (ii) heat pump drying (Erbay & Icier, 2009b), (iii) solar drying (Nourhène et al. 2008), and (iv) freeze drying (Martinho et al. 2019) have shown to be highly effective in exerting the liberation of endogenous active compounds from olive leaves.

Selection of a suitable drying approach is important due to the presence of high moisture content in plant leaves that is a key factor in developing perishability and susceptibility to spoilage (Babu et al. 2018). It is preferable to increase shelf-life by optimal drying operation to render the moisture unavailable for microbial/enzymatic activity and oxidation reaction (Babu et al. 2018). Efficient drying, in part, exhibits optimal separation and purification of bioactive compounds, and thus the potential of maximizing exploitation of olive leaves (Erbay & Icier, 2009b). Drying operation parameters primarily include drying temperature and time, and their effect on drying performance (Babu et al. 2018). High temperature with prolonged drying operation may destroy or alter the structure of bioactive ingredients. Considerable degradation of oleuropein during elevated drying temperature at 60 °C has been reported by Malik and Bradford (2008). However, Ahmad-Qasem et al. (2013), supported the applicability of hot air drying to olive leaves (120 °C for 12 min). The authors of this study found significant concentrations of phenolic compounds, particularly oleuropein ($p < 0.05$) in the extract. Another important factor is the selection of a suitable air circulation system to ensure that a consistent air flow enables optimal moisture removal from samples (Babu et al. 2018).

1.3.2. Conventional extraction Techniques

Classical extraction techniques, commonly employed for the recovery of active compounds from olive leaves, involve a large proportion of organic solvents, using agitation and/or high temperature to maximize diffusivity/mass transfer of the desired compounds from food matrix (Rahmanian et al. 2015). Examples are percolation (Rahmanian et al. 2015), solid-liquid extraction (e.g., maceration, Soxhlet extraction) (Romero-García et a. 2016), distillation, and heat reflux (Žuntar et al. 2019). The choice of solvent is crucial to optimize the solubility and diffusivity of target ingredient/s (Žuntar et al. 2019). Ethanol, methanol, and hydroalcoholic solvents are the most typical solvents used for extraction of a wide range of hydrophilic and lipophilic compounds in olive leaves. The conventional solid–liquid extraction methods, although offering some advantages (including effectiveness of maceration methods for heat-

susceptible compounds, and the accuracy/reproducibility of Soxhlet methods), represent the downsides of (i) inefficient diffusivity and a slow/prolonged extraction process (Žuntar et al. 2019; Lama-Muñoz et al. 2019), (ii) using a prodigious amount of organic solvents (with potential toxicity), (iii) lengthy extraction time, and (iv) poor processing adaptability (Rahmanian et al. 2015). Other disadvantages inherent in both thermal and non-thermal conventional extractions are the high energy use, high production cost, and the potential incidence of degradation, arising from (i) prolonged subjection to solvents, e.g., in maceration (Lama-Muñoz et al. 2019), and (ii) the use of a high temperature involved in thermal operation (Rahmanian et al. 2015; Žuntar et al. 2019).

1.3.3. Alternative strategies to improve extraction performance

Many attempts have been made to develop modified/advanced technologies for the efficient extraction of bioactive compounds from olive leaves (Lama-Muñoz et al. 2019; Rahmanian et al. 2015). In the literature, a range of emerging techniques have been proposed to date as alternative tools to replace traditional/inefficient processing systems. A summary of the typical methods investigated for the extraction of olive leaves is provided in **Table 1.2**.

The research of Lama-Muñoz et al. (2019) (**Table 1.2**), on pressurized liquid extraction (PLE), using olive leaves with 5% moisture content and 80% ethanol at 190 °C during a 5 min extraction time, demonstrated that this method optimally increased the release of oleuropein (63.35 g/kg leaves) and luteolin-7-O-glucoside (2.71 g/kg leaves). Pressurized liquid extraction relatively helps address the hurdle of slow processing inherent in traditional methods, although it entails large capital investment but it offers advantages of low running/input cost (Lama-Muñoz et al. 2019).

The steam explosion method has been studied by Romero-García et al. (2016) to optimize the extraction of sugars and antioxidant compounds from olive tree leaves. Although sugars were the main compounds recovered (70% from the initial content), the authors reported significant extraction of oleuropein, hydroxytyrosol and flavonoids, that, respectively, represented 1613 mg, 260 mg, and 123 mg per 100 g dry leaves, with the extraction temperature of 180 °C for 4 min.

Recovery of olive mill leaves, using ultrasound assisted extraction (UAE) method, with ethanol solvent within a 30 min extraction time, improved the concentration (30%) of oleuropein in extracted leaves (Contreras et al. 2020). The principal limitations of UAE include non-selective extraction (Varelis et al. 2018), variability of ultrasound energy, and possible alteration of molecular structure of active components (Panzella et al. 2020).

Da Rosa et al. (2019), assessed extraction of total phenolic compounds from olive leaves using three methods (**Table 1.2**). By comparing the efficacy of the extraction methods examined in their

research, microwave assisted extraction (MAE) was found to be the most efficient approach (86 °C for 3 min). Their study demonstrated that MAE, where water was used as solvent, provides efficacy over the conventional (maceration) method. An increase of 82% recovery of total phenolics was observed. These authors suggested the potential efficacy of microwave assisted extraction when incorporated in pre-treatment step during ultrasonic assisted extraction. Sánchez-Ávila et al. (2009) also worked on optimization design of MAE for the quantification of triterpene derivatives in olive leaves and confirmed the efficacy of this method in the extraction yield. The optimum conditions of independent variables determined in their study included: (i) irradiation time (5 min), (ii) irradiation power (180 W), and the special focus on (iii) solvent ratio, optimally ethanol/water (80:20). Application of MAE is beneficial in terms of (i) short extraction time, ii) extraction efficiency (Da Rosa et al. 2019; Mandal et al. 2015), (iii) appealing physicochemical quality, and (iv) no waste of heat energy to environment (Mandal et al. 2015). However, the extraction yield is constrained by the type of solvents, entailing dielectric (dipole) solvents capable of absorbing microwave energy (Mandal et al. 2015). Further, the scale-up of MAE systems may represent major drawbacks, as the length of penetration of microwaves is rather limited.

Table 1. 2 Summary of existing techniques proposed for olive leaf extraction.

Nature of Study	Extraction Method	Advantage	Reference
Optimizing extraction of total phenolic compounds (including oleuropein, flavonoids, luteoline-7-O-glucoside)	Pressurized liquid-extraction (optimized by Response Surface Methodology) Solvent: ethanol/water	Efficient method for isolation of phytochemicals; low operation/input cost; high speed; greater recovery	Lama-Muñoz et al. (2019)
Study of various extraction methods and assess the influence of designed parameters on recovery of total phenolics and antioxidant activity	Microwave-assisted extraction (MAE) Ultrasonic assisted extraction (UAE) Conventional (maceration)	MAE: Most efficient method; less time consuming UAE: Lower temperature provides better quality and greater yield	da Rosa et al. (2019)]
Determination of increasing oleuropein isolation	Hybrid extraction technique: aqueous ethanolic extract (from liquid–solid extraction) was subjected to supercritical antisolvent extraction, to obtain precipitated/concentrated oleuropein	Enables precipitation and formation of oleuropein powder; efficient method. Only traces of ethanol remain in final extract	Baldino et al. (2018)
Investigation of total phenolic compounds through developing supercritical fluid extraction	Supercritical fluid extraction	High-speed; automated means; requires less solvent; selective isolation	Le Floch et al. (1998)
Effective development of phenolic extraction using high voltage electrical discharges compared to conventional method	High voltage electric discharges (characterization analysis performed by ultra-performance liquid chromatography-tandem mass spectrometry)	Use of organic solvents; effective extraction method (high extractability)	Žuntar et al. (2019)

As indicated in **Table 1.2**, in another study by Baldino et al. (2018), using an integration of liquid–liquid extraction (to extract olive leaves using ethanol), and supercritical antisolvent extraction (to recover oleuropein from aqueous ethanolic extract), demonstrated around 36% isolated (powder) oleuropein, with an extraction pressure of 150 bar at 35 °C. Le Floch et al. (1998) employed supercritical fluid extraction (SFE), using 10% methanol of CO_2 with the extraction pressure of 334 at 100 °C for 140 min (**Table 1.2**). In their research, total phenolic compounds (TPC), using Folin–Ciocalteu reagent assay, was assessed, and compared to the sonication-assisted liquid solvent extraction. The concentration of TPC obtained by SFE was higher than other solvents used in sonication, including n-hexane, but the percentage yield of TPC was lower than that extracted with methanol which represented a 45% yield.

Supercritical extraction using CO_2 has shown efficacy in isolation of plant bioactive constituents (Baldino et al. 2018). The main advantages include (i) using lower temperature (Paulo & Santos, 2020), (ii) allowing extraction selectivity, and (iii) enabling rapid extraction (Xynos, 2012). However, due to relative polarity of phenolics, the process needs the inclusion of polar solvent, namely, a modifier to help increase solubility (in CO_2), and extraction yield (Paulo & Santos, 2020).

Electric field-assisted extraction is also regarded as an emerging aid which encompasses both thermal and non-thermal extraction systems and has great potential for the extraction of active compounds from olive tree leaves. Moderate electric fields and the associated ohmic heating (where heat is generated due to the application of an electric field to a semi-conductive sample) may be used which allows (i) internal, uniform and fast heating, enabling favorable decrease in processing time with minimum thermal degradation of compounds), (ii) partial cell wall permeabilization, and (iii) minimum energy and time consumptions (Pereira et al. 2016). On the other hand, pulsed electric fields uses high voltage electric fields applied in extremely short pulses (to avoid heating) to destroy the cell membrane structure, improving the release of the bio-compounds to be extracted (Pereira et al. 2016). In this context, the experiment of Žuntar et al. (2019), demonstrates that a non-thermal high voltage electrical discharges (**Table 1.2**) is a viable technique for phenolic extraction from olive leaves. It enables greater rate of diffusivity by exerting the cell rupture and forcing the diffusion of mass transfer from internal part of cells to the external surrounding/solution, and thus assisting in the increment of yield within less time. However, other important factors inherent in the potential development of chemical residue and corrosivity of system components (e.g., when using less expensive electrodes) are yet to be identified. In addition, the upscaling operation system entails large investment, mainly for apparatus and processing inputs (Žuntar et al. 2019).

1.4. Significance of stability of olive leaf extract

It is important to take the factor of preservation and delivery of the final extracts into consideration. This is mainly because many bioactive compounds, including phenolics, are inherently susceptible to heat, light and oxygen, together with (i) variability in water solubility, (ii) high metabolic rate, and (iii) tendency of being expelled instantaneously from the body. This may substantially reduce biological availability and functionality of polyphenols (Parisi et al. 2014). In addition, non-phenolic compounds such as triterpenoids are likely to be less bioavailable, due to their poor solubility/hydrophilicity which in turn hinders their biological functions (Bishayee et al 2011). Therefore, and besides the necessary caution during harvest and processing mentioned in the previous section, post-processing olive leaves

by-products stabilization is also of utmost importance. The most common approaches described for this purpose involve different types of encapsulation techniques, allowing the protection of the extracts against various environmental stresses as well as controlled release of the bioactivity, at the desired time and place.

Application of various techniques based on nanotechnology, such as nano-structuring and polymeric nanoparticles, helps improve solubility, and functionality of these compounds (Bishayee et al. 2011). Encapsulation of olive leaf extract, e.g., by freeze drying, using cyclodextrin for encapsulation (Mourtzinos et al. 2007), or by water in oil (W/O) nano-emulsion using soybean oil, has been considered a markedly useful technique in this regard (Mohammadi et al. 2016). In a research, carried out by Urzúa et al. (2017), through examining microencapsulation ability in preservation of phenolics and their bioactive potency, olive leaf extracts were encapsulated with inulin by means of spray drying. The outcome of their study showed that microencapsulation was highly efficient in preserving active compounds (oleuropein yielded above 87% in the encapsulated leaf extract). Additionally, Soleimanifar et al. (2020), in their research on nanoencapsulation of olive leaf phenolics by whey protein electro-spraying (using low temperature without solvent), postulated the use of this technique as a propitious approach for protecting functional constituents of olive leaves.

1.5. Potential industrial applications of olive leaf extracts

Olive leaves, together with having great potential for medicinal, cosmetic (Tsimidou & Papoti, 2010), and pharmaceutical uses, could find applications in functionalizing/developing a wide range of food products (Erbay & Icier, 2010). The addition of olive leaf extract to vegetable oils is believed to be effective in promoting oxidation stability and retaining nutritive components of oils, leading to a longer shelf-life (Sanchez de Medina et al. 2011). Ammar et al. (2017), conducted research on the effect of addition of olive leaves in extra virgin olive oil (added prior to oil extraction) on phenolic profile of the extracted oil samples from Tunisian cultivars. These authors found a significant increment of phenolic content in the recovered oil, from Chétoui cultivar, treated with 3% of olive leaves. Incorporation of olive leaves (3%) to Tunisian olive oils (Oueslati cultivar) has shown to increase the concentration of polyphenols (44%), chlorophyll (around 67%), and carotenoids (around 62%) in the oil (Tarchoune et al. 2019). Sevim et al. (2013) also described that inclusion of olive leaves in olive oil has potential to promote the bioactive profile of the oil stored for 18 months. Significant increment of alpha-tocopherols, phenolics, and chlorophyll proportions as well as antioxidant activity were observed in the leave-treated oils $p < 0.001$.

The significance of developing oxidative stability of cooking oils, particularly when using deep frying or a high temperature, is primarily due to oxidative susceptibility of vegetable oils during cooking process rendering unappealing changes in the food structure with potential degradation of nutritional/sensory quality (Nunes et al. 2016). Addition of olive leaf extracts to sunflower, palm, and olive oil (TPC concentrations of extracts: 120 and 240 mg/Kg oil) was examined by Chiou et al. (2007) for antioxidation properties in the fried potato chips (French fries). It was found that polyphenolic concentration significantly increased as much as three to 60-fold greater than corresponding compounds in potato chips fried with commercial oils samples. Their study also indicated that oleuropein represented the prevailing phenolic constituent in the leaf extract (1.25 g/Kg leaves). Effectiveness of upgrading stabilization of refined olive oil to the extent of virgin olive oil is potentially achievable when olive leaves (1 Kg) is incorporated in 50 to 230 L refined oil. However, this amount has a huge reliance on variations in cultivars, sampling/harvesting time, and chemical features of the extracted oil (Paiva-Martins et al. 2007).

Inclusion of phenolic-rich olive leaf extract in the food system, as an alternative functional source, compared to the expensive purified biomolecules such as oleuropein, offers the advantage of being a low-cost processing means as it eliminates the need for the purification step, while being considerably effective. In this regard, Zoidou et al. (2017) investigated the effectiveness of incorporation of oleuropein-rich olive leaf extract in yogurt, through adding leaf extract to the milk ingredient (0.43 mg extract per mL milk) during processing of yogurt production. These authors postulated the great potential of olive leave extract (rich in oleuropein), as an inexpensive functional material, in the enrichment of yogurt products.

1.6. Future trends

Maximizing extraction efficacy (considering both yield and functionality) while minimizing overall cost is evidently a desired approach. Many studies on the analysis and exploitation of olive leaves have been published to date but further details on the exploration of viable approaches based on environmental and economic sustainability are needed to fill the knowledge gap currently exists. Among the challenges involved in the isolation of phytochemicals from olive leaves are the following: (i) variability in quantification and characterization, (ii) achieving reproducibility and flexibility of extraction approach, (iii) enabling simplicity and adaptability to various industrial applications, including functional foods and nutraceutical supplements (iv) substantiating stability and potency of phenolic extracts, and (v) ensuring up-scalability.

Another challenge, when using a new technique, is the factor of chemical residue that brings about toxic hazards to the environment. There is a growing trend towards researching in this area, via minimizing toxic processing inputs, to present a competitively innovative/non-conventional alternative, or to optimally complement the traditional methods. Furthermore, current in vivo studies related to the toxicological measures of olive leaves appear to be limited, and thus, this requires further research to conclusively determine this, even though olive leaves have been generally considered natural/safe products. From the perspective of oleuropein, the median lethal dose in rodents is reportedly greater than 3000 mg/Kg (following oral administration), and greater than 1000 mg/Kg (following intraperitoneal injection) (Zoidou et al. 2017).

Additionally, analytical challenges (that are partly attributed to the variability in phenolic biosynthesis and bioactivity among various cultivars) need to be taken into consideration. The innovative analytical methods such as metabolic fingerprinting, as a useful means, effectively helps classify cultivars based on their molecular profile that can be used as chemical markers. Di Donna et al. (2010), in their study to determine whether the selected cultivars are distinguished/classified within different growing sites, performed a supervised pattern analysis, including soft independent modelling of class analogy that was found to be effective in the predictive analysis of cultivars and growing sites. Furthermore, it is essential to understand the efficacy of adequate proportions of active components in olive leaf extracts to optimally enable the exertion of their functionality in food/non-food product developments.

Olive leaves, although endowed with bioactivity which are of benefit to developing added-value products, the inherently low recovery yields, and complexity of active compounds, may hamper expensive/demanding downstream processing. On the other hand, current studies point to applying integrated approaches that ensure maximum re-use of the available biomass, preferably towards the whole use of different olive-based residue fractions. The United Nations' 2030 Agenda for Sustainable Development has emphasized specific goals on sustainability and responsible production side-by-side with economic growth and industrial innovation. In this context, application of a diverse range of biomass/by-products, generated in agricultural and industrial processing of olives, helps contribute to economic and environmental sustainability. Future perspectives seemingly incorporate deeper analysis of (i) flexible on-site or near-site processing of plants, (ii) biomass stabilization, (iii) transportation needs, (iv) exploitation of various types of residues/by-products of plants, and (v) sequential extraction of various recoverable fractions.

1.7. Conclusions

A large proportion of olive leaves, the by-products of the agricultural and processing methods of olive crops, does not end up being utilized and their disposal represents huge economic and environmental impacts. These residues are abundant in valuable bio-compounds and their inclusion in the food/dietary system is decidedly a preferable alternative to using synthetic counterparts. They have great market potential for industrial applications as functional/natural ingredients and high-added value products. However, there are principal factors involved in the level of extractability and biological potency of phytonutrients. Among the major determinants, the type of extraction system has been emphasized in many studies. The selection of a viable and inexpensive processing approach to accommodate an optimum liberation of desired molecules, with especial attention to environmental sustainability, is challenging work. In the literature, numerous advanced methodologies have been examined to develop sustainable processing techniques to replace the conventional/expensive extraction systems currently used. Many research studies have postulated efficient processing designs to increase recovery of biomolecules and decrease: (i) toxic extraction solvents, (ii) fossil fuel energy, (iii) manufacturing cost, and (iv) processing time. These findings are potentially propitious but, having said that, olive leaves have currently found limited commercial use and the industrial applications are still unable to adapt the new/proposed techniques, in part because of the operation/system complexity and overall production expenses. This may hamper broader scientific efforts to provide a feasible means, with minimum challenges, for valorization of olive leaves which enables (i) minimum operation costs, (ii) simple and flexible processing protocol, and (iii) accessible system components. Performance of a sustainable bio-processing strategy helps reach the goal of zero disposal/green processing of olive leaf by-products while assuring an acceptable quality of functional/natural products to satisfy both consumers and industrial needs.

1.8. References

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CHAPTER 2

EFFECT OF OHMIC HEATING ON THE EXTRACTION YIELD, POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY OF OLIVE MILL LEAVES

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Effect of Ohmic Heating on the Extraction Yield, Polyphenol Content and Antioxidant Activity of Olive Mill Leaves

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Abstract: This study examined the influence of ohmic heating (*OH*), compared to the conventional heating (*Conven*) and *Control* (solvent) methods, on the extraction of olive mill leaves. The main extraction parameters were: (i) solvent ratio (aqueous ethanol; 40%, 60%, and 80%, *v/v*), and (ii) extraction temperature; 45–75 °C (for *OH* and *Conven*), and room temperature (for *Control*). The selected response variables were extraction yield (%), total phenolic content (TPC), and antioxidant activity (ABTS and DPPH). The ohmic system, compared to *Conven* and Control, exhibited the greatest effects ($p < 0.001$) on increasing (i) extraction yield (34.53%) at 75 °C with 80% ethanol, (ii) TPC at 55 °C (42.53, 34.35, 31.63 mg GAE/g extract, with 60%, 40%, and 80% ethanol, respectively), and (iii) antioxidant potency at 75 °C detected by DPPH and ABTS, in the range of 1.21–1.04 mM TE/g, and 0.62–0.48 mM TE/g extract, respectively. Further, there were relatively similar trends in TPC and antioxidant activity (both methods), regardless of solvent ratios, $p < 0.001$. These findings demonstrate the potential of ohmic heating, as a green processing tool, for efficient extraction (15 min) of olive leaves. To date, no literature has described ohmic application for olive leave extraction.

Keywords: ohmic heating; olive leaves; residual biomass; polyphenols; antioxidant activity; green solvent; sustainable extraction.

2.1. Introduction

Olive leaves constitute a large proportion of the residual biomass generated from agro-industrial activities of olive crops and, while being abundant in high-added-value ingredients, currently have found low-value applications in folk/traditional medicine (Makowska-Wąs et al. 2017) and animal feed (Rahmanian et al. 2015). Polyphenols are among the key antioxidants in olive leaves, markedly prized for their bio-functional potentials, and this fact has prompted continuous research studies to devise optimum processing design to maximize their recovery at a low-cost/durable system. The conventional methods, such as maceration, Soxhlet, and percolation, although being rather simple, are inherently (i) inefficient in the processing/extraction system, (ii) solvent/energy intensive, and (iii) time consuming. To address the challenges associated with the existing technology, the emphasis is often placed on the optimization of emerging/green technologies, including microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), supercritical fluid extraction (SFE), pulsed electric field (PEF), and high-voltage electric discharges (HVED).

In the research of Da Rosa et al. (2019), the extraction efficiency between MAE, UAE, and maceration (conventional) was compared and the results confirmed that the use of MAE, particularly over the conventional method, was significantly productive. Le Floch et al. (1998) detected a higher concentration of total phenolics using SFE compared to that obtained by sonication-assisted liquid solvent extraction (with the exception of methanol solvent). The increased phenolic recovery from olive leaves by means of HVED was well justified in the study of Žuntar et al. (2019), highlighting that the use of this method exerts effects on the increased cell rupture and mass transfer within a short extraction time through using high-voltage pulsed electric field. Pappas et al. (2021) found increased total phenolics through the extraction of olive leaves by PEF (using 25% ethanol v/v and 10 μs pulse duration). Research also demonstrates a significant influence of UAE on phenolic liberation from olive leaves (Giacometti et al. 2018).

Although the recent findings show great potential for optimal extraction of olive leaves, there is no single processing benchmark for a sustainable extraction system. This is partly because the suitability of an ideal design relies heavily on the nature/concentration of extraction solvents, solid-to-solvent ratio, extraction time/temperature, plant origin/cultivar, and physicochemical characteristics of bio-phenols, together with others.

Among the green technologies is the ohmic heating which has been viewed as a competitively ideal approach for the eco-extraction of phytonutrients from various food matrices (Coelho et al. 2019; Pereira et al. 2016). Ohmic heating (Joule heating or electrical resistance heating) mainly refers to the conversion

of electrical energy to heat energy in foods. This phenomenon comes about when the electric current travels through the interior of the food (that is, resistant to electric flow) which, in turn, by the effect of their electric resistance, results in the temperature increase within the product (Coelho et al. 2019; Al-Hilphy et al. 2015; Sakr & Liu, 2014). The favorable features of ohmic heating primarily include (i) exertion of internally uniform heat supply within the food, in a short period of time, and (ii) low energy/running cost (Pereira et al. 2016; El Darra et al. 2013). The potential effectiveness of this method developed the idea of performing this research to examine its feasibility on olive leaf extraction. In this respect, the purpose of this study was to evaluate the effect of ohmic extraction, compared to the conventional heating and Control (solvent) methods, on the extraction yield, total polyphenol content, and antioxidant activity of olive mill leaves (using different concentrations of aqueous ethanol at different temperatures). Until now, no published report has examined the efficacy of the ohmic system on the extraction of olive leaves. The present research lays down the preliminary basis for expanding the knowledge of the ohmic technology to potentially enable sustainable re-utilization and valorization of olive leaf residues.

2.2. Materials and methods

2.2.1. Plant materials and chemicals

Olive mill leaves of "Picual" cultivar, were kindly supplied by "Center for Advanced Studies in Energy and Environment", University of Jaén, Campus of Las Lagunillas, Jaén, Spain. The trees, within the same age range, 40–60 yr, were managed under the same agricultural condition. The leaves were delivered to the University of Minho, Portugal, manually cleaned, washed, dried, and ground. The dry leaves (with 3.6% moisture content) were stored between 0 °C to 4 °C prior to the experiments.

The following chemicals (of analytical grade) were purchased from Sigma-Aldrich (Saint Louis, MO, USA): ethanol (99.8%), sulfuric acid (≥95%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu, anhydrous gallic acid (≥98.0%), anhydrous sodium carbonate (≥99%), hydrochloric acid, D-(+)-Glucose (≥99.5%), potassium persulfate (≥99%), 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox).

2.2.2. Experimental design

In this study, primarily, the efficacy of ohmic technique on the extraction of olive mill leaves was investigated. As shown in **Figure 2.1**, the leaves were initially cleaned (to eliminate stems/foreign objects), washed, and dried at 37 °C for 48 h. The dried leaves were size reduced with a grinder to pass through a 0.3 mm mesh and vacuum packed in polypropylene bags and refrigerated (0–4 °C) for a

maximum of two weeks. The dry leaves, prior to the extraction study, were initially examined for proximate analysis.

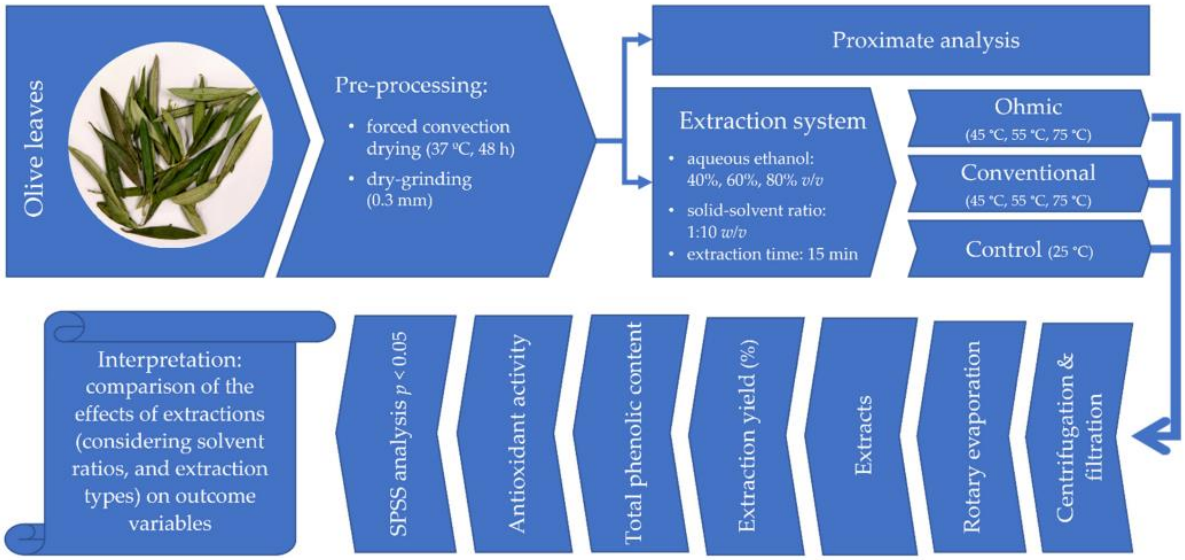


Figure 2. 1 Summary of the experimental design.

2.2.3. Proximate analysis

The dried/ground leaves were initially assessed for proximate composition as follows:

2.2.3.1. Moisture content

Moisture content (%) of dried leaves was determined thermogravimetrically using an electronic moisture analyzer Radwag®, MAC 50/1/NH (Radom, Poland).

2.2.3.2. Total ash

Dry ashing was carried out according to the official method of Association of Official Analytical Chemists (AOAC, 2000). The total ash content (%) of dry leaves was determined using the following equation:

$$Ash\ content\ (\%) = \frac{Weight\ of\ ash}{Initial\ weight\ of\ dry\ sampe} \times 100 \quad --- Equation\ 1$$

2.2.3.3. Total fat

Crude fat was determined using a Soxtec automated extraction system (Foss SoxtecTM 8000), a Randall adaptation of Soxhlet approach (Anderson 2004; Thiex et al. 2003) , and calculated using the following equation:

$$Total\ fat\ (\%) = \frac{Weight\ of\ fat\ in\ sample\ (g)}{Initial\ weight\ of\ dry\ sampe} \times 100 \quad --- Equation\ 2$$

2.2.3.4. Crude protein

Total protein was determined by means of an automated Kjeldahl analyzer (FOSS Kjeltec™ 8400). Briefly, an aliquot (1 g) of dry leaves was transferred into a Kjeldahl flask (containing two copper catalyst tablets) and heated using sulfuric acid (15 mL) to complete the digestion process. The nitrogen content (%) was automatically calculated (in accordance with the consumption of volumetric standard solution). The total protein content was calculated using the conversion factor of 6.25.

2.2.3.5. Crude fiber

Crude fiber was determined according to the official method of AOAC (2000). Into a conical flask containing 200 mL of 0.128 M sulfuric acid, sample (2 g) was added and boiled for 30 min with periodic agitations. The solution was filtered into a discard conical flask though a muslin cloth. The filtered solid (residue) was washed into another conical flask with 200 mL base solution (0.313 M sodium hydroxide) and processed for boiling basic solution (30 min) and filtration. The filtered solid was then collected into a dry crucible, and, after drying the fiber in hot-air oven at 130 °C for 2 h, the crucible with dried fiber was weighed and incinerated in muffle furnace at 550 °C for 2 h. The weight of crucible with ash was recorded and the concentration of crude fiber (%) was calculated using the following equation:

$$Crude\ fibre\ (\%) = \frac{W1 - W2}{Ws} \times 100 \quad --- Equation\ 3$$

where: W1 = weight of crucible with dried fiber, W2 = weight of crucible with ash, Ws = weight of sample (g).

2.2.3.6. Total carbohydrate

Total carbohydrate was determined using anthrone method (Sadasivam & Manickam, 2005). Briefly, around 100 mg of sample was hydrolyzed in 5 mL of hydrochloric acid (2.5 N) in a boiling water bath for 3 h. After cooling at room temperature, the samples were centrifuged at 8000 rpm for 3 min. The supernatant was transferred into allocated test tubes (for sample analysis). The working standard was prepared by diluting 10 mL of stock solution (standard glucose) to 100 mL with distilled water. A series of Standards were prepared in different concentrations by pipetting 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1 mL into designated test tubes and made the final volume of 1 mL with distilled water. Into each test tube (samples and standards), 4 mL anthrone reagent (containing100 mL ice-cold concentrated sulfuric acid and 200 mg anthrone) was added and boiled for 8 min. The absorbance reading was measured at 630 nm. The carbohydrate concentration was calculated using the standard calibration curve (plotting the concentration of glucose concentration versus absorbance).

2.2.4. Extraction system

The extraction of olive leaves was assessed through: (i) ohmic heating (*OH*), (ii) conventional heating (*Conven*), and (iii) *Control* (solvent) methods. *Instrumental setup*—the main components of a bench-scale ohmic heater used in this study (**Figure 2.2**) were: (i) a Pyrex glass reactor chamber (10 cm height, 9 cm i.d, with 100 mL capacity) equipped with two titanium electrodes (with 5 cm distance in between), and a K-type thermocouple to be positioned in the center, (ii) a function generator (Protek 2MHz Sweep Function Generator) enabling waveform adjustment, (iii) an amplifier from which the electrodes received signals, (iv) a hand-held oscilloscope (Industrial ScopeMeter® 125/S 40 MHz, Fluke, Everett, WA, USA), and (v) a data acquisition system (LabVIEW 7 Express system software) to monitor the temperatures of the food received from the thermocouple sensor.

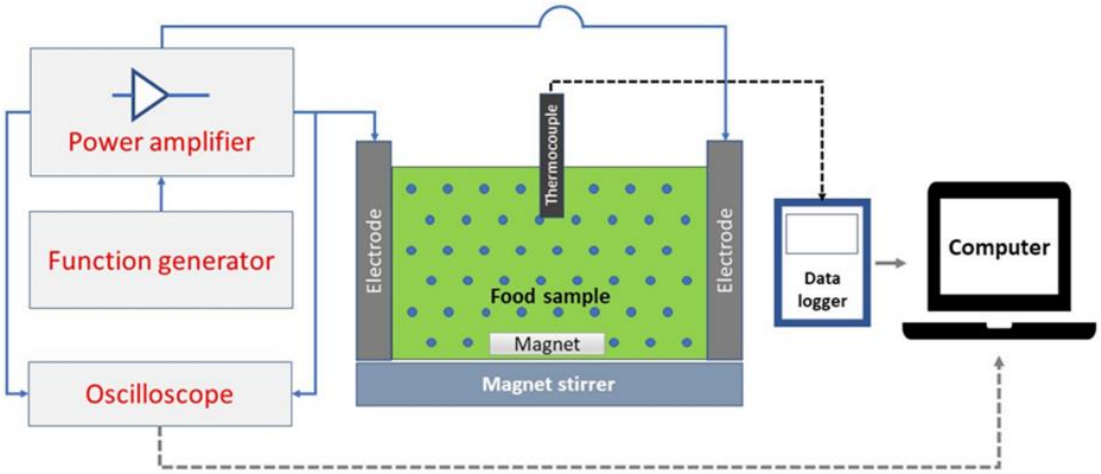


Figure 2. 2 Layout of ohmic heating system used for olive leaf extraction.

Ohmic extraction – five grams of sample (dried/ground leaves) mixed with 50 mL of the selected concentration of aqueous ethanol (40%, 60%, and 80% EtOH, v/v) and transferred into the reactor chamber (the electrical conductivity of the extraction solvents were intensified, using sodium chloride, to obtain: 4.2 mS/cm, 3.1 mS/cm, and 3.4 mS/cm for 80%, 60%, and 40% aqueous ethanol, respectively). The extraction was processed, using the selected extraction temperature (45 °C, 55 °C, and 75 °C separately for each experiment) for 15 min. The homogeneity of sample solution and uniformity of heat transfer during the extraction process were achieved by means of a magnetic stirrer positioned in the reactor chamber stirred at 150 rpm. The temperature variations in the sample were measured with a thermocouple, equipped with a data logger operating through a software computer system for data acquisition. The frequency and electrical voltage range remained constant, with 25 kHz and 1–10 V/cm, respectively.

The hydroethanolic extract was centrifuged at 5000 rpm for 15 min. The supernatant was filtered through a Whatman filter paper. The filtrate was then subjected to rotary evaporation at 40 °C for 1 h. The extract was nitrogen flushed, transferred to an amber glass bottle and stored at −20 °C until analytical experiments.

Conventional heating extraction — to effectively assess the influence of the ohmic method, a conventional thermal extraction was performed, under the same conditions/system components applied for the ohmic with the exception that in the glass reactor (containing the sample) only thermocouple was used without using electrodes. The system was equipped with a circulating/thermostat water bath enabling thermal/temperature control.

Control (solvent) extraction — in addition to the abovementioned methods, a solvent extraction was carried out as Control through the same solvent conditions with the exception that no heat treatment was applied (using only agitation at room temperature, 25 °C).

2.2.5. Extraction yield

The extraction yield (%), based on thermal gravimetric analysis, was evaluated through drying of 1 mL aliquot of extract at 105 °C and the weight measurement was monitored in two-hour intervals until it reached equilibrium point/constant weight. The results were calculated as follows and expressed as gram extract per 100 g of dry leaves:

$$Yield\ of\ extracted\ leaves\ (\%) = \frac{We}{Wi} \times 100 \quad ---\ Equation\ 4$$

where: Wi = Initial weight of dry sample (g), and We = Weight of dry extract (g).

2.2.6. Total phenolic content (TPC)

The gross quantification of total phenolics was determined using Folin–Ciocalteau assay, described by Singleton et al. (1999) with some modifications. Into a 10 mL volumetric flask, 100 µL of extract, 6 mL distilled water and 500 µL of Folin–Ciocalteau reagent were added. The mixture was vortex mixed and allowed to stand at 25 °C for 8 min before adding 1.5 mL of 20% sodium carbonate. The mixture was made up to 10 mL final volume with distilled water and incubated in dark/cool place for 60 min. The absorbance was measured at 760 nm using UV-vis spectrophotometer. Total phenolic content was calculated against gallic acid standard curve and reported as mg of gallic acid equivalents/g extract dry weight (mg GAE/g extract d.w.).

2.2.7. Antioxidant activity

2.2.7.1. ABTS radical scavenging activity

The ability of bioactive compounds in extracts to inhibit ABTS radical activities was assessed using the method described by Re et al. (1999) with slight modifications. The radical cation ($ABTS^{\bullet+}$) was liberated through the reaction of ABTS stock solution (7 mM) with potassium persulfate (2.45 mM). After incubating in dark for 16 h, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. An aliquot of sample (properly diluted) was then added to the solution (1:10 v/v), mixed, and the absorbance was measured at 734 nm. The absorbance readings were calculated against Trolox calibration curve and expressed as mM Trolox equivalents/g extract (mM TE/g extract d.w.).

2.2.7.2. DPPH radical scavenging activity

The DPPH radical scavenging assay was carried out following the method of Brand-Williams et al. (1995) with slight modifications. An aliquot of 3.9 mL DPPH working solution (36 µg/mL ethanol) and 100 µL of extract (properly diluted) were added into a test tube and made up to volume (10 mL) with ethanol and vortex mixed for 10 s. The mixture was incubated in the dark/room temperature for 60 min. The absorbance readings (517 nm) were calculated against Trolox standard curve and the results were expressed as mM TE/g extract d.w. Additionally, the following equation was used to determine the percent inhibition of radical activity:

$$\%\ Inhibition\ of\ DPPH\ radical = \frac{A0\ - A1}{A1} \times 100\ \ ---\ Equation\ 5$$

where: A0 = Absorbance of control (DPPH solution without extract), and A1 = Absorbance of sample (DPPH solution mixed with extract).

2.2.8. Statistical analysis

Each experiment was carried out in triplicate and the results were recorded as the mean values ± SD. The significant differences ($p < 0.05$) between the mean values (± SD) of all determinations were statistically assessed via Analysis of Variance (ANOVA) using SPSS software, version 27.0. The quality parameters (response variables) of the extracts were individually analyzed using factorial ANOVA (two-way ANOVA) to assess the interactive effects of two independent variables (solvent ratio and extraction method) and their joint effects on the mean values of dependent variables individually. The assumption of homogeneity of equal variance was assessed through the Levene's test (homogeneity of variance assumption was satisfied/not violated when P-value was greater than 0.05).

2.3. Results and discussion

The selection of the extraction system plays a significant role in the overall extraction yield and quantity/functionality of recovered bioactive compounds. Food and nutraceutical producers require a viable/affordable extraction design to sustainably reuse/valorize the byproduct streams, including olive leaves. A range of methodologies are being researched, encompassing both innovative and conventional methods. While being highly effective, various extraction methods represent variabilities in phenolic quantities/bioactivities; reflecting their variations in diffusivity, solubility, polarity, and heat sensitivity, along with others. In this regard, the extent of phenolic recovery/bioactivity rests highly on the selected extraction design/parameters that may favorably/unfavorably affect the food microstructure and the solute mass transfer.

In the present study, the investigation into the efficacy of ohmic application, using varied solvent concentrations and extraction temperatures, was carried out for olive leave extraction, with special attention to (i) extraction yield, (ii) total phenolic content, and (iii) antioxidant activity of extracts. It is noteworthy that ethanol, as a preferred extraction solvent, is considered a green/biodegradable solvent with minimum toxicity (typically produced in the course of fermentation of sugars from plants/algae) and its ability to favorably extract phenolics from olive leaves has been substantiated in recent studies.

2.3.1. Proximate analysis

The selected macromolecules present in dry/ground leaves were determined to evaluate their main elemental characteristics. The proximate composition of olive leaves (g/100 g d.w.) was examined in terms of moisture, ash, total fat, crude fiber, crude protein, and carbohydrates (**Table 2.1**).

The dry ash, that is the inorganic residue remaining after combustion, represents the mineral content in the food. It is routinely performed as part of the characterization of chemical/nutritional attributes of the food of interest. The ash content measured in this experiment (10.82%) is relatively close to that reported by Contreras et al. (2019), where the olive mill leaves (from the same cultivar/growing region used in this study) exhibited around 10.04%. Doménech et al. (2021) also experimented on olive leaves from the same growing region and found 9.1% ash in the raw biomass. The concentration of ash is partly determined by (i) pre-harvest/agricultural conditions, (ii) sample origins/collection methods, and (iii) pre-processing approaches, such as blanching and size reduction, together with others. Caballero et al. (2020) reported 8.22% ash in Spanish olive leaves, obtained from the olive mill (pneumatically separated from olive drupes, washed, dried, and ground). Zeitoun et al. (2017) explored 5.80%, 5.77%, and 4.58% ash content in blanched/dried, solar-dried, and oven-dried leaves, respectively. Further, Cavalheiro et al. (2015), through their experiment, found 4.65%, 6.00%, 4.37%, 4.85%, and 5.36% ash

in tree-harvested leaves from Arbosana, Ascolano, Grappolo, Koroneiki, and Negrinha do Freixó, respectively.

Table 2. 1 Proximate composition (mean ± SD) of dried/ground olive leaves.

Component g/100 g Dry Leaves	Mean ± SD
Moisture	3.57 ± 0.18
Total ash	10.82 ± 0.8
Crude fat	4.13 ± 0.02
Crude protein	8.02 ± 0.13
Crude fiber	35.41 ± 0.35
Carbohydrate	37.65 ± 1.30

As shown in **Table 2.1**, the dry leaves in this study contained 8.02 ± 0.13 g/100g crude protein, which is as close as that reported by Contreras et al. (2019), where 8.10% protein was found in olive mill leaves from the same cultivar/region. The protein content in olive leaves varies among different studies; partly due to the variations in growing regions and soil fertility, among others. Examples are 4.95%, (Zeitoun et al. 2017), 5.45% (Erbay and Icier 2009), 6.9%–8.1% (Contreras et al. 2020), and 10.6% (Ibrahim et al. 2016), 7.8% (Doménech et al. 2021).

The fat content determined in this study was 4.13 ± 0.02 g/100 dry leaves. There is also a broad spectrum of the reported total lipids in olive leaves. Examples are 2.29% (Ismail et al. 2016), 6.54% (Erbay & Icier, 2009), 9.9% (Contreras et al. 2020) , and 9.13%–9.80% (Cavalheiro et al. 2015). One of the main deciding factors affecting the fat content, beyond the cultivar variations and growing regions, is the particle size of samples, which is dependent on the selected milling method. The dry leaves, ground to pass through a 60-mesh screen, reportedly contained 7.9% total lipids (Ibrahim et al. 2016). In another study, the pulverized leaves with 1 mm particle size contained 3.21% total fat (Martin García et al. 2003).

Example are 2.29% (Ismail et al. 2016) and 6.54% (Erbay & Icier, 2009), 9.9% (Contreras et al. 2020), and 9.13%–9.80% (Cavalheiroa et al. 2015). One of the main deciding factor affecting the fat content, beyond the cultivar variations and growing regions, is the particle size of samples; which typifies the importance of the selected milling method to size reduce dry leaves prior to proximate analysis. Ibrahim et al. (2016) found 7.9% total lipids in olive leaves (d.w.) pre-ground through a 6-mesh screen. Martin García et al. (2003) obtained 3.21 g/100 g fat in dried olive leaves with 1 mm particle size.

Total carbohydrates constituted around 37.65% in this study. Among the published reports include 36.75% and 45.96% (in oven-dried and solar-dried leaves, respectively (Zeitoun et al. 2017); 29.20% and 25.4% (in non-blanched and blanched leaves, respectively) (Sucharitha et al. 2019) ; and 27.58% (Erbay & Icier, 2009). Moreover, focusing on different varieties, Cavalheiro et al. (2015) obtained 8.74%,

11.60%, 12.75%, 16.70%, and 32.63% in olive leaves from Negrinha do Freixó, Koroneiki, Ascolano, Arbosana, Grappolo cultivars, respectively.

The crude fiber accounted for 35.41% in the leaves of this study. Examples in previous studies are: 14.5% in oven-dried/60-mesh powdered leaves (Ibrahim et al. 2016); 32.83% in oven-dried leaves freshly harvested from trees (Ismail et al. 2016); 7.0% in heat-pump-dried leaves (Erbay & Icier, 2009); and 4.71% in oven-dried leaves (Zeitoun et al. 2017).

2.3.2. Extraction yield

The yield percentage of the residual dry matter (the extractable matter) was determined though convection drying. The extraction ability of ohmic heating was examined compared to *Conven* and *Control*. The mean values of the extraction yield (%) obtained from different extraction methods are shown in **Table 2.2**.

Table 2. 2 Mean values (± SD) of yield and functional properties of extracted olive mill leaves – extraction yield (g/100g dry leaves), total phenolic content (mg GAE/g extract), TEAC assays (ABTS and DPPH radical scavenging activity mM TE/g extract), and % inhibition of DPPH and ABTS radicals.

Extraction System		Extraction yield (g/100 g dry leaves)	TPC (mg GAE/g extract)	Antioxidant Activity			
Method	% EtOH (v/v)			ABTS·⁺ inhibition (mM TE/g extract)	ABTS·⁺ inhibition (%)	DPPH· inhibition (mM TE/g extract)	DPPH· inhibition (%)
OH 45 °C	40	22.02 ± 0.15	33.73 ± 0.21	0.44 ± 0.01	67.06 ± 1.03	0.93 ± 0.07	85.23 ± 0.10
OH 45 °C	60	28.30 ± 0.12	38.37 ± 0.32	0.45 ± 0.02	68.93 ± 0.18	0.96 ± 0.01	86.67 ± 0.19
OH 45 °C	80	30.80 ± 0.11	30.45 ± 0.39	0.49 ± 0.10	69.60 ± 0.41	1.08 ± 0.05	87.70 ± 0.25
OH 55 °C	40	23.21 ± 0.15	34.36 ± 0.36	0.44 ± 0.10	70.45 ± 0.47	0.93 ± 0.10	89.94 ± 0.13
OH 55 °C	60	30.20 ± 0.14	42.53 ± 0.31	0.49 ± 0.11	74.72 ± 0.45	1.01 ± 0.06	90.77 ± 0.52
OH 55 °C	80	31.10 ± 0.17	31.63 ± 0.43	0.55 ± 0.02	77.56 ± 0.36	1.15 ± 0.01	92.55 ± 0.12
OH 75 °C	40	27.53 ± 0.13	34.06 ± 0.23	0.48 ± 0.01	73.95 ± 0.39	1.04 ± 0.50	90.85 ± 0.44
OH 75 °C	60	28.50 ± 0.12	41.13 ± 0.40	0.54 ± 0.05	76.79 ± 0.17	1.11 ± 0.30	91.56 ± 0.56
OH 75 °C	80	34.53 ± 0.41	30.23 ± 0.35	0.62 ± 0.15	78.72 ± 0.48	1.21 ± 0.04	92.80 ± 0.57
Conven 45 °C	40	19.41 ± 0.54	23.92 ± 0.16	0.44 ± 0.02	67.08 ± 0.08	0.96 ± 0.04	86.54 ± 0.07
Conven 45°C	60	21.39 ± 0.55	28.44 ± 0.31	0.47 ± 0.03	68.60 ± 0.35	1.05 ± 0.01	88.33 ± 0.13
Conven 45 °C	80	21.18 ± 0.27	26.75 ± 0.32	0.48 ± 0.02	70.10 ± 0.94	0.98 ± 0.03	87.21 ± 0.14
Conven 55 °C	40	19.63 ± 0.16	23.48 ± 0.29	0.45 ± 0.17	73.99 ± 0.22	1.03 ± 0.03	89.40 ± 0.31
Conven 55 °C	60	21.40 ± 0.12	32.86 ± 0.41	0.49 ± 0.05	75.78 ± 0.39	1.09 ± 0.06	90.31 ± 0.13
Conven 55 °C	80	22.52 ± 0.54	24.67 ± 0.28	0.48 ± 0.03	74.79 ± 0.20	1.00 ± 0.11	89.10 ± 0.09
Conven 75 °C	40	19.34 ± 0.49	24.75 ± 0.18	0.47 ± 0.18	74.34 ± 0.27	0.98 ± 0.01	88.91 ± 0.08
Conven 75 °C	60	22.40 ± 0.13	31.56 ± 0.20	0.52 ± 0.10	76.07 ± 0.10	1.10 ± 0.03	91.39 ± 0.19
Conven 75 °C	80	22.20 ± 0.42	28.94 ± 0.30	0.49 ± 0.09	75.66 ± 0.30	1.04 ± 0.01	90.40 ± 0.20
Control 25 °C	40	19.47 ± 0.41	19.75 ± 0.28	0.27 ± 0.18	59.07 ± 1.12	0.65 ± 0.07	75.49 ± 0.19
Control 25 °C	60	20.29 ± 0.43	25.26 ± 0.23	0.32 ± 0.01	65.74 ± 0.32	0.69 ± 0.12	76.54± 0.11
Control 25 °C	80	19.90 ± 0.20	23.25 ± 0.19	0.30 ± 0.01	63.88 ± 0.10	0.73 ± 0.01	78.06 ± 0.14

The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) at different temperatures (45 °C, 55 °C, and 75 °C) for 15 min. The *Conven* represents groups of samples with conventional heating extraction using the same solvent ratios/temperatures as those in *OH* system. The *Control* represents extraction at room temperature using the same abovementioned solvent concentrations. TEAC, Trolox equivalent antioxidant capacity; TPC, total phenolic content (mg gallic acid equivalents/g extract d.w.).

The results from the post-hoc test (comparing different mean values of the extraction yield between different groups of extraction methods) showed that the extent of difference was significantly great between the *OH* groups (particularly 75 °C) and other groups of extraction methods. The difference was more pronounced between *Control* and OH groups ($p < 0.001$). As illustrated in **Figure 2.3**, the extraction solvent with 80% ethanol showed the highest values of yield across all groups of extractions (except for

Control and *Conven*, 75 °C and 45 °C), particularly in *OH* groups exhibiting 34.53%, 31.10%, and 30.80% with 75 °C, 55 °C, and 45 °C, respectively.

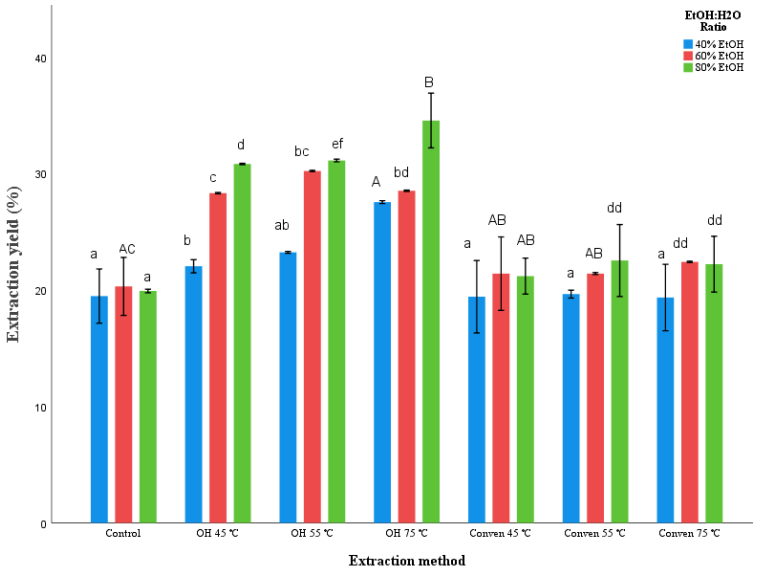


Figure 2. 3 Extraction yield (g extract/100 g dry olive leaves) obtained through various extraction methods. The *Control* represents the extraction with no heat treatment (25 °C). The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C), The *Conven* represents groups of samples with conventional heating extraction using the same solvent ratios/temperatures as those employed for *OH* samples. Mean values of % yield are shown with standard deviation error bars for each category of extraction method. Different letters (a–f, A–C) above the bars indicate statistically significant differences between means ($p < 0.05$).

The statistical significance of interactions between the two main factors (where the extraction methods were individually compared vs. solvent ratios) was studied through a pairwise comparison, which confirmed whether/not the extraction methods are significantly dependent on the solvent concentrations ($p < 0.05$). It was evident that the mean differences between the solvent ratios in *OH* groups were highly significant ($p < 0.001$), compared to *Conven* and *Control*. In other words, the reliance of ethanol concentration was greatest in *OH* groups that showed the highest effects on the extraction yield ($p < 0.001$). It was also found that in Control (between 40% and 80%), *Conven* 45 °C (60% and 80%), and *Conven* 75 °C (60% and 80%), the mean differences were not statistically significant ($p > 0.05$).

The extraction yield of olive leaves has been researched in numerous studies. Şahin Sevgili et al. (2019) examined different solvent proportions within a selected temperature/time range. Among the main findings, the extraction yields (50 °C for 60 min) accounted for 322.33 mg/g and 328.82 mg/g using 50% ethanol and 100% methanol, respectively. In the study of Lama-Muñoz et al. (2019), using 80% ethanol with 1:6 solid/solvent ratio, the highest extraction yield (dry residue of leave biomass) was

around 27.55%. Further, among the key results reported by Doménech et al. (2021), total extraction yield (%) of olive leaves, using water, was found to be around 35.0%. The rate of extraction yield is greatly reliant on the extraction methods/condition. However other contributing factors may decidedly affect the increase/decrease in extraction yield, which include variations in sample collections among different agro-industrial practices, cultivars, geographical origin, tree life time, storage conditions, etc.

2.3.3. Total phenolic content (TPC)

The concentration of polyphenols is among the key factors responsible for antioxidant activity/capacity in food. In this study, TPC was measured through the reaction of Folin–Ciocalteu reagent with total phenols available in the extracts. **Figure 2.4**, illustrates the mean values of the TPC measured for all extraction groups (mg GAE/g extract d.w.). In respect of extraction methods, ohmic heating represented the highest values, notably at 55 °C, subsequently with 75 °C and 45 °C. Samples extracted with *OH* 55 °C yielded 42.53, 34.36, and 31.63 mg GAE/g extract using 60%, 40%, and 80% ethanol, respectively.

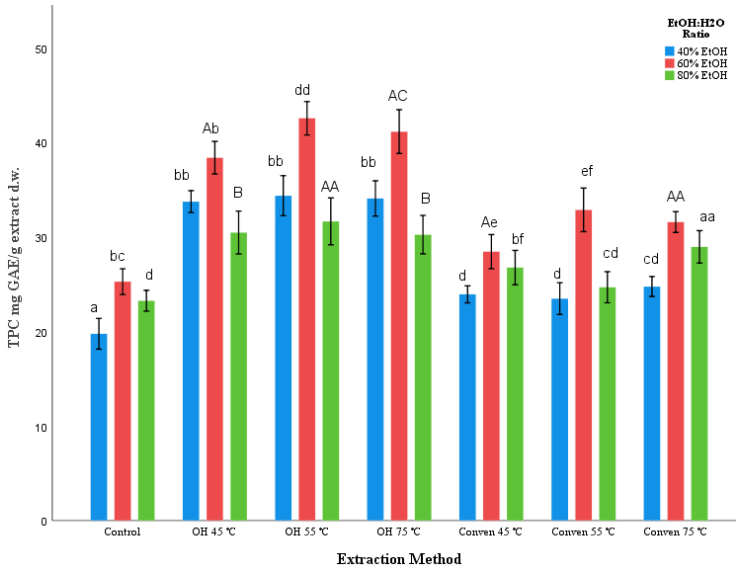


Figure 2. 4 Total phenolic content (TPC) mg GAE/g extract d.w. through various extraction methods are presented with standard deviation bars. The *Control* represents the extraction with no heat treatment (25 °C). The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperatures as those applied for ohmic system. Different letters (a–f, A–C) above the bars indicate statistically significant differences between means ($p < 0.05$).

Overall, 60% ethanol exerted the greatest effect on increased TPC in all extraction groups. The magnitude of difference between 60% and 80% (in *OH* groups), and 60% and 40% (in *Conven* and *Control* groups) was significantly large ($p < 0.001$).

In previous research experiments, using different extraction techniques/designs, various concentrations of TPC have been reported. Goldsmith et al. (Goldsmith et al. 2014) obtained 32.4 mg GAE/g using aqueous extraction at 90 °C for 70 min, and 1:60 g/mL solid–solvent ratio. In their study, Lee et al. (2009) detected that olive leaves extracted with 80% ethanol contained phenolics around 148 mg/g tannic acid equivalents. Research unveiled the potential of the optimized UAE (43.61% ethanol for 59.99 min at 34.18 °C) for increased phenolic recovery up to 43.825 mg GAE/g dry leaves (Şahin et al. 2015). Sánchez-Gutiérrez et al.(2021) found higher total phenolics (76.1 mg GAE/g d.w.) in Soxhlet-extracted leaves compared to those extracted by MAE with 80% ethanol for 10 min (54.0 mg GAE/g d.w.). On the other hand, Da Rosa (2019) described that the optimized MAE (using water at 86 °C for 3 min) is more effective, compared to the maceration, in the increase in phenolic liberation (by 82%). Another study demonstrated that olive leaves (dried at 60 °C for 120 min), through supercritical extraction, contained 36.1 mg GAE/g dry leaf (Canabarro et al. 2019).

The performance of extraction solvents in diffusivity may vary due to the phenolic complexity (in respect of solubility and polarity), and this constitutes a challenge to select the appropriate solvent(s) to ensure maximum extraction of bio-phenols while maintaining their bio-functionalities. It may not be ideally practical to use 100% of a single solvent and the use of excessive polar or non-polar solvents may work poorly on the release of phenols. Further, the choice of solvents comes with a challenge of choosing green/non-toxic ones, such as water and ethanol. There is much research investigating the effects of solvent nature/ratios on phenolic recovery from olive leaves. Şahin Sevgili et al. (2019) conducted research on various solvents/solvent concentrations, together with considering extraction temperature/time. Among the main findings, the samples with 100% methanolic extracts (50 °C for 60 min) yielded around 328.82 mg/g extract that was greater than those obtained by 100% ethanol (176.42 mg/g extract) under the same time/temperature conditions. However, the proportional use of ethanol showed improved extraction ability, as using 50% ethanol yielded 322.33 mg/g extract under the same temperature/time conditions. In another study, the extraction of olive leaves (from Koroneiki cultivar) with 50% ethanol, using maceration and microwave-assisted extractions, yielded phenolics of around 69.027 mg TAE/g and 88.298 mg TAE/g d.w., respectively (Rafiee et al. 2011).

Other crucial factors affecting polyphenol content include pre-processing approaches, such as blanching, drying, and grinding methods. Ahmad-Qasem et al. (2013) observed that hot-air drying at higher temperature for shorter drying time (120 °C for 12 min) enabled increased phenolic content (59 mg GAE/g d.w.), compared to that obtained through lower temperatures for a longer time (45 mg GAE/g d.w., using 70 °C for 50 min). Research also demonstrated that drying olive leaves (Chemlali cultivar)

with an infrared drying method at 70 °C and 40 °C exhibited around 5.14 and 2.13 g caffeic acid/100 g (d.w.), respectively (Boudhrioua et al. 2009).

2.3.4. Antioxidant activity

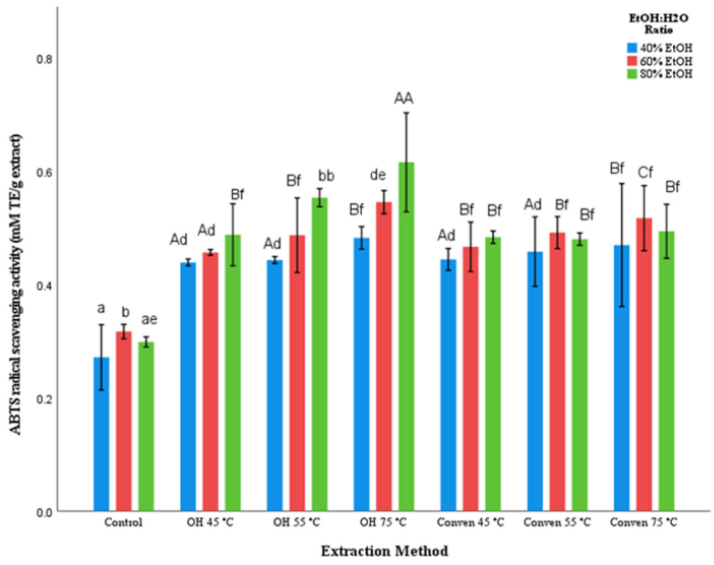
The endogenous polyphenols in olive leaves potentially exert antioxidant activity via deactivating/stabilizing free radicals. The following experiments enabled identification of the protective ability of the extracts against oxidative damage of free radicals.

2.3.4.1. Trolox equivalent antioxidant capacity (TEAC)

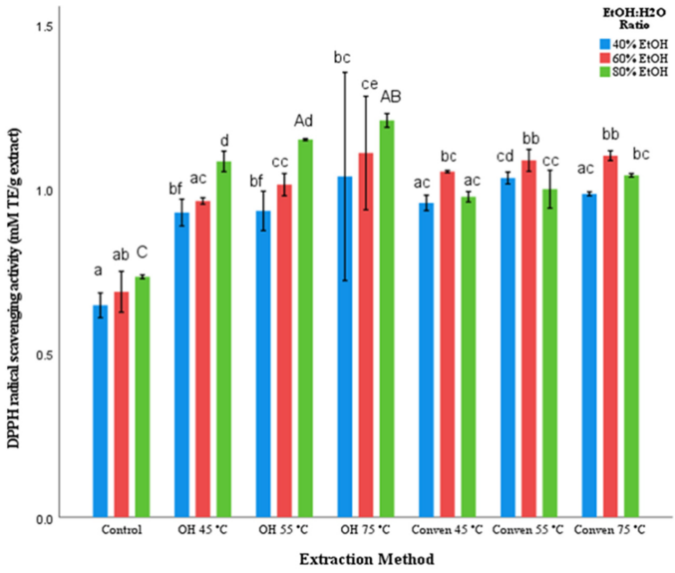
The free radical scavenging activity of extracts was determined using two different *in vitro* antioxidant assays (DPPH and ABTS). The method relies on the electron-transfer mechanism, that is, the potential of antioxidants to inhibit radical activities via transferring electrons.

As shown in **Figure 2.5**, the OH groups showed relatively similar pattern in both DPPH and ABTS assays (exhibiting the highest values with 80%, and the lowest values with 40% ethanol). The different patterns between them were as follows: (i) *Control* groups – in DPPH, the 80% exhibited the highest value (0.73 mM TE/g), while, in ABTS, the 60% showed the highest value (0.32 mM TE/g), (ii) *Conven* 45 °C – where 60% represented the highest level in DPPH (1.05 mM TE/g), and the 80% showed the highest in ABTS (0.48 mM TE/g), and (iii) *Conven* 55 °C — antioxidant capacity in descending order: 60%, 40%, and 80% in DPPH results, and 60%, 80%, and 40% in ABTS results.

The mean values of antiradical activity detected by DPPH assay were evidently greater than the corresponding values identified by ABTS method. Results confirmed, from both assays, that ohmic heating represented the highest antioxidant potency compared to *Conven* and *Control*, particularly in *OH* 75 °C, ranging from 1.21 to 1.04 mM TE/g extract (with DPPH), and 0.62 to 0.48 mM TE/g extract d.w. (with ABTS).



(a)



(b)

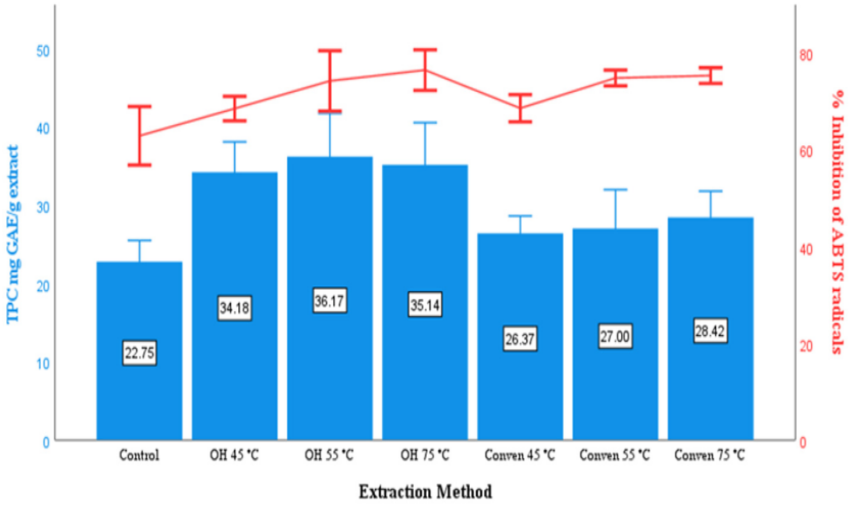
Figure 2. 5 Mean values of Trolox equivalent antioxidant capacity (TEAC) in olive leaf extracts. **(a)** ABTS radical scavenging activity (mM TE/g extract d.w.). **(b)** DPPH radical scavenging activity (mM TE/g extract d.w.). Different letters (a–f, A–C) above the bars indicate statistically significant differences between means ($p < 0.05$). The *Control* represents the extraction with no heat treatment (25 °C). The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperatures as those applied for ohmic system. Different letters (a–f, A–C) above the bars indicate statistically significant differences between means ($p < 0.05$).

In previous studies, the TEAC values differed largely among various processing methods/conditions, cultivar/growing regions, antioxidant assays, and sample collections (tree-picked, pruning biomass, or olive mill leaves).

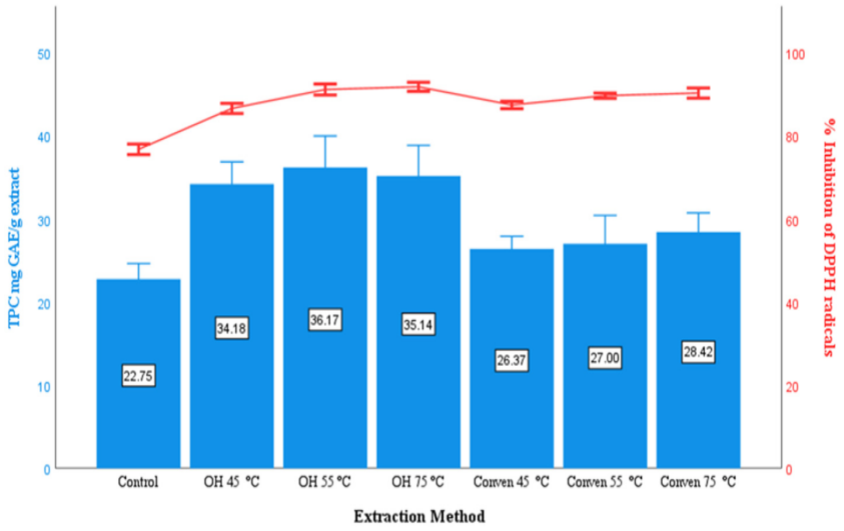
Herrero et al. (2011), through their experiment on the extraction of olive leaves (residues of olive oil industry, Spain), employed pressurized liquid extraction and noted that using water (200 °C) and ethanol (150 °C) exhibited 2.661 mM TE/g and 0.677 mM TE/g, respectively. In their research, Lins et al. (2020) observed around 0.215 and 0.148 g TE/g extract d.w. in DPPH and ABTS, respectively. Abaza et al.(2011), focusing on the effect of solvent natures on the extraction of olive leaves from Chétoui cultivar, found ABTS radical inhibiting values in a range of 629.87–1064.25 μmol TE/g d.w., with 80% methanol giving the greatest antiradical potency. Goldsmith et al. (2014), through optimization of aqueous extraction, detected around 85.26 mg TE/g using the DPPH method. Nicolì et al. (2019) used 60% ethanol for the extraction of fifteen varieties of Italian olive leaves. Among their findings, the DPPH radical inhibition (8.67–29.89 μmol TE/mg extract, d.w.) in leaves from Minerva and Itrana cultivars represented the lowest and the highest ranking, respectively. Also, the ABTS radical scavenging activities have been reported in (i) olive mill leaves, in a range of 18,234–25,459 μmol TE/100 g, and (ii) tree-picked olive leaves, of around 59,651 μmol TE/100 g (Contreras et al. 2019). The research of Orak et al. (2019) discovered a range of TEAC values in the selected olive leaves with different genotypes cultivated in Turkey, which ranged from 0.7 to 1.01 mM TE/g extract, with Esek Zeytini and Uslu genotypes giving the highest and the lowest ABTS scavenging effects, respectively. Moreover, in the research of Hayes et al. (2011), the commercial olive leaf extract exhibited around 37.93 g TE/100 g extract d.w.

2.3.4.2. Relationship between antioxidant activity (%) and TPC

To present more informative data, the results were further evaluated to compare the % inhibition of free radicals with the mean values of TPC for each extraction group (**Figure 2.6**). Regardless of solvent ratios, comparatively, similar trends were seen in the results of TPC and antioxidant activity (both methods). However, the highest mean values in TPC were observed in OH 55 °C, while the highest values of antiradical activity in both assays belonged to *OH* 75 °C. In addition, the magnitude of mean differences of TPC between *OH* groups and *Conven* groups was much greater compared to the corresponding groups in ABTS and DPPH.



(a)



(b)

Figure 2. 6 Comparison between TPC and antiradical activity: **(a)** Relationship between % ABTS radical scavenging activity and total phenolic content. **(b)** Relationship between % DPPH radical scavenging activity and total phenolic content. The values were obtained from various extraction methods (*OH*, *Conven*, and *Control*). Error bars represent the standard deviation ($p < 0.05$).

It was determined, overall, that the relation between total polyphenol content and antiracial activity (DPPH and ABTS) was statistically significant ($p < 0.001$). By reason of their biological activity, the greater proportion of polyphenols in plant tissues is expected to be positively correlated with higher antioxidant activity. The association between these two outcome variables in olive leaf extracts have been discussed in several studies. It is noteworthy that, due to the variability and complexity of phenolic compounds in different olive leaves (in terms of molecular structure, polarity, solubility, and concentration), the findings may not present a unique pattern for all types of olive leaves (even from the same cultivar/growing region). In the research of Papoti et al. (2018), olive leaf extracts with higher total phenolic content

exhibited considerable DPPH radical scavenging capacity (%), and it was highlighted that some of the examined cultivars, such as Atsilochou, Asprolia, Chrysophilli, and Pikrolia, represented particularly significant antiradical potential, ranging from 89 to 92% and 91 to 94%, in ethanolic and methanolic extracts, respectively. Monteleone et al. (2021) examined Biancolilla leaves, collected from tree pruning, and discovered that hydroalcoholic extracts yielded higher TPC, while the DPPH radical inhibiting values (%) were relatively close in all types of both aqueous and ethanolic/methanolic extracts (range: 88.90%, 90.85%, and 91.20%, using water (90 °C), 70% ethanol, and 70% methanol, respectively). Kiritsakis et al. (2010) described that the use of methanol (60%) for successive extraction of different cultivars (Koroneiki, Kalamon, and Megaritiki) enabled total phenolics around 6196, 5579, and 6094 mg GAE/kg dried leaves, respectively. These authors confirmed the high association between polyphenols and DPPH radical inhibiting potency. Irakli et al. (2018) explored positive correlation, particularly in the leaves extracted by the ultrasound-assisted method, using 50% acetone, 50% methanol, and 50% ethanol. Sánchez-Gutiérrez et al. (2021) found positive linearity in Soxhlet-extracted leaves using 50% ethanol (around 76.1 mg GAE/g with 78.01 mg TE/g), 75% ethanol (around 71.9 mg GAE/g with 72.043 mg TE/g), and water (around 67.6 mg GAE/g with 65.765 mg TE/g d.w.).

In this study, ohmic heating proved significantly useful for greater recovery of total polyphenols and, correspondingly, higher antioxidant activity in olive leaves. This information would further benefit from extensive research assessments to compare the ohmic with a range of green/competing extraction methods (focusing on various operating/processing parameters). For example, it is of value to compare ohmic method with MAE (e.g., in terms of frequency and energy efficiency) which has been demonstrated to be competitively effective in phenolic extraction and, using less energy/extraction time, it enables uniform heat transfer from interior of the food and exerts effects on rupturing the cell walls which assists in the release/extraction of desired bio-compounds (Vilas-Boas et al. 2020). Additionally, the extraction potency of ohmic can ideally be compared with non-thermal emerging techniques, such as PEF and HVED, e.g., in respect of electric strength, distance of electrodes, and frequency. Research, investigating the effects of green technologies (HVED, PEF, and UAE) on polyphenolic extraction from blueberry pomace, highlighted the weighty influence of electric strength and energy input on the rate of permeabilization of cell walls, liberation of intracellular molecules, diffusivity, and selective extraction of bioactive compounds (Lončarić et al. 2020). Optimization of ohmic heating, through an in-depth study of the effects of key independent variables on the response variables (extraction yield, total/individual phenolics, and bioactivity) potentially provides a value-added processing device for sustainable extraction of olive leaves.

2.4. Conclusions

Ohmic heating is considered a competitively viable approach for eco-sustainable extraction of biomolecules from food by-products. Using an inside-out heat generation, it overcomes the downsides inherent in conventional heating methods. The evidence presented in this study concludes that the ohmic technique is highly effective, compared to the conventional heating and *control* (solvent) methods, in increased extraction yield, total phenolics content, and antioxidant activity of olive leaves ($p < 0.001$). Of particular interest is that similar trends were seen in the results of TPC and antioxidant activity, irrespective of solvent ratio. Additional work will be needed to further understand the effects of different processing/operating parameters through an extensive comparative study between ohmic and competing/emerging methods. The ohmic technique, following optimization process may be employed as a competing benchmark for optimum extraction of olive leaves, enabling maximum/selective recovery of endogenous bio-phenols that can be used as high-value ingredients for bio-functional and nutraceutical applications. Moreover, through a zero waste/sustainable process, it may address the challenges in current system for exploitation of olive leave residues which may highly assist in the improvement of the return of investment for agricultural/food producers.

2.5. References

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CHAPTER 3

EXTRACTABILITY OF OLEUROPEIN, HYDROXYTYROSOL, TYROSOL, VERBASCOSIDE AND FLAVONOID-DERIVATIVES FROM OLIVE LEAVES USING OHMIC HEATING (A GREEN PROCESS FOR VALUE ADDITION)

This work has been submitted to the Royal Society of Chemistry (RSC):

Safarzadeh Markhali, F., and Teixeira, J.A., 2023. Extractability of Oleuropein, Hydroxytyrosol Tyrosol, Verbascoside and Flavonoid-Derivatives from Olive Leaves using Ohmic Heating (A Green Process for Value Addition). *Sustainable Food Technology*.

Abstract

The aim of this study was to determine the influence of ohmic heating (*OH*) as a green processing device on the extraction of principal polar phenols from olive leaves, with special attention to oleuropein content. The concentrations were quantified through using ultra-high-performance liquid chromatography (UHPLC). Other target analytes including total flavonoid content (TFC) and total condensed tannins (TCT) were also assessed as the secondary response variables. The key predictor variables were: (i) extraction method: ohmic heating (*OH*) and conventional heating (*Conven*), (ii) solvent ratio (40–80% aueous ethanol), and (iii) extraction temperature: 45 –75 °C. In addition, *Control* samples were prepared through solvent extraction at room temperature using the same solvent ratios applied for OH and Conven. The length of extraction time (15 min) was the same for all extraction methods. Overall, the data showed that the intensified extractability of individual phenols is readily achievable by ohmic heating rather than other aforementioned methods ($p < 0.05$). Importantly, the recovery of oleuropein through the ohmic system reached up to 26.18 mg/g extract at 75 °C with 80% ethanol, compared to the *Conven* (7.98–14.55 mg/g extract) and *Control* (8.64–10.81 mg/g extract). Other major polyphenols that reached the maximum levels, via the ohmic approach, were as follows: (i) luteolin 7-O-glucoside (4.12 mg/g extract), apigenin 7-O-glucoside (3.47 mg/g extract), rutin (3.78 mg/g extract), and tyrosol (0.34 mg/g extract) using 60% ethanol at 55 °C (ii) verbascoside (1.04 mg/g extract) using 80% ethanol at 75 °C, and (iii) hydroxytyrosol (1.38 mg/g extract) using 80% ethanol at 55 °C. The findings of this study brings to light that ohmic heating is potentially a method of preference for efficient recovery of representative phenols of olive leaves.

Keywords: Olive leaf extract, ohmic heating, extractability, principal polyphenols, oleuropein, hydroxytyrosol, verbascoside.

3.1. Introduction

Olive mill leaves (the biomass residues of olive oil industry) contain significant levels of polyphenols; some of which are prized for their distinctive antioxidant properties. These include oleuropein (a secoiridoid glycoside, that is exclusively intrinsic to Oleaceae plants), verbascoside, hydroxytyrosol, and flavonoids (Safarzadeh Markhali et al. 2020). In this regard, sustainable recovery of such valuable compounds from olive leaf residues (presently underexploited), may find high-value applications in food and pharmaceutical products; which in turn can respond to the current market requirements (the growing demands for bio-based and health-promoting food ingredients). In food applications, the leaf extracts rich in the abovementioned polyphenols have great potential to be incorporated into a diverse range of food products to: (i) enhance their oxidative stability and shelf-life, and (ii) promote their nutritional and health benefits. In particular, oleuropein-rich extracts have additional benefits as, beyond having potential for food formulation, it can act as a natural substrate for hydroxytyrosol formation (upon successful hydrolysis from which phenolic alcohol such as hydroxytyrosol can be generated. Development of this application can be of value for the industry to produce natural hydroxytyrosol in place of the costly synthetic ones.

At present, the existing extraction system is commonly based on conventional/classic modes which represent weak efficiency (high consumption of fossil fuel energy/solvents for prolonged extraction time). This has prompted scientists to investigate on innovative mechanical approaches to address the downsides inherent in the conventional/current system. In effect, numerous emerging techniques have been researched through a broad range of operating conditions/processing designs. Typical examples are: microwave-assisted extraction, ultrasound-assisted extraction (UAE) (da Rosa et al., 2019), supercritical fluid extraction (SFE) (Le Floch et al. 1998), high-voltage electric discharges (HVED) (Žuntar et al. 2019), and pulsed electric field (PEF) (Pappas et al. 2021) have been researched.

Another green solution is making use of ohmic heating which is primarily based on an inside-out thermal generation; enabling an instantly uniform heat supply within the food with minimum energy use. This method was practiced in our previous study (Safarzadeh Markhali et al. 2022) dealing with the extraction of olive leaves, via a series of temperatures (45, 55, and 75 °C) and solvent ratios (using aqueous ethanol). As a result, the ohmic system, compared to the conventional approach, enabled significantly higher: (i) extraction yield (up to 34.53%) at 75 °C with 80% ethanol, and (ii) total phenolic content (TPC) up to 42.53 mg GAE/g extract, at 55 °C with 60% ethanol, and (iii) antiradical capacity at 75 °C with 80% ethanol (1.21 mM TE/g by DPPH), and 0.62 mM TE/g extract by ABTS). Above all, the data on the total phenols across the extraction methods showed relatively similar trends of changes as

those observed in radical scavenging activities, which can in part annotate the roles of polyphenol concentrations in dictating antioxidant activities of the resulting extracts.

However, limitations exist in the previous research, as it was a preliminary study. As example, the quantification of polyphenols was limited to total compounds and the assays were based on spectrophotometric methods. Hence there needs to be further investigation, particularly, on the quantities of the principal endogenous phenols of the *OH* extracts via instrumental analyses such as high-performance liquid chromatography (HPLC). This is especially important due to the fact that there are variations in phenolic structures and biochemical routes, and thus the performance of each phenolic component may be markedly different under various processing conditions. Above all, data are particularly needed on oleuropein content that is among the principal bioactive compounds in olive leaves. Oleuropein belongs to secoiridoid glycosides (the compounds exclusively intrinsic to Oleaceae plants) and is highly valued for having strong antioxidant potential (Safarzadeh Markhali et al. 2020). In this line, to further evaluate the extraction efficacy of ohmic heating, the present study extended the previous work to examine the olive leaf extracts for the content of the chief polyphenols comprising oleuropein, hydroxytyrosol, verbascoside, tyrosol, and flavonoid-derivatives (luteolin 7-O-glucoside, Apigenin 7-O-glucoside, and rutin). Other selected analytes chosen for this study include total flavonoids, total condensed tannins, and antioxidant capacity (using three different assays).

3.2 Materials and methods

3.2.1 Plant materials and chemicals

Plant materials – Olive mill leaves from the same cultivar/growing site as that applied in our earlier work (Safarzadeh Markhali et al. 2022) were kindly provided by "Center for Advanced Studies in Energy and Environment", University of Jaén, Campus of Las Lagunillas, Jaén, Spain.

Chemicals – all chemicals used in this study were identical to those practiced in our earlier work (Safarzadeh Markhali et al. 2022). Additionally, the following chemicals (Sigma-Aldrich, Saint Louis, MO, USA) used for the current study: (i) analytical-grade chemicals – aluminum chloride, sodium nitrite, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), sodium hydroxide, methanol, quercetin, vanillin, and catechin, (ii) HPLC-grade standards/reagents – oleuropein, hydroxytyrosol, verbascoside, tyrosol, luteolin 7-O-glucoside, apigenin 7-O-glucoside, rutin, formic acid, and acetonitrile.

3.2.2. Extraction design

Prior to samples being extracted, olive leaves were pre-processed following precisely the same procedure as that practiced earlier (Safarzadeh Markhali et al. 2022). Briefly, the leaves upon arrival were cleaned,

and dried at 37 °C for 48 h. The dried leaves were ground to 0.3 mm, vacuum packed in polypropylene bags and refrigerated (0–4 °C) for a maximum of two weeks prior to extraction experiments. The extraction of dry ground olive leaves were conducted (solid-solvent ratio of 1:10) via the following techniques for a 15-min processing time.

3.2.2.1. Ohmic heating

The ohmic extraction was performed using a bench-scale ohmic heater following the same procedure previously reported (Safarzadeh Markhali et al. 2022). Prior to the extraction, the electrical conductivity of the extraction solvents were intensified, using sodium chloride, to obtain: 4.2 mS/cm, 3.1 mS/cm, and 3.4 mS/cm for 80%, 60%, and 40% aqueous ethanol, respectively. The frequency and electrical voltage range remained constant, with 25 kHz and 1–10 V/cm, respectively. The main extraction parameters were (i) solvent ratio (in the range 40–80% ethanol-water *v/v*), and (ii) extraction temperature (in the range 45–75 °C).

3.2.2.2. Conventional heating

The conventional (*Conven*) extraction of dry ground leaves was performed following the same process design practiced earlier (Safarzadeh Markhali et al. 2022). The extraction process was under the same solvent ratio/temperature conditions using the same operation components as applied for the ohmic with the exclusion of electrodes in the reactor chamber.

3.2.2.3. Solvent extraction (Control)

As the control samples, the dry leaves were extracted through the constant agitation at room temperature (without heating) using the same aforementioned solvent ratios used for the *OH* and *Conven* methods.

3.2.3. Colorimetric analyses of phenolic content and antioxidant capacity of olive leaf extracts

3.2.3.1. Total flavonoid content (TFC)

Total flavonoid content was measured using aluminum trichloride assay as described by Qawasmeh et al. (2012). Into a 5 mL volumetric flask, 500 µL of extract mixed with 150 µl of 5% sodium nitrite solution and incubated at room temperature for 5 min prior to adding 150 µL of 10% aluminum trichloride solution. The solution was vortex mixed and allowed to stand for 1 min. After adding 1 mL of 1 M sodium hydroxide, the mixture was made up to 5 mL final volume with distilled water, vortex mixed and measured at 510 nm using UV-vis spectrophotometer. Total flavonoid content was calculated against quercetin standard curve and reported as mg of quercetin equivalents/g extract dry weight (mg QE/g extract d.w.).

3.2.3.2. Total condensed tannins (TCT)

The content of total condensed tannins in olive leaf extracts were quantified using the method of Julkunen-Tiitto (1985) with minor modifications. Briefly, an aliquot (50 µL) of extract or standard solution was added to 1 mL of reagent solution (containing 4% vanillin and 8% hydrochloric acid mixed with methanol, 1:1 *v/v*). After 20 min incubation at room temperature, the absorbance was measured at 500 nm. Total tannins were calculated against standard curve and the results were expressed as mg catechin equivalents/g extract (mg CE/g extract d.w.).

3.2.3.3. Total phenolic content (TPC)

Total phenolic content was determined for further validation in this model study. The method of Singleton et al. (1999) was carried out with some modifications as described previously (Safarzadeh Markhali et al. 2022). The data were calculated against gallic acid standard curve and the results were recorded as mg of gallic acid equivalents/g extract (mg GAE/g extract).

3.2.3.4. Antioxidant capacity (*in vitro*)

The antioxidant capacity of extracts (*in vitro*) was determined based on Trolox equivalent antioxidant capacity (TEAC) assay via three different analytical methods listed below to minimize potential errors of interpretations inherent in the complexity of the biological/molecular structure of plants and/or potential formation of reaction intermediates/transient species.

DPPH radical scavenging activity – the antiradical potential of the extracts to diminish 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radicals was examined according to the method of Brand-Williams et al. (1995), with slight modifications as described in our previous report (Safarzadeh Markhali et al. 2022). The absorbance readings (515 nm) were calculated against Trolox standard curve and the results were expressed as mM Trolox equivalents/g extract d.w. (mM TE/g extract).

ABTS radical scavenging activity – the antiradical potential of the extracts to deplete 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) free radicals was determined according to the method of Re et al. (1999) with slight modifications as detailed in our previous study (Safarzadeh Markhali et al. 2022). The absorbance was measured at 734 nm and calculated against Trolox calibration curve and expressed as mM TE/g extract d.w.

Ferric reducing antioxidant capacity – The assay of ferric reducing antioxidant power (FRAP) was performed to determine the antiradical potential of extracts for reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). The experiment was adapted from the methods of Benzie & Strain (1996) and Hayes et al. (2011). The FRAP reagent was initially prepared by mixing: (i) acetate buffer (300 mM/L, pH 3.6), and (ii) a

mixture of 10 mM/L 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), in 20 mM/L hydrochloric acid and 20 mM/L ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), (mixture ratio: 10:1:1 v/v/v). The prepared reagent was incubated at 37 °C for 10 min. Into a test tube containing 950 µL of FRAP reagent, an aliquot of extract (100 µL) was then added, vortex mixed and incubated at 37 °C for 30 min. A blank sample containing only FRAP reagent was also prepared. The absorbance readings of samples were measured (against the blank) at 593 nm. The values were calculated against Trolox standard curve and the results were reported as mM TE/g extract.

3.2.4. HPLC analyses – determination of the recovery of principal polyphenols from olive leaf extracts

The chromatographic separations of target polyphenols were carried out through ultra-high-performance liquid chromatography (UHPLC) (Shimadzu Nexera X2 UHPLC) connected to a diode array detector (Shimadzu SPD-M20A), and an integration system (Shimadzu LabSolutions software, Kyoto, Japan). using a reverse-phase Aquity UPLC BEH C-18 column (100 mm x 2.1 mm, 1.7 µm, Waters Corporation). The gradient elution program (**Table 3.1**) used in this study was based on the method described by Quero et al. (2022) with slight modifications. A flow rate of 0.3 mL/min was selected and the injection volume was 5 µL. The column temperature maintained at 40 °C. In advance of running the HPLC, the eluents (mobile phase) were filtered through 0.22 µm membrane nylon filters, and the samples/standards were filtered through 0.22 µm syringe filters. The chromatograms were registered at a wavelength of 280 nm and the content of the desired phenol was identified with reference to the retention time of the standard and the UV spectra detector. The standard calibration curve was plotted (peak area vs. concentration) and the selected phenolic content in the extracts was quantified against the linear calibration curve of the corresponding standard and the results were reported as mg target phenol/g extract. d.w.

Table 3. 1 Gradient elution program used for the HPLC analysis of target phenolic compounds in olive leaf extracts.

Time Period (min)	**Flow (mL/min)**	**Mobile phase A (%)** Water/formic acid (99.9/0.1 v/v)	**Mobile phase B (%)** Acetonitrile
0 – 5.5	0.3	95	5
5.5 – 17	0.3	40	60
17 – 18.5	0.3	0	100
18.5 – 30 Column equilibrium	0.3	95	5

3.2.5. Statistical analysis

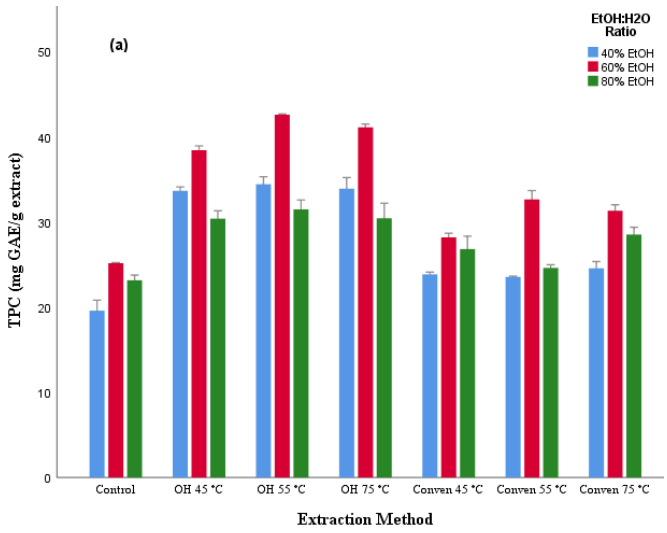
Significant differences ($p < 0.05$) between the mean values (± SD) of all determinations were statistically assessed via Analysis of Variance (ANOVA) using SPSS software, version 27.0. The dependent variables (response variables) of the extracts were individually analyzed using two-way ANOVA to assess

the interactive effects of two independent variables (solvent ratio and extraction method) on the mean values of each dependent (response) variable individually. Pearson correlation was used to evaluate the correlation between dependent variables. A difference was considered statistically significant when $p < 0.05$. The assumption of homogeneity of equal variance was assessed through the Levene's test (homogeneity of variance assumption was satisfied when p-value was greater than 0.05).

3.3. Results and discussion

3.3.1. Total flavonoids, total condensed tannins, and total phenolic content

As shown in **Figure 3.1**, the values from total flavonoid content (TFC), and total condensed tannins (TCT), varied largely between *OH* and *Conven* groups. For the same solvent ratio at the same extraction temperature the *OH* extracts significantly improved the recoveries, compared to the corresponding groups of *Conven* extracts. Indeed, the *OH* extract with 60% ethanol at 55 °C enabled the maximum recoveries of TFC, TCT that reached 20.45 mg QE/g (for flavonoids) and 10.92 mg CE/g extract (for Tannins). Regardless of the extraction method, the use of 60% ethanol showed the highest extractability of TFC for the same extraction experiment.



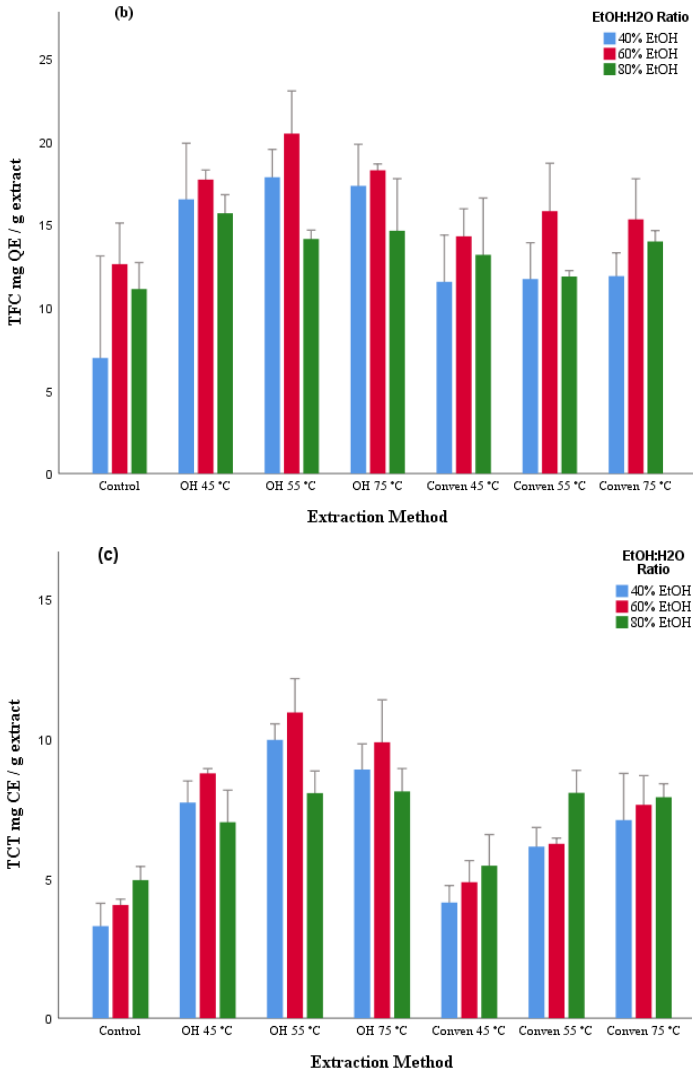


Figure 3. 1. Effect of extraction methods on: **(a)** total phenolic content (TPC) mg GAE/g extract, **(b)** total flavonoid content TFC (mg QE/g extract), and **(c)** total condensed tannins (TCT) mg CE/g extract. Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH *v/ v*) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* methods.

Flavonoids form a significant proportion of polyphenols in olive leaves, predominately in glycosidic form rather than in aglycon (free) form. The variations in flavonoid content reported in different studies confirm that the extractability of bio-phenols is heavily affected by the parameters involved in throughout the processing systems. In the study of Lee et al. (2009), through 80% ethanol solvent extraction, the total flavonoids yielded 58 mg naringin equivalents/g extract. Ghelichkhani et al. (2019) detected 396.4 mg QE/g and 298.16 mg QE/g in olive leaf extracts spray dried and freeze dried, respectively). In their research, Abaza et al. (2015), observed the highest recovery of flavonoids from olive leaves extracted by 50% acetone through ultrasound-assisted for 10 min at 60 C using at 60 °C. Agatonovic-Kustrin et al. (2021), described the effectiveness of fermentation extraction with lactic acid-forming bacteria using ethyl

acetate in the improved yield of flavonoids that reached up to 238 µg rutin equivalents/20 µL of olive leave extract. The study of Lins et al. (2018), explored that the commercial micronized dry olive leaves (superfine ground) extracted with 80% methanol produced 19.4 mg quercetin equivalents/g olive leaves.

Figure 3.1.(c) illustrates the total tannin content of the extracts. Tannins, the complex polymeric groups of phenolics, possess a range of bio-functional properties, and are known to have antioxidant, antimicrobial, and anticarcinogenic activities. In the literature, the amount of total condensed tannins (the catechin-class tannins) in olive leaves is variable; reportedly as follows: 8.30 g/Kg leaves d.w. (García et al. 2003), 9.49 g/Kg leaves d.w. (Molina-Alcaide & Yáñez-Ruiz, 2008), 0.46% leaves d.w. (Olfaz et al. 2018), and 1.07% fresh leaves (Fegeros et al. 1995). In their research, Mansour-Gueddes et al. (2020) through using maceration at room temperature for two days reported variations in condensed tannins of the aqueous extracts of olive leaves from three growing sites in Tunisia (0.24–0.84 mg CE/g (d.w.). In a recent paper Guebebia et. al. (2022) observed significant effects of genotypes and extraction approach. These authors found that tannin content of leaves (Chemlali variety) extracted by maceration yielded greatest proportion (23.03 mg CE/100g d.w.) compared to the leaves (Zarrazi variety) extracted by ultrasonic assisted system (8.32 mg CE/100g d.w.). Together with the key factors such as cultivars, climate, leaf life-time, handling/storage conditions, other factors such as pre-processing operations can be influential in the content of condensed tannins. Molina-Alcaide & Yáñez-Ruiz (2008) through their research on comparing various drying methods/temperatures of olive leaves found 10.0, 9.92, 7.90, 6.24, 9.57 g/Kg total condensed tannins in fresh, freeze dried, air-dried, forced convection oven dried (60 and 100 °C), respectively.

3.3.2. Antioxidant capacity

In the present study, antioxidant capacity of extracts was assessed using three different TEAC assays based on FRAP, DPPH, and ABTS detections. As shown in **Table 3.2**, relatively consistent with our earlier work, the *OH* extraction system at 75 °C displayed the highest values in all experimented assays. The values reached the summit by using 80% ethanol levels (1.56 mM TE/g by FRAP), (1.27 mM TE/g by DPPH), and (0.68 mM TE/g by ABTS).

Table 3. 2 Mean values (±SD) of Trolox equivalent antioxidant capacity (TEAC) of olive leaf extracts detected by FRAP, DPPH, and ABTS radical scavenging activity (mM TE/g extract d.w.).

Extracts		Radical Scavenging Activity (mM TE/g)		
Extraction technique	**% EtOH (*v/v*)**	**FRAP**	**DPPH**	**ABTS**
OH 45 °C	40	0.93 ± 0.03 ^{a}	0.91 ± 0.09 ^{a}	0.44 ± 0.05 ^{a}
	60	0.99 ± 0.04 ^{a}	0.96 ± 0.01 ^{a}	0.46 ± 0.02 ^{a}
	80	1.28 ± 0.10 ^{c}	1.08 ± 0.05 ^{b}	0.49 ± 0.11 ^{b}
OH 55 °C	40	1.04 ± 0.10 ^{d}	1.03 ± 0.10 ^{a}	0.45 ± 0.01 ^{a}
	60	1.17 ± 0.09 ^{f}	1.01 ± 0.09 ^{a}	0.56 ± 0.08 ^{b}
	80	1.37 ± 0.21 ^{ad}	1.15 ± 0.13 ^{dd}	0.55 ± 0.03 ^{b}
OH 75 °C	40	1.35 ± 0.14 ^{d}	1.13 ± 0.02 ^{b}	0.53 ± 0.09 ^{b}
	60	1.47 ± 0.20 ^{cb}	1.19 ± 0.16 ^{af}	0.60 ± 0.02 ^{c}
	80	1.56 ± 0.05 ^{dd}	1.27 ± 0.02 ^{de}	0.68 ± 0.05 ^{ef}
Conven 45 °C	40	1.10 ± 0.10 ^{d}	0.96 ± 0.05 ^{a}	0.45 ± 0.03 ^{a}
	60	1.16 ± 0.13 ^{f}	1.05 ± 0.08 ^{b}	0.47 ± 0.02 ^{b}
	80	1.12 ± 0.30 ^{d}	0.98 ± 0.06 ^{a}	0.44 ± 0.09 ^{a}
Conven 55 °C	40	1.15 ± 0.22 ^{f}	1.02 ± 0.11 ^{a}	0.46 ± 0.02 ^{a}
	60	1.21 ± 0.08 ^{b}	1.09 ± 0.20 ^{b}	0.49 ± 0.01 ^{b}
	80	1.08 ± 0.15 ^{d}	1.01 ± 0.24 ^{b}	0.48 ± 0.02 ^{b}
Conven 75 °C	40	1.13 ± 0.07 ^{f}	0.98 ± 0.06 ^{a}	0.47 ± 0.04 ^{b}
	60	1.18 ± 0.05 ^{f}	1.10 ± 0.12 ^{b}	0.52 ± 0.09 ^{aa}
	80	1.14 ± 0.10 ^{f}	1.04 ± 0.11 ^{a}	0.49 ± 0.05 ^{b}
Control 25 °C	40	0.77 ± 0.06 ^{ac}	0.65 ± 0.05 ^{ac}	0.27 ± 0.02 ^{bc}
	60	0.82 ± 0.03 ^{ac}	0.69 ± 0.08 ^{ac}	0.32 ± 0.02 ^{cd}
	80	0.86 ± 0.05 ^{de}	0.73 ± 0.12 ^{ae}	0.30 ± 0.01 ^{cd}

Different letters down the column indicate significant differences ($p < 0.05$) on radical scavenging activities amongst the extract samples.

In general, the concentration of polyphenols is expected to have a positive correlation with antioxidant capacity. In other words, the values may increase correspondingly with the rise of phenolic content (particularly the aglycones which are the free phenols). In our earlier report (Safarzadeh Markhali et al. 2022), the antiradical activities of olive leaf extracts were examined using two types of *in vitro* assays. Comparing their values with the phenolic values, similar trends of variations were detected in the TPC results and radical scavenging activities (DPPH and ABTS), irrespective of solvent ratio.

In the current study, the comparison data (taking into consideration the extraction methods, regardless of solvent ratios) between antiradical activities and TPC (**Figure 3.2**) also support our earlier work as the TEAC values showed similar upward/downward trends with the corresponding groups of TPC values. The data showed that the *OH* extraction was significantly effective in raising TPC as well as antiradical activities ($p < 0.05$), compared to the conventional methods. However, there were slight dissimilarities as the maximum TPC values belonged to *OH* 55 °C, while the maximum antioxidant activity belonged to *OH* 75 °C ($p < 0.05$).

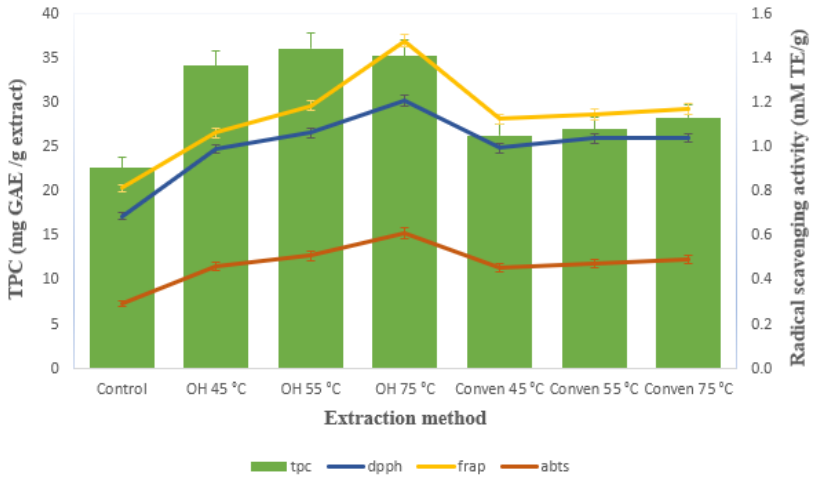


Figure 3. 2 Relationship between DPPH, FRAP, and ABTS radical scavenging activities (mM / g extract) and total phenolic content (TPC), as a function of extraction method. The values were obtained from various extraction methods: *OH* (ohmic heating), *Conven* (conventional heating), and *Control* (solvent) through various temperatures. Error bars represent the standard deviation ($p < 0.05$).

3.3.3. Recovery of principal polyphenols from olive leaves – chromatographic analysis

The concentrations of the target polyphenols in olive leaf extracts (mg/g extract) were determined through chromatographic analysis.

3.3.3.1. Oleuropein

As shown in **Figure 3.3**, compared to the *Conven* & *Control* extracts, the *OH* groups yielded the highest content of oleuropein for all corresponding temperature/solvent ratios ($p < 0.05$). The maximum level of oleuropein (26.18 mg oleuropein/g extract) belonged to *OH* extracts at 75 °C with 80% ethanol. Intriguingly, this particular approach (*OH* 75 °C, 80% ethanol) also favored maximum values of radical scavenging activities in terms of DPPH, FRAP, and ABTS (**Table 3.2**). Given the fact that antiradical activities in the plant matrix are heavily influenced by the content of certain bio-phenols, and the fact that oleuropein has great antioxidant potential, the data here may suggest that oleuropein acted as a radical scavenger dose-dependently for each extract.

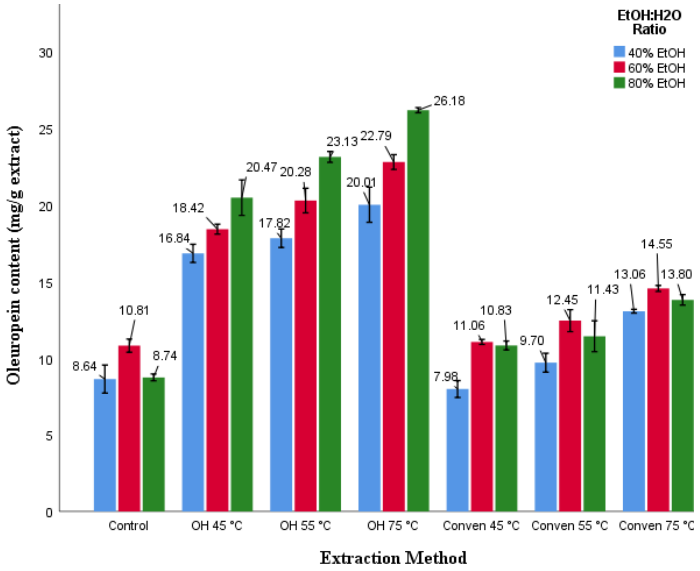


Figure 3. 3 Effect of extraction methods on oleuropein content (mg/g extract). Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* extractions.

The data on oleuropein content of olive leaves vary largely among studies depending on growing sites, seasonal/collection times, and processing strategies. Having said that, despite the lack of consistency of results, the majority of studies described that oleuropein is among the chief phenolic secoiridoids present in olive leaves. The research performed by Contreras et al. (2019), using the same variety and growing region as those in the present study, showed oleuropein content varied broadly in response to three sequent extraction steps, from which those extracted with UAE prior to alkaline extraction represented highest values 12,694 and 1790 mg/100 g extracts from tree-picked olive leaves and olive mill leaves, respectively. Şahin et al. (2012) reported that Arbequina olive leaves cultivated at various regions in Texas (sampled in February) represented different proportions, among which, those grown in Santa Fe yielded the highest proportion (71.53 µg /mg fresh leaves). In another study, steam-blanched olive leaves following an optimized multiple extraction system (85 °C for 30 min) contained significantly greater oleuropein (103.1 mg / g olive leaves d.w.) compared those extracted by conventional method (4.6 mg/g, at 40 °C for 48 h) (Stamatopoulos et al. 2014a). In their research, Xie et al. (2015) observed high recovery of oleuropein in olive leaves (Frantoio cultivar) when processed with ultrasound-assisted combined with reduced pressure extraction approach that enabled 92.3% efficiency through a single attempt of the extraction. Irakli et al. (2018) reported improved concentration of oleuropein (10.65%) tree-picked olive leaves (north Greece) having being extracted by UAE optimized with

50% acetone at 60 °C for 10 min. In a recent paper, using olive mill leaves (Arbequina variety), Márquez et al. (2022) found a great effectiveness of homogenizer-assisted extraction approach in the recovery of oleuropein (4,345 mg/100 g). Moreover, Ahmad-Qasem et al. (2013a) detected high recovery of oleuropein (108.6 mg/g d.w.) in hot air dried olive leaves (120 °C for 12 min) extracted in shaking water bath at 22°C for 24 h.

3.3.3.2. Hydroxytyrosol

As a simple phenol, hydroxytyrosol is among the distinguishing hydrolysates produced from the ring cleavage of oleuropein in the course of acidic or enzymatic (endogenous and exogenous) hydrolysis. This compound is valued as being one of the many powerful antioxidants present in plant species. The production of synthetic counterpart is generally costly and/or intricate. In effect, some studies have turned to finding efficient solutions to isolate this compound from plant origin. Among which, olive leaves have potential to serve such a purpose; provided that they are rich in oleuropein compounds and not being oxidized under severe processing conditions. If that being the case, through various strategies, hydroxytyrosol may be freed from the decomposition of oleuropein.

The mean values of hydroxytyrosol obtained by different extraction approaches are highlighted in **Figrure. 3.4**, Examining the *OH* groups here, it is clear that the use of ohmic heating with 80% ethanol (for the same extraction temperature) shows great potential for the diffusion of hydroxytyrosol from the cell walls. In particular, at 55 °C which favored the highest recovery in the *OH* extract that reached up to 1.38 mg/g extract. On the other hand, comparably, the use of 40% ethanol in OH groups favored the least amount of recovery; particularly at 45 °C (0.77 mg/g extract) . Further, no significant difference was found between *Conven* 45 °C and *Conven* 55 °C for the same solvent ($p > 0.05$).

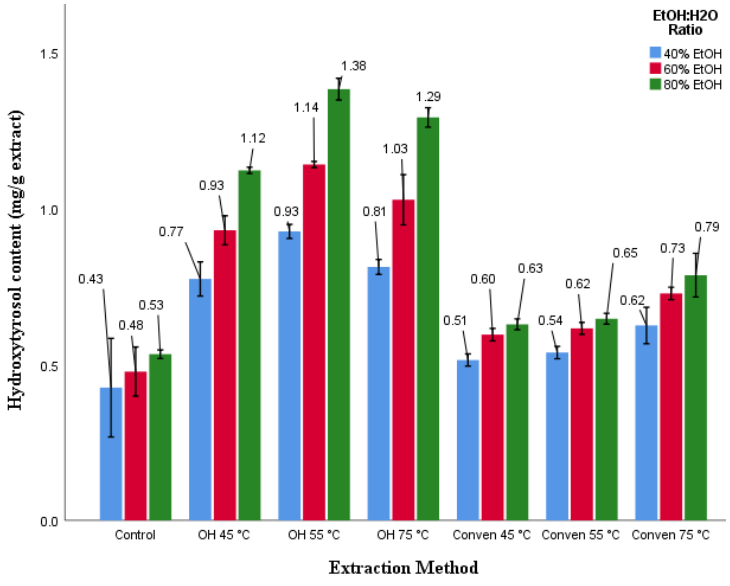


Figure 3. 4 Effect of extraction methods on hydroxytyrosol content (mg/g extract). Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* extractions.

In previous research studies, the results from hydroxytyrosol concentrations tend to vary largely among different experiments through various approaches/conditions. In the research of Ghomari et al. (2019) using 80% ethanol and distilled water, the concentrations of hydroxytyrosol were 15.17 and 27.20 mg/g (through sonication), and 0.02 and 0.25 mg/g (through a single-run maceration). Ortega–García & Peragón (2010) through their investigation on the effect of Spanish cultivars (including Picual), detected that there was a correlation between catalytic activities of regulatory enzymes [phenylalanine ammonia lyase (PAL), and polyphenol oxidase (PPO)], and endogenous phenolic content as it was found that each cultivar responded differently. Among which, the Picual cultivar exhibited low hydroxytyrosol content in response to the low activity of PAL and high activity of PPO. In another research conducted by Orak et al. (2019), using different genotypes of olive leaves from Izmir, Turkey, the hydro-methanolic extracts (65 °C, 15 min) exhibited variations in the range 1.33–4.03 mg/g with Uslu and Esek Zeytini cultivars being the lowest and the highest, respectively. Caballero et al. (2020), through using supercritical fluid extraction of olive leaves, observed the highest recovery of hydroxytyrosol (1.35 mg/g extract) with the pressure at 300 bar. Further, a recent study demonstrated the effectiveness of ultrasound-assisted extraction using natural deep eutectic solvent (citric acid/glycine/water) in yielding significantly higher amount of hydroxytyrosol (87 ppm); that was over fourfold increase compared to those provided by convention method with water (Zurob et al. 2020).

3.3.3.3. Tyrosol

As illustrated in **Figure 3.5**, the maximum content of tyrosol observed in this study belonged to the *OH* 75 °C with 80% ethanol (0.39 mg/g extract). Tyrosol is a phenylethanoid (a phenolic alcohol) with relatively similar molecular structure as that of hydroxytyrosol but with one less hydroxyl group. Although present in a trace amount in olive leaves, tyrosol is known to have a considerably high stability against auto-oxidation (its antioxidant potential remains stable greater than other major phenolic compounds) (Karković Marković et al. 2019).

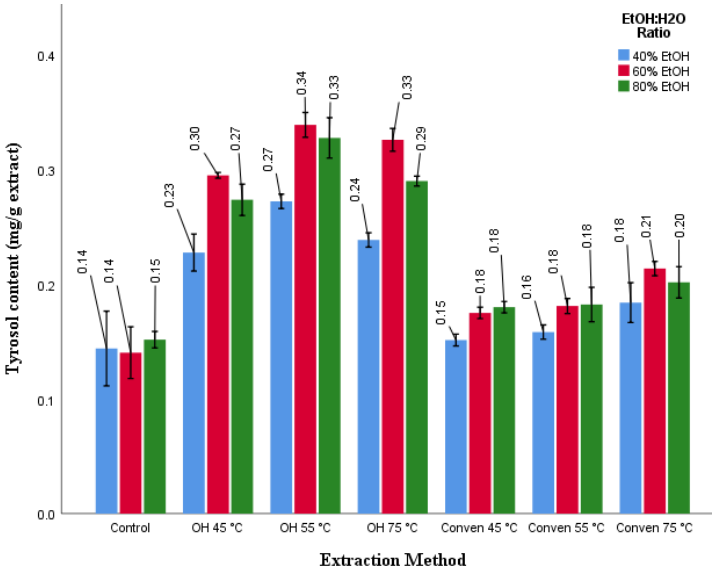


Figure 3. 5 Effect of extraction methods on tyrosol content (mg/g extract). Results are presented as mean values with standard deviation error bars. The OH represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The Conven represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The Control represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for OH and Conven extractions.

The extractability of tyrosol from olive leaves in part depends on the processing parameters of the extraction system. In a recent report, Akli et al. (2022) proposed an optimized extraction protocol using deep eutectic solvents mixed with different amino acids including lysine which represented 53420.23 µg/g tyrosol in the extracts that was significantly higher than that obtained by conventional solvent processed with %70 ethanol (19398.64 µg/g). These authors observed that the content of tyrosol was greater than hydroxytyrosol and oleuropein (that is typically different from what can be expected) and explained that the probable reasons can be the high stability of tyrosol and solvent suitability for tyrosol recovery. In another research, olive leaves extracted by maceration through ethanol-methanol and ethanol-water contained 0.49 and 1.95 mg/g, respectively (Ghomari et al. 2019).

3.3.3.4. Verbascoside

As a phenylethanoid glycoside, this compound is among the valuable bioactive molecules with strong antioxidant, anti-carcinogenic, anti-inflammatory, and anti-microbial effects (Alipieva et al. 2014). It is structurally characterized by a heterosidic ester of caffeic acid and hydroxytyrosol. As shown in **Figure 3.6**, the maximum level of verbascoside (1.04 mg/g extract) yielded when the ohmic heater was applied at 75 °C with 80% ethanol. Interestingly, as mentioned earlier (**Section 3.3.3.1**) oleuropein content reached the highest value through the same extraction parameters. This may indicate that both

oleuropein and verbascoside potentially contributed to the rise in antioxidant capacity since the levels (in all three antiradical assays) correspondingly reached the summit of antiradical activities (**Section 3.3.2**).

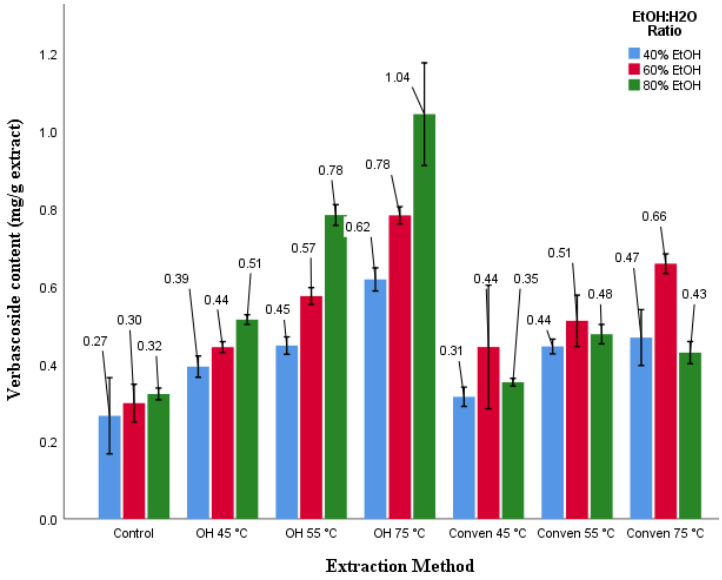


Figure 3. 6 Effect of extraction methods on verbascoside content (mg/g extract). Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH *v/v*) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* extractions.

The level of extractability of verbascoside from olive leaves may partly depend on the type of cultivar and collection time. A recent study on characterization of phenolic compounds in different varieties of olive leaves grown in Italy (collection time: April and November), the greatest amount of verbascoside was detected in samples from Coratina cultivar (142.2 mg/100 g extract) (Difonzo et al. 2022). In another research performed by Orak et al. (2019), the quantified verbascoside showed significantly large variations across nine different genotypes (range: 0.45–21.07 mg/g leaf extract).

Another influential factor in the recovery of verbascoside from olive leaves evidently is the processing approach. Of particular examples of the values reported in previous studies are: 18.5 mg/g using ultrasound-assisted extraction (Ahmad-Qasem et al. 2013b); 22.9 ppm using cloud point extraction based on salting-out phenomenon (Stamatopoulos et al. 2014b); 3.90 mg/g in Picual leaf extracts using microwave extraction with water (Martínez-Navarro et al. 2021).

3.3.3.5. Flavonoids

Flavonoids in olive leaves, are predominately in glycosidic form which are clustered into different sub-classes. The following are among the most commonly found flavonoid-derivatives in olive leaves:

Luteolin 7-O-glucoside – luteolin belongs to flavones, the representative sub-class of flavonoids in olive leaves. This phenol is mostly in glycosidic linkage form in olive leaves; among which includes luteolin 7-O-glucoside that has been regarded as one of the principal polyphenols in olive leaves (Lama-Muñoz et al. 2019; Kiritsakis et al. 2018). The values observed in this study (**Figure 3.7**) revealed that ohmic heating, despite variations, exhibited significantly higher concentrations in the range 3.02–4.17 mg/g, with the OH 55 °C using 60% ethanol being the most effective for the liberation of this compound.

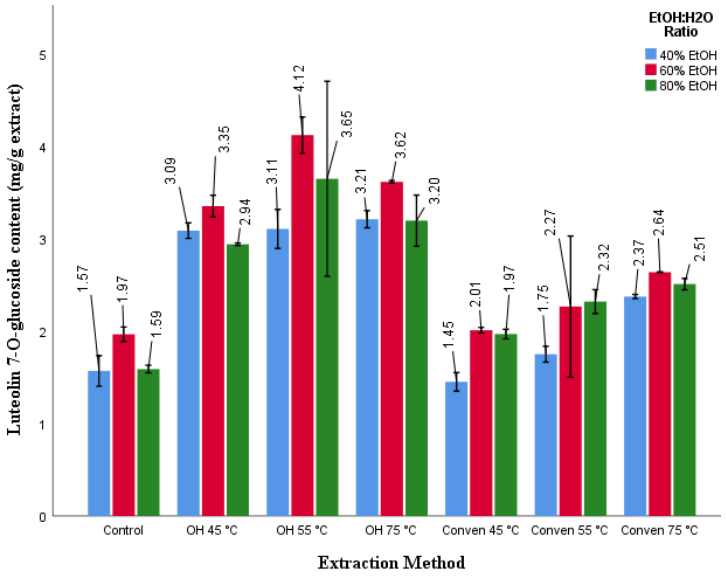


Figure 3. 7 Effect of extraction methods on luteolin 7-O-glucoside content (mg/g extract). Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* extractions.

The composition data from previous studies shows a large variations in luteolin 7-O-glucoside content which partly explains the contributing effects of processing/extraction methods. Contreras et al. (2019) reported 237–338 mg per 100 g of extracts from olive mill leaves. Olive leaves extracted by ultrasound-assisted and conventional (with agitation) methods represented around 11.0 and 9.7 mg luteolin-7-O-glucoside per gram, respectively (Ahmad-Qasem et al. 2013b). In another study, an optimized design of pressurized liquid extraction (using 80% aqueous ethanol, 190 °C, 5 min,) has shown to improve the recovery of this compound (2.71 g/kg dry olive leaves) (Lama-Muñoz et al. 2019). Moreover, Kashaninejad et al. (2020) through their research on UAE and conventional methods, found that freeze-dried extracts using 80% ethanol favored an increase in the content of phenols including luteolin 7-O-glucoside (1.4% *w/w*).

Apigenin 7-O-glucoside – another major flavone-derived phenol is apigenin that is typically in glycosidic bond form in olive leaves. Of all the constituents, apigenin 7-O-glucoside is among the pronounced glycosides of apigenin. As shown in **Figure 3.8**, the level of this compound reached up to 3.47 mg/g extract using ohmic heating at 55 °C, 60% ethanol.

Together with others, the extractability of this particular phenol from olive leaves may highly rest on the selected cultivar. In the study of Lukić et al. (2020), the factor of cultivar type notably became influential in proportions of phenols including apigenin 7-O-glucoside that showed large variations across six selected cultivars of olive leaves (27.79–91.75 mg/100 g).

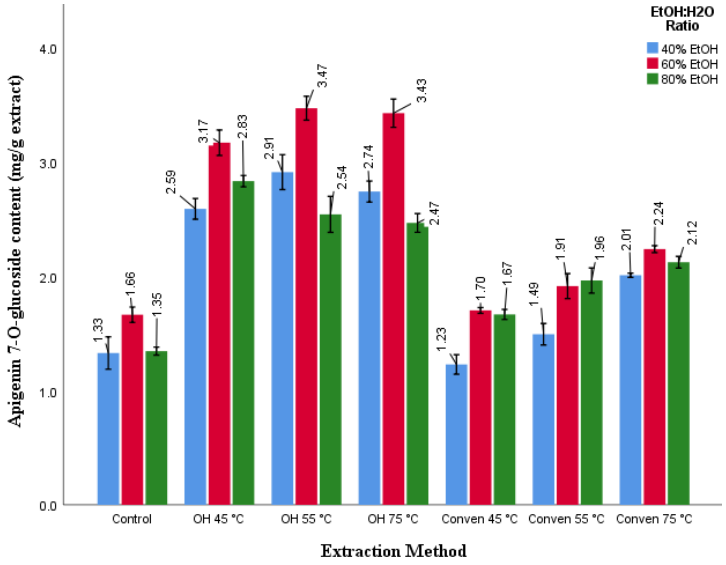


Figure 3. 8 Effect of extraction methods on apigenin 7-O-glucoside content (mg/g extract). Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* extractions.

Rutin – similar to other flavonoid phenols examined thus far, the maximum rutin content (3.78 mg/g extract) was detected in ohmic extract at 55 °C, 60% ethanol (**Figure 3.9**). The extractability of rutin is reportedly influenced by the nature and/or proportion of the extraction solvent(s). A typical example is the research of Akli et al. (2022) wherein olive leaves exhibited discrepancies in rutin proportions depending on the extraction solvents as the extraction by conventional solvent (70% ethanol) represented 714.63 µg rutin/g, while the extraction by eutectic solvents enabled 268.59–463.06 µg/g.

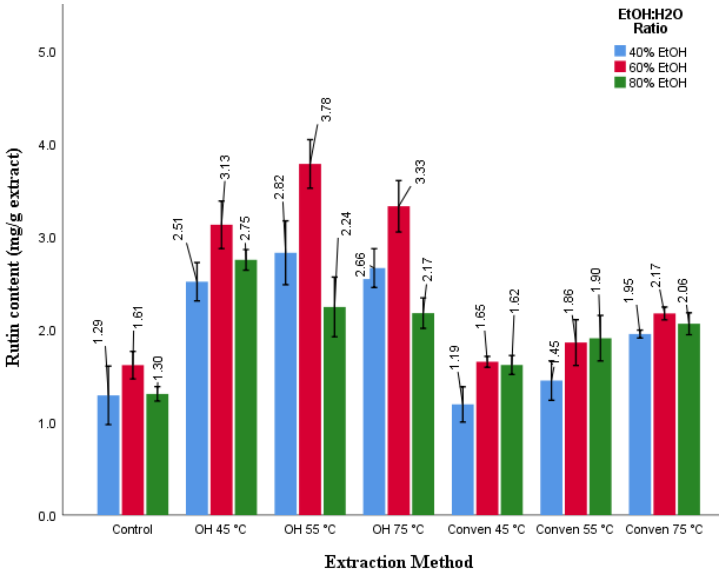


Figure 3. 9 Effect of extraction methods on rutin content (mg/g extract). Results are presented as mean values with standard deviation error bars. The *OH* represents ohmic extraction using different concentrations of aqueous ethanol (40%, 60%, and 80% EtOH v/v) and different temperatures (45 °C, 55 °C, and 75 °C). The *Conven* represents groups of samples with conventional heating using the same solvent ratios/temperature as those applied for ohmic system. The *Control* represents the extraction with no heat treatment (25 °C) using the same solvent ratios as those applied for *OH* and *Conven* extractions.

Moreover, in the study of Kashaninejad et al. (2020), olive leaves from the same cultivar as used in the current study (Picual), showed dissimilarities in rutin content when different solvent ratios were examined. The data ranged from 0.026 (100% ethanol) to 0.5 mg/g (50% ethanol). Likewise, different concentrations of rutin have been reported in the leaves of various cultivars. Talhaoui et al. (2014), observed variations in the concentrations of rutin from Picual, Sikitita, and Arbequina giving 0.289, 0.319, and 0.651 mg /g leaves d.w., respectively.

Overall the data shows that the use of ohmic extraction favored the highest recovery of individual phenols of this study, when compared to *Control* and *Conven* methods. It is also noteworthy that the extractability of each phenolic group differed with solvents/temperatures as the data showed that at *OH* at 55 °C with 60% ethanol favored the highest levels of flavonoid phenols (rutin, apigenin 7-O-glucoside, and luteolin 7-O-glucoside), while the *OH* at 75 °C with 80% ethanol enabled maximum values of oleuropein and verbascoside. These discrepancies can be explained by their inherently diverse structural features in terms of (i) chemical composition, (ii) locations within the cells, and (iii) molecular bindings. Taking a cue of this data, it can be concluded that using ohmic heating that proved useful in the current study for the extraction of polyphenols, potentially entails appropriate customization for solvent ratio and extraction temperature when the isolation of specific polyphenol(s) is of interest.

3.4. Conclusions

The concentrations of target phenolic compounds in olive leaf extracts varied across different extraction methods. Combining results from this study show that the ohmic heating was found to be significantly effective in the increment of all polyphenols. By comparison, the magnitude of differences between *OH* groups and *Conven/Control* groups were significantly high ($p < 0.05$). The use of ohmic heating with 80% ethanol enabled the highest content of oleuropein, hydroxytyrosol, tyrosol, and verbascoside, while the 60% ethanol represented optimum values of luteolin 7-O-glucoside, apigenin 7-O-glucoside, and rutin. Overall, comparing the various phenolic groups quantified in this study, the proportions in descending order is as follows: oleuropein > luteolin 7-O-glucoside > rutin > apigenin 7-O-glucoside > hydroxytyrosol > Verbascoside > tyrosol.

The data received in this study in part shed light on significant effectiveness of ohmic heating, compared, in the recovery of the aforementioned phenolic groups that characteristically are among the major antioxidant compounds in olive leaves. This study can be extended in further research work to evaluate an up-scalable process benchmark for application of an ohmic heater to optimally extract the desired bio-phenols from olive leaf residues. More research work is needed to ascertain adaptations of the extraction parameters, particularly in terms of solvent ratio and extraction temperature when the isolation of certain types of phenols is of particular interest.

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CHAPTER 4

EFFECT OF STORAGE, THERMAL, AND PH CONDITIONS ON THE PRESERVATION OF OLEUROPEIN CONTENT OF OLIVE LEAF EXTRACTS

Abstract

For oleuropein to exert sustainable antiradical effects it needs to remain potent in an adequate amount in every part of the valorization system. In this regard, this study investigated the stability of olive leaf extracts obtained by ohmic heating (*OH*), having them subjected to different surrounding conditions: (i) storage temperatures (25, 4, and –20 °C) over time points (week), (ii) thermal conditions (70, 90, and 110 °C) over time points (min), and (iii) pH conditions (3–9). Special attention was placed on the changes in oleuropein content, and its potential bioconversions into hydroxytyrosol under the abovementioned conditions. Other aspects including total phenolic content (TPC) and antioxidant capacity were also evaluated. The data from the storage stability revealed that the storage temperature of –20 °C conferred optimal condition for the stability of the extracts, while the storage at 25 °C was the least desirable condition particularly for oleuropein, TPC and antioxidant capacity. The effect of heat on the extracts showed that exceeding high temperature can be detrimental to oleuropein content. Ideally, if the heating temperature reached up to 90 °C, the heating time should not exceed 20 min. Also, the pH of 5 as the optimal value for all response variables, narrowly resembled to the pH values observed at the initial point of the extracts (4.8–5.2). The data support the preference of ohmic extraction method over the conventional one because substantial levels of polar phenols and antioxidant capacity remained after the *OH* extracts were stored and heated over eight weeks. In the case of oleuropein, the ohmic heating at 75 °C, competitively was the most favorable approach wherein the values continued to stay at the highest levels upon the surrounding conditions/over times.

Keywords: Olive leaf extract, stability, storage, thermal, pH, oleuropein, polyphenols, antioxidant activity.

4.1. Introduction

Polyphenols when recovered from olive leaves have great potential for being incorporated into food, nutraceutical, and medicinal applications. However, before such applications could be approached, it is essential to evaluate the stabilities of the extracts in order to ascertain the threshold of their resistance upon different surrounding environments over a course of time period. For example, during their inclusion in food applications, foods may be processed at high temperature, exposed to high/low pH, or stored at different temperatures. Therefore the extracts need to sustain such conditions with minimum compositional changes, otherwise, the value-added application may lose its relevance. This is highly important in view of the content of principal polyphenols particularly oleuropein that significantly imparts bio-functional potential of olive leaves (Delgado-Pertíñez et al. 1998; Acar-Tek & Ağagündüz, 2020; Omar, 2009; Safarzadeh Markhali et al. 2020). For oleuropein to deliver antioxidant effects, it needs to be present in a considerable amount throughout the life of the extract. Previous research studies, through using different operating conditions, found that the stability of oleuropein in olive leaf extracts is affected by: (i) the pH conditions (Stamatopoulos et al. 2014; Gonzalez-Ortega et al. 2022), (ii) the storage temperature/duration (Martínez-Navarro et al. 2021; Ahmad-Qasem et al. 2016; Malik & Bradford, 2008), and (iii) the heating temperatures (Stamatopoulos et al. 2014; Mohammadi et al. 2016; Gonzalez-Ortega et al. 2022).

In the exploratory study of our previous work (**Chapter 3**), the extraction by ohmic heating (*OH*) proved significantly useful in efficient recovery of principal polyphenols, particularly oleuropein, from olive mill leaves. Given the fact that, the concentration of polyphenols may decline significantly under various surrounding conditions (including ambient temperature, heat, and pH conditions), a need exists to examine the extent of their stabilities under such conditions. This has prompted the performance of the present study to address this issue by monitoring the tolerance of *OH* leaf extracts under the following environmental conditions: (i) storage temperatures over time points, (ii) thermal conditions over time points, and (iii) pH conditions. Special attention was placed on the variations of oleuropein under the selected conditions. The content of hydroxytyrosol was also studied throughout the stability trials to partly evaluate the possible assumption that the chemical depletion of oleuropein may occur concurrently with the increment of hydroxytyrosol under the same executed conditions. Other aspects, as the secondary response variables, assessed in this study include total phenolic content (TPC) and *in vitro* antioxidant capacity.

4.2. Materials and methods

4.2.1. Plant materials and chemicals

Olive mill leaves from the same cultivar/growing area as that applied in our earlier experiment (see **Chapter 2** & **3**) were kindly supplied by "Center for Advanced Studies in Energy and Environment", University of Jaén, Campus of Las Lagunillas, Jaén, Spain. Upon arrival, they were cleaned, dried and ground using the same methods as practiced in our earlier work (Safarzadeh Markhali et al. 2022).

The following chemicals and standard reagents purchased from Sigma-Aldrich (Saint Louis, MO, USA): (i) analytical-grade chemicals – methanol, ethanol, Folin–Ciocalteu, anhydrous gallic acid, anhydrous sodium carbonate, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), (ii) HPLC-grade standards/reagents – oleuropein, hydroxytyrosol, formic acid, and acetonitrile.

4.2.2. Sample preparation

4.2.2.1. Extraction system

The extraction processing design in this study was based on the same procedures as practiced in our previous experiments (see **Chapter 2 & 3**). Briefly, olive leaves were initially cleaned, washed, and dried at 37 °C for 48 h. The dried leaves were ground and size reduced to 0.3 mm. The dry ground olive leaves (solid-solvent ratio of 1:10) were then subjected to the extraction process for 15 min. The selected extraction methods were based on: (i) ohmic heating (*OH*), (ii) conventional heating (*Conven*), and (iii) *Control* (agitation without heating). The aqueous ethanol was used as the extractant (with 40%, 60%, and 80%, *v/v*) for all executed methods. The extraction temperatures for *OH* and *Conven* ranged 45 °C, 55 °C, and 75 °C. For the *Control*, solvent extraction was applied at room temperature (25 °C).

4.2.2.2. Stability of olive leaf extracts

The stability of polyphenols (total and individual phenols) and antiradical capacity of leaf extracts were assessed under the selected surrounding environments as shown in **Table 4.1**.

Table 4. 1 Surrounding conditions of the extracts selected for this study to determine the stability of polar phenols and antioxidant capacity of olive leaf extracts.

Surrounding environment		Specification
Storage temperature (°C)	4	The leaf extracts were stored at each defined temperature over a course of 8 week duration. The analytical assessments were performed at weekly intervals from week 0 to the end of week 8.
	25	
	–20	
Heating temperature (°C)	70	The leaf extracts were heated at each defined temperature over a total of 60 min. The assessments were performed after 0, 20, 40, and 60 min.
	90	
	110	
pH	3	The leaf extracts were adjusted to the given pH values with the prepared buffer solutions and incubated for one week prior to analyses.
	5	
	7	
	9	

4.2.3. Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined using the method of Singleton et al. (1999) with some modifications as described previously (Safarzadeh Markhali et al. 2022). The results were recorded as mg of gallic acid equivalents/g extract (mg GAE/g extract).

4.2.4. Determination of antioxidant capacity (*in vitro*)

The antioxidant capacity of extracts (*in vitro*) was determined based on Trolox equivalent antioxidant capacity (TEAC) assay through three different methods as follows:

4.2.4.1. DPPH assay

The antiradical potential of the extracts to reduce 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radicals was examined according to the method of Brand-Williams et al. (1995), with slight modifications as reported previously (Safarzadeh Markhali et al. 2022). The absorbance readings (515 nm) were calculated against Trolox standard curve and the results were expressed as mM Trolox equivalents/g extract (mM TE/g extract).

4.2.4.2. ABTS assay

The antiradical potential of the extracts to deplete 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS) free radicals was determined according to the method of Re et al. (1999) with slight modifications as reported previously (Safarzadeh Markhali et al. 2022). The absorbance readings were measured at 734 nm and calculated against Trolox calibration curve, and the results expressed as mM TE/g extract.

4.2.4.3. FRAP assay

The antiradical potential of the extracts to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was examined via ferric reducing antioxidant capacity assay as reported by Benzie & Strain (1996) and Hayes et al. (2011) with slight modifications as described in our earlier work (see **Chapter 3)**. The absorbance readings (593 nm) were calculated against Trolox standard curve and the results were reported as mM TE/g extract.

4.2.5. HPLC analyses of the target polar phenols

The content of oleuropein and hydroxytyrosol of the extracts as a function of stability was measured through UHPLC (Shimadzu Nexera X2 UHPLC) connected to a diode array detector (Shimadzu SPD-M20A), and an integration system (Shimadzu LabSolutions software, Kyoto, Japan). The separations of target polar phenols were performed using a reverse-phase Aquity UPLC BEH C18 column (100 mm x 2.1 mm i.d., 1.7 μm particle size, Waters Corporation) following the method of Quero et al. 2022 with slight modifications as described in previous experiment (**Chapter 3**).

4.2.6. Statistical analysis

Significant differences ($p < 0.05$) between the mean values (± SD) of all determinations were statistically assessed via Analysis of Variance (ANOVA) using SPSS software, version 27.0. Factorial Repeated Measures ANOVA was used to analyze significant differences between the means of the levels within a subject (times within storage; temperatures within heating; values within pH) and for interactions between factors. Multiple comparison between the mean values of dependent variables of different extraction groups were analyzed by running a Post hoc test. Pearson correlation was used to evaluate the correlation between dependent variables. The assumption of homogeneity of equal variance was assessed through the Levene's test (homogeneity of variance assumption is satisfied/ not violated when p-value is greater than 0.05).

4.3. Results and discussion

In this present study the stability of polyphenols and antioxidant capacity of olive leaf extracts obtained by ohmic heating (*OH*), conventional heating (*Conven*), and solvent/no heating (*Control*), with special attention to oleuropein content of the extracts under (i) the storage temperatures over time points, (ii) heating temperatures over time points, and (iii) pH conditions.

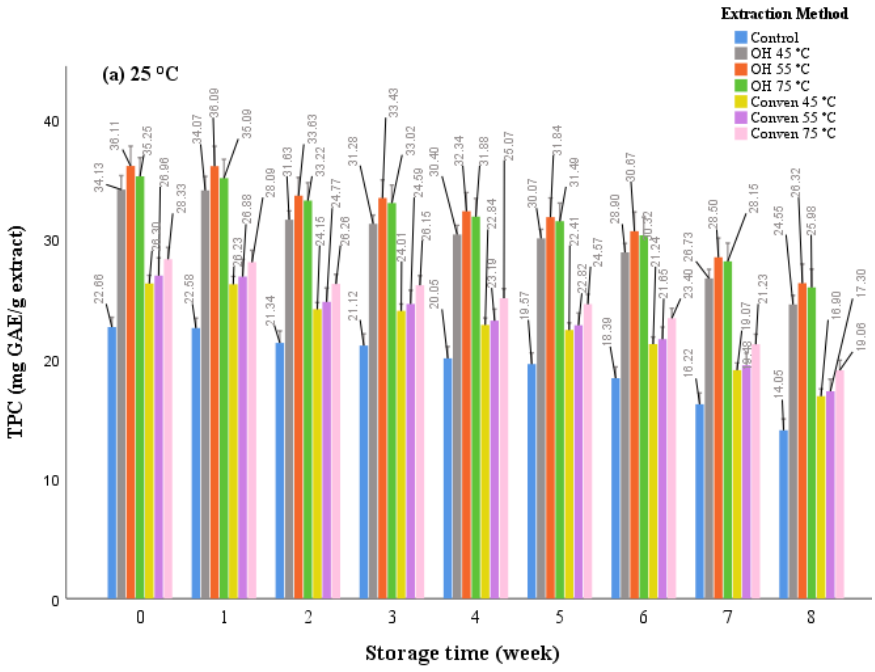
Preliminary statistical assessment – using the pairwise comparisons to determine whether the extraction methods are dependent on the solvent ratios, in all experiments, the interactions between

extraction methods and surrounding environments (storage/heat/pH) were independent of the factor of solvent ratio. In other words, the effect of solvent ratio was not statistically significant for the same experiment $p > 0.05$. Taking a cue of this, the data from all examined dependent variables discussed below are based on the stability changes occurred for each extraction method (*OH*, *Conven*, and *Control*) irrespective of the factor of solvent ratio.

4.3.1. Effect of storage conditions on the stability of polyphenols and antioxidant capacity of olive leaf extracts

4.3.1.1. Changes in total phenolic content

As shown in **Figure 4.1**, across all extraction groups, the content of total polyphenols at 25 °C remained relatively unchanged following the first week, then slightly decreased after the second week and thereafter sharply decreased over an 8 week period. At 4 °C the decrease in TPC started after week 4 until the end of the storage (week 8) but the magnitude of losses was less significant compared to those stored at 25 °C. The storage at –20 °C represented the most prefered condition for TPC preservation that showed minimum depletion of polyphenols, in each extraction group, over the time points, and overall the values remained more stable than those stored at other executed temperatures.



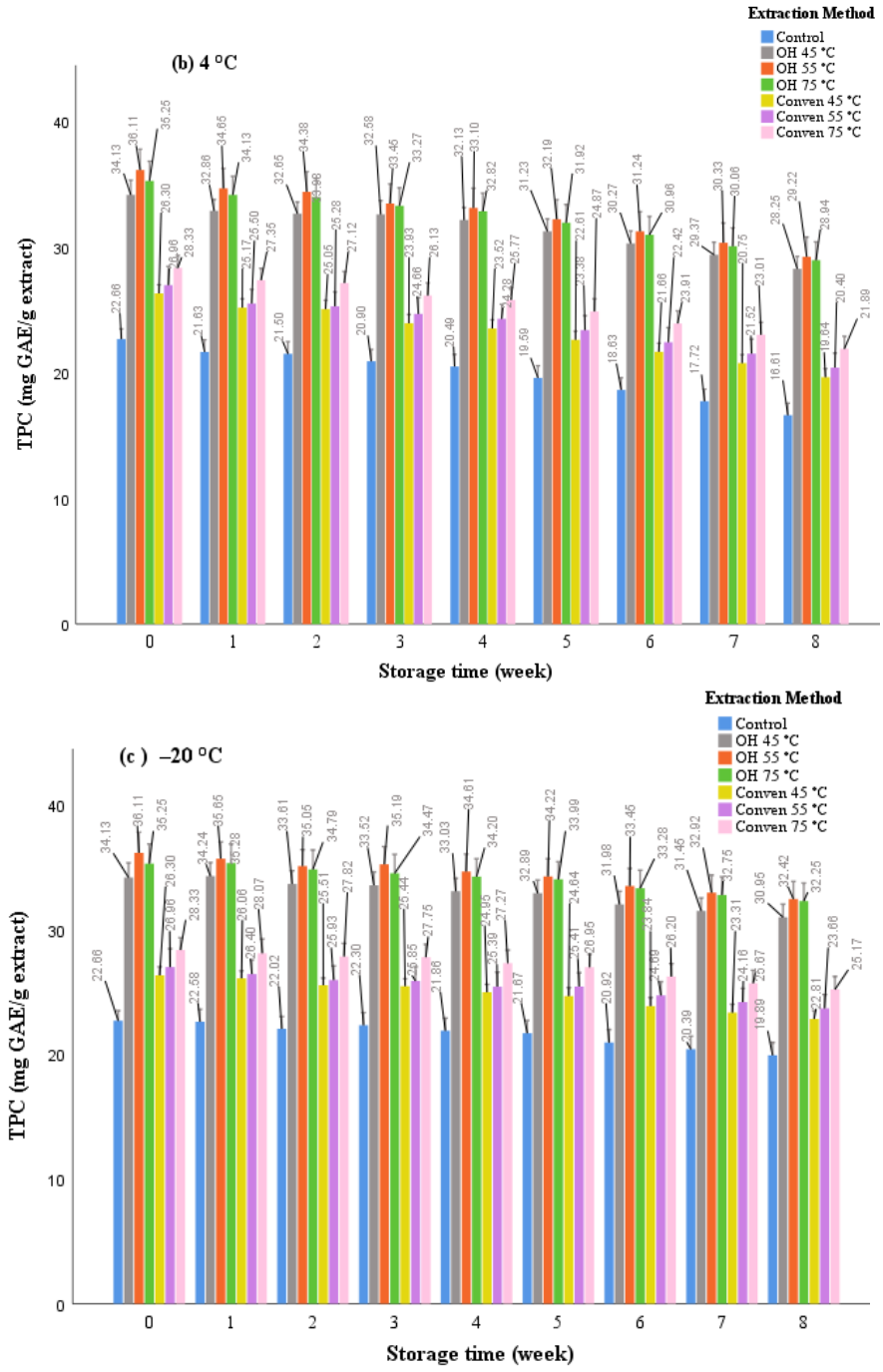


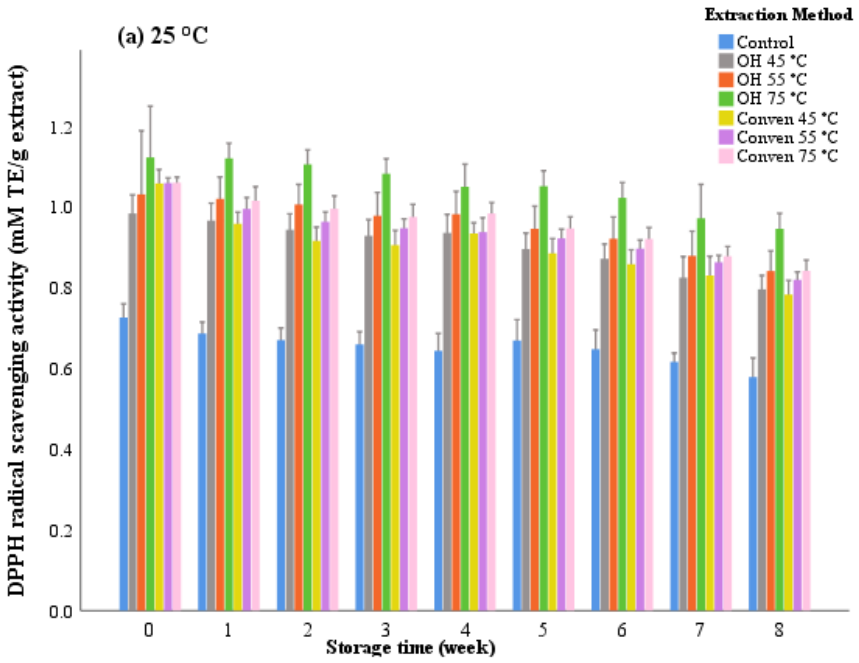
Figure 4. 1 Changes in total phenolic content (TPC) of olive leaf extracts (mg GAE/g extract) stored over time points (week) at (a) 25 °C, (b) 4 °C, and (c) –20 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in OH method. The *Control* represents solvent extraction without heating at 25 °C.

Comparing the extraction methods, for the same storage temperature, the trends of decrease in TPC over time points followed comparatively the same pattern in all corresponding extraction groups. However, quantitatively, the *OH* groups contained significantly greater amounts after an eight week storage particularly at –20 °C (30.95–32.42 mg GAE/g extract). This can be explained by the reason of

their richness in TPC at the initial point of extraction (week 0) which, despite being affected proportionally throughout the storage, the numbers represented considerably high over the course of eight weeks. Therefore, compared to *Conven* and *Control* groups, the extraction by ohmic heating (particularly at 55 °C) conferred benefits for the conservation of total polyphenols under the storage conditions assessed in this study, with the freezing point (–20 °C) being the temperature of preference.

4.3.1.2. Changes in antiradical activities

For the same storage temperature, the variations of radical scavenging activities detected by DPPH, FRAP, and ABTS (**Figures 4.2–4.4**) were relatively similar between extraction methods. However, the reactivities of free radicals after week 2 were more accelerated in ABTS results (**Figure 4.4**). Significant drops from week 2 to week 8 was detected in all extract groups stored at 25 °C. There were marginal increments at some points of storage conditions, particularly for *Control* groups (with DPPH at 25 °C following week 5, and at 4 °C and –20 °C following week 6). This may suggest the potential release of free phenols (aglycons) from glycosidic bonds that possibly acted as radical scavengers at such points. Overall, the storage temperature of –20 °C can be preferable for antioxidant capacity of all groups of extracts.



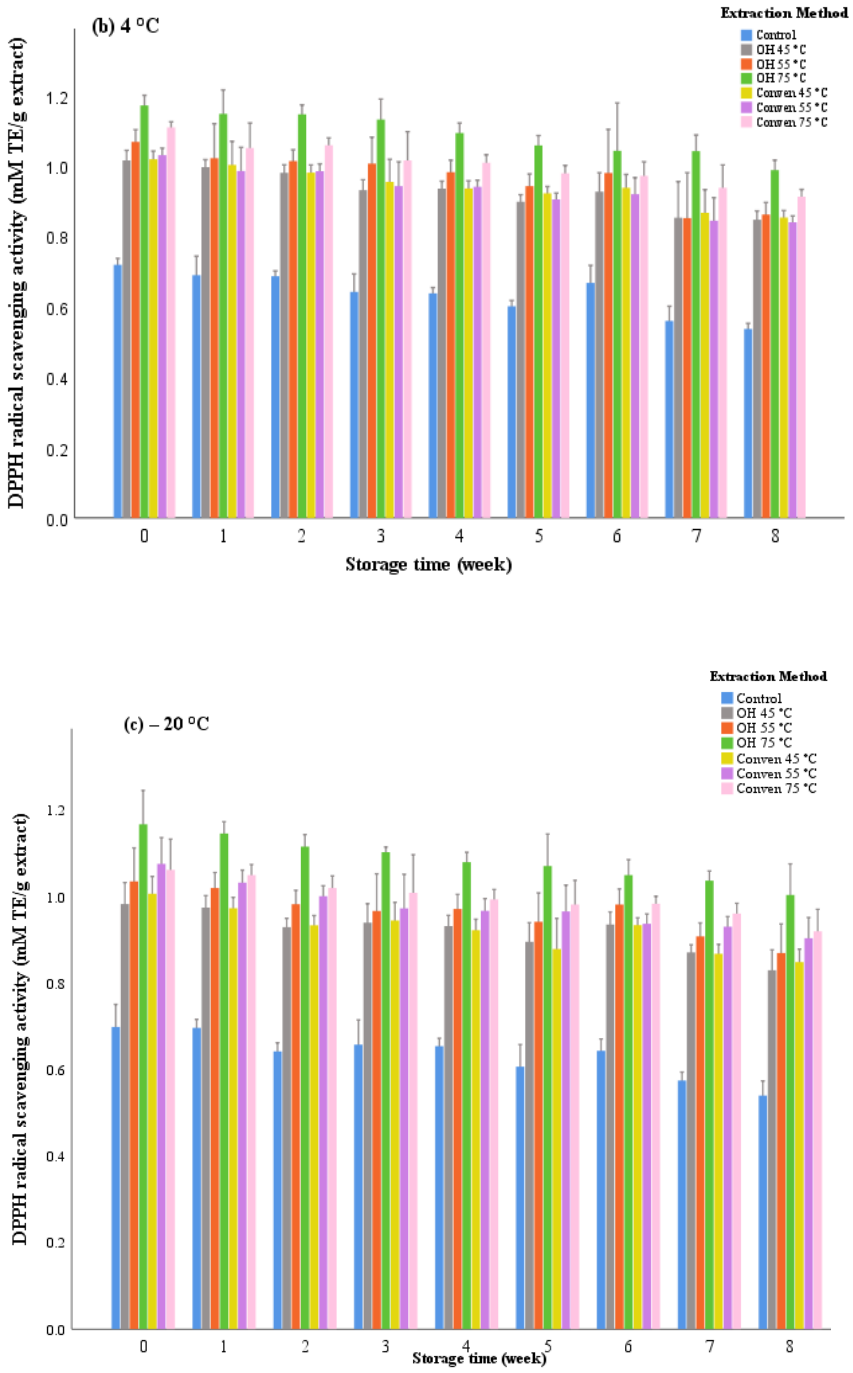
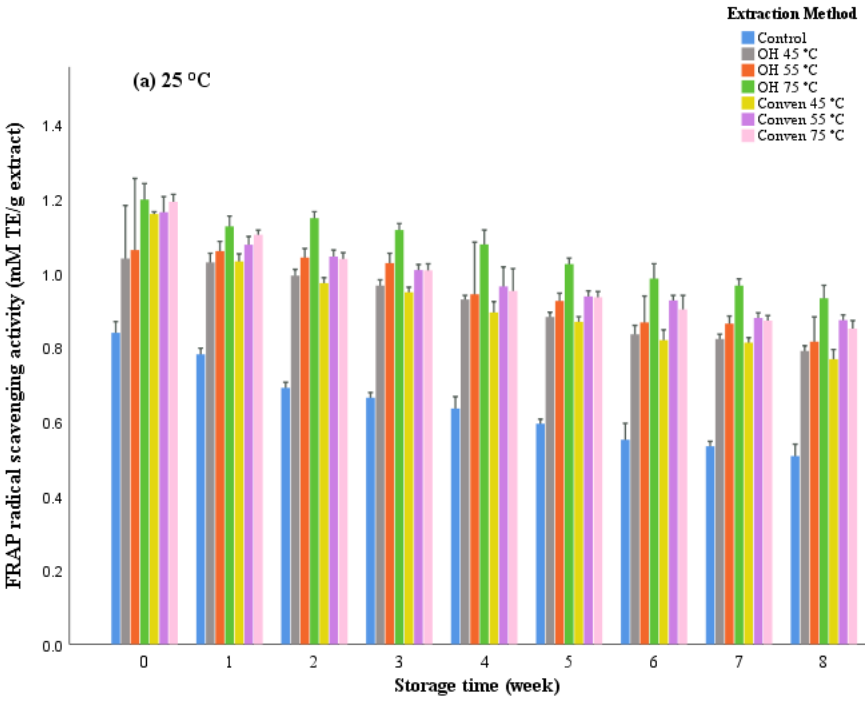
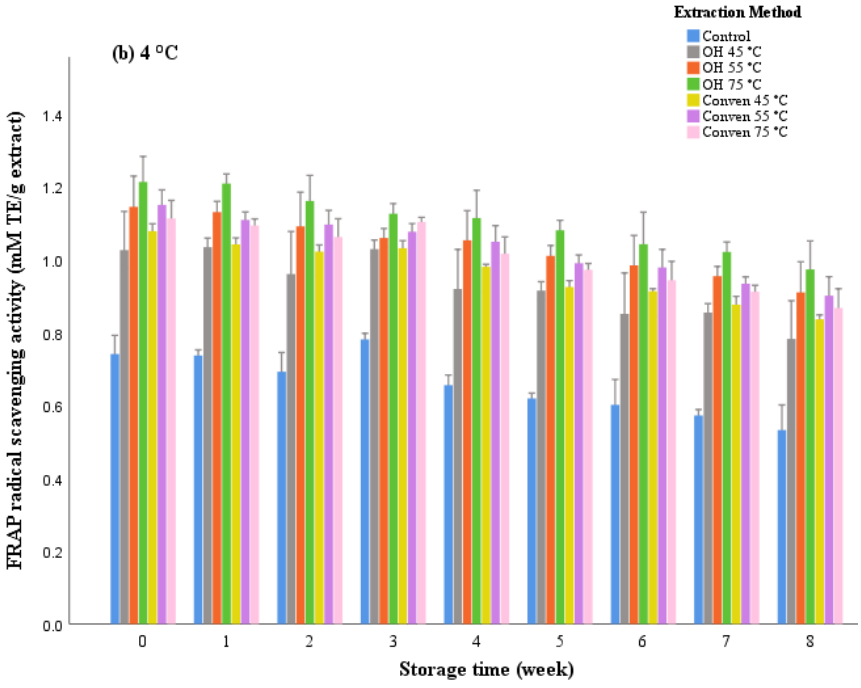


Figure 4. 2 Changes in DPPH radical scavenging activities of olive leaf extracts (mM TE/g extract) stored over time points at (a) 25 °C, b) 4 °C, and (c) – 20 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.


(a) 25 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
FRAP radical scavenging activity (mM TE/g extract)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
1
2
3
4
5
6
7
8
Storage time (week)


(b) 4 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
FRAP radical scavenging activity (mM TE/g extract)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
1
2
3
4
5
6
7
8
Storage time (week)

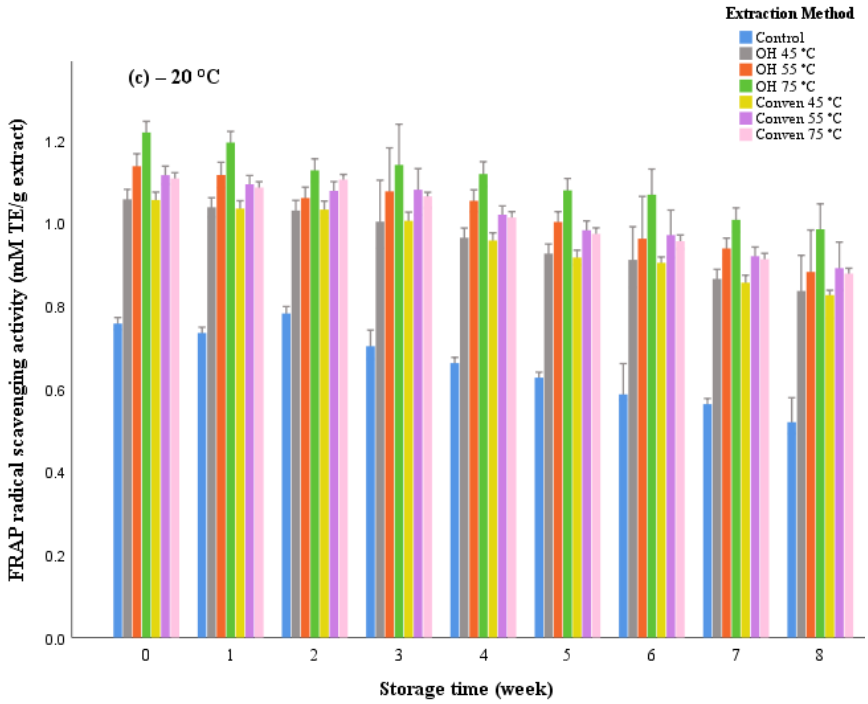
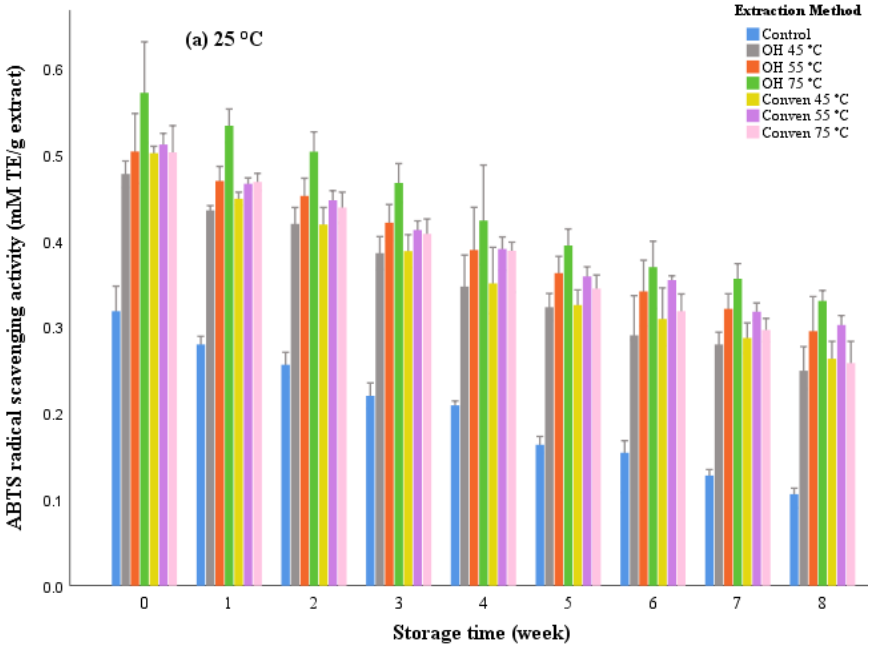


Figure 4. 3 Changes in FRAP radical scavenging activities of olive leaf extracts (mM TE/g extract) stored over time points (week) at (a) 25 °C, (b) 4 °C, and (c) – 20 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.



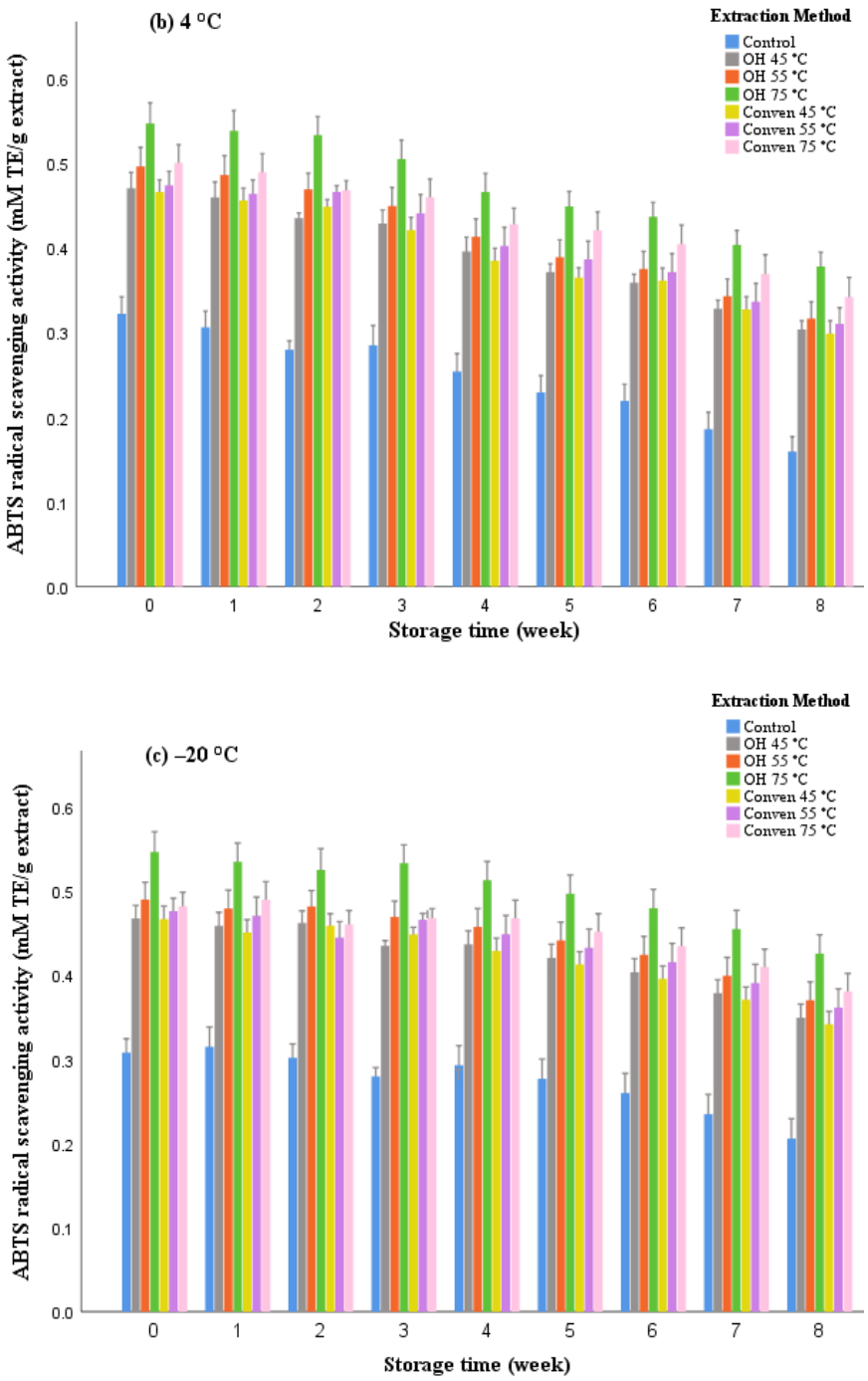


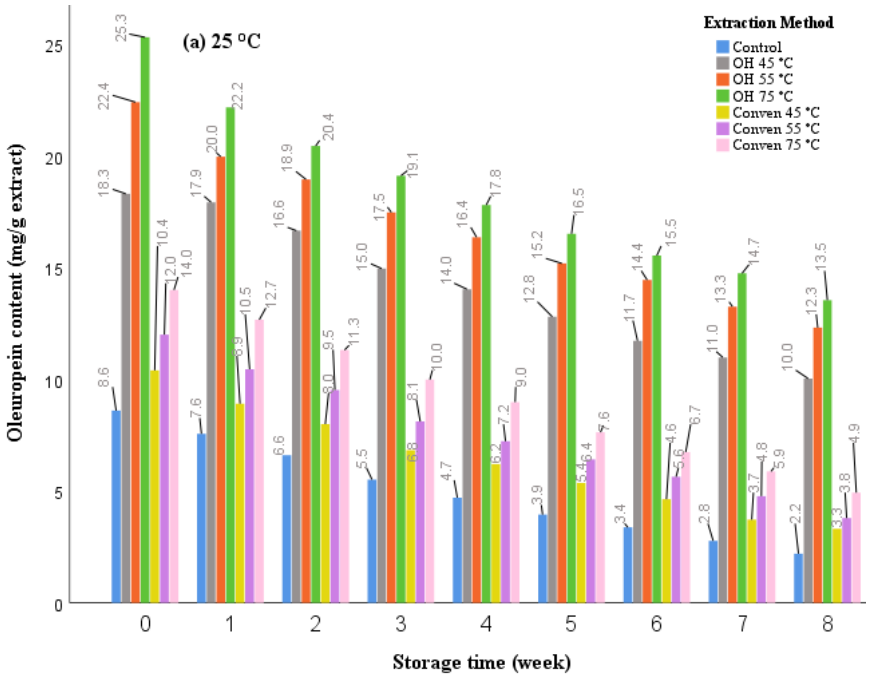
Figure 4. 4 Changes in ABTS radical scavenging activities of olive leaf extracts (mM TE/g extract) stored over time (week) at (a) 25 °C, (b) 4 °C, and (c) – 20 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

In all three antioxidant assays, the use of ohmic heating at 75 °C represented maximum antiradical capacity for the same storage temperature. As mentioned in our earlier experiment (Safarzadeh Markhali et al. 2022), the antioxidant capacity of the extracts can be partly influenced by the content of polyphenols of the extract. Comparing the antiradical data of the extracts here with those observed with TPC (see

Figure 4.1), relatively similar trends of changes exist for the same storage temperature. However there were some dissimilarities as the highest values of TPC were detected in the ohmic extracts of 55 °C whereas the highest antiradical activities here were detected in ohmic extracts of 75 °C (**Figure 4.2–4.4**). Also, the data from TPC showed greater magnitude of differences between *OH* and *Conven* than those observed in antiradical scavenging activities. In our earlier study (**Chapter 3**), the antioxidant capacity of the extracts seemed to be more dictated by the content/performance of individual/representative groups of polyphenols, particularly oleuropein that showed similarity with antiradical data (the maximum values belonged to the same extraction group ohmic with 75 C). In this respect, the following data (changes of oleuropein content) provide further insight into it.

4.3.1.3. Changes in oleuropein content

Examining the changes in oleuropein content (**Figure 4.5**), the trends of declines in all extraction groups were found to be relatively similar over time points for the same storage temperature. At 25 °C, the concentrations in all extracts started to decrease sharply from the first week of storage and the numbers continued to drop significantly to the end of storage. Compared to the intense effect of room temperature, the storage at –20 °C followed by 4 °C caused less detrimental effects on oleuropein content, although the numbers decreased (after week 2 at –20 °C, and after week 1 at 4 °C), the levels remained rather stable over storage.



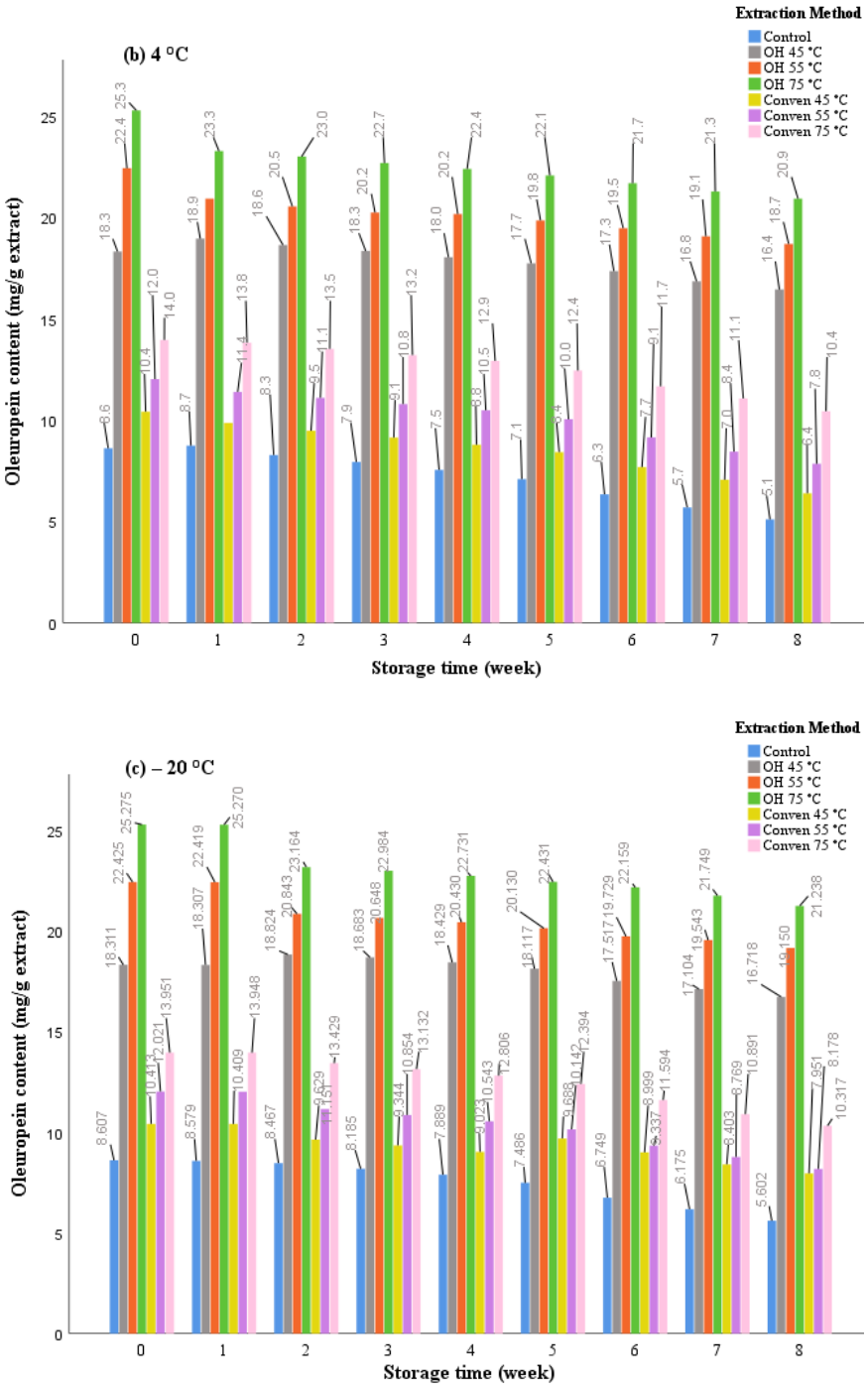


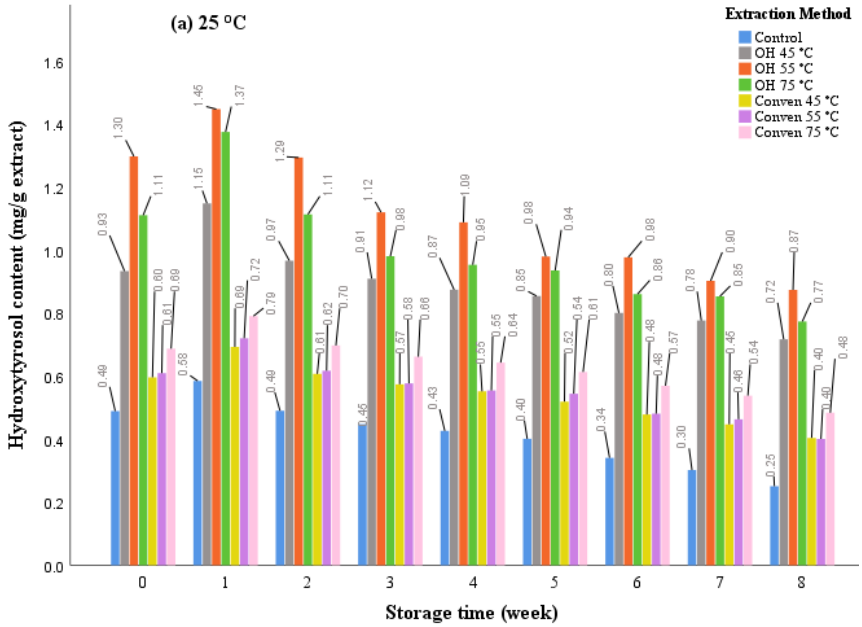
Figure 4. 5 Changes in oleuropein content of olive leaf extracts (mg/g) stored over time points (week) at (a) 25 °C, (b) 4 °C, and (c) – 20 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in OH method. The *Control* represents solvent extraction without heating at 25 °C.

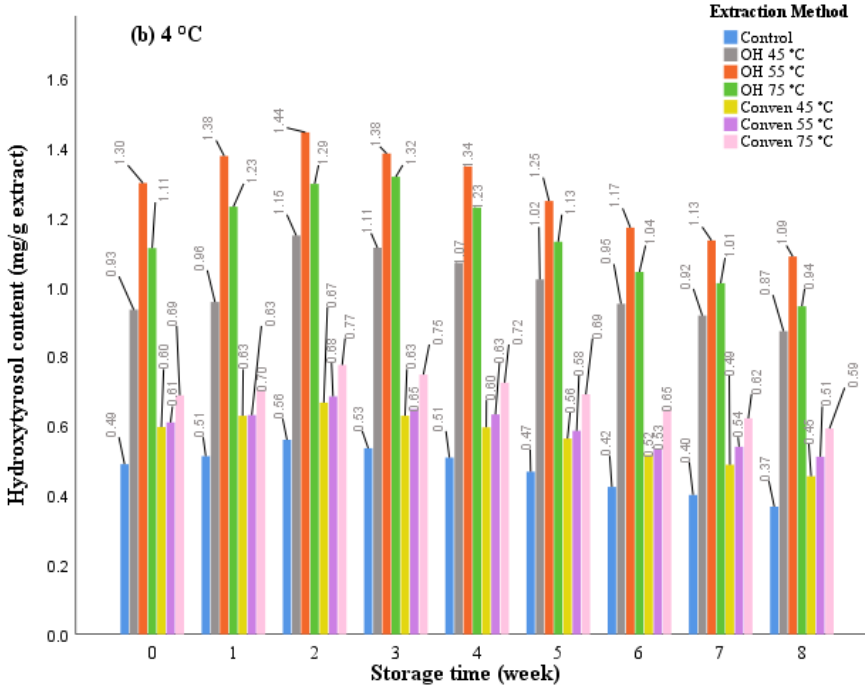
Polyphenols upon their recovery from olive leaves need to be preserved properly all around the shelf life of the extracts. Importantly, for oleuropein to exert sustainable antiradical effects, it needs to be available in adequate proportions over the life of the extract. The loss of oleuropein content unavoidably occurs during storage; partly due to the oxidation induction caused by catalytic enzymes such as

polyphenol-oxidase through their exertions to decompose oleuropein to the secondary oxidized products (De Leonardis et al. 2015). In this regard the choice of extraction method becomes a crucial factor for the initial recovery of analytes through the extraction process. As it can be seen in **Figure 4.5**, ohmic heating, compared to conventional heating and Control methods, enabled substantial recovery of oleuropein upon extraction (week 0), thus, being originally rich in oleuropein, the extracts possessed considerable quantities over the storages (especially at –20 °C) despite being suffered from the ambient temperatures over time points. The suitability of ohmic heating for oleuropein keepability (under the same storage condition) followed the order: 75 °C > 55 °C > 45 °C. That being the case, the extraction by ohmic heating at 75 °C is a preferred extraction method for oleuropein conservation at –20 °C (throughout an eight-week storage) Moreover, this particular extraction method (ohmic at 75 °C) also favored improved antiradical capacity of the extracts (**Figures 4.2** & **4.3**) which may be explained by the presence and ability of oleuropein that can potentially act as a powerful radical scavenger.

4.3.1.4. Changes in hydroxytyrosol content

As shown in **Figure 4.6**, Hydroxytyrosol slightly increased at the early stage of storage (after first week at 25 °C , and after second week at 4 °C and –20 °C). The initial increments of hydroxytyrosol may be due to the concurrent reduction of oleuropein (**Figure 4.5 a–c)**, assuming that the mechanism of action favored the formations of simple phenols such as hydroxytyrosol. In this case the fall in oleuropein content stems from hydrolytic reaction (rather than oxidative reaction) by the activities of enzymes such as esterase that is reportedly responsible for the breakage of the ester bonds with subsequent liberation of phenolic alcohols including hydroxytyrosol (De Leonardis et al. 2015). Regardless of the variations in the experimental conditions, the data on simultaneous rise in hydroxytyrosol content and drop in oleuropein content determined in this study support previous research studies (Feng et al. 2021; Martínez-Navarro et al. 2021).


(a) 25 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
Hydroxytyrosol content (mg/g extract)
Storage time (week)


(b) 4 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
Hydroxytyrosol content (mg/g extract)
Storage time (week)

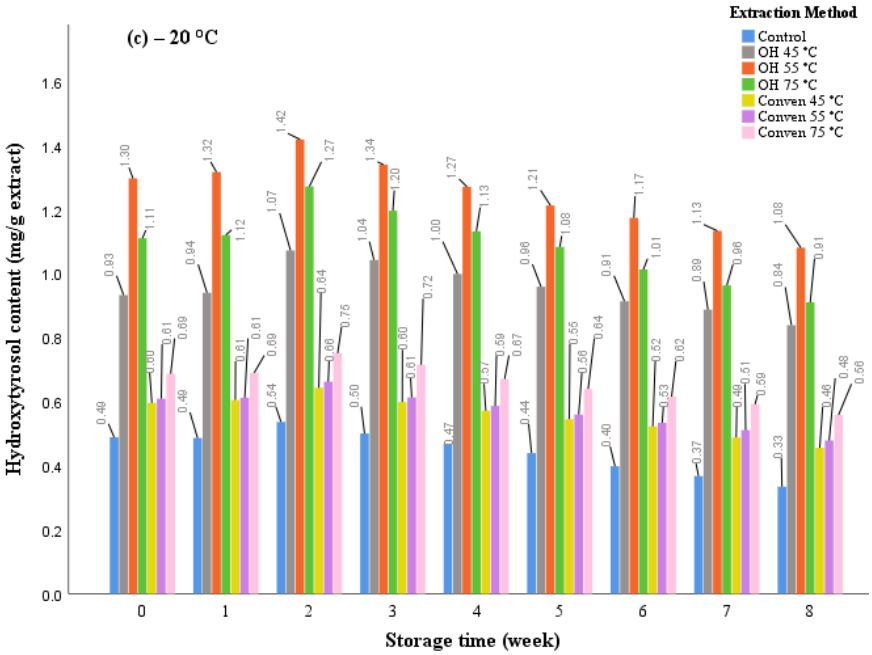


Figure 4. 6 Changes in hydroxytyrosol content of olive leaf extracts (mg/g) stored over time points (week) at (a) 25 °C, (b) 4 °C, and (c) – 20 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

Although a short-term storage (one week at 25 °C, two weeks at 4 °C and –20 °C) favored the extracts for hydroxytyrosol content, the degrading impact of extended storage was more significant on hydroxytyrosol content compared to those observed for oleuropein content and antioxidant activity. The decrease in hydroxytyrosol during the storage period can be due to the adverse effects of intrinsic enzymes such as polyphenol-oxidase (Martínez-Navarro et al., 2021).

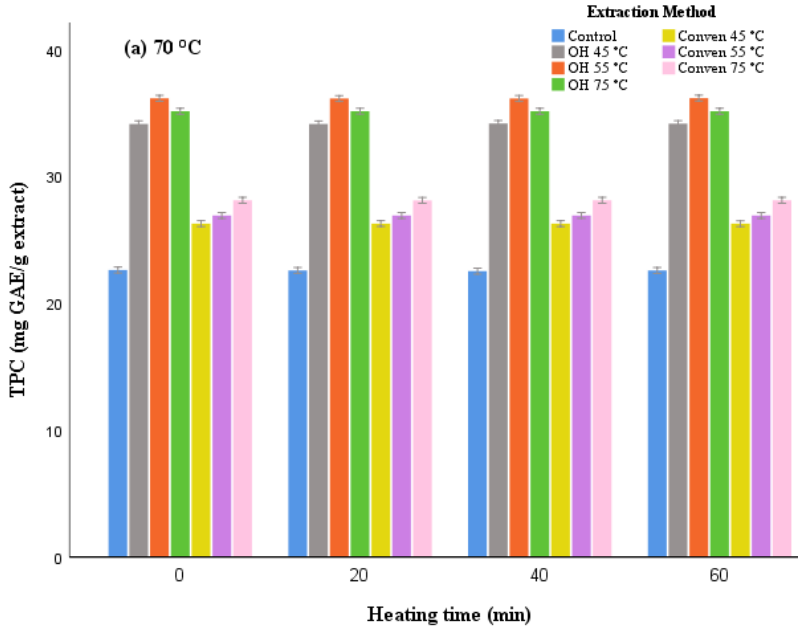
The data demonstrate that the extraction by ohmic heating is preferable over conventional heating, not only in terms of the recovery (week 0) but also for alleviating the severity of storage by keeping substantial levels of hydroxytyrosol over the storage period (8 weeks) when stored at 4 °C and –20 °C. The suitability of the temperature of ohmic heating for this particular compound follows the order: 55 °C > 75 °C > 45 °C.

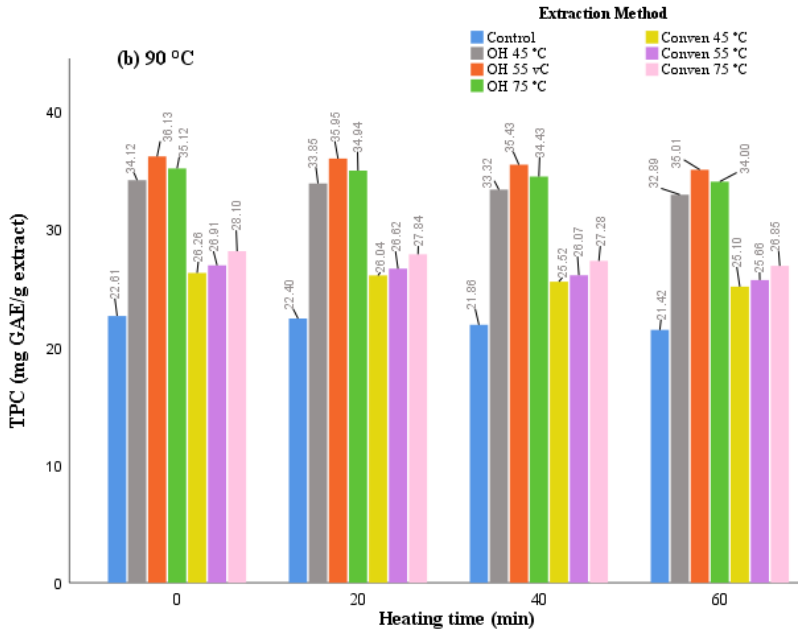
4.3.2. Effect of heating over time points on polyphenols and antioxidant capacity of olive leaf extracts

4.3.2.1. Changes in total phenolic content

The thermal stability of leaf extracts was studied at different temperatures (70, 90, and 110 °C) over different time points (20, 40, and 60 min). The obtained results from total phenolic content show that over-exposure to high temperature may compromise the stability of total phenolic content. As seen in **Figure 4.7**, the values of TPC in all extraction groups remained stable at 70 °C and to a lesser extent

at 90 °C. After a 20-min heating process, progressive degradations of TPC occurred with around 5% loss after 60 min heating. Heating at 110 °C was significantly detrimental to total phenolic content in all extracts with relatively sharp declines over times, up to around 12–14% losses in the end.





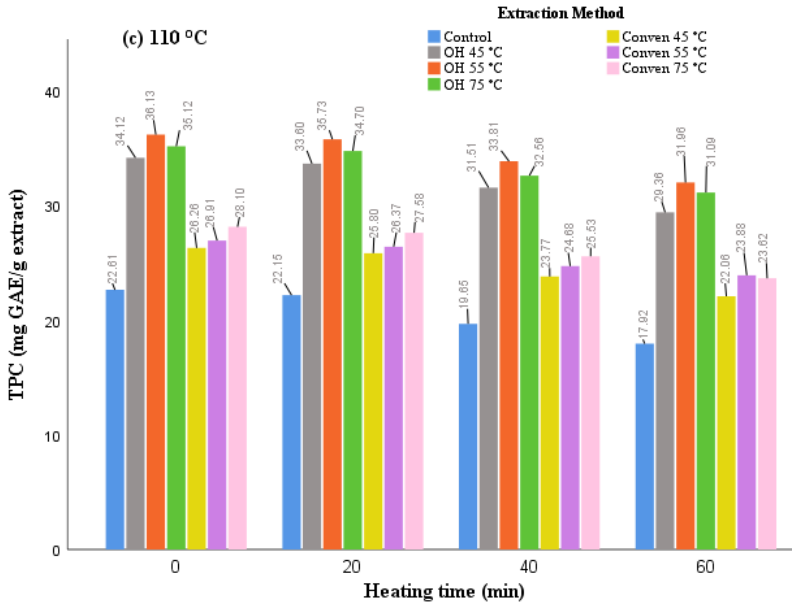
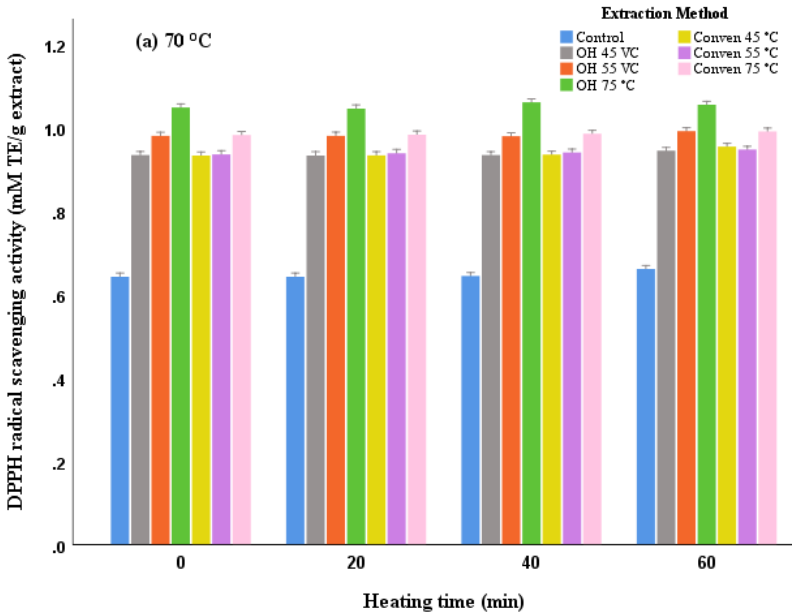


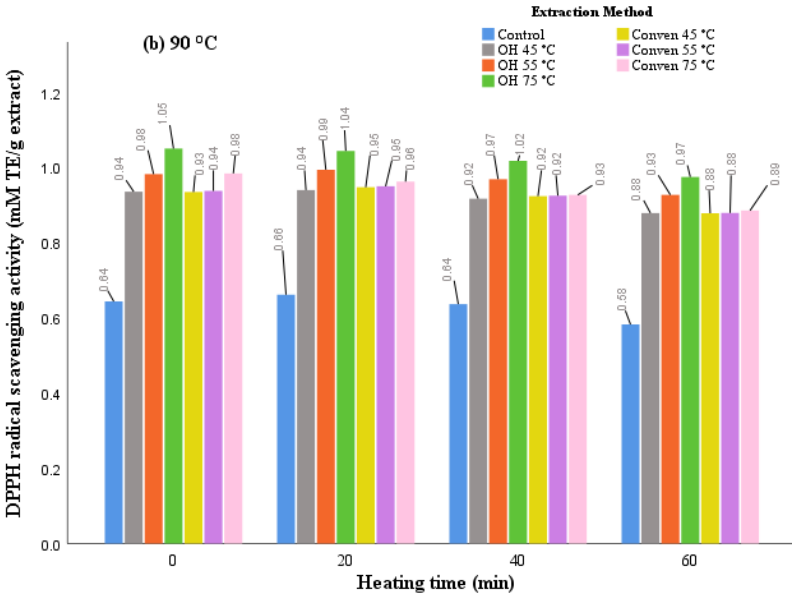
Figure 4. 7 Changes in total phenolic content (TPC) of olive leaf extracts (mg GAE/g) as a function of heating over time points at (a) 70 °C, (b) 90 °C, and (c) 110 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

Overall the extraction groups showed relatively similar rhythm of changes in TPC over time points for the same heating temperature. Quantitatively, the extracts of ohmic heating represented the ideal candidates in terms of thermal stability as they contained the highest concentrations of total phenols from initial point (0 min) to the end of thermal periods (60 min). Indeed, the magnitude of differences between ohmic groups and the corresponding *Conven*/ *Control* groups, under the same thermal condition, were significantly large over time points $p < 0.05$.

4.3.2.2. Changes in antioxidant capacity

Examining the influence of thermal stability on radical scavenging activities detected by DPPH, FRAP, and ABTS (**Figures: 4.8–4.10**), it was found that the heating of extracts at 70 °C caused no changes but there were moderate reductions over time points at 90 °C (over 60 min), and rather significant reductions at 110 °C (over 40 and 60 min). The trends of variations in the corresponding extraction groups were relatively similar for all TEAC assays. Of all groups, the ohmic extracts at 75 °C showed the highest antiradical activities over heating times for the same heating temperature.


(a) 70 °C
Extraction Method
Control
OH 45 VC
OH 55 VC
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
DPPH radical scavenging activity (mM TE/g extract)
1.2
1.0
.8
.6
.4
.2
.0
0
20
40
60
Heating time (min)


(b) 90 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
DPPH radical scavenging activity (mM TE/g extract)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.64
0.94
0.98
1.05
0.93
0.94
0.98
0.66
0.94
0.99
1.04
0.95
0.95
0.96
0.64
0.92
0.97
1.02
0.92
0.92
0.93
0.68
0.88
0.93
0.97
0.88
0.88
0.89
0
20
40
60
Heating time (min)

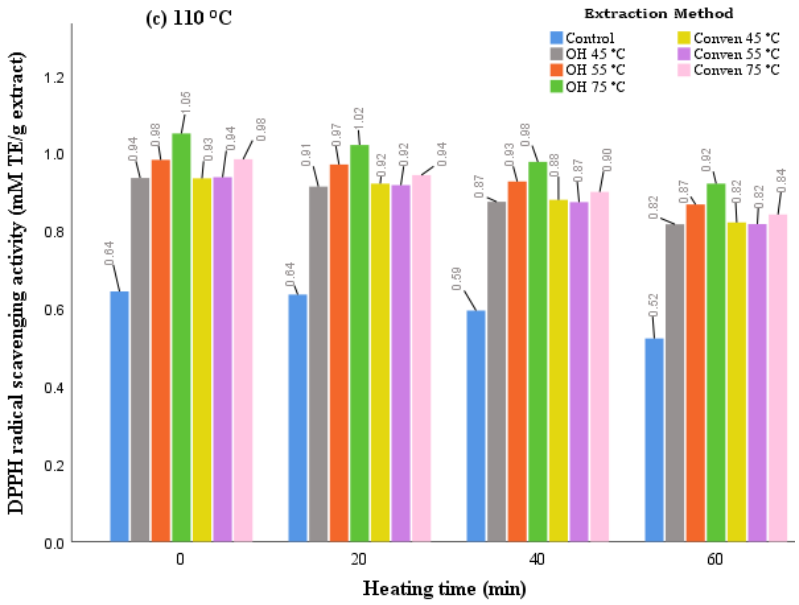
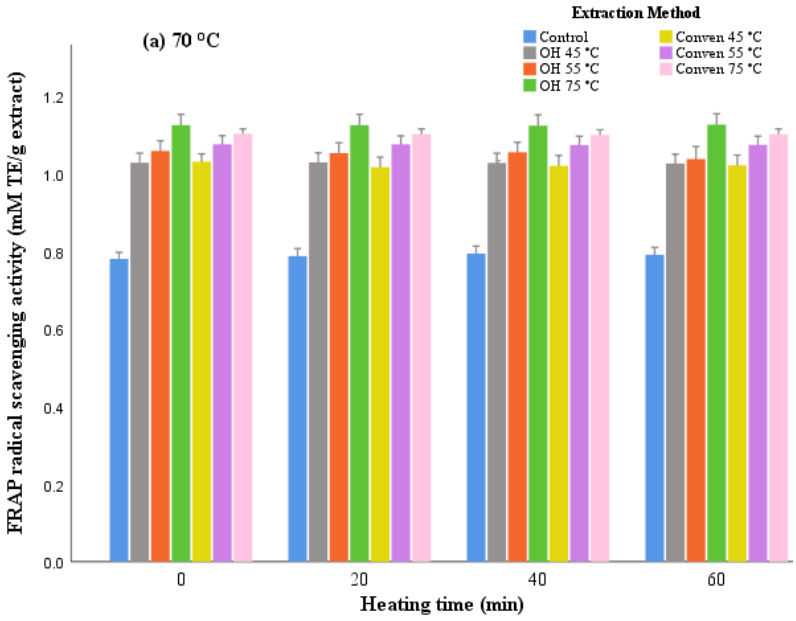


Figure 4. 8 Changes in DPPH radical scavenging activity of olive leaf extracts (mM TE/g) as a function of heating over time points at (a) 70 °C, (b) 90 °C, and (c), and (c) 110 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.



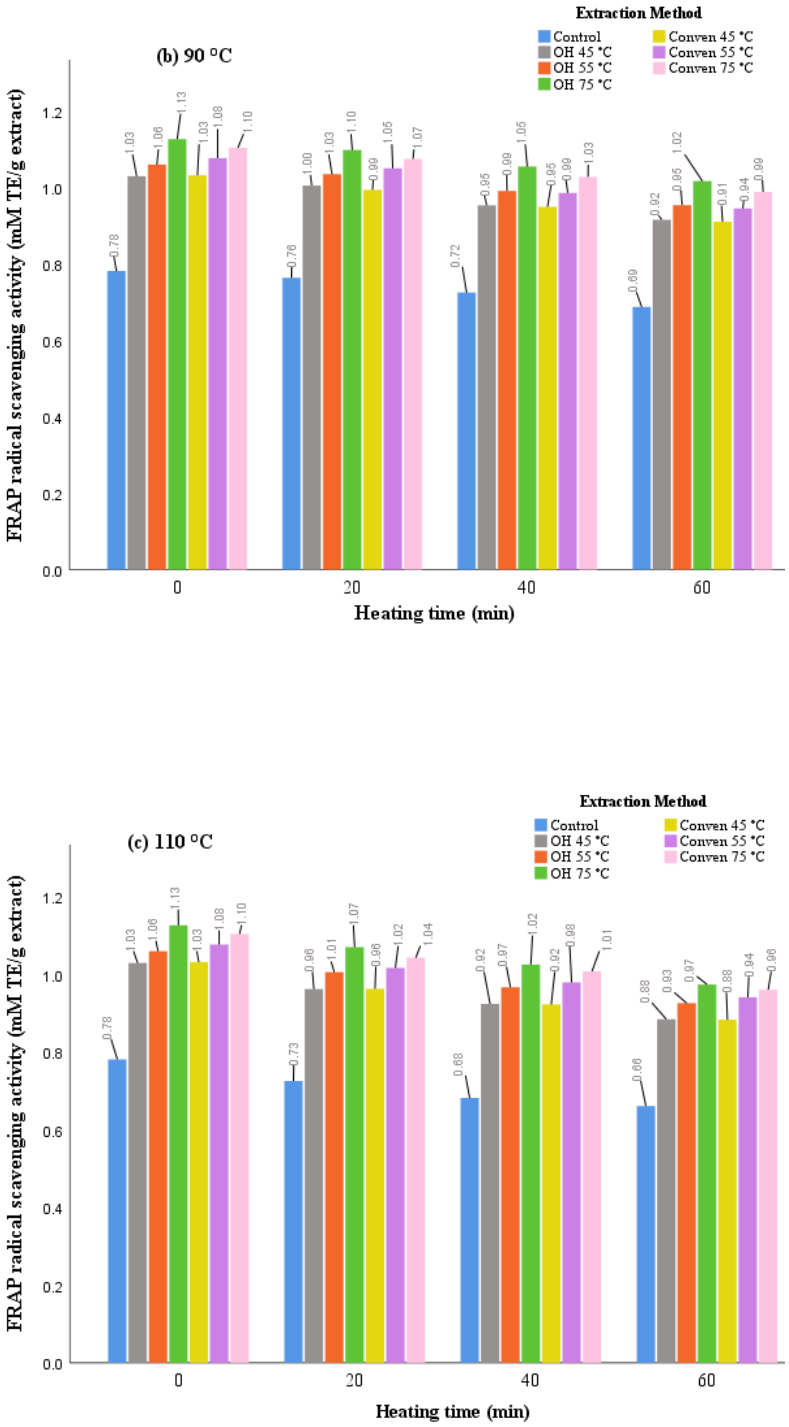
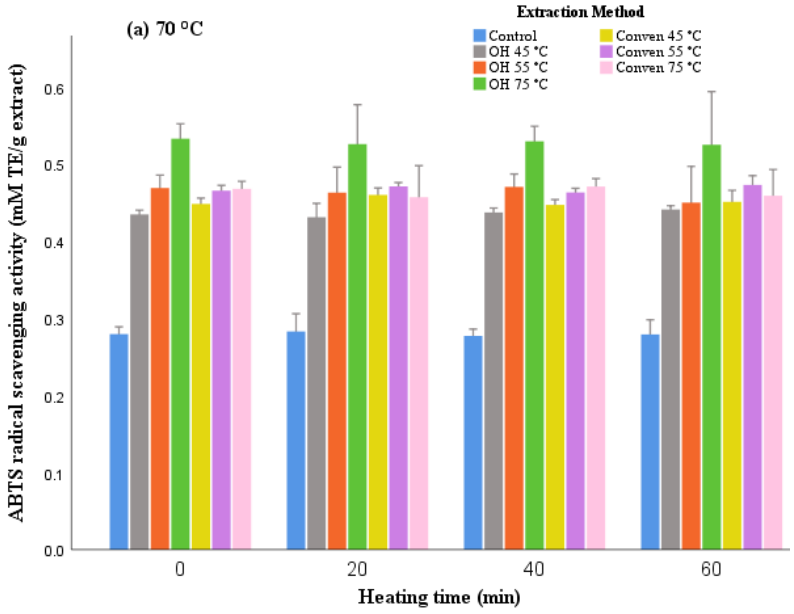
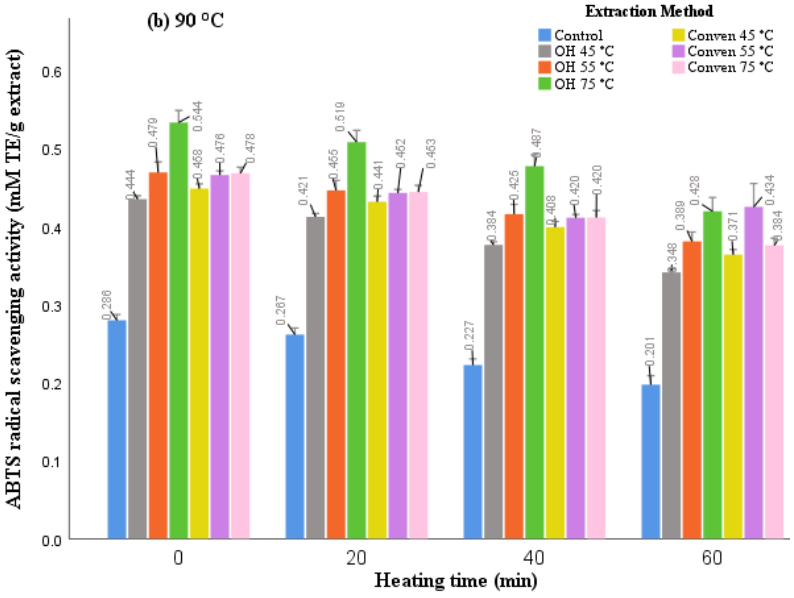


Figure 4. 9 Changes in FRAP radical scavenging activity of olive leaf extracts (mM TE/g) as a function of heating over time points at (a) 70 °C, (b) 90 °C, and (c), and (c) 110 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.


(a) 70 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
ABTS radical scavenging activity (mM TE/g extract)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
Heating time (min)


(b) 90 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
ABTS radical scavenging activity (mM TE/g extract)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.288
0.444
0.479
0.544
0.458
0.476
0.478
0.267
0.421
0.465
0.519
0.441
0.462
0.463
0.227
0.384
0.426
0.487
0.408
0.420
0.420
0.201
0.348
0.389
0.428
0.371
0.434
0.384
0
20
40
60
Heating time (min)

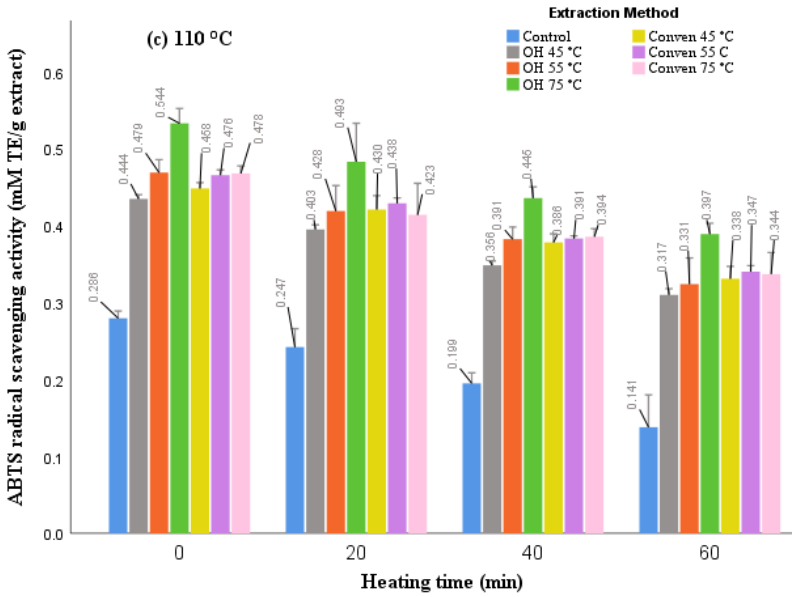
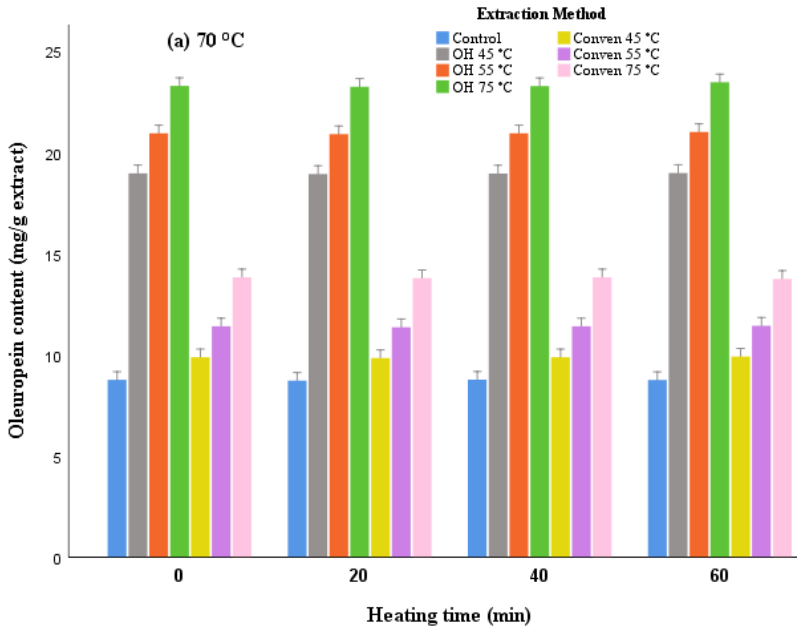


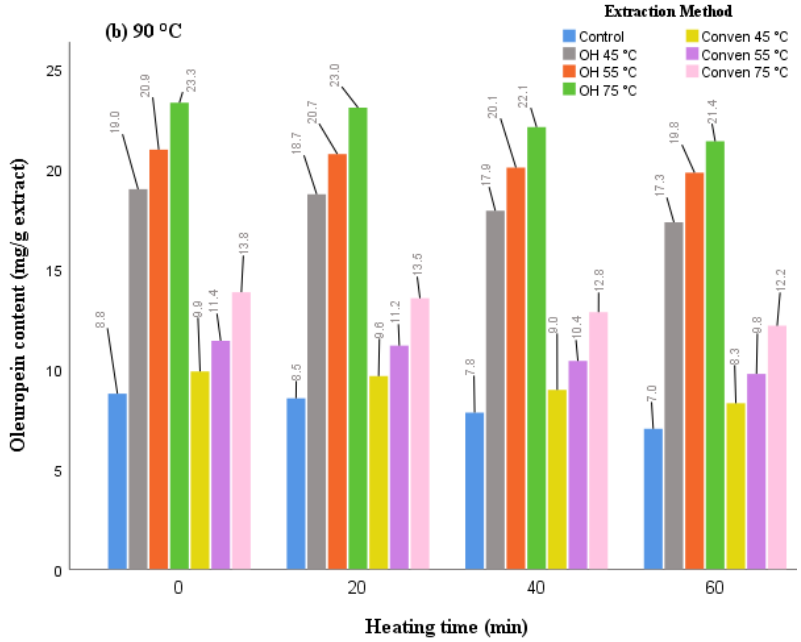
Figure 4. 10 Changes in ABTS radical scavenging activity of olive leaf extracts (mM TE/g) as a function of heating over time points at (a) 70 °C, (b) 90 °C, and (c), and (c) 110 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

Overall, the least detrimental conditions were: (i) when the surrounding temperature did not exceed 70 °C for no longer than 60 min, and (ii) when the heating temperature did not exceed 90 °C for no longer than 20 min. As mentioned above, the *OH* 75 °C represented the highest antiradical activities at each time points in all heating experiments. Similar case was also observed in the storage stability trials (**Section 4.3.1.2**) as this particular method (ohmic at 75 °C) was found to best justify the suitability for the storage of total polypheols.

4.3.2.3. Changes in oleuropein content

As shown in **Figure 4.11**, oleuropein was stable at 70 °C (for 60 min) and 90 °C (for 40 min), but showed little stability at 110 °C particularly during extended heating time (60 min). This may indicate that the increased elevated temperature potentially caused an infusion of other structural compounds which can react with the target phenols over heating time. In general, the biochemical degradation of oleuropein can be due the exertion of catalyzing enzymes including: (i) polyphenol-oxidase through the oxidation reaction, (ii) esterase through the hydrolysis reaction, and (iii) β-glucosidase through de-glycosylation (Bounegru & Apetrei, 2022; Cavaca et al. 2020; De Leonardis et al. 2015).


(a) 70 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
Oleuropein content (mg/g extract)
0
5
10
15
20
25
0
20
40
60
Heating time (min)


(b) 90 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
Oleuropein content (mg/g extract)
8.8
19.0
20.9
23.3
9.9
11.4
13.8
8.5
18.7
20.7
23.0
9.8
11.2
13.5
7.8
17.9
20.1
22.1
9.0
10.4
12.8
7.0
17.3
19.8
21.4
8.3
9.8
12.2
0
5
10
15
20
25
0
20
40
60
Heating time (min)

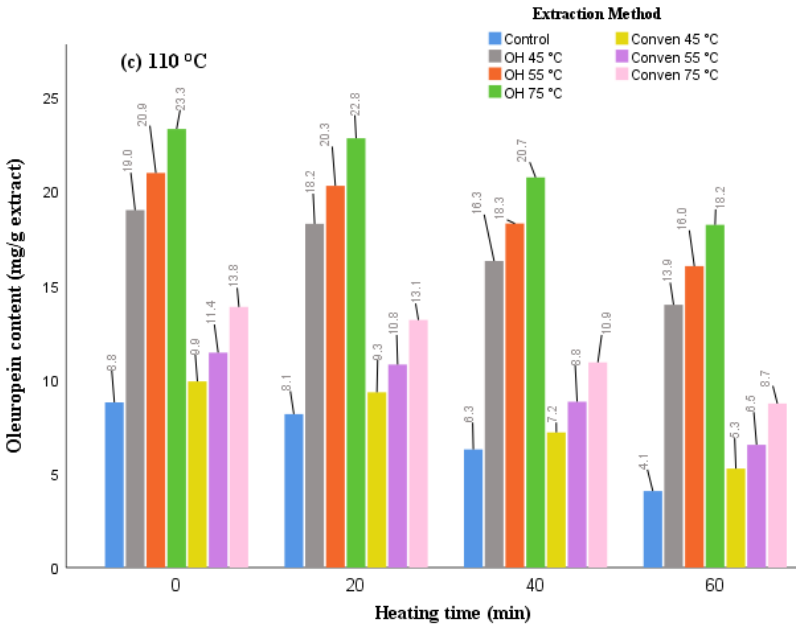


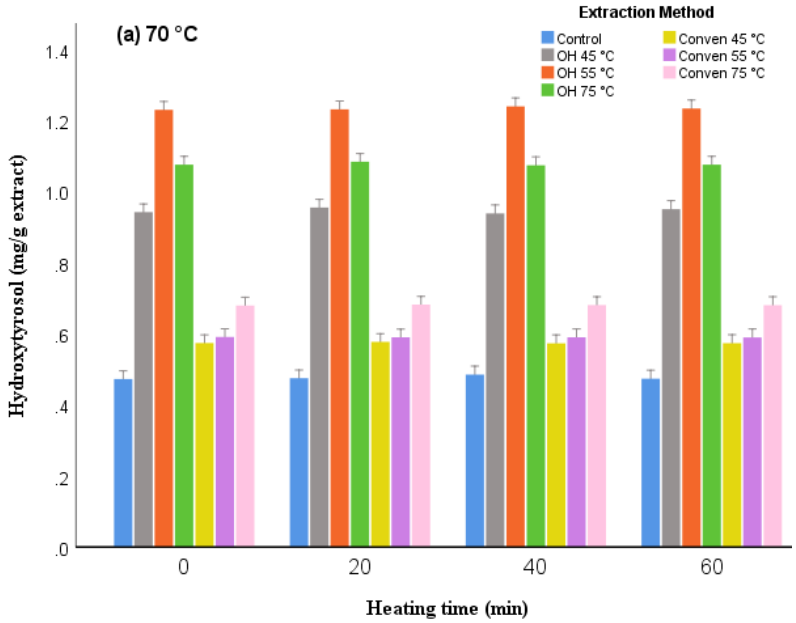
Figure 4. 11 Changes in oleuropein content of olive leaf extracts (mg/g) as a function of heating over time points at (a) 70 °C, (b) 90 °C, and (c), and (c) 110 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

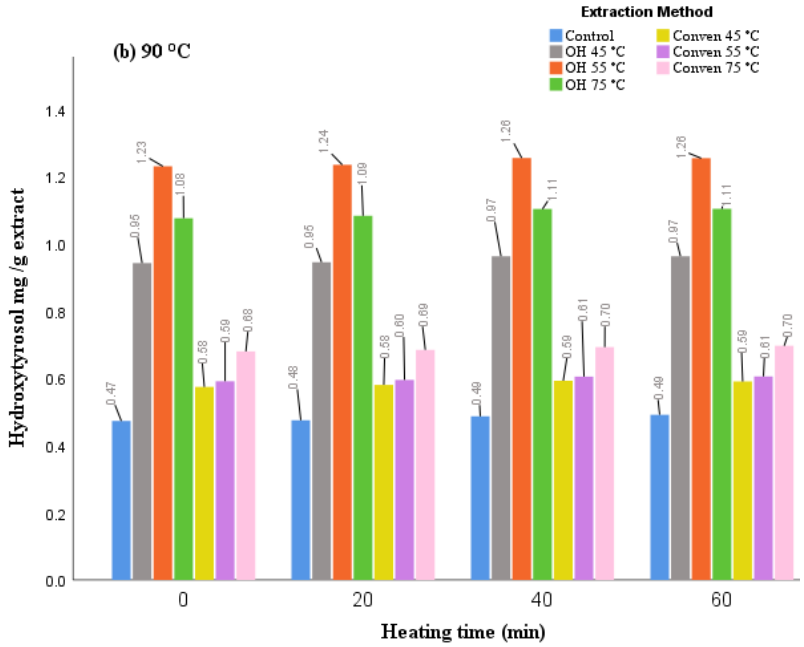
As seen in **Figure 4.11**, the ohmic extracts contained significantly the highest levels of oleuropein from initial recovery point (min 0) up to the end of 60 min. This was the case for all heating temperatures examined in this section (**Figure 4.11a –c**). Among the *OH* groups, the ones at 75 °C showed the highest content for the same heating temperature, which makes it an appropriate method for better conservation of oleuropein in the extract as a function of heating temperature/time.

Overall, the extracts should not be exposed to too high temperature to suppress unfavorable degradation of bound polyphenols. This is particularly the case when the key phenolics such as oleuropein are the products of interest in the extract. In this regard, the heating preferably should not exceed 90 °C for more than 40 min.

4.3.2.4. Changes in hydroxytyrosol content

As shown in **Figure 4.12**, in all extraction groups the hydroxytyrosol remained stable at 70 °C (over the heating time points from 0 to 60 min), and at the early stage of 90 °C (20 min). Heating for 40 min at 90 °C showed slight increase (2–3%) and remained relatively unchanged when the heating extended (60 min). At 110 °C, the concentrations declined slowly by around 3–4% over a 60 min heating period.


(a) 70 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
Hydroxytyrosol (mg/g extract)
1.4
1.2
1.0
.8
.6
.4
.2
.0
0
20
40
60
Heating time (min)


(b) 90 °C
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
Hydroxytyrosol mg /g extract
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.47
0.95
1.23
1.08
0.58
0.59
0.68
0.48
0.95
1.24
1.09
0.58
0.60
0.69
0.49
0.97
1.26
1.11
0.59
0.61
0.70
0.49
0.97
1.26
1.11
0.59
0.61
0.70
0
20
40
60
Heating time (min)

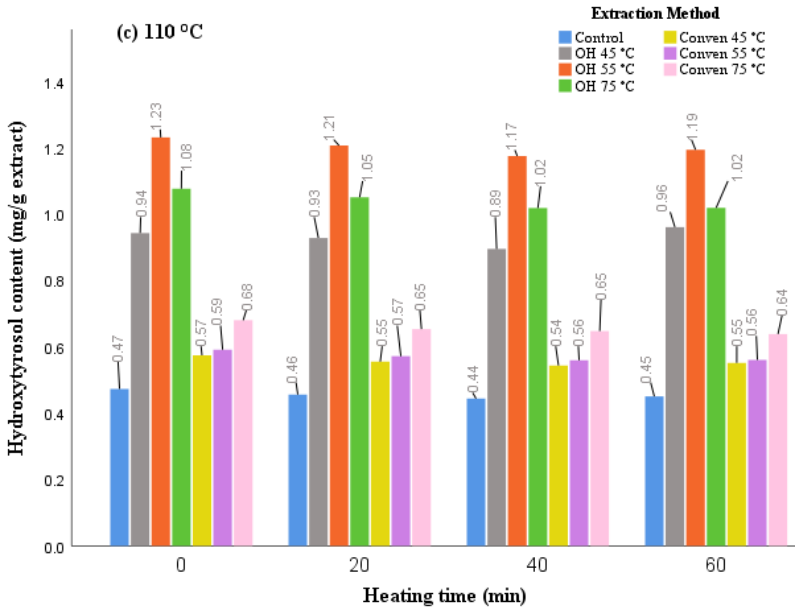


Figure 4. 12 Changes in hydroxytyrosol content of olive leaf extracts (mg/g) as a function of heating over time points at (a) 70 °C, (b) 90 °C, and (c), and (c) 110 °C. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

Hydroxytyrosol, compared to oleuropein, showed more stable fluctuations during heating trials. Minor degradation was observed at 90 °C and 110 °C (60 min). The increase in hydroxytyrosol (90 °C, 40 min) is potentially related to the concurrent degradation of oleuropein, rather than the performance/type of the extraction system. In the case of extraction methods, the *OH* extracts showed significantly higher content of hydroxytyrosol over the time points for each heating temperature. Examining the extraction temperature applied during ohmic process, the suitability of this method (*OH*) for the thermal stability of hydroxytyrosol follow the order: 55 °C > 75 °C > 45 °C.

4.3.3. Effect of pH on polyphenols and antioxidant capacity of olive leaf extracts

The pH environment of the extract is among the major factors responsible for the fate of polyphenols and their antiracial capacity as evidenced previously in research experiments (Roy & Urooj, 2003; Palvai et al. 2014; Krungkri & Areekul, 2019; Chen et al. 2008; Chethan & Malleshi, 2007; Friedman & Jürgens, 2000; Arabshahi et al. 2007; Amendola et al. 2010; Katanić et al. 2015).

In the present study, the pH stability of live leaf extracts was examined in term of polyphenolic and antioxidant capacity as follows:

4.3.3.1. Changes in total phenolic content

The changes in total phenols of olive leaf extracts as a function pH is shown in **Figure 4.13**. As it can be seen, the pH of 5 can be an ideal condition for the preservation of TPC. However, all extraction

groups showed stability to pH variations as the mean differences were not statistically significant $p > 0.05$.

Comparing the extraction groups, the *OH* extracts represented the maximum TPC values for the same pH level, particularly, the ohmic heating at 55 °C. The optimum pH condition evaluated here (pH 5) relatively corresponds with the pH values measured originally (4.8–5.2) prior to pH adjustments. This partly explains that the pH of the media of the *OH* extracts can be suitable for the protection of polyphenols, hence no further pH adjustments/modifications may be needed in this respect.

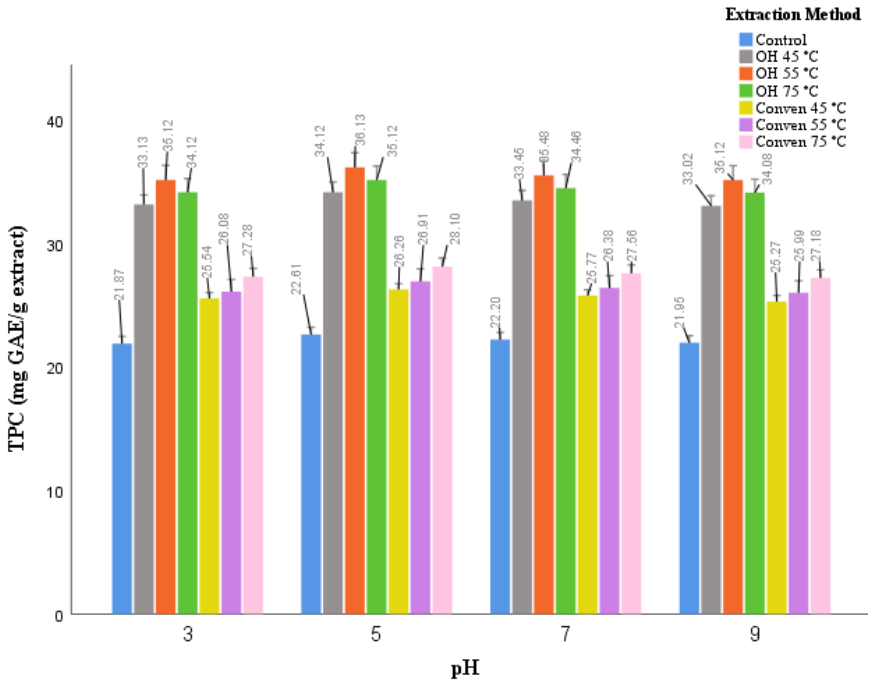
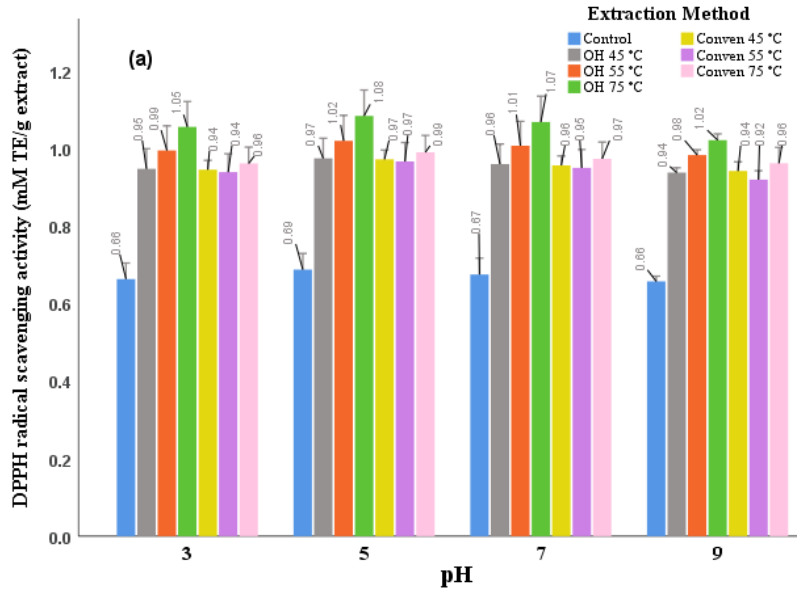
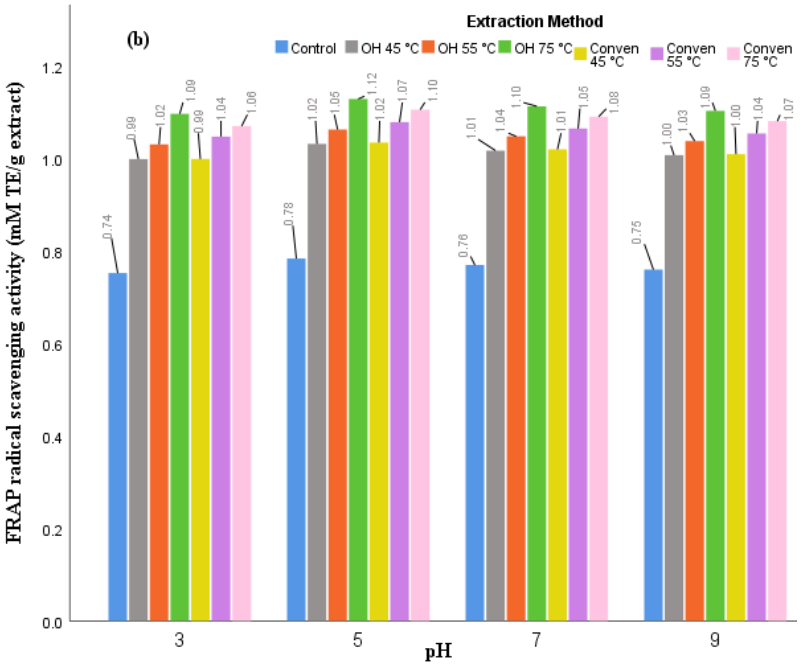


Figure 4. 13 Changes in total phenolic content (TPC) of olive leaf extracts (mg/g) as a function of pH. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

4.3.3.2. Antioxidant capacity

As can be seen in **Figure 4.14,** the radical scavenging activities detected by FRAP, DPPH, and ABTS in all corresponding groups of extracts showed more stability in slightly acidic and neutral pH conditions. As mentioned above the highest concentrations of TPC were detected at pH 5 (**Section 4.3.3.1**). These data are relatively consistent with the antiradical results. This may suggest the major role of pH in the proportional stability of total phenols, that in turn affects the overall status of antioxidant capacity of the extract (Medina et al. 2007; Devi et al. 2021).


(a)
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
DPPH radical scavenging activity (mM TE/g extract)
pH


(b)
Extraction Method
Control
OH 45 °C
OH 55 °C
OH 75 °C
Conven 45 °C
Conven 55 °C
Conven 75 °C
FRAP radical scavenging activity (mM TE/g extract)
pH

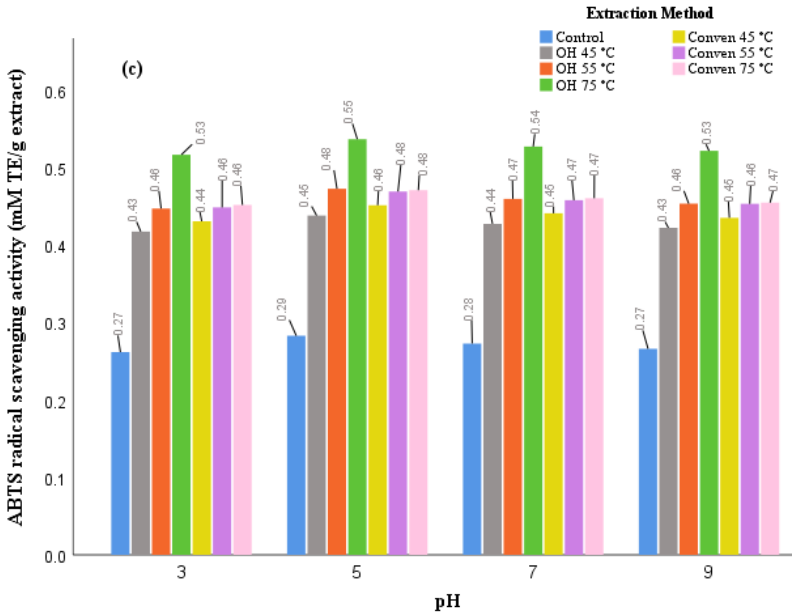


Figure 4. 14 Changes in radical scavenging activities: [**(a)** DPPH, **(b)** FRAP, **(c)** ABTS] of olive leaf extracts as a function of pH. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

4.3.3.3. Changes in oleuropein content

The degree of acidity or basicity can in part dictate the release and functional abilities of the target polyphenols (Tsao, 2010). The rate at which the solubilization occurs is influenced differently by the structure of each phenolic class within the cell; particularly the position and numbers of their hydroxyl groups.

Comparing of the extraction methods, the *OH* 75 °C evidently favored most for the stability of oleuropein in the extracts over the pH environments examined in the current study. The highest content of oleuropein was observed in samples with the pH of 5 for all extraction groups (**Figure 4.15**). The numbers also responded almost favorably in acidic medium (pH 3) which can be due to the phenomena of protonation of phenol hydroxyl groups and glycosidic disruption (Tagrida & Benjakul, 2020; Ghosh et al. 2015). In alkaline environment (pH 9) the least values were obtained. This could be attributed to potential de-protonation of the hydroxyl groups of phenols through breaking their ester bonds. Therefore, the exposure of extracts to high pH solutions during various applications may cause depletion in oleuropein content.

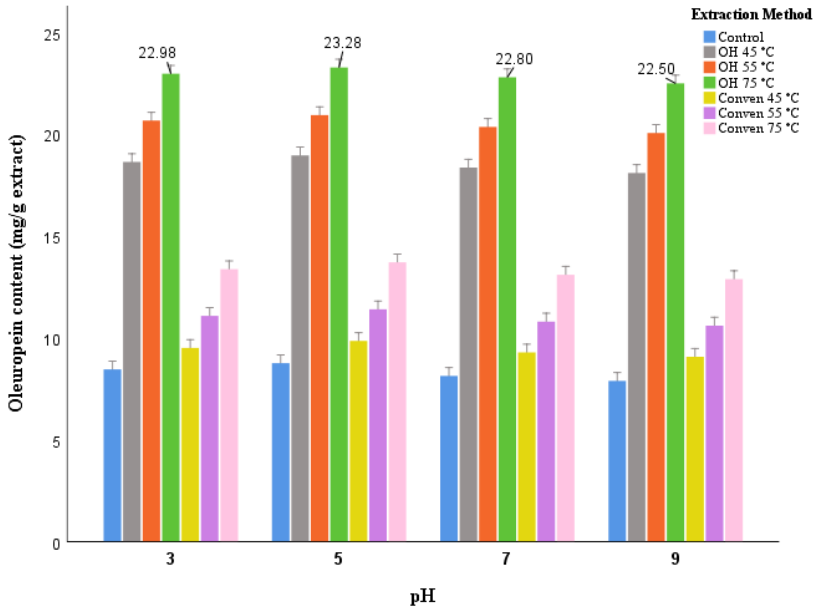


Figure 4. 15 Changes in oleuropein content of olive leaf extracts (mg/g) as a function of pH. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

4.3.3.4. Changes in hydroxytyrosol content

The pattern of changes for hydroxytyrosol content at pH of 5 rather resembled to those with oleuropein (**Figure 4.16**). In other words, it was likely that oleuropein (glycosidic or aglycone group) was only partially or negligibly the precursor of the increased hydroxytyrosol under the same pH condition; which means that other hydroxytyrosol-content phenols including oleuropein derivatives such as de-carboxymethyl oleuropein and oleuropein ligstrosides potentially served as the major substrates for the generation of hydroxytyrosols under such a condition (pH 5).

In summary, pH 3 and pH 9 were found to be less desirable conditions for hydroxytyrosol. In other words, the exposure of extracts to slight acidic condition was most favorable to the stability of this compound rather than too acidic/too alkaline conditions.

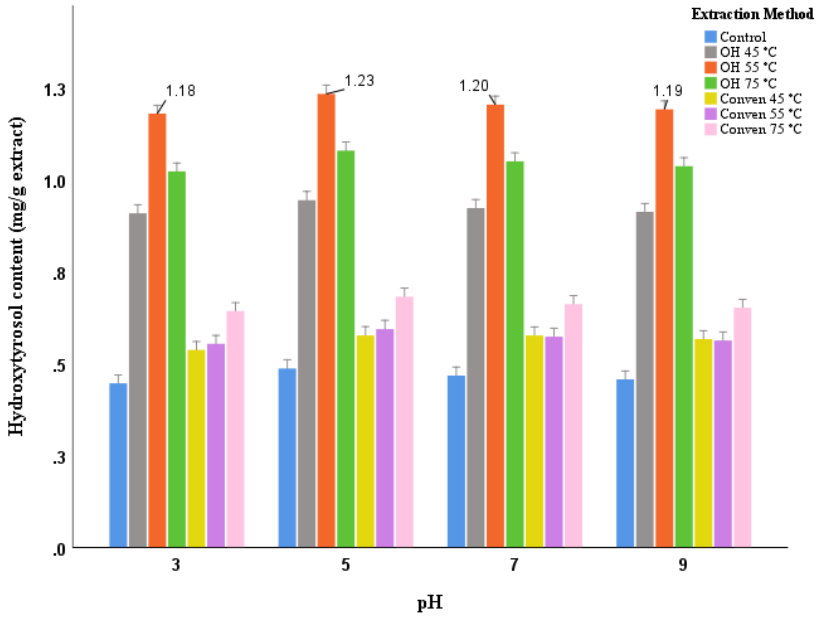


Figure 4. 16 Changes in hydroxytyrosol content of olive leaf extracts (mg/g) as a function of pH. The *OH* represents ohmic heating extraction at 45, 55, and 75 °C. The *Conven* represents conventional heating extraction using the same temperatures as in *OH* method. The *Control* represents solvent extraction without heating at 25 °C.

Overall, the pH of 5 seemed to be an ideal condition for the ohmic extracts, because it offered advantages of higher polyphenols and antioxidant capacity, as well as being favorable for both hydroxytyrosol and oleuropein content. In conclusion, the optimum pH condition relatively corresponds with those in original/initial extracts (4.8–5.2) measured prior to pH modifications. This can explain that the pH status during the extraction process can be suitable, particularly for the protection of target phenols (oleuropein as well as hydroxytyrosol).

4.4. Conclusions

The current study showed that the changes in polyphenolic content (total phenols, oleuropein, and hydroxytyrosol) and antioxidant capacity of the extracts (regardless of the extraction methods) are relatively in similar patterns under the given environmental conditions (storage, heating, and pH) over time. The storage at 25 °C was found to be the least desirable temperature for the extended period while the storage at –20 °C was evidently the most favorable condition that enabled significantly better conservation of the extracts over the course of eight weeks. Moreover, the ohmic extracts maximally retained their oleuropein content for a short term at –20 °C.

Heating at higher temperature (110 °C) for the extended times was detrimental to TPC, oleuropein and, correspondingly the antioxidant capacity of the extracts. The results also confirmed that the initial pH conditions of the extracts were relatively close to the optimum value (pH 5) determined for the

protection of polyphenols especially oleuropein. The alkaline condition (pH 9) was the least desirable medium for oleuropein stability.

Overall, the data justify the significance of ohmic heating as a value-added processing technique because after the storage/thermal/pH trials, significant values of oleuropein, antioxidant capacity (extraction temperature of 75 °C), total polyphenols and hydroxytyrosol (at 55 °C) remained in the *OH* extracts. However, further research is needed to ascertain an optimum condition for the conservation of total/individual polyphenols of the leaf-extracts using various surrounding conditions (through a larger set of parameters) over times, before deciding on re-directing the extracts for potential bio-functional applications.

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Part II

Extractability of Polyphenols from Olive Leaves through Pre-processing System and Possibilities for Sustainable Processes in Olive Oil Industry

CHAPTER 5

ROLES OF DRYING, SIZE REDUCTION, AND BLANCHING IN SUSTAINABLE EXTRACTION OF PHENOLICS FROM OLIVE LEAVES

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Safarzadeh Markhali, F., 2021. Roles of drying, size reduction, and blanching in sustainable extraction of phenolics from olive leaves. *Processes*, *9*(9), 1662. https://doi.org/10.3390/pr9091662

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Review

Roles of Drying, Size Reduction, and Blanching in Sustainable Extraction of Phenolics from Olive Leaves

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Abstract: It is now known that olive leaves contain a sizable portion of polyphenols and there is much research highlighting that these natural ingredients favorably exhibit bio-functional activities. In this regard, many studies have focused on the exploration of optimum conditions involved directly in the extraction process. These investigations, while being highly valuable, may somewhat cast a shadow over other contributing factors such as those involved in the preprocessing of leaves, including size reduction, drying, and blanching. The use of these unit operations under appropriate conditions, together with other benefits, potentially exert improved surface area, homogeneity, and diffusion/mass transfer which may help develop the liberation of target bio-compounds. The research work in this area, particularly size reduction, is relatively limited. Although in various experiments they are incorporated, not many studies have focused on them as the main predictor variables. The performance of further research may help ascertain the magnitude of their effects. Consideration of the operational parameters in preprocessing step is equally important as those in the processing/extraction step that may comparably influence on the extraction efficiency. This review provides an overview of the potential roles of drying, size reduction, and blanching in the extraction efficiency of phenolics from olive leaves.

Keywords: preprocessing; size reduction; grinding; drying; blanching; olive leaves; by-products; phenolics; sustainable extraction.

5.1. Introduction

Olive leaves form a large proportion of biomass residues derived from (i) agricultural practices (mainly at pruning stage) (Rahmanian et al.; Talhaoui et al. 2015), and industrial processing of olive oil and table olives (Contreras et al. 2020; Gullon et al. 2020; Şahin & Bilgin, 2017). The abundance and bio-functional potential of the endogenous phytonutrients, such as polyphenols, in these residues, have progressively led to increasing global interest in developing extraction techniques to optimally re-use/re-direct these valuable natural components for high added-value applications. The emerging extraction technologies such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), etc., have shown potential for higher efficiency. Although the selection of extraction types/methodologies is greatly important, the achievement of sustainable processing rests decisively on the integration of associated factors before, during, and after extraction processing that may enable recovery of target biomaterials within an eco-friendly processing system. Among the key factors include the operational parameters associated with the preprocessing/pretreatment step which are comparably as important as those involved during the actual extraction. These include size reduction, drying, and blanching that are among the most common approaches applicable to olive leaves. They can be ideally incorporated into the processing flow, at the preliminary stage prior to the actual extraction process (**Figure 5.1**). Recent studies on the extraction of bioactive compounds from olive leaves observed improved extraction yield/bioactivity of the desired biomolecules using various extraction designs. In many experiments, olive leaves are typically preprocessed (e.g., dried and ground) but limited studies have examined their effects on the extraction efficiency. Special attention often seems to be towards the processing conditions (as the main independent variables), which rather overshadows the significance of those involved in the preprocessing step (particularly, from the perspective of size reduction).

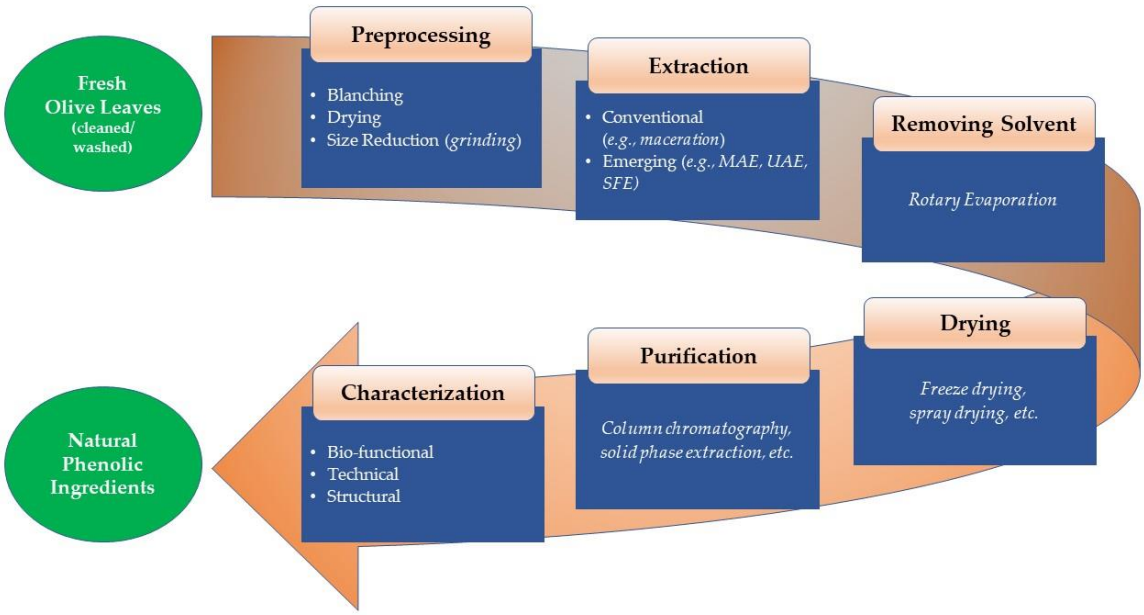

Figure 5. 1 Typical steps associated with phenolic extraction from olive leaves.

The appropriate integration of preprocessing with processing means potentially reinforces the overall extraction efficiency in a sustainable manner. Depending on the selected types, they may significantly contribute to (i) reducing extraction time, (ii) increasing extraction yield and quality of nutritive molecules, and (iii) reducing input energy. For instance, particle size reduction, using dry grinding, which is often considered as an indispensable operation at the preliminary stage of exploitation of olive leaves, on its own plays a decisive role in the extraction efficiency, quantitatively and qualitatively. Other preprocessing means, drying and blanching, are comparably important and their operations may favorably/unfavorably affect the extractability and functionality of target biomolecules. This review discusses the roles of particle size reduction, drying, and blanching on the extraction efficiency of phenolic compounds from olive leaves. Given the fact that many studies tend to stress the importance of the main processing/extraction parameters, together with the potential practicalities of the abovementioned pretreatments, it may be worthwhile to highlight an overview of the effects of these unit operations and emphasize that there is a need to take them into account when addressing the challenges involved in the extraction/bioactivity of phenolic compounds from olive leaves.

5.2. Typical preprocessing means applicable to olive leaf extraction

5.2.1. Drying

Drying fresh olive leaves at the preliminary stage prior to the extraction process is partly a deciding factor affecting the rate of extraction/bio-functionality of the released biomolecules. The dehydration of

olive leaves may be achieved by different drying methods. Among the most common methods include (i) thermal drying through natural convection (such as shade and open sun drying), and forced convection (such as oven drying, solar drying, and heat pump drying (Babu et al. 2018), and (ii) special forms of drying such as freeze-drying (Babu et al. 2018; Espeso et al. 2021), microwave drying (Babu et al. 2018; Espeso et al. 2021), infrared drying (Espeso et al. 2021), and greenhouse drying (Babu et al. 2018).
The agro-industrial system often uses thermal energy for drying operations (Ekechukwu, 1999;, Yang et al. 2020), which exerts mainly two parallel phenomena: (i) heat flow from the driving force to the inside of the food, and (ii) mass transfer where moisture moves from the inside to the exterior/surface of the food and is then evaporated in the air (Gowen et al. 2006; Norton, 2012; Ndukwu et al. 2017), which enables the reduction in the food moisture content to a certain proportion (Iqbal et al. 2019; Ekechukwu & Norton, 1999; Tomar et al. 2017). The resulting moisture reduction may confer improved preservation and bioavailability of the endogenous phytonutrients including phenolic constituents via (i) protecting them against oxidative (Ahmad-Qasem et al. 2013) and enzymatic activities (Babu et al. 2018), and spoilage microorganisms (Afaneh et al. 2015; Orphanides et al. 2013; Saifullah et al. 2019), (ii) enhancing the stability of large scale samples for further processing including extraction, (iii) enabling cellular destruction through rupturing the cell membrane that potentially gives rise to the liberation of bound phenolics (Ahmad-Qasem et al. 2013; Saifullah et al. 2019). The magnitude of the effects of drying on the extraction yield partly rests on the drying method and the type/physicochemical characteristics of the foods to be dehydrated. In the research of Nambiar et al. (2013), the polyphenolic concentrations in fresh, air-dried, and oven-dried drumstick leaves represented around 141.59, 158.82, 185.32 mg/100 g, respectively.

Drying also finds applications for dehydrating the liquid extracts to form them into powders (**Figure 5.1**) which can partly assist in promoting the storage stability of the extracts, together with others (Kiritsakis et al. 2018). The performance of polyphenols over drying operations has been discussed in previous studies. Kiritsakis et al. (2018) justified the effectiveness of a short-term spray drying of aqueous olive leaf extracts through which the total phenols were not adversely influenced (6109 and 6985 μg/kg in Chalkidiki and Koroneiki, respectively). In their research, Kashaninejad et al. (2020) showed that freeze-drying of aqueous extracts of olive leaves is effective in the improved recovery of oleuropein (above 11% w/w) and luteolin-7-O-glucoside (1.4% w/w).

It is important to select an appropriate type of drying that may come with challenges specially when selective extraction is needed. Freeze-drying (lyophilization) is generally credited as being highly efficient in retaining the nutritive and sensory qualities of the dried products. Lyophilization, among other benefits,

enables (i) minimal thermal damages to the food tissue, makes it a prime candidate for the protection of thermolabile compounds (Shukla, 2011), and (ii) a porous structure that allows an increased penetration of the extraction solvents (if applicably used), and hence a greater chance of recovery of phytonutrients (Saifullah et al. 2019; Harnkarnsujarit et al. 2016). However, due to its limitations, freeze-drying may need optimization to ensure the retention of endogenous phenolic constituents over drying application. Among the operational disadvantages includes the impact of ice crystals with potential damage to the cell structure of the food tissues (Ahmad-Qasem et al. 2013). Research demonstrates that olive leaf extracts, pretreated with a hot air drier (120 °C), represented higher phenolic recovery when compared to those pretreated with a freeze dryer (loss of polyphenols reached up to 39% d.w.) (Ahmad-Qasem et al. 2013). On the other hand, in some research studies, freeze-drying has shown significant potential for the increased liberation of phenolics from the cell wall matrices. The research of Ghelichkhani et al. (2019) demonstrated that freeze-dried olive leaves have great potential for extraction of total phenolic content (TPC) up to around 446.63 mg gallic acid equivalents (GAE) per gram. Martinho et al. (2019) compared freeze-dried and non-dried (fresh) olive leaves and found polyphenolic concentrations in the range of 7.72–24.65, and 2.09–8.44 mg GAE/g leaves, respectively.

It should be pointed out that the temperature is indeed among the prominent parameters in the drying operation that may favorably/unfavorably exert influence on the microstructure of the food (depending on the types and molecular characteristics of target bio-phenols). For example, research demonstrates that the use of hot air drying at a higher temperature (120 °C) has shown a better potency for the extraction of some phenolic compounds in olive leaves when compared to samples dried at 70 °C (through which the extraction yields of oleuropein and verbascoside decreased by 36% and 44%, respectively) (Ahmad-Qasem et al. 2013). This may be attributed to the effect of high temperature on the decrease in drying time, while the lower/moderate temperature may need lengthier drying time to accommodate a decrease in the moisture content to reach the final/certain level in the dried products (Ahmad-Qasem et al. 2013). However, other studies suggest that heating may be unfavorable/less effective when the intention is to retain some bioactive compounds including oleuropein. Afaneh et al. (2015) found more effective extractability of oleuropein when olive leaves were dried at room temperature (25 °C), yielding 10.0 mg/g dry olive leaves) that was greater than those obtained by other groups of leaf samples (dried leaves at 50 °C and dry leaves harvested from the olive tree, yielding 1.7 and 2.5 mg/g of olive leaves d.w., respectively). These authors also indicated that drying operation is preferable due to its potential for improved oleuropein extraction as it was evidenced that fresh leaves contained negligible amounts of oleuropein (less than 0.1 mg/g). The fact that the extraction yield of oleuropein is

generally greater in dried leaves compared to fresh ones is potentially due to the formation of oleuropein from oleuropein glucoside exerted by the enzymatic action of beta-glucosidase (Afaneh et al. 2015).

Further, Erbay and Icier (2009) optimized the drying performance of a tray drier on olive leaves and reported that at a temperature of 51.16 °C, air velocity 1.01 m/s, and drying time of 298.68 min, the TPC loss in dried leaves reached up to 10.25% with a moisture content of 6.0%. Bahloul et al. (2009) performed research on the roles of solar drying on the quality and functionality of olive leaves. In their research, it was found that the depletion of TPC (arising from the increase in drying time) is potentially minimized with a drying temperature of 50 °C and air velocity of 1 m/s. An optimized geothermal drying approach investigated by Helvaci et al. (2019) described that drying at a temperature of 50 °C and air velocity of 1 m/s is effective in lessening the depletion of polyphenols in dried olive leaves. Nourhene et al. (2008) investigated the drying kinetics of olive leaves from four Tunisian cultivars through solar drying (using an indirect forced convection) and reported that the rate of moisture diffusivity (ranging from 2.95×10^{-10} to 3.60×10^{-9} m^2/s) relies on drying temperatures as well as cultivar variations. The considerable effect of temperature variations during infrared drying on the yield recovery of polyphenols from olive leaves has been observed by Boudhrioua et al. (2009). Among the main findings of their research includes the extraction yield of the TPC in non-blanched leaves from Chemlali cultivar that increased with the rise in drying temperature (from 40 to 70 °C representing 2.13 and 5.14 g/100 g, d.w., respectively). The lowest concentration in the same cultivar was found in the non-dried/fresh leaves, 1.38 g/100 g of dry leaves.

The selection of suitable drying technique is of benefit to designing an efficient extraction system. Drying performance is partly interdependent on numerous deciding factors, among which include other complementary preprocessing operations. The drying process of olive leaves may be followed by grinding operation, and/or preceded by blanching process. In addition to the need for optimizing the parameters of each operation involved in preprocessing, the evaluation of their potential intercorrelations is equally significant particularly when considering a selective extraction of phenolics from olive leaves.

5.2.2. Size reduction

Dried olive leaves are often subjected to dry grinding or milling process which in part plays a crucial role in the resulting extraction efficiency. The main advantages of grinding are as follows: (i) intensified surface area via reducing the particle size that potentially improves the physicochemical and bio-functional activities (Oyinloye & Yoon, 2020), (ii) improved bulk density, and (iii) increased flow rate and porosity (Bitra et al. 2009). An ideal particle size reduction potentially enables improved dissolution and

extractability which may in part address the poor solubility inherent in solvent extraction system (Chemat et al. 2020).

In agri-food system the traditional grinders use milling operation based on a diverse range of devices including knife mills, disk mills, hammer mills, and ball mills (Karam et al. 2016). Among the main disadvantages inherent in the conventional grinders include the possible increase in temperature (due to the friction and energy used to exert particle size reduction) and uneven distribution of particles (Karam et al. 2016). The operating conditions, however, continue to be improved. For example, lately, ball milling has become more advanced, using a temperature control system that makes it a viable milling approach applicable to thermolabile compounds (Gao et al. 2020; Karam et al. 2016).

As mentioned in **Section 5.2.1**, the process of drying may be followed by the operation of grinding. These two phenomena are both influential in the efficiency of further processing, and their appropriate operations may jointly complement the extraction system. Drying may give a further boost to grinding performance, and grinding, together with other benefits, helps increase the surface area which in part improves/reinforces the efficiency of further processing such as the drying performance involved after extraction to powder aqueous extracts. **Table 5.1** highlights a summary of the studies on phenolic extraction from olive leaves wherein drying and particle size reduction were employed prior to the extraction process (although mostly not selected as the predictor variables).

Much research has investigated the extractability and bioactivity of endogenous phenols from the perspective of solvent selection, in which aspects such as polarity, solubility, diffusivity, and non-toxicity were taken into consideration. The appropriateness of extraction solvents is of great importance as, together with other factors, the behavior of each phenolic group is different due to their variations in molecular and physicochemical properties. In this regard, the use of optimal size reduction may partly help complement the performance of extraction solvents. In the research of Stamatopoulos et al. (2014), particle size (ranging 0.05, 0.1, 0.2, 0.315, and 1.0 mm) was assessed as one of the main factors to optimize a multistage extraction process of olive leaves (**Table 5.1**). It was found that the particle size reduction, up to about 0.315 mm, is highly effective in the exertion of phenolic liberation from dried olive leaves.

Table 5. 1 Examples of drying and size reduction approaches used prior to phenolic extraction of olive leaves.

Main Processing Factors for Phenolic Extraction	Drying & Size Reduction Prior to Extraction	Key Finding(s)	Reference
Leaves (pre-blanched) assessed as follows: - Optimization via a single-stage extraction (particle size was among the key independent variables) - Further optimized via multistage extraction system (compared to conventional method 40 °C, 48 h)	Drying: oven-dried with an air tray oven (60 °C, 4 h). Size reduction: dry ground and sieved through 0.05, 0.1, 0.2, 0.315, and 1.0 mm.	- Single-stage extraction: Optimized conditions include 0.315 mm particle size, 70% ethanolic extraction, solid-liquid ratio of 1:7 - Multi-step extraction: Optimization with three stages (30 min, 85 °C) improved TPC (166.6 mg/g); Oleuropein (103.1 mg/g); luteolin-7-O-glucoside (33.7 mg/g); verbascoside (16.0 mg/g); apigenin-7-O-glucoside (13.8 mg/g). - Multistage extraction enabled a 10-fold higher antioxidant activity compared to conventional extraction.	Stamatopoulos et al. 2014
Steam blanching and hot water blanching (blanching time and particle size of fresh leaves accounted for the key parameters through optimization of blanching)	- For blanching optimization: particle size of fresh leaves ranged: above 20 mm, 20–11 mm, and 3–1 mm. - For extraction: leaves (optimally blanched), air dried (60 °C for 4 h), and ground to 1 mm.	Optimized steam blanching (10 min, 20–11 mm particle size) improved oleuropein extraction (8.28 g/kg leaves d.w.), and antioxidant effects (4 to 13-fold increase, compared to those obtained from non-blanched ones).	Stamatopoulos et al. 2012
Extraction solvents (methanol, ethanol, water, and acetone)	Drying: dried at room temperature in the dark. Size reduction: dry ground to pass through a 20-mesh screen.	- Leaves extracted with 80% methanol exhibited higher TPC (392 mg GAE/g extract); total flavonoids (71 mg rutin equivalent/g); total tannins (18 mg GAE/g). - Leaves extracted with ethanol (80%) exhibited DPPH antiradical activity (IC_{50} = 1082.35 µg/mL). Total antioxidant activity (via linoleic acid system) was 76.36% with 2400 µg/mL extract.	Al-Marazeeq et al. 2016
Combining supercritical fluid extraction (with CO_2) and pressurized liquid extraction (PLE)	Drying: dried in the shade (ventilated). Size reduction: dry ground to 3 mm particle size.	Oleuropein reached 10.44%, 9.5%, and 9.9%, with DPPH scavenging effects of 127.3, 145.3, and 138.6 µg/mL in defatted residues, using water (150 °C), ethanol (60%, 50 °C), and water (50 °C), respectively.	Xynos et al. 2012
Freezing (conventional and liquid nitrogen) and drying (hot air drying and freeze drying) techniques	Drying: hot air-dried (70 °C for 50 min, 120 °C for 12 min) and freeze-dried. Size reduction: dry ground to 0.05 mm particle size.	Using hot air drying (120 °C): - Increased phenolics particularly oleuropein (108.6 mg/g d.w.). - Antioxidant capacity via ferric reducing antioxidant power (FRAP) reached 109 mg Trolox equivalents (TE)/g d.w.	Ahmad-Qasem et al. 2013
Hybrid extraction protocol (conventional ethanol extraction subsequent with supercritical fluid antisolvent extraction)	Olive leaves with 8% moisture content ground at room temperature to 1 mm particle size.	Concentrated yield of oleuropein powder reached up to 36% (35 °C, 150 bar).	Baldino et al. 2018
Optimization of aqueous extraction using water	Drying: dried at 120 °C for 90 min. Size reduction: dry ground to 0.1 mm.	Maximum TPC (32.4 mg GAE/g) yielded through extraction at 90 °C for 70 min, solid/solvent ratio of 1:60 g/mL Antioxidant capacity, using DPPH and FRAP, reached 85.26 and 91.03 mg TE/g, respectively.	Goldsmith et al. 2014
Optimization of UAE	Drying: air-dried at 40 °C Size reduction: dry ground to a 0.5 mm	Increased yield of oleuropein (10.65%) using 50% acetone, 60 °C, 10 min.	Irakli et al. 2018

Main Processing Factors for Phenolic Extraction	Drying & Size Reduction Prior to Extraction	Key Finding(s)	Reference
Extraction methods (solvent extraction, UAE, and reduced pressure extraction)	Drying: dried at ambient temperature (no exposure to solar radiation). Size reduction: ground with a high-speed crusher to pass through a 40–60 mesh.	Increased oleuropein via combined UAE and reduced pressure extraction (92.3% extraction efficiency in a single run).	Xie et al. 2015
Olive leaves (dried and fresh) from different cultivars	Drying: freeze-dried Size reduction: ground to 0.1 mm	- TPC ranged 7.72–24.65 and 2.09–8.44 mg GAE/g in dried and fresh leaves, respectively. - Effective in inhibiting proliferation of human carcinoma cell line (e.g., freeze dried leaves ranged from 0.07 to 2.40 µg phenolic constituents/well).	Martinho et al. 2019
- Extraction methods (MAE, Soxhlet) - Extraction solvents	Drying: open air-dried in the dark. Size reduction: ground and sieved (<2 mm)	Higher TPC (76.1 mg GAE/g), and antioxidant activity (78.0 mg TE/g) in Soxhlet extracted leaves (50% ethanol). Oleuropein was the key component. MAE was comparably effective.	Sánchez-Gutiérrez et al. 2021
Extraction methods (MAE, UAE, maceration)	Drying: oven-dried (24 h, 40 °C). Size reduction: ground to pass through a 60-mesh.	MAE extracts (86 °C, 3 min) exhibited higher TPC (104.22 mg GAE/g), with 90.03% antioxidant activity.	Da Rosa et al. 2019
- Preprocessing leaves: drying, non-drying (fresh leaves) - Solvent variations	Drying: freeze-dried (–50 °C, 36 h, 0.08 mbar); hot air oven dried (120 °C, 8 min). Moisture content < 1%. Grinding: milled using a blender	- Hot air-dried leaves extracted by 30% ethanol exhibited highest TPC (151 mg/g d.w.), with DPPH-scavenging activity of 922 µmol TE/g. - The use of water (100%) comparably effects on increased TPC (144 mg/g) of hot air-dried leaves.	Difonzo et al. 2017
- Successive extraction techniques - Samples: Olive mill leaves and collected leaves from olive trees	Drying: air-dried Size reduction: ground to 1 mm particle size	- TPC in extracts from olive mill leaves: 4476–6167 mg GAE/100 g. - Extracts from Olive tree leaves (UAE prior to alkaline extraction) contained TPC around 13,108 mg GAE/100 g; oleuropein (12,694 mg/100 g); luteolin 7-O-glucoside 903 mg/100 g; with antioxidant efficiency of 59,651 µmol TE/100 g - The highest concentration of oleuropein in olive mill leaves was 1790 mg/100 g extract.	Contreras et al. 2019
Optimization of UAE extraction	Dried leaves were ground to 0.9–2.0 mm prior to extraction	- Extraction with 43.61% ethanol, 34.18 °C, 59 min exhibited increased TPC (43.825 mg GAE/g dried leaves). - Total flavonoids (31.992 mg catechin equivalents/g dried leaves) through 70% ethanol, 34.44 °C, 60 min. - DPPH inhibiting capacity ranged 89.3%–90.5%	Şahin et al. 2015

Main Processing Factors for Phenolic Extraction	Drying & Size Reduction Prior to Extraction	Key Finding(s)	Reference
- Extraction solvents (ethanol, methanol, acetone, and water) -Extraction methods (MAE and maceration)	Drying: dried in the shade Size reduction: ground to pass through a 60-mesh size screen	TPC using ethanol (50%) represented 88.298 and 69.027 mg GAE/g extract d.w. via MAE and maceration, respectively.	Rafiee et al. 2011
Pressurized liquid extraction using water and ethanol	Drying: dried at ambient condition (not exposed to solar radiation) for about 50 days (depending on relative humidity). Size reduction: cryogenically ground using liquid nitrogen.	- TPC yielded 58.7 and 45.8 mg GAE/g, using water (200 °C) and ethanol (150 °C), respectively. - Through water extraction, hydroxytyrosol was the principal phenolic component (up to 8.542 mg/g extract). Through ethanol extraction, oleuropein was the principal component (up to 6.156 mg/g extract). - Extraction with water (200 °C), and ethanol (150 °C) showed effective DPPH scavenging activities (EC_{50} = 18.6 and 27.4 µg/mL, respectively).	Herrero et al. 2011
Solvent extraction (80% methanol)	Dried/micronized olive leaves (commercial powders)	- Extraction enabled TPC up to 131.7 mg GAE/g leaves d.w.), total flavonoids with 19.4 mg quercetin equivalents/g, and oleuropein 25.5 mg/g Antioxidant effects: 281.8 mg TE/g, and EC_{50} 13.8 µg/mL using FRAP and DPPH, respectively.	Lins et al. 2018
Effect of drying on supercritical extracts	Drying: conveyer belt dryer (air temperatures range: 50, 60 and 70 °C; residence time: 180, 120 and 60 min). Size reduction: ground with a knife mill for 5 min, and sieved (274 µm particle mean diameter).	Drying at 60 °C for 120 min presented higher TPC (36.1 mg GAE/g d.w.) in supercritical extracts, with 73% DPPH inhibiting activity, EC_{50} = 1.1 µg/mL	Canabarro et al. 2019
- Microencapsulation of olive leaves - Frying methods: starch gluten fried dough added with microencapsulated leaves	Drying: pre-blanched leaves dried in force air oven (at 45 °C for 18 h). Grinding device: windmilled.	- Olive leaf extract: TPC was 25.7 mg GAE/mL extract; oleuropein was 28.4 mg/mL extract: EC_{50} = 0.15 mg GAE/mL extract (DPPH) and 109 µmol TE/mL extract (FRAP). - Highest TPC in atmospheric fried dough containing microencapsulated leaves.	Urzúa et al. 2017
Olive leaf extract (80% ethanol) and fractions	Drying: dried at 40 ± 5 °C for 6 h Size reduction: ground to pass through a 20–30 mesh	- Ethanolic extract (80%) contained TPC (148 mg/g); total flavonoids (58 mg naringin equivalents/g); oleuropein (the main phenol, 102.11 mg/100 g). Rutin, vanillin, and caffeic acid (minor phenols) represented 1.38, 0.66, and 0.31 mg/100 g, respectively. - Among the fractions: butanol fraction showed greatest antioxidant activity with highest TPC (175 mg/g), and flavonoids (75 mg/g).	Lee et al. 2009
Optimization via UAE extraction	Drying: air-dried at 25 °C for 7 days. Size reduction: coarsely ground using mortar and pestle.	Compared to maceration, oleuropein increased (30%) with UAE (70% ethanol, 25 °C, 2 h, solid: solvent ratio of 1:5).	Cifá et al. 2018

Main Processing Factors for Phenolic Extraction	Drying & Size Reduction Prior to Extraction	Key Finding(s)	Reference
Extraction kinetics and temperature with UAE and conventional	Dried in a tunnel microwave dryer (70 °C, 1200 W, 10 min) and ground prior to extraction.	- Oleuropein, TPC, and antioxidant capacity increased with the rise of temperature (through both UAE and conventional). - Oleuropein ranged from 6.48 to 6.65 g/100 g d.w.) through UAE that enabled 88% oleuropein extraction in the 1st min. - Using UAE at low temperature (10 °C) competitively exhibited higher oleuropein (5.71 g/100 g d.w.) in 10 min, compared to the conventional (5.15 g/100 g d.w.).	Khemakhem et al. 2017
Drying of aqueous extracts (freeze-drying and spray-drying)	Leaves (after being washed) kept in the shade (48 h), and ground (80-mesh screen).	- Freeze-dried extracts: TPC (446.63 mg GAE/g d.w.), total flavonoids (298.16 mg quercetin/g), tannins (117.32 mg GAE/g), with 96.57% antioxidant activity. - Spray-dried extracts: TPC (442.84 mg GAE/g d.w.), flavonoids (396.4 mg quercetin/g), tannins (128.71 mg GAE/g), with 96.05% antioxidant activity.	Ghelichkhani Et al. 2019
Optimization of extraction conditions including drying methods and solvent types/ratio	Drying methods: shade-drying; microwave (2450 MHZ, 80 sec); and vacuum (– 0.5 bar, 55 °C, 24 h). Size reduced by grinding.	- Microwave drying of fresh leaves provided the highest TPC (6.45 g GAE/100 g dried leaves). - Favorable extraction conditions (40% ethanol 60 °C, 120 min) enabled high antioxidant activity (IC_{50} = 18.92 µg/mL), with a TPC around 6.63 g/100 g.	Wissam et al. 2016

Among the advanced milling methods includes superfine grinding (micronization), which has shown to have great potential for increased surface area, uniform size reduction, bulk density, and flowability. This type of grinding, compared to the conventional methods, may exert greater physicochemical changes, dispersion, and solubility (Gao et al. 2019). Particle size reduction, driven by superfine grinders are typically in the range of 100 μm to 0.001 μm (Gao et al. 2020). Among the milling devices commonly used for micronization include jet mills, ball mills, vibration mills, agitated media, and roller mills (Gao et al. 2019). Numerous studies demonstrate the significant potential of superfine grinding for improved bio-functional and physicochemical properties of phytonutrients in a broad range of foods. Example: olive pomace byproducts (Speroni et al. 2019), red grape pomace (Zhao et al. 2015), pomegranate peels (Zhong et al. 2016), rice bran (Zhao et al. 2018), persimmon by-products (Ramachandraiah et al. 2016), Quercus salicina Blume leaves (Hong et al. 2021), and ginseng species (Jiang et al. 2020).

Research demonstrates that finely ground stevia leaves (with a particle size of 200 μm) facilitate high recovery of the target bioactive compounds during ultrasound extraction within a shorter time, which may signify the occurrence of mass transfer during grinding prior to the extraction process (Alsaud & Farid, 2020). On the other hand, in the research of Chen et al. (2015), there was no significant difference between flavonoid content in the superfine ground mulberry leaves and those obtained from coarsely ground leaves. This may be indicative of the ineffectiveness of the grinding temperature used (45 °C) on the extractability of these phenolics (Chen et al. 2015). Some studies suggest that the micro-size pulverization may have adverse effects on the extraction rate and bioactivity of some functional nutrients. This in part depends on the nature of the food and the milling conditions, together with others. For instance, green tea represented reduced proportions of catechins, and total phenols when it was ground to a finer particle size (Hu et al. 2012). In their research, Tchabo et al. (2018) through optimization of phytonutrient properties of aqueous extracts of mulberry leaves, found that the particle size less than 2 μm may bring about oxidation; partly due to the exposure of the released biomolecules to the oxidizing agents.

In the case of olive leaves, there is limited information on micro-size particles and their effects on extraction efficiency. Lins et al. (2018), through their research on antioxidant activity (in vitro), used dried micronized olive leaves (**Table 5.1**) extracted by 80% methanol (agitated for 170 rpm at 25 °C). Among the main findings of their study include the improved concentration of total phenolics (131.7 mg GAE/g leaves d.w.), oleuropein (25.5 mg/g leaves d.w.), and flavonoids (19.4 mg quercetin equivalents/g leaves d.w.). On the other hand, different from what is generally expected, the particle size reduction less than 0.2 mm is reportedly disadvantageous for olive leaf extraction, causing a reduction in the extractability

(Stamatopoulos et al. 2014). This may be attributed to the potential agglomeration of primary particles that adversely affect solvent permeation, and hence render the extraction difficult (Stamatopoulos et al. 2014).

Cryogenic grinding, namely freezer grinding or cryomilling (Gao et al. 2020), is among the advanced grinding methods that allows finer particle size via grinding operation at extremely low temperature, often making use of liquid nitrogen (Junghare et al. 2017; Saxena et al. 2013). Compared to traditional methods, it offers advantages, among which include enabling (i) very small, evenly distributed particle size, (ii) increased yield, (iii) economically viable milling operation, (iv) no thermal damage—makes use of a cryogenic mechanism and develops embrittled/fractured products (Karam et al. 2016; Saxena et al. 2013; Saxena et al. 2016), and (v) hindrance to the formation of oxide layer on the particle surface (Gao et al. 2020; Bellik et al. 2019). Numerous research studies performed investigations on the effect of cryogenic grinding on the extraction yield and bio-functional activity. Saxena et al. (2016), found improved concentration of total phenolics in the cryogenically ground fenugreek seed extracts (ranging 75.72–94.03 mg GAE/g depending on the genotype variations) compared to the non-cryomilled samples. Sharma et al. (2015) observed enhanced recovery of polyphenols from cryomilled ajwain seeds and the maximum recovery of TPC (in genotype samples extracted by dimethyl sulfoxide) was around 168.0 mg GAE/mL of crude seed extract. Saxena et al. (2013), through their investigation on the quality and antioxidative profile of coriander, observed total phenolic recovery that, depending on the genotypes examined, ranged between 32.44 to 92.99 mg GAE/g crude seed extract of cryomilled samples.

Among the limited studies on cryogenic grinding of olive leaves includes the research of Herrero et al. (2011), wherein olive leaves were cryomilled in advance of pressurized liquid extraction (PLE) using water (200 °C) and ethanol (150 °C) and yielded TPC around 58.7 and 45.8 mg GAE/g extract, respectively (**Table 5.1**). The principal components following extractions with water and ethanol were hydroxytyrosol and oleuropein, respectively. In another research study, the cryo-ground olive leaves, prior to subcritical water extraction at 200 °C, contained TPC of around 77.84 mg GAE/g extract (Herrero et al. 2012). The evaluations of these studies are significantly valuable; having said that, there is still uncertainty regarding the effects of the milling method (cryogenic) on the resulting extraction of each class of phenolics.

Selection of the milling operation for particle size reduction seems to play a decisive part in the quality and extractability of bio-compounds. The process of extraction kinetics, particularly through the washing phase and diffusion phase, is greatly reliant on the changes that occur in the cellular structure (disruption/intactness of the tissue cells). The rate of extraction during the washing process is potentially

accelerated by the increased surface area of the food tissue. Khemakhem et al. (2017) indicated that the enhanced extraction rate during the washing phase is partly attributed to the effects of the particle size reduction in olive leaves that were initially milled prior to the extraction process (**Table 5.1**).

5.2.3. Blanching

Blanching, as a thermal pretreatment, can be used at the initial point of olive leave extraction, often in advance of the drying operation. Blanching helps alter/weaken the cellular structure of food tissue and potentially enhance the overall extraction efficiency (Xiao et al. 2017; Otero et al. 2020).

Among various thermal blanching are conventional (hot water blanching), steam blanching, ohmic heat blanching, microwave blanching, and superheated steam impingement blanching (Deng et al. 2019). The potential effectiveness of blanching in promoting the extraction yield is partly indicative of inactivating polyphenol oxidase, the enzyme prominently responsible for the reduction/oxidation of bio-phenols (Zeitoun et al. 2017). The study by Zeitoun et al. (2017) describes that the blanching of olive leaves (90–95 °C for 20 s), extracted with 70% ethanol, exerts effects on the liberation of total phenols (593.0 μg GAE/g, around 61.70% increase) which was also positively correlated with greater antioxidant activity.

Hot water blanching of olive leaves, as an efficient thermal pretreatment, has a potential for improved extraction of phenolics such as oleuropein that is reportedly 8-fold higher compared to that released from non-blanched leaves (Stamatopoulos et al. 2012). In a recent study, Sucharitha et al. (2019) observed an improved extraction of oleuropein up to 35–38% from hot water-blanched olive leaves (50–70 °C for 10 to 30 min). However, blanching by hot water may cause leaching or dissolution of some phytonutrients (particularly hydrophilic compounds), together with other downsides inherent in generating wastewater and the need for increasing the drying time (Stamatopoulos et al. 2012).

Steam blanching is generally considered a better alternative to hot water blanching. Stamatopoulos et al. (2012), from their experiments, used steam blanch pretreatments on olive leaves for 10 min, followed by solvent extraction (70% ethanol) at 40 C for 30 min, and observed increased oleuropein extraction that reached up to 35-fold greater than the corresponding components from non-steam-blanched samples. The authors also investigated the particle size of fresh olive leaves as the main predictor variable to optimize steam blanching (**Table 5.1**) from which a higher amount of oleuropein (8.28 g/kg leaves d.w.) was obtained from the leaves with 20–11 mm particle size blanched for 10 min. This technique appears to have great potential. The performance of broader investigations to assess its correlation with other preprocessing factors (e.g., drying and size reduction) may help devise a viably scalable design that potentially (i) allows minimal loss of water-soluble molecules, and (ii) enables

optimum efficiency during drying, via reducing the drying time and suppressing excessive break-up of the cell membrane that may otherwise render the moisture mobility difficult during the drying operation.

Blanching and drying may favorably/unfavorably affect the rate of phenolic extraction interdependently. Nobosse et al. (2017) examined the influences of steam blanching and drying methods on the composition and bioactivities of phytonutrients in Moringa leaves. Their study suggested that drying (solar and electric drying) may have adverse effects on the loss of phenolics in both blanched and fresh leaves (the extent of loss was greater in blanched/dried samples). The blanched/undried samples contained TPC of around 3.40% while the blanched leaves/solar dried and blanched/electric dried represented 2.32% and 2.67%, respectively. Moreover, it was found that the fresh leaves (non-blanched/undried) represented TPC of around 3.28% which was rather close to those in blanched/undried ones.

A novel study patented by Musco et al. (2017) describes the production of supreme quality olive leaf powder through infrared dry blanching (concurrent use of blanching and drying) to produce olive leave powders with an improved yield of soluble polyphenols and greater antioxidant capacity, applicable to various industrial (food/non-food) uses. Boudhrioua et al. (2009), through their research, used blanching in advance of drying of four types of olive leave cultivars (Chetoui, Chemlali, Zarrazi, and Chemchali) and highlighted that the variability of phenolic concentrations across different cultivars is partly dependent on the temperature variations of drying (infrared). For instance, the increment of polyphenols at a lower drying temperature (40 °C) was achieved in all groups of leaves (excluding Chetoui). It was also reported that the non-blanched/dried samples (Chemlali cultivars) required less drying time, compared to the blanched/dried ones, to reach the final/desired moisture content.

Olive leaves may have bio-functional potential for enrichment of other types of foods, such as edible/vegetable oils. Research by Majetic Germek et al. (2019) compared the effects of adding fresh and dry/steam blanched olive leaves to rapeseed oil. It was observed that the addition of fresh leaves showed potential for phenolic increment, while the dry/steamed blanched leaves had great ability to increase chlorophylls in the oil. Another study by Gonzáleza et al. (2019) assessed phenolic properties from encapsulated olive leaf extracts (using sodium alginate through spray drying), wherein the leaves were initially blanched at 95 °C for 4.5 min and dried at 45 °C prior to extraction with 50% ethanol. Although the blanching factor was not considered as the target predictor variable for their study, it was found that the encapsulated extracts had significant potential for bio-accessibility and bioavailability (58% and 20%, respectively).

5.3. Future perspectives

A sizeable proportion of research has emphasized the importance of various parameters associated with phenolic extraction from olive leaves. There are numerous challenges involved to achieve a sustainable extraction. Among other factors is the importance of size reduction, drying, and blanching which have been somewhat less studied (as the main predictor variables) compared to other substantial factors. More extensive investigations can help determine the magnitude of their effectiveness in the extractability and quality of the desired components. There needs to be clear conclusions on the optimal conditions of particle size reduction, which may entail a comparative study between coarse particles and micronized particles, and to identify their effects (benefits or impacts) on the extraction.

It may also be reasonable to perform research based on an interface between drying and grinding, to determine the effect(s) of particle size reduction on the efficiency of further processing performance, e.g., the secondary drying employed after the extraction process (to dry aqueous extracts). Moreover, the choice of blanching approach is potentially dependent on the type of the selected drying, and vice versa. This can further annotate the reason for gaining further knowledge of the intercorrelations between various unit operations involved at the pre-extraction step. In addition, a deeper knowledge may be required from the perspective of determining the rate of phenolic content in pretreated leaves before the actual extraction process and comparing it with the corresponding compounds obtained after completion of the extraction process. This information may be of great value due to the possibility of a significant release of compounds on pretreated samples (without being subjected to the extraction) that may be quantitatively comparable to the yield(s) after the extraction process.

Many research studies have found novel solutions to optimally valorize olive leaves through various emerging extraction means. One of the main factors considered in numerous experiments is the appropriateness of extraction solvent type/ratio which comes with a significant challenge. The use of water as an eco-friendly solvent is undoubtedly desirable, primarily due to its non/less environmental/health hazard, non-toxicity, and low expenditure (Castro-Puyana et al. 2017; Ghoreishi & Shahrestani, 2009). Although using water for phenolic extraction may be less preferred due to its inherently weaker extraction efficiency when the extraction temperature is low (Difonzo et al. 2017), it is reportedly effective in the increased extraction of total/some phenolics at higher temperatures. For example, using water at 90 °C enhanced phenolic extraction from grape seeds (Paladino & Zuritz, 2011), and increased recovery of oleuropein and hydroxytyrosol from olive leaves (Monteleone et al. 2021). As shown in **Table 5.1**, the total phenolic content in hot air-dried olive leaves (120 °C) extracted by water was comparable to those by ethanol solvents (Difonzo et al. 2017). In this respect, it may be of value to

perform deeper research to interact the extraction ability of water solvents with the key parameters in preprocessing operations, including the temperature(s) selected for drying fresh leaves.

Future studies should potentially involve a broad range of evaluations on the valorization of olive leave residual biomass, among which may include an integration of preprocessing and processing that may partly help formulate a feasible processing system to deliver better extraction performance and greater sustainability for olive leave reutilization.

5.4. Conclusions

The extraction of bioactive compounds such as polyphenols from olive leaves has been largely investigated. In this regard, many research studies have focused on factors involved directly in the extraction process and their effects on the optimum extractability of natural nutrients from olive leaves. These studies, while being significantly valuable, seem to have relatively overshadowed the importance of the roles of preprocessing means in the overall extraction efficiency and quality of the final product. Drying and grinding are often considered vital unit operations and have been incorporated into many experiments of olive leave extraction, but there is limited information available in the literature specifying their influences on the extraction.

Preprocessing of olive leaves, depending on the selected extraction approach, may play a decisive role in the improvement of extraction efficiency. Among others, they exert improved surface area, mass transfer, and extraction yield/acceleration. Size reduction is a major preprocessing operation that requires further research to gain a deeper understanding of the effect(s) of grinding parameters. Drying and blanching, although have been the topic of some assessments, need further evaluations that can help provide a better understanding of the magnitude of their effects. Moreover, a comparison study before and after extraction can potentially help understand the extraction yield through the grinding (prior to the extraction stage) and after the extraction process. This may help reinforce the overall extraction system, regardless of the extraction method (conventional or emerging).

Given the limited information available on the effects of size reduction, drying, and blanching on the overall extraction of phenolics from olive leaves, together with the possibility that the extraction of bio-compounds may be significantly influenced by these operations, more extensive research studies are needed in this respect. The findings may partly contribute to the development of innovation patterns for the valorization of olive leaves wherein a more advanced processing system may enable sustainable extraction of bio-functional materials such as polyphenols from this residual biomass generated from both agricultural and industrial activities of olives.

5.5. References

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CHAPTER 6

EFFECT OF PROCESSING ON PHENOLIC COMPOSITION OF OLIVE OIL PRODUCTS AND OLIVE MILL BY-PRODUCTS AND POSSIBILITIES FOR ENHANCEMENT OF SUSTAINABLE PROCESSES

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Review

Effect of Processing on Phenolic Composition of Olive Oil Products and Olive Mill By-Products and Possibilities for Enhancement of Sustainable Processes

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Abstract: The bio-functional properties of olive oil products and by-products rely greatly on the proportions and types of the endogenous phenolics that may favorably/unfavorably change during various processing conditions. The olive oil industrial activities typically produce (i) olive oils, the main/marketable products, and (ii) olive mill by-products. The mechanical processing of olive oil extraction is making progress in some areas. However, the challenges inherent in the existing system, taking into consideration, the susceptibilities of phenolics and their biosynthetic variations during processing, hamper efforts to ascertain an ideal approach. The proposed innovative means, such as inclusion of emerging technologies in extraction system, show potential for sustainable development of olive oil processing. Another crucial factor, together with the technological advancements of olive oil extraction, is the valorization of olive mill by-products that are presently underused while having great potential for extended/high-value applications. A sustainable re-utilization of these valuable by-products helps contribute to (i) food and nutrition security and (ii) economic and environmental sustainability. This review discusses typical processing factors responsible for the fate of endogenous phenolics in olive oil products/by-products and provides an overview of the possibilities for the sustainable processing to (i) produce phenolic-rich olive oil and (ii) optimally valorize the by-products.

Keywords: processing; phenolics; olive oil products; olive mill by-products; sustainability; extraction.

6.1. Introduction

Polyphenols, the secondary metabolites and the predominant groups of phytonutrients in plants, are highly valued for their bio-functional properties and defense mechanisms. Among other benefits, they confer antioxidative (Swallah et al. 2020) and antimicrobial activities (Tanase et al. 2019; Pandey & Rizvi, 2009). Of all the plant-derived foodstuff, the unique healthful and organoleptic attributes of olive oil products have noticeably caught the global attention, signifying their distinctive chemical and molecular characteristics (Banilas & Hatzopoulos, 2013). In this regard, the agro-industrial activities of olive crops, which have been traditionally and economically substantial particularly in the Mediterranean region, have progressively increased primarily for olive oil production (Khdair & Abu-Rumman, 2020; Ghanbari et al. 2012). Olive oil, on top of being a good source of unsaturated fatty acids (around 72% monounsaturated fatty acids primarily oleic acids, and 14% polyunsaturated fatty acids (Barjol, 2013), is highly valued as a source of minor bioactive compounds including phenolics (Agrawal et al. 2017; Cicerale et al. 2012). The bio-functional potential of phenolics, however, relies greatly on their proportion, molecular structure/interactions, and chemical metabolism (Balasundram et al. 2006). In the olive oil industry, there are different classes of olive oil products, typically characterized by the specified/standard quality parameters. The main groups, among others, include extra virgin olive oil (EVOO),virgin olive oil (VOO), and refined olive oil (Barjol, 2013). Of all designated groups, EVOO is known as the highest quality, containing total polyphenols in the range of 50 to 1000 mg/Kg, and acidity level below 0.8 g/100 g (Jimenez-Lopez et al. 2020). The functional potency of olive oil partly relies on the quantities of specific groups of endogenous phenols (Medina et al. 2006) that are increased/decreased depending on the various pre/postharvest activities. In this respect, there has been a growing research trend towards the exploration of the fate of endogenous bioactive molecules, particularly polyphenols, in olive oil and its by-products, through various agro-industrial steps. Together with other factors, the industrial processing and storage conditions are highly influential in the degradation/formation of polyphenols (Jimenez-Lopez et al. 2020, Diamantakos et al. 2020). The initial point of the optimum preservation of endogenous phytonutrients relies on a good handling of raw materials that may be achievable through a minimum/careful storage time. Ideally, the storage time for the harvested crops to be processed for olive oil extraction should not exceed 48 h (Boskou, 2008). The prolonged storage of harvested olives may give rise to spoilage (oxidative and/or hydrolytic) in the final product (Boskou, 2008). Together with the storage factors, the processing parameters during olive oil extraction, particularly malaxation temperature/time, plays crucial roles in the phenolic composition of the extracted oil, which is further outlined in more details in **Section 5.2**. Olive tree farming, in addition to the target/marketable products,

generates a range of waste stream/by-products (**Figure 6.1**) that is considered to be a good source of bio-functional molecules but they are currently underused for limited/low-value applications, signifying the inefficiency of the processing system currently used for their extraction/recovery.

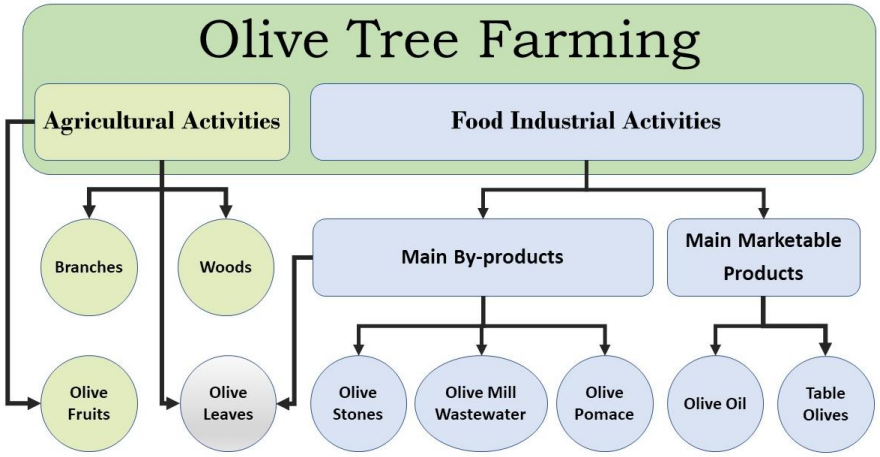

Figure 6. 1 Major products and by-products produced from agro-industrial activities of olive fruits.

Green technologies show potential for delivering optimal processing of olive oil products, and sustainable valorization of olive mill by-products, Although, there is a trend towards the processing developments in olive oil industry but, having said that, the proposed emerging methods have not quite reached the production system. The existing mechanical operations commonly used in both olive oil production and by-product reutilization often represent (i) less efficiency of the processing of the main products, and (ii) low value applications of the processed by-products. This review describes the role of the processing on the fate of major phenolics in olive oil products and by-products and emphasizes the possibilities for a sustainable processing system that may enable achievement of the increased recovery of health-promoting phenols in olive oil, as well as improved valorization of olive mill by products/residues that have the potential for high-value applications across various industrial sectors.

6.2. Olive oil products

Overview of Characteristics of Typical Endogenous Phenolics and the Effect of Processing Conditions on Their Loss/Gain

The phenolic constituents of olive oil typically include oleocanthal, oleacein, oleuropein, hydroxytyrosol, and tyrosol. Of all endogenous phenolic groups, oleocanthal (decarboxymethyl ligstroside aglycone), a representative secoiridoid, is considered distinctively beneficial, particularly with respect to its anti-inflammatory effects, which are comparable to those exerted by ibuprofens (Beauchamp et al. 2005; de Medina et al. 2017; Demopoulos et al. 2015; Karković Marković et al. 2019). Oleocanthal potentially plays a part in curtailing the activities of inflammation-induced enzymes such as

cyclooxygenase-2 (COX-2) (Beauchamp et al. 2005; Karković Marković et al. 2019; Perona & Botham, 2013; Carpi et al. 2019). A daily consumption of 50 g of extra virgin olive oil, which typically contains around 10 g oleocanthal, is reportedly proportionate to 10% of the low/daily dosage of ibuprofen recommended for adult consumption to treat soreness (Cicerale et al. 2012). Moreover, like other COX-2 inhibitors, it has been suggested that the regular consumption of this natural ingredient competitively confers protection against chronic disorders including cardiovascular and some types of carcinogenic diseases (Cicerale et al. 2012). Further, this healthful ingredient is potentially inhibitory against progressive brain disorders such as Alzheimer's disease (Parkinson & Cicerale, 2016).

Another representative secoiridoid in olive oil is oleacein (3,4-dihydroxyphenylethanolelenolic acid dialdehyde), which possesses a molecular structure relatively similar to that in oleocanthal but slightly differs in terms of the hydroxyl group (—OH), where oleacein bears one further —OH (Demopoulos et al. 2015). This component significantly exhibits antioxidation (Demopoulos et al. 2015) and anti-inflammation (Filipek et al. 2020), and it reportedly contributes to the suppression of platelet aggregation, partly through hindering the action of 5-lipoxygenase (5-LOX) enzymes (Agrawal et al. 2017). The antioxidative activity exerted by oleacein has been justified in the literature in a way that it may competitively be greater than that exhibited by hydroxytyrosol and oleuropein (de Medina et al. 2017; Paiva-Martins et al. 2009). From the perspective of sensory attributes in extra virgin olive oil, oleacein contributes more to the bitterness (Demopoulos et al. 2015) rather than the pungency/peppery sensation that is more attributable to oleocanthal (Cicerale et al. 2012). Oleacein generally manifests low storage stability and has less heat stability compared to oleocanthal (Demopoulos et al. 2015).

In the literature, much research has been carried out to re-evaluate various processing conditions to optimize recovery of phytonutrients in the extracted oil. The proportions and discrepancies of intrinsic phenolic constituents rely partially on the mechanical operations used for the extraction of olive oil (Servili et al. 2014). The extraction of olive oil primarily involves the exertion of oil release that is predominantly present in the mesocarp and to a lesser extent in endocarp of the olive fruits (Di Giovacchino, 2013). An optimum oil extraction may be achieved following (i) disintegration of the cell membrane of mesocarp through the crushing step, and (ii) progression of the oil droplet coalescence through the malaxation (mixing) step (Di Giovacchino, 2013; Kalogianni et al. 2019). A typical example highlighting the significant roles of the processing in phenolic composition is the variability of oleocanthal concentrations among various extra virgin olive oils, which can be as little as 0.2 mg/kg or as great as 498 mg/kg oil (Cicerale et al. 2012). Through their research Cicerale et al. (2012) observed that inclusion of pits during crushing and malaxation result in lower concentration of oleocanthal (around 43.8 mg/kg oil). These authors

reported that the extracted oil through the crushing of de-stoned pulp represented higher percentage of oleocanthal (around 54.8 mg/kg oil). The pressure of crushed stones in olive paste may unfavorably cause peroxidase activity and oxidative damage, which brings about reduction of the phenolic proportion in the extracted olive oil (Servili et al. 2007). Oleocanthal is also susceptible to the light exposure, which occurs often during storage (Demopoulos et al. 2015).

The proportion of each phenolic compound also varies in different classes of olive oil. Hydroxytyrosol, a phenylethanoid, yields considerably greater in EVOO compared to those in refined olive oil, representing around 14.32 and 1.74 mg/kg oil, respectively (Martínez et al. 2018). Hydroxytyrosol, even being present in low concentration in olive oil (Romani et al. 2019), is highly prized for its pronounced bioactive effects and exhibits relatively high oxidative stability in virgin olive oil (Nissiotis & Tasioula-Margari, 2002), while some other groups of endogenous antioxidants such as tyrosol and ligstroside aglycone exhibit less potency in this respect (Boskou, 2008). The antioxidative potency of hydroxytyrosol has also been reported to be greater than that exerted by butylated hydroxytoluene (BHT) (Papadopoulos & Boskou,1991), which further merits its utilization as a natural antioxidant alternative. The bioconversion of tyrosol into hydroxytyrosol has been studied by numerous researchers. Bouallagui & Sayadi (2018) performed research on the catalyzing influence of the whole strain of bacterial cells of Pseudomonas aeruginosa, grown on tyrosol using a fermenter, on the conversion of tyrosol to hydroxytyrosl, finding 86.9% bioconversion recovery. Azabou et al. (2007) observed optimized recovery of hydroxytyrosol (64.36%) through using photocatalytic oxidation of tyrosol.

The industrial processing of olive oil commonly uses thermal application during malaxation of olive paste, which is effective in gaining an improved extraction of oil from olive pulp through the action of coalescence of oil droplets (Diamantakos et al. 2020). The proportion of phenolics in olive oil partly reflects the rate of their solubilization and chemical/enzymatic reactions, which are highly affected by the processing conditions including malaxation parameters (Diamantakos et al. 2020). Chemical alterations occur when olive paste is in contact with the air during malaxation, which may adversely develop degradation/oxidation of aglycones (the non-sugar groups of glycosides), typically by enzymatic activities (Boskou, 2008).

The increased malaxation temperature may foster catalyzing reactions of some oxidative enzymes such as polyphenol oxidase and peroxidase (Diamantakos et al. 2020). The use of heating during mixing process may be favorable or detrimental to the resulting phytonutrients of the end products. Some phenolics such as oleocanthal are relatively heat-stable, and this is particularly the case when significant concentration of oleocanthal is present at the early stage of recovered extra virgin olive oil (Demopoulos

et al. 2015). de Torres et al. (2018) found increased proportion of oleocanthal (289.4 mg/kg), as well as hydroxytyrosol (7.83 mg/kg), with the rise of temperature of up to 60 ◦C. By contrast, concentrations of phenolics such as oleuropein, ligstroside aglycone (Lukić et al. 2018), luteolin, and flavonoids (de Torres et al. 2018) have shown reverse correlation with the temperature increase.

The malaxation time comparably is a decisive factor for phenolic status in the oil (Diamantakos et al. 2020). Gomez-Rico et al. (2009) observed a 70% increase of C6 aldehydes (a distinctive volatile component of olive oil (Karagoz et al 2017), predominantly (E)-2-hexenal (that is characterized by green leaf and apple sensory attributes), when the malaxation time was exceeded from 30 to 90 min. Using an extended kneading time provides an opportunity for the increased dispersion of the oily phase and liberation of the volatile compounds (Gomez-Rico et al. 2009). The study of Miho et al. (2020) demonstrated an increased concentration of oleacein and oleocanthal in virgin olive oil with the increase of malaxation time. However, these authors reported a noticeable reduction of isomers of oleuropein aglycone (3,4-DHPEA-EA) and ligstroside aglycone (p-HPEA-EA) in the oil after using a prolonged malaxation. Likewise, total phenolic concentration is reportedly reduced within lengthy malaxation time (Diamantakos et al. 2020).

The main processing steps involved in olive oil production consist of cleaning of harvested olives, crushing of olives, mixing of olive paste, and phase separating. The separation of olive oil uses mechanical operations either based on (i) the conventional approach, using discontinuous pressing system, or (ii) the modern approach, using continuous centrifugation system (Di Giovacchino, 2013; Zbakh & Abbassi, 2012). **Table 6.1** provides a comparison between the main separating techniques often used for the extraction of olive oil. The conventional approach is typically equipped with millstones that favorably use less crushing time via gentle/low-speed spinning, and thus the incidence of emulsion is potentially impeded, enabling improved coalescence and increased oil recovery (Di Giovacchino, 2013). However, together with other downsides inherent in the labor intensity and inefficient system operation, including low working capacity, include the potential contamination of the filter mats and the need for a strict hygiene routine (Di Giovacchino, 2013).

The modern centrifugation system often uses either a two-phase or a three-phase decantation (Di Giovacchino, 2013; Vekiari et al. 2007; Khdair et al. 2015). The system commonly employs metal crushers that help increase extractability of the oil and recovery of total phenolics (Di Giovacchino, 2013). However, the emulsion intensity and development of bitter taste in the extracted oil are of typical disadvantage. In general, application of the two-phase decanters in place of a three-phase centrifugation system eliminates/minimizes the use of water addition to olive paste and enables obtaining an increased

recovery of polyphenols in the extracted oil (Khdair et al. 2015; Ammar et al. 2014; Kalogeropoulos & Tsimidou, 2014).

Table 6. 1 Comparison of phase separating techniques for olive oil extraction.

Phase Separation Method *(Principle and Outputs)*		Benefits	Downsides	Reference
Conventional Approach *Discontinuous system*	**Pressing** (***using millstones***) *Outputs*: - Pomace - oil - Wastewater	Less crushing time Less emulsion Improved coalescence Increased oil recovery Low energy consumption Low moisture-content pomace	Labor intensity Discontinuous/inefficient system Low working load Needs strict hygienic routines	Khdair & Abu-Rumman, 2020 Di Giovacchino, 2013 Ouazzane et al. 2017
Modern Approach *Continuous centrifugation decanting*	**Two-phase** *Outputs*: - Wet pomace - Olive oil	Eliminates use of water Greater phenolic recovery High quality/yield of oil Less use of energy No generation of wastewater	High moisture-content pomace Lower working load Organoleptic acceptance (too bitter/pungent)	Di Giovacchino, 2013 Zbakh & Abbassi, 2012 Khdair et al. 2012
	Three-phase (***Addition of water***) *Outputs*: - Olive cake - Olive oil - Wastewater	High working load Automated system Less labor/production cost High quality/recovery of oil Moderate moisture-content pomace	Need additional use of water and energy Generation of wastewater Lower phenolic recovery Wastewater management cost	Di Giovacchino, 2013 Zbakh & Abbassi, 2012

Furthermore, other accountable factors such as the appropriateness of analytical methods selected for the quantification of endogenous phenols play a decisive part in the precision and reliability of the obtained results. Application of high-performance liquid chromatography (HPLC), as an official method to measure individual phenolics of olive oil, uses polar solvents including methanol and water. This method may not be an ideal approach for accurate analysis of dialdehyde phenols including oleocanthal, oleacein, and derivatives of hydroxytyrosol and tyrosol due to their potential reactions with the above-mentioned solvents, giving rise to peak broadening and possible developments of hemiacetal and acetal derivatives (Karkoula et al. 2012; Karkoula et al. 2014; Killday et al. 2014; Melliou et al. 2015). However, the research of de Medina et al. (2017) examined the possibility of conversion of oleocanthal and oleacein into hemiacetal and acetal derivatives through liquid chromatography with tandem mass spectrometry (LC–MS/MS) and found that the methanol/water had no/little effects on the formation of these artifacts. Indeed, a slight proportion was detected when methanol gradients were applied under acidic conditions at the stage of the chromatographic separation. Their investigation postulated the use of acetonitrile for the extraction step and methanol-based gradients for the chromatographic separation step as a suitable approach for measuring oleocanthal and oleacein in olive oil.

Further, nuclear magnetic resonance (NMR) spectroscopy, using deuterated solvents, has been viewed as an ideal analytical method for accurate measurements of secoiridoid aldehydes (Diamantakos

et al. 2020; Karkoula et al. 2012; Killday et al. 2014). The use of NMR has shown effectiveness and selectivity in the detection of some isomeric aglycones that may not be detectable through a normal and/or reversed-phase chromatography as they inherently transform to other types of aglycone isomers. This is potentially indicative of the interaction of extracted oil with the silica-based stationary phase (Diamantakos et al. 2020; Diamantakos et al. 2015). Diamantakos et al. (2020), in their research on investigation of some key factors (including malaxation time/temperature) on the concentrations of phenolics of EVOO from various cultivars, used NMR for the quantification of secoiridoid phenolics. Together with oleocanthal and oleacein, other major secoiridoid derivatives including oleomissional and oleokoronal were detected and quantified. These compounds are the recently known isoforms associated with oleuropein aglycone and ligstroside aglycons which are well detected by means of NMR method (Diamantakos et al. 2020; Diamantakos et al. 2015). In this regard, it is essential to employ appropriate analytical techniques for phenolic measurements because using inapplicable methods may lead to misinterpretation of phenolic proportions in olive oil.

6.3. By-products of olive oil industry

The growing industrial production of olive oil that is partly in response to the increased global desire (Khdair et al. 2015), has resulted in a massive generation of a varied range of byproducts/waste streams (**Figure 6.1**). Among others, they include liquid waste (olive mill wastewater), and solid waste (olive pomace) (Khdair et al. 2015). The discharge of these processing biomass, particularly the liquid effluent, represents (i) environmental impact, causing toxicity, contamination, and pollution (Khdair et al. 2015; Dutournié et al. 2019), and (ii) economic damage to the respective manufacturers (Gullón et al. 2020).

6.3.1. Functional potential and processing considerations for extraction

The bioactive potency of natural phenols present in olive mill by-products has been well reviewed in the literature. Their phenolic concentration is reportedly much greater than those remain in olive oil products, representing around 98% and 2%, respectively (Alu'datt et al. 2010). Their exploitation not only helps address the environmental issue but is of benefit to provide natural bio-ingredients with value addition potentials that enable a sustainable re-use for food or non-food applications (Rodrigues et al. 2015). As an example, research has demonstrated the favorable effectiveness of incorporating the extracted phenols such as hydroxytyrosol and oleuropein into other food products such as vegetable oils to promote their functional/nutritional properties (Gullón et al. 2020). However, the achievement of a sustainable exploitation system is highly dependent on the appropriateness of the mechanical extraction techniques (Rodrigues et al. 2015). This is partially because the optimum recovery of target phenolics

may be hampered due to (i) complexity of molecular structure of phenolics, as they are often attached to glycone (sugar) or protein groups, and (ii) variability of biochemical pathway and the possibility of unfavorable formation of some phenolic derivatives that may impede the optimum recovery of desired compounds (Brglez Mojzer et al. 2016).

The traditional types of phenolic extraction include Soxhlet, hydro-distillation (Gullón et al. 2020), and solvent extraction methods (Alu'datt et al. 2010). These methods are still widely used in the agro-industrial system partly because of their simplicity, flexibility, and versatility (Brglez Mojzer et al. 2016). Application of innovative technologies, which are somewhat being adopted in some areas, potentially enables the achievement of (i) operation efficiency (Lama-Muñoz et al. 2019), (ii) improved quality, (iii) productivity with lower cost, and (iv) environmental sustainability (Brglez Mojzer et al. 2016; Lama-Muñoz et al. 2019). Among the key processing paraments of extraction include extraction time/temperature and solvent type/ratio (Brglez Mojzer et al. 2016). Together with other disadvantages of the conventional system inherent in the need for prolonged extraction time and/or high temperature is the need for using relatively high proportion of solvents (such as methanol and acetone). To date, numerous emerging methods such as microwave-assisted extraction (MAE) (Gullón et al. 2020), ultrasound-assisted extraction (UAE) (Gullón et al. 2020; Brglez Mojzer et al. 2016), infrared-assisted extraction (IR-AE), membrane separation, and supercritical fluid extraction (SFE) (Gullón et al. 2020) have been recommended to overcome some of the challenges associated with the conventional/existing methods. A typical advantage, among other things, includes using safe/organic solvents such as supercritical carbon dioxide, water, and ethanol (Tyśkiewicz et al. 2018).

The suitability of the extraction system to enable optimum recovery of the given compounds also relies on the solubility and polarity of target molecules (Tyśkiewicz et al. 2018). For example, some organic solvents such as hexane may not be applicable for the extraction of polar phenolics, producing poor solubility and low yield recovery (Tyśkiewicz et al. 2018). Moreover, using a single organic solvent may act inadequately on the efficiency of the diffusion rate/mass transfer of polar compounds. To tackle this hurdle, researchers have suggested some alternatives, such as using selective solvents proportionally, e.g., water mixed with ethanol or acetone (Brglez Mojzer et al. 2016). The functional potential and processing considerations for the main types of olive oil by-products are outlined as follows:

6.3.1.1. Olive leaves

Olive leave by-products are not only accumulated during the agricultural/pruning activities but are massively generated through the industrial activities of olive oil production, which account for 5% (Guinda et al. 2015) up to 10% (Fki et al. 2020) of overall weight of olives harvested for processing. These biomass

residues are presently underexploited and usually being re-used as animal feed (Moreno et al. 2020; Rahmanian et al. 2015) while having a great potential for high-value addition owing to their good source of bioactive compounds.

Olive leaves are known to be markedly rich in oleuropein (a phenolic secoiridoid) in part because they do not undergo the extraction processing of olive oil. Indeed, they are typically removed at the preliminary stage of olive oil extraction, prior to the milling/crushing operations. In this regard, oleuropein constituents that are liable to be degraded/hydrolyzed during oil extraction potentially remain intact in olive leaves (Zoidou et al. 2017). The presence of valuable natural phytonutrients such as oleuropein (around 14% dry basis) and oleanolic acid (around 3% dry basis) in olive leaves (Guinda et al. 2015) has currently led to the extensive research studies, with particular attention to the design formulation of extraction methods/parameters to ideally liberate the antioxidants of interest (Souilem et al. 2017). During an ideal extraction process, the release of functional molecules is exerted, which may favorably increase the chance of their bioavailability. For example, the incidence of decomposition/hydrolysis of oleuropein molecules that gives rise to production of hydroxytyrosol and elenolic acid can be favorable if the intention is to liberate hydroxytyrosol. The degradation of oleuropein, together in presence of acids and metal ions, may occur through enzymatic reactions and high temperature (Yuan et al. 2015). These phenomena can be undesirable particularly if the main purpose of the extraction is to isolate oleuropein. On this account, the extraction parameters need to be designed cautiously to ensure no/minimum detrimental effect incurs on the molecular structure of the target compounds.

Devising sustainable extraction techniques is of paramount significance, particularly when considering the susceptibility of phenols to high temperature (Rahmanian et al. 2015) and oxygen (Wang & Bohn, 2012). The inclusion of appropriate pre-treatments, such as blanching through olive leave extraction, has been found to be influential in obtaining increased recovery of phenolics. Zeitoun et al. (2017) observed improved recovery of total phenolic compounds, up to about 61.70% when the leaves were subjected to blanching using hot water at 90 °C for 20 min. The types of sample preparation and storage parameters of leave samples prior to the extraction process are also accountable to the level of depletion/recovery of the representative phenolics, such as oleuropein and verbascoside. Malik and Bradford (2008) carried out an investigation on various processing parameters on the loss/preservation of phenolics in the extracted olive leaves and found significant efficiency of recovery of oleuropein and verbascoside when fresh leave samples were dried at 25 °C, whereas drying of leaves at elevated temperature (60 °C) lowered the concentration of total phenolic compounds. The authors of this study

also observed that defrosting frozen olive leaves within 5 min and 2 min lowered the recovery of oleuropein up to 57.7% and 53.5%, respectively.

6.3.1.2. Olive pomace

Olive pomace, namely, olive cake, refers to the residual solid by-product that is made up of olive pulp (up to 90%) and olive stones (Arvanitoyannis et al. 2007; Skaltsounis et al. 2015), which remains after processing of olive oil extraction through centrifugation or pressing (Ghanbari et al. 2012; Clodoveo et al. 2015). The solid residue that is produced from a two-phase centrifugation system is known as "two-phase olive pomace", also termed olive mill solid waste, which contains around 65% (Sygouni et al. 2019), up to 70% moisture (Skaltsounis et al. 2015). The pomace generated from a three-phase decanter contains a lower amount of water, around 45% moisture (Skaltsounis et al. 2015).

Olive pomace is abundant in an array of phenolic compounds (Rodríguez-López et al. 2020), including hydroxytyrosol (around 1.8%, Skaltsounis et al. 2015); oleuropein; verbascoside; tyrosol (Ghanbari et al. 2012), making it a valuable candidate for bio-functional and value-added applications. Many studies have been carried out on the processing design (including extraction time, temperature, and solvent types) to optimally extract nutritive components of this by-product. Vitali Cepo et al. (2017) observed efficiency of using ethanol (60%) in the extraction of total phenolics (3.62 mg gallic acid equivalent/g pomace) and oleuropein (115.14 mg/kg pomace) through the extraction temperature at 70 °C for 2 h. The use of methanol as the extraction solvent has also been reported to notably facilitate the extraction yield of total phenolics from olive cake, as evidenced through the extraction process (i) at 70 °C for 12 h using 80% methanol (Alu'datt et al. 2010), and (ii) at 70 °C for 3 h using 40% methanol to extract olive pomace (Böhmer-Maas, et al. 2020).

The lipid/oily phase of olive pomace is commonly extracted using solvents and subjected to a refining process to make it edible, where it is then mixed with virgin olive oil (around 5% to improve its quality/sensory attributes), being commercially known as refined olive pomace oil (Clodoveo et al. 2015). It is crucial to deliver these processed residues promptly to the pomace oil manufacturing to prevent/minimize possibility of oxidation/rancidity in the final oil product.

6.3.1.3. Olive mill wastewater

The generation of olive mill wastewater (OMWW) as the liquid effluent occurs particularly through a three-phase centrifugation system that accounts for a large amount, about 50% of the total yield of process output, after each extraction process (Khdair & Abu-Rumman 2020). The endogenous polyphenols in OMWW (ranging from 0.5 to 24.0 g/L wastewater (Rahmanian et al. 2015) may

significantly exhibit health benefits such as antiradical and antimicrobial activities (Ghanbari et al. 2012). The recovery of bio-phenols from OMWW is achievable through a variety of extraction methods. Solvent extraction technique is a relatively more commonly used method but, due to its drawback inherent in the need for a sizable portion of solvents, can be ideally replaced by a supercritical extraction system (although it comes with capital/apparatus expenditure) (Takaç & Karakaya, 2009). The membrane filtration method is considered as a potentially advantageous technique for valorization of OMWW, which, among others, include reducing energy use and eliminating additive use (Takaç & Karakaya, 2009). The main classifications, other than the conventional membrane technique, include nano-filtration, ultrafiltration, microfiltration, and reverse osmosis membranes that are regarded as highly effective means due to their great applicability for the recovery and isolation of target molecules (Takaç & Karakaya, 2009). Zagklis et al. (2015) performed a study on the extraction of phenolics from OMWW—using membrane filtration (comprising reverse osmosis concentrate, nanofiltration, and ultrafiltration), and isolation of the recovered compounds—using resin adsorption/desorption. In their research, the concentrated phenolic compounds represented 378 g gallic acid equivalent per liter as compared to those in the original/non-filtered OMWW (2.64 g/L). As described in **Section 5.3.1.1**, the biosynthesis of hydroxytyrosol may come about when oleuropein is hydrolyzed/decomposed during processing of oil extraction, and it is largely accumulated in olive mill wastewater. Hydroxytyrosol is highly treasured for its bio-functional qualities and has the market potential/industrial demand in the food and dietary system, particularly when considering the expensive and complicated process to synthesize this component (Bouallagui & Sayadi, 2018; Rahmanian et al. 2015).

Olive oil industries (particularly in Spain) continue to adopt the replacement of the three-phase centrifuge decanter by the two-phase system (namely ecological) that generates a significantly lower amount of OMWW (Rodrigues et al. 2015; Sygouni et al. 2019; Azbar et al. 2010). A sustainable reutilization of OMWW provides a good marketing potential for value addition/nutraceutical applications in nutrition and food system, particularly when a feasible methodology is designed to gain optimum extraction yield. Optimization of process design that serves both effluent treatment and valorization of OMWW has been the topic of research studies, and some proposed methods are outlined in this review (**Section 5.4**).

6.3.1.4. Olive stones

Olive Stones, namely, the pits, refer to the endocarps of olive fruits (García Martín et al. 2020) that account for around 10% (Rodrigues et al. 2015) or up to 27% of total weight of fruit (Ordoudi et al. 2018). Olive stones and/or their kernel (the seeds surrounded by endocarps) are a good source of phenolic

compounds with bio-functional potentials (Ordoudi et al. 2018). Among them are hydroxytyrosol (0.4–1.9 g/100 g of whole stone, d.w.) (Rodríguez et al. 2008), tyrosol (0.1–0.8 g/100 g of whole stone, d.w.) (Rodríguez et al. 2008; Fernández-Bolaños et al. 1998), and oleuropein (0.1–0.2 g/100 g of whole stone, d.w.) (Rodríguez et al. 2008). Specific types of phenolics such as verbascoside are only present in the kernel part of the stone (0.4–0.8 g/100 g dry kernel) (Rodríguez et al. 2008). Elbir et al. (2015) in their experiment reported various concentrations of total phenolics as follows: 11.32, 4.55, and 3.56 mg gallic acid equivalent per gram dry basis of olive stones extracted from Moroccan olives of Picholine, Haouzia, and Menara cultivars, respectively.

Olive stones as the natural lignocellulosic biomass are also a weighty source of polymers including lignin and cellulose that are valued for direct/indirect fields of applications such as combustion for bioenergy uses (Rodríguez et al. 2008). The ability to devise an applicable process formulation via innovative means, promptly affects the ability to re-use the whole olive stones and convert the desired components into high-added value products sustainably. For instance, using pre-treatment via a steam explosion prior to the isolation and fractionation of target biomolecules helps ensure isolation of components, which is otherwise likely to be hurdled due to the physical and chemical characteristics of these products (Rodríguez et al. 2008).

6.4. Sustainable processing system in olive oil industry—an overview of optimizing value

6.4.1. Olive oil

In the olive oil industry, there is a trend towards evolving the processing technologies to enhance both quality and extraction yield. A typical example is the replacement of the three-phase centrifugation system by the two-phase decanters, which is reportedly more effective in the increased concentration of total phenolics as well as individual/specific phenolic groups such as oleuropein aglycone, hydroxytyrosol, and tyrosol. However, the current advancements of the processing technologies used in olive oil production may not be sufficient, although virgin olive oil is endogenously abundant in bioactive compounds; however, given its richness of unsaturated fatty acids (Li & Wang, 2018), the oxidation of oil during processing/storage, e.g., as a result of prolonged storage (Li & Wang, 2018) and light exposure (Li & Wang, 2018; Caponio et al. 2005) is often expected, which in turn brings about the degradation of endogenous phenolics. Furthermore, some phenolics are inherently more liable to be diminished/degraded through various steps of processing, e.g., decomposition of oleuropein when the temperature is increased. Research studies have suggested alternative means to overcome oxidative deterioration of olive oil. As an example, the addition of olive leaves in advance of crushing step to enhance olive oil quality. The isolated phenols from olive leave by-products can be generally used as

propitious value-added products through their incorporation into lipid-based foodstuff with poor oxidative stability such as refined olive oil (Ammar et al. 2017; Sanchez de Medina et al. 2011).

In general, each group of phenolic components is influenced differently during the processing of olive oil, and this may hamper identification of a decisive pattern for the synthetic routes/formation of each class of phenols during different processing conditions (Diamantakos et al. 2020). Although parameters such as malaxation temperature may favorably correlate with the increase of some phenolics/total phenolics, but this may not be applicable to the production of virgin olive oil in terms of the sensory acceptability that is likely to be adversely affected by the rise of temperature (de Torres et al. 2018; Inarejos-García et al. 2009). Another study reported that the rise of temperature may be favorable in terms of yield improvement, but this can bring about deterioration/oxidative rancidity (Guerrini et al. 2019) with resulting impact on the phenolic profile/potency. Possible solutions to upgrade the conventional malaxation operation are (i) inclusion of chemical aids such as pectolytic and cellulolytic enzymes that may assist in rupturing the cellular structure and liberating the phenols into the oil fraction (Ghanbari et al. 2012; Puértolas & Marañón, 2015), and (ii) olive paste treatment using emerging technologies during malaxation to complement the extraction efficiency. Puértolas and de Marañón (2015) through their investigation on the treatment of olive paste using pulsed electric field during malaxation of olive oil, reported an increased recovery of total phenolics (11.5%) as well as yield percentage of the extracted olive oil (13.3%) when compared to the samples without treatment. Other types of emerging techniques, with the potential to overcome the challenges inherent in the malaxation operation, include microwave heating, ultrasound technologies (Clodoveo, 2013; Pérez et al. 2021), and high-pressure processing (Pérez et al. 2021).

6.4.2. Olive mill by-products

The enormous generation of olive mill by-products has prompted the idea among scientists to propose green solutions for high-value applications to optimally recover and re-use these valuable substances and enable sustainable marketability in food/dietary and non-food system (Azbar et al. 2010). Currently olive mill by-products have found rather low/moderate value applications for direct/indirect uses in the agriculture and industrial systems (Berbel & Posadillo, 2018). Some applications use de-fatted pomace (Di Giovacchino, 2013; Petruccioli et al. 2011) for natural renewable energy sources, via thermochemical decomposition of organic compounds (such as cellulose and lignin), through pyrolysis and combustion, as well as gasification (Arvanitoyannis et al. 2007). Other uses have reached as far as animal feed and compost (Berbel & Posadillo, 2018), and to some extent, have found applications in the

pharmaceutical and cosmetic sectors but they are predominantly under-exploited while being valuable as health-giving products (Rodrigues et al. 2015).

The ability to deliver the high-value applications of olive mill by-products entails consideration of numerous factors associated with the green valorization process, which partly highlights the importance of (i) the selected extraction method, with special attention to gaining higher recovery of bio-functional compounds, as well as their conversion into value added ones; (ii) the challenges involved in meeting the sustainability criteria; and (iii) high-value applicability of the recovered products in the industrial sectors to increase marketing opportunities. An overview of the typical factors involved in a green valorization of residual biomass generated in the olive oil industry is illustrated in **Figure 6.2**.

Figure 6. 2 Summary of the associated factors accountable for the sustainable valorization of olive mill by-products.

The treatment of olive mill wastewater comes with a great challenge due to its phytotoxicity (Khdair & Abu-Rumman 2020; Sakarika et al. 2020). Numerous waste management strategies have been recommended in this respect, most of which favorably enable the liberation of bioactive molecules. Sygouni et al. (Sygouni et al. 2019) postulated the effectiveness of membrane filtration as an ideal ecofriendly method for the treatment of OMWW as well as the achievement of phenolic recovery. Other

methods to achieve purification of functional ingredients from OMWW include solvent extraction (widely used), chromatographic, and enzymatic-assisted extraction methods (Bonetti et al. 2016).

Kachouri and Hamdi (2004) justified the influence of incorporation of olive mill wastewater (fermented by Lactobacillus plantarum) to olive oil that facilitated the decrease of phenolics in wastewater residue and increase of phenolics in olive oil. This is primarily attributed to the abilities of Lactobacillus plantarum to de-polymerize high-molecular-weight phenolics in OMWW that potentially enables their movement from wastewater to the oil (Kachouri & Hamdi, 2004). These authors found a significant increase of polyphenol content in the oil with the inclusion of OMWW with fermented L. plantarum compared to the oil with plain/non-fermented OMWW, representing 703 and 112 mg/L oil, respectively. A similar pattern in this study was also observed for individual phenolic component, particularly oleuropein content, which represented 401.8 and 140.4 mg/L in oil samples with and without fermented L. plantarum, respectively. Furthermore, the isolated active molecules from OMWW such as hydroxytyrosol and oleuropein have markedly found applications in the cosmetic industry (Berbel & Posadillo, 2018).

The olive oil industry generates a great amount of olive pomace that can represent, on its own, considerable environmental/economic challenges. The re-utilization of this type of by-product is somewhat progressing in various applications. Examples are the commercial applications for direct uses such as edible vegetable oil and animal feed. The de-oiled fraction of pomace, namely, exhausted pomace, has found uses in agricultural applications including compost and soil amendment (Petruccioli et al. 2011). The de-fatted fraction also finds application in animal feed, commonly after being subjected to de-stoning (Di Giovacchino, 2013) and pretreatment to decrease the lignin content (Petruccioli et al. 2011), the high-molecular-weight polymers with water insolubility constituting roughly about 37% dry basis (Battista et al. 2016).

6.5. Conclusions and future prospects

In recent years, the distinctive value of olive oil products has been globally appreciated nutritionally and economically. However, the maintenance of the nutritive attributes is of great reliance on the fate the endogenous phenolics in the final product, which is highly dependent, favorably and adversely, on the types/parameters of processing and storage. To overcome the disadvantages inherent in malaxation parameters, researchers have proposed incorporation of auxiliary processing means based on green methodologies. However, given the challenges involved in gaining the increased yield of (i) desired

phenolics and (ii) olive oil, together with other factors such as acceptability of organoleptic attributes, more extensive research work may be needed.

The intensification of nutritive quality of olive oil is of paramount importance, but this alone may not suffice in meeting the sustainability of the processing system in the olive oil industry. The huge generation of the biomass residues necessitates using efficient approaches to deal applicably with each type of waste stream generated during olive oil extraction which potentially enable (i) optimum waste management, particularly in the case of liquid effluent, and (ii) sustainable valorization of functional biomolecules.

Olive mill by-products are often under-utilized—most have found low/moderate value applications while having an appreciably high added-value potential in various industrial sectors with great marketability. The mechanical processing techniques used for the extraction play decisively in the recovery of phenolics of interest. Researchers have made much effort to enable delivery of an ideal extraction system to optimally isolate target phenolics from the waste stream, which, together with other things, involves a great challenge due to the structural complexity of polyphenols and the inconsistent rhythms of their biosynthesis in various conditions. The inclusion of emerging techniques in the processing system has shown to be significantly effective in the increment of phenolic recovery, and there seem to be a movement towards adoption of these new technologies in some areas/applications. However, the prevalence of traditional/inefficient methods, entails more research efforts to enable scalability of the extraction design, affordability, and simplicity of process operations.

Application of the green technologies in the olive oil industry, when the sustainable criteria are adequately fulfilled, helps achieve optimum production of olive oil and valorize the olive mill by-products that may (i) deal with the challenges associated with food/nutrition security, (ii) address the environmental issues, (iii) develop production/consumption of natural and healthy products, (iv) enable broader applications in the food/non-food system, and (v) improve marketability/investment return that can be of great value for the industrial system.

6.6. References

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CHAPTER 7

COMPARATIVE STUDY OF DIFFERENT PARTICLE SIZES OF ADDED OLIVE LEAVES FOR THE CONTENT OF TARGET POLYPHENOLS IN VIRGIN OLIVE OIL

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Comparative study of different particle sizes of added olive leaves for the content of target polyphenols in virgin olive oil

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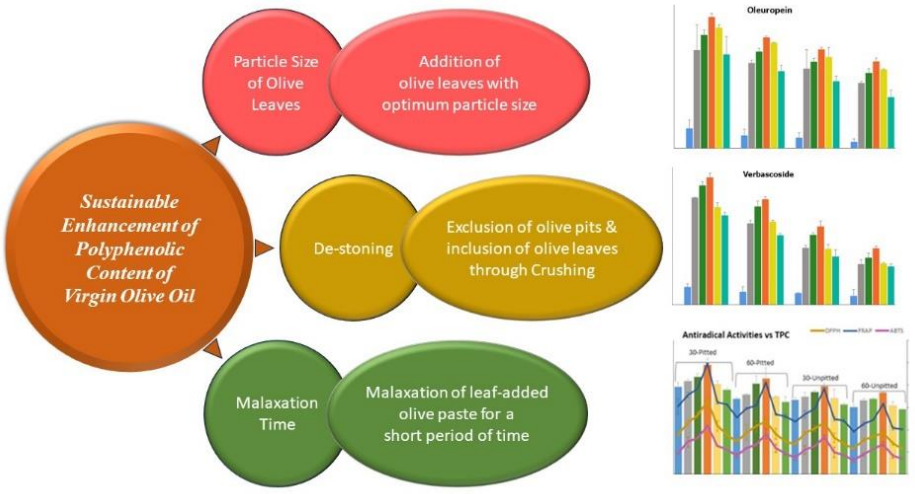
Abstract: The addition of olive leaves during processing of olive oil has been studied and reviewed from different perspectives but there is a paucity of information on the roles of particle sizes of the added leaves in phenolic content of the oil. Dry ground olive leaves with a range of particle size fractions (0.07–3.0 mm) were added to crushed olives prior to the malaxation to compare their effects on: (i) the content of total and selected polyphenols (particularly oleuropein and verbascoside), (ii) antioxidant capacity (in vitro), and (iii) physicochemical quality and the yield of the extracted olive oil. Besides particle size, that was the main factor of study, olive pitting and malaxation time (30 and 60 min) were also considered as independent variables. Olive leaves with 0.3 mm favorably exhibited significant effects ($p < 0.001$) in all assays. Indeed, the oil samples with 0.3 mm leaves produced from the pitted olives showed maximum values when the malaxation time was (i) 30 min – for oleuropein (5.85 mg per kg oil), verbascoside (4.02 mg per kg oil), luteolin (15.44 and mg per kg oil), and total phenolic content (TPC) (368.01 mg per kg oil), and (ii) 60 min – for hydroxytyrosol (19.14 mg per kg oil) and tyrosol (16.89 mg per kg oil). These findings indicate that the particle size of added olive leaves can play a significant role in the content of principal polyphenols of the resulting olive oil; a topic that has not been approached in the literature.

Graphical Abstract



Keywords: Olive leaves, particle size, polyphenols, olive oil, oleuropein, verbascoside.

7.1. Introduction

Fresh olive fruit, from which virgin olive oil (VOO) is mechanically produced, contains a large proportion of phenolic antioxidants that are decidedly responsible for the nutritional quality and stability of its extracted oil. The question arises whether these valuable compounds are proportionally recovered and preserved in the oil following the extraction process. Research studies have demonstrated that this is not the case as polyphenols are prone to degradation/oxidation especially during malaxation of olive paste as well as during the storage of oil. In particular, certain types of phenols including oleuropein (a secoiridoid glycoside) and verbascoside (a phenylethanoid glycoside), the highly prized antioxidants, may suffer from biochemical degradations due to enzymatic and chemical reactions throughout the processing of the oil. As a result, their concentrations may decline significantly, and hence only trace quantities may be found in the resulting oil. In the literature, numerous processing techniques have been discussed to intensify the transfer of polyphenols to the extracted olive oil. These include the addition of olive leaves during processing to compensate phenolic loss and support the stability of olive oil. Indeed, a large volume of research, dealing with valorization of olive leaves in olive oil processing, exhaustively investigated a broad range of factors including (i) mechanical processing parameters of oil extraction such as malaxation time/temperature, pitting (de-stoning of olives), types of crushers, etc., (ii) fruit ripeness, (iii) dose-dependency of added leaves, and (iv) cultivars/growing regions. Having said that, there is no clear evidence on the efficacy of particle size of added leaves, while a substantial body of evidence reports that size reduction is among the key mechanical factors responsible for the physicochemical properties (associated with enhanced surface area and diffusivity/mass transfer) and extractability of the desired phytonutrients (Chemat et al. 2020; Safarzadeh Markhali, 2021a). It is therefore important to take the particle size of olive leaves into consideration when addressing sustainable exploitation of these biomass residues, especially when selective extraction of bio-molecules is of interest. Given this, the present study aimed at investigating the effect of particle size of dry ground olive leaves on olive oil quality when added to the crushed olives in advance of malaxation process. The specific objectives were as follows: (i) *primarily* – to evaluate the influence of different particle sizes of dry ground leaves on principal polyphenols and antioxidant capacity of the leaf-added virgin olive oil. Special attention was placed on the content of oleuropein and verbascoside, and (ii) *secondarily* – to validate the assumption of favorable effects of shorter malaxation time (30 min) and pitting of olive paste (particularly before malaxation) on the increment of oleuropein and verbascoside in the oil enriched with optimal particle size of olive leaves.

7.2. Materials and methods

7.2.1. Plant materials and chemicals

Olive leaves (the residues from olive oil processing) and olive fruits of Picual cultivar were kindly supplied by "Center for Advanced Studies in Energy and Environment", University of Jaén, Campus of Las Lagunillas, Jaén, Spain. Upon arrival to the University of Minho, Portugal, the plant materials were prepared as follows: (i) the leaves were manually cleaned (to remove stems and foreign objects), washed, and dried at 37 °C for 48 h. The dried leaves were then ground and passed through a range of sieves (200–7 mesh) to separate them into five particle fractions (0.07, 0.15, 0.3, 1.6, and 3.0 mm). Each fraction was vacuum packed in polypropylene bags, refrigerated (4 ± 2 °C) for further processing within two weeks. (ii) Olive fruits, were manually cleaned to remove foreign objects/bruised olives, laid flat on food-grade/dry trays, refrigerated, and processed for oil extraction within two weeks.

The following reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA): (i) *analytical grade* – Folin–Ciocalteu, anhydrous gallic acid (≥98.0%), anhydrous sodium carbonate (≥99%), hydrochloric acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride ($FeCl_3$), ethanol (99.8%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), acetic acid (99.8%), chloroform (99%), potassium iodide (≥99.0%), hexane (≥99%), and methanol (99%), (ii) *HPLC grade* – phenolic standards (oleuropein, verbascoside, luteolin, hydroxytyrosol, apigenin, and tyrosol with 98% w/w purity), formic acid, and acetonitrile (≥99.9%).

7.2.2. Study design

In this study, a range of particle size fractions of dry ground olive leaves were added to pitted olives during crushing, to compare their effects on the quality of the extracted olive oil. The response variables (dependent variables) consisted of: (i) total phenolic content (TPC), (ii) principal polyphenols (oleuropein, verbascoside, hydroxytyrosol, tyrosol, luteolin, and apigenin), (iii) antioxidant capacity (in vitro), (iv) physicochemical quality comprising free fatty acids (FFA), peroxide value, pigments (carotenoids and chlorophylls), and the yield (%) of the resulting olive oil. The key independent variable (factor) was the particle size of the added olive leaves. Additionally, in order to extract further data, the following auxiliary factors were investigated: (i) malaxation time (30 and 60 min), and (ii) pitting/un-pitting of olives in advance of malaxation to assess their interactive effects on the response variables. The quality of leaf-added oil samples was compared to that of control samples (oils processed without the addition of olive leaves). The selected cultivar, growing region, and handling/storage conditions were the same for all experiments.

7.2.3. Mechanical processing – extraction of olive oil

The mechanical operations employed for the extraction of virgin olive oil (**Figure 7.1**) consisted of: (i) *crushing* – the olives (100 g) were initially crushed using a blender equipped with a rotation speed/temperature adjustment (Vorwerk Bimby® TM6, Germany) at 1500 rpm for 2 min. The blender was stopped and the stones were removed (using a pair of stainless steel forceps tweezers). Thereafter, dry ground leaves (3%) were added (separately for each fraction) to the crushed olives and further blended at 2000 rpm for 4 min (the machine stopped in between and stirred manually every 1 min interval). Final crushing (pressing) was then applied for 4 min using a ceramic mortar and pestle, to confirm the release of oil from the cell tissues driven by the pressure exerted on the food matrix, (ii) *Malaxation* – the crushed olive paste was then mixed under slow spinning for various times (30 and 60 min for each experiment), with the same blending appliance, to coalesce the oil droplets through which the extraction of free oil is enabled, and (iii) *two phase separation (centrifugation decanting)* – the mixed olive paste was centrifuged at 3500 rpm for 5 min in Thermo-IEC polypropylene centrifuge bottles at 27 °C. The oil fraction was separated, weighed, bottled, nitrogen flushed, capped, and stored in dark, cool/ventilated place until experimental analysis. The same procedure was repeated for unpitted samples (stones remained in the paste) for each malaxation time separately. Likewise, the control samples (free of olive leaves) were obtained accordingly for both pitted and unpitted experiments. Temperature remained constant at 27 °C throughout the extraction. The choice of adding dry leaves rather than fresh ones was based on the extensive studies reviewed previously (Safarzadeh Markhali 2021a,b).

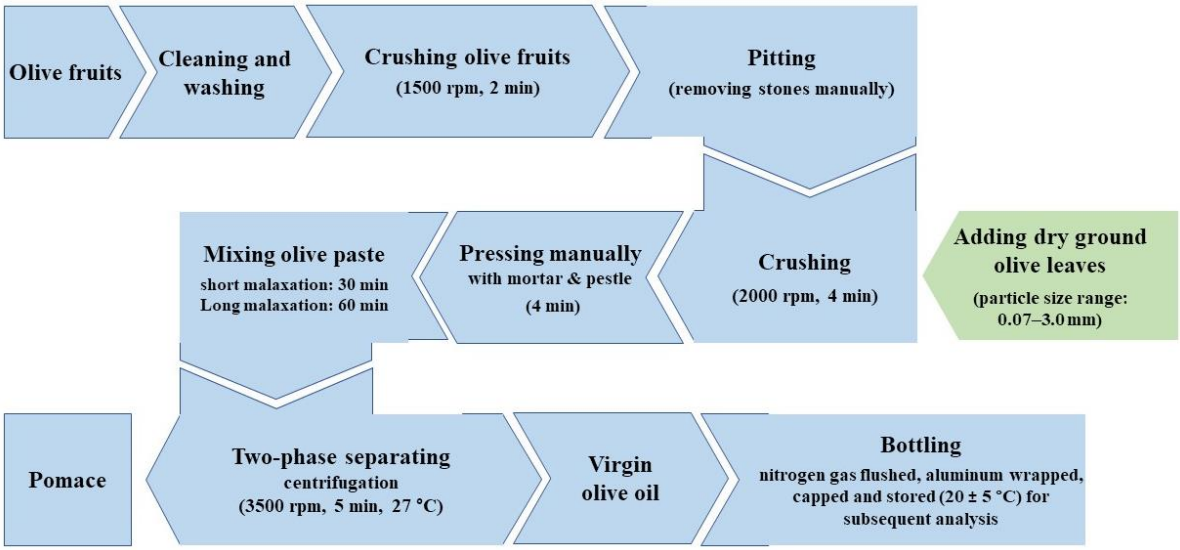


Figure 7. 1 Summary of the mechanical processing technique used in this study for the extraction of virgin olive oil.

7.2.4. Analysis of lipid matrix (olive oil samples prior to phenolic extraction)

Olive oil samples with/without olive leaves were initially analyzed for physicochemical quality parameters as follows: Peroxide value and free acidity. The quality of olive oil samples in respect of peroxide value and free fatty acids was measured according to the official method of American Oil Chemists Society (AOCS, 1998) with minor modifications.

7.2.4.1. Peroxide value

The peroxide value of oil that represents the degree of oxidative rancidity was assessed through iodometric titration according to the official method of American Oil Chemists Society (AOCS, 1998) with minor modifications. Briefly, one gram of oil was dissolved with 10 mL acetic acid-chloroform (3:2 v/v), gently shaken before adding 2 mL saturated potassium iodide. The mixture was incubated in the dark for 5 min to enable the release of iodine from saturated potassium iodide. After adding 10 mL distilled water (shaken for 1 min) the mixture was then titrated with 0.01 N sodium thiosulphate standard solution (the initial burette reading was recorded). One mL starch solution (1.5%) was used as indicator. The titration was continued until the color changed from dark to pale/white. The final burette reading was recorded. The peroxide value was calculated according to following equation. The results were expressed as milliequivalents of active oxygen per kilogram oil (mEq. O_2/kg).

$$\text{Peroxide value } (\text{mEq. } O_2/\text{kg}) = \frac{V \times N \times 1000}{Ws} \quad --- \text{ Equation 1}$$

Where: *Ws* = weight of sample (g),
V = volume of sodium thiosulphate (mL) that is: (final burette reading – initial burette reading),
N = normality of sodium thiosulphate (0.01 N).

7.2.4.2. Free fatty acids (FFA)

The percentage of free fatty acids was determined by titration (AOCS, 1998) where the oil samples were dissolved with ethanol/ether (1 : 1, v/v) and 0.1 M sodium hydroxide. Phenolphthalein was used as an indicator. The results were expressed as percentage of oleic acids (g /100 g, w/w).

7.2.4.3. Extraction yield of olive oil

The yield percentage (%) of the extracted oil (extractability of the oil) was calculated according to the following equation.

$$\text{Yield of extracted oil } (\%) = \frac{Wo}{Wi} \quad --- \text{ Equation 2}$$

where: *Wo* = weight of extracted oil, *Wi* = initial weight of olive fruits (g)

7.2.4.4. Pigment determination.

The content of chlorophylls and carotenoids was determined spectrophotometrically by dissolving 7.5 g of oil with 25 mL of cyclohexane (Minguez-Mosquera et al. 1991). The absorbance readings were measured at 670 nm (for chlorophyll) and 470 nm (for carotenoid), and the results were expressed as mg per kg oil, respectively.

7.2.5. Analysis of phenolic extracts of olive oil samples

In this section, the polar fraction of olive oil was extracted and assessed for the content of total and individual polyphenols, as well as antioxidant capacity.

7.2.5.1. Sample preparation – isolation of phenolic extracts

The fraction of polyphenols from olive oil was separated through a liquid–liquid extraction method using the method described by Lozano-Castellón et al. (2021) with minor modifications. Briefly, 2 g of oil was mixed with n-hexane (2 mL) and methanol/water (4 mL), vortex mixed (1 min) and centrifuged (3000 rpm for 5 min). The polar phase was taken and the hexane phase (lipophilic fraction) was re-extracted thrice to obtain an oil-free solution. The extracted polar phase was then subjected to a rotary evaporator, dissolved in HPLC-grade methanol/water (50/50, v/v),nitrogen flushed (using Reacti-therm™ Heating Module, Pierce), filtered through a 0.22 µm polytetrafluoroethylene membrane and stored at −20 °C before analysis.

7.2.5.2. HPLC determination of selected phenolic compounds

The chromatographic separations of target polyphenols were carried out on a reverse-phase Aquity UPLC BEH C-18 column (100 mm × 2.1 mm i.d., 1.7 µm particle size, Waters Corporation) using UHPLC (Shimadzu Nexera X2 UHPLC) connected to a diode array detector (Shimadzu SPD-M20A), and an integration system (Shimadzu LabSolutions software, Kyoto, Japan). The gradient elution program was based on the method reported by Quero et al.(2022) with slight modifications as described in our earlier work (Safarzadeh Markhali & Teixeira, 2023). The solvent flow rate and the injection volume were 0.3 mL/min, and 5 µL, respectively.

Prior to the analysis, the eluents (mobile phase) were initially filtered through 0.22 µm membrane nylon filters, and the samples/standards were filtered through 0.22 µm syringe filters. The chromatograms were registered at 280 nm and the target phenols of the samples were identified by referring to the retention times of the commercial standards and the UV spectra detector. The standard calibration curve was plotted (peak area *vs.* the known concentration) and the selected phenolic content

was quantified against the linear calibration curve of the corresponding standard. The results were reported as mg target phenol per kg oil.

7.2.5.3. Antioxidant activity

The reactivity of free radicals, due to their unpaired-electron structure, can bring about lipid oxidation in edible oils which is a major issue as it can render the oil nutritionally and organoleptically degraded. In this regard. antiradical activity of olive oil can be an ideal marker to determine the potential of the endogenous antioxidants for reducing free radicals. In this study, phenolic extracts of oil samples were examined by Trolox Equivalent Antioxidant Capacity (TEAC) assay through the following *in vitro* methods:

DPPH radical scavenging activity – the ability of phenolic extracts of oil samples to diminish activities of free radical 2,2- diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) was assessed using the method of Brand-Williams et al. (1995) with slight modifications as described in our previous study (Safarzadeh Markhali et al. 2023). This stable radical (a dark purple color) can be reduced/neutralized by the reaction of antioxidants usually through their electron transfer (Brand-Williams et al. 1995). The data from the decrease in absorbance (515 nm) were calculated against Trolox standard curve and the results were expressed as mM Trolox equivalents/kg oil (mM TE/kg oil).

FRAP radical scavenging activity – the assay of ferric reducing antioxidant power (FRAP) was performed following the method of Benzie & Strain (1996) with slight modification. It determines the antioxidant potential of samples for reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). The FRAP reagent was initially prepared by mixing: (i) acetate buffer, and (ii) 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in hydrochloric acid and $FeCl_3 \cdot 6H_2O$ (10 : 1 : 1 v/v/v). The mixture was incubated (37 °C, 10 min). Into a test tube containing FRAP reagent, an aliquot of sample was added, vortex mixed and incubated (37 °C, 30 min). A blank containing only FRAP reagent was also prepared. The absorbance readings were measured at 593 nm and the values were calculated against Trolox standard curve and the results were reported as mM TE/kg oil.

ABTS radical scavenging activity – the *ABTS* radical scavenging assay was carried out according to the method of Re et al. (1999) with slight modifications as described in our previous study (Safarzadeh Markhali et al. 2022) The principle behind this assay: (i) initial generation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) $ABTS^{\bullet+}$ through the reaction of ABTS and potassium persulfate (in the absence of antioxidants), and (ii) subsequent decolorization and reduction of $ABTS^{\bullet+}$ after adding antioxidants, changing from bluish green to colorless. Following this assay, the data from reduction of absorbance at 734 nm were calculated against Trolox calibration curve and expressed as mM TE/kg oil.

7.2.5.4. Total phenolic content (TPC)

Total phenolic content of olive oil was determined using Folin–Ciocalteau assay as described by Singleton & Rossi (1965) with slight modifications. Into 1 mL phenolic extract of oil, the following reagents were added: one mL of 20% sodium carbonate (freshly prepared) and 1 mL of Folin– Ciocalteau reagent. The solution was gently mixed and incubated in the dark for 30 min to develop a blue color prior to centrifugation (2500 rpm for 1 min). The blue color fraction was collected for absorbance measurement at 765 nm and the total phenolic content was calculated against gallic acid standard curve. The results were reported as mg of gallic acid equivalents/kg oil (mg GAE/kg).

7.2.6. Statistical analysis

The significant differences ($p < 0.05$) between the mean values (±SD) of all determinations were statistically assessed via Analysis of Variance (ANOVA) using SPSS software, version 27.0. The effect of independent variables on each dependent variable was analyzed through factorial ANOVA (two-way ANOVA). The interactions between independent variables: (i) particle size *vs.* malaxation time, and (ii) particle size *vs.* pitting/un-pitting, were assessed using pairwise comparisons. The assumption of homogeneity of equal variance was assessed through the Levene's test (homogeneity of variance assumption was not violated when p-value was greater than 0.05). Each experiment was carried out in triplicates.

7.3. Results and discussion

7.3.1. Effect of particle size of added leaves on physicochemical quality and yield of extracted oils

7.3.1.1. Peroxide value (PV)

The measurement of peroxide value in olive oil is vital as it reflects the status of oxidative rancidity (at the primary stage of oxidation) in olive oil. The lower values of PV thus represents higher stability and longer shelf-life. As shown in **Figure 7.2**, peroxide values ranged from 5.82 to 9.36 mEq. O_2/kg oil. According to the quality criteria designated for the trade standards, the peroxide value of extra virgin olive oil (EVOO) must not exceed 20.0 mEq. O_2/Kg [International Olive Council (IOC) 2019; Commission of the European Communities, 1991]. In this respect, the results here meet the standard criteria suggesting that they did not undergo the primary oxidation during and after oil processing. In all malaxation groups, the addition of 0.3 mm leaves showed significantly the most effectiveness in lowering peroxide values ($p < 0.05$).

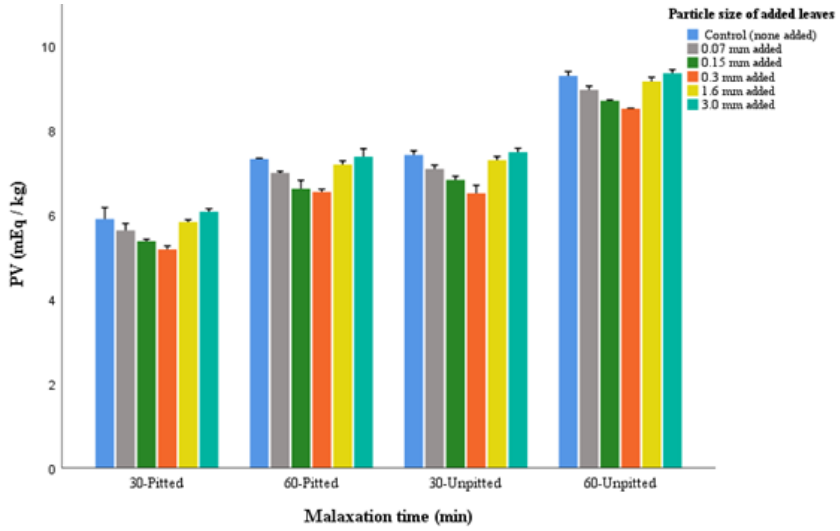


Figure 7. 2 Effect of particle size of added olive leaves on peroxide value (PV) (mEq O2/Kg) of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min, respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

In all processing groups, with the exception of 0.3 mm and 0.15 mm leaves, the addition of leaves did not greatly change the peroxide values compared to the control samples. Examining the interactive effects of the auxiliary factors (pitting and malaxation time), it was found that the pitted groups represented significantly lower peroxides compared to the unpitted corresponding samples. Likewise, shorter malaxation in both pitted and unpitted groups represented significantly lower PVs. This can be explained by the fact that olive stones are a source of peroxidase enzymes. If the stones remain in the paste, particularly during longer malaxation, their endogenous enzymes are activated causing oxidation and degradation of bioactive compounds including polyphenols. Thus, pitting and short malaxation may both contribute to complementing the efficacy of the preferred particle size of added leaves (0.3 mm) in reducing PV in the extracted oil. In previous studies, there is no clear consensus on the ideal addition of olive leaves that may assist in the reduction of peroxides. Considering the factor of dosage of added olive leaves, some studies observed that the addition of 2% and 3% leaves exerted effect on the reduction of peroxides (Sari & Ekinci, 2017; Sanmartin et al. 2019) while others found 3% and 1–10% addition of leaves raised the numbers in the resulting extracted oil (Sonda et al. 2014; Malheiro et al. 2013).

7.3.1.2. Free fatty acids (FFA)

Free fatty acids (also known as free acidity) in olive oil refers to the broken-off fatty acids within the fat molecules. It is commonly measured as a marker of hydrolytic rancidity that may arise from the reaction of triglycerides with oxygen and water. There was no significant interaction between malaxation time and particle size of leaves, suggesting that the acidity level for corresponding oil samples was not

reliant on mixing time (**Figure 7.3**). However, there was significant interaction between pitting factor and particle size as it was found that the free acidity of each pitted sample (produced from the same malaxation time) was significantly lower than the corresponding sample from unpitted paste. In all groups of experiments, the acidity values did not exceed the limited criteria (0.8%, grams of oleic acid/100 g oil) approved by IOC (2019); hence, in this respect, can be acceptable as extra virgin olive oil. Among all groups, the oil with 0.3 mm leaves obtained from pitted olives (30 min) represented the lowest values (0.14%).

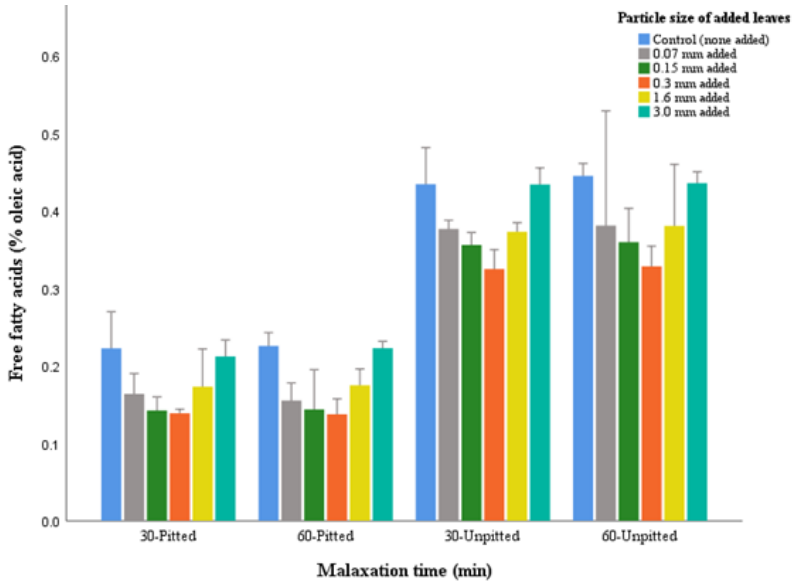


Figure 7. 3 Effect of particle size of added olive leaves on free fatty acids (% oleic acid) of the extracted olive oil from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min, respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

7.3.1.3. Yield of the extracted olive oil (%)

The yield percentage of the extracted olive oils were measured to determine the extent of the effect of leaf particle size and its interaction with other two factors (pitting and malaxation time) on the extractability of the oil. As seen in the obtained results (**Figure 7.4**,) there were no significant differences between malaxation times for corresponding samples, although the extended malaxation (60 min) enabled slightly higher amount of oil. Indeed, malaxation may solely assist in easier separation of oil phase from solid phase (during centrifugation) and greater extractability of oil through the following phenomena: (i) coalescence of small oil droplets, (ii) destabilization of oil/water emulsion (as occurs during crushing operation), and (iii) flocculation of larger oil drops to the surface of the olive paste. The highest yield percentage was found in oils with 0.3 mm leaves processed from unpitted olives (13.19%) that was relatively higher than that obtained from the pitted olives (12.13%). However, regardless of the fact that the unpitted samples yielded slightly greater portions of oils compared to the pitted ones,

malaxation in the presence of stones has been viewed as an issue due to the potential incidence of phenolic degradation induced by the peroxidase enzymes present in stones. In addition, as described thus far (see **Figure 7.2**) the peroxide values in unpitted samples were significantly higher than oils obtained from the pitted ones. Therefore, the marginal increase in the oil extractability may not give grounds for the need to leave the stones in the paste during malaxation.

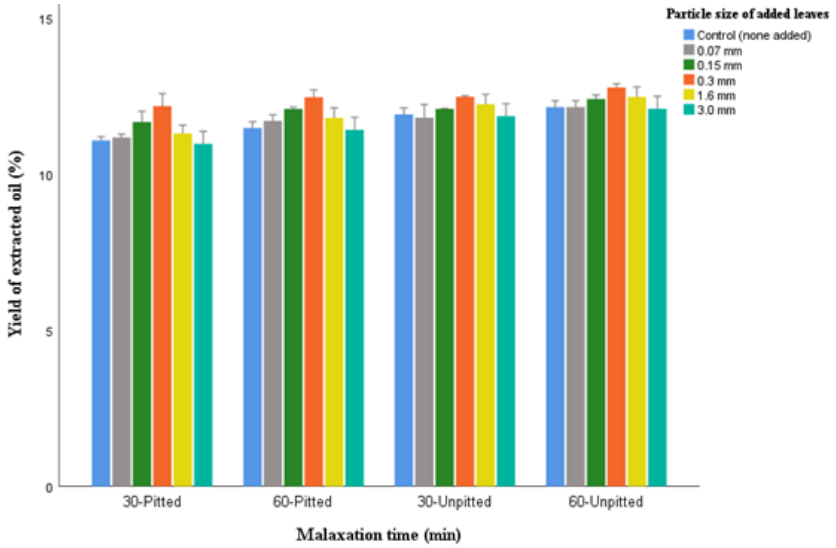


Figure 7. 4 Effect of particle size of added olive leaves on the yield (%) of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min, respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

7.3.1.4. Pigments

The combined data from chlorophylls and carotenoids (**Figure 7.5**) show that the addition of olive leaves significantly increased the content of both pigments in all groups of malaxations and it was evident that the pitted samples contained significantly more pigments compared to unpitted corresponding samples. Moreover, for all malaxation trials, the effect of particle sizes for each pigment was correspondingly comparable. However, the maximum levels belonged to the pitted samples with 0.3 mm leaves (60 min malaxation) that reached up to 16.71 mg/kg (for chlorophyll) and 8.74 mg/kg (for carotenoid).

Chlorophyll and carotenoid both account for a large proportion of pigments in olive leaves (Safarzadeh Markhali et al. 2020). Owing to their characteristic bio-functionalities, in terms of antioxidant effects (providing that products are not exposed to light) and natural color enhancements, they are considered as value-addition ingredients in the food system such as in olive oil products (Sevim et al. 2013).

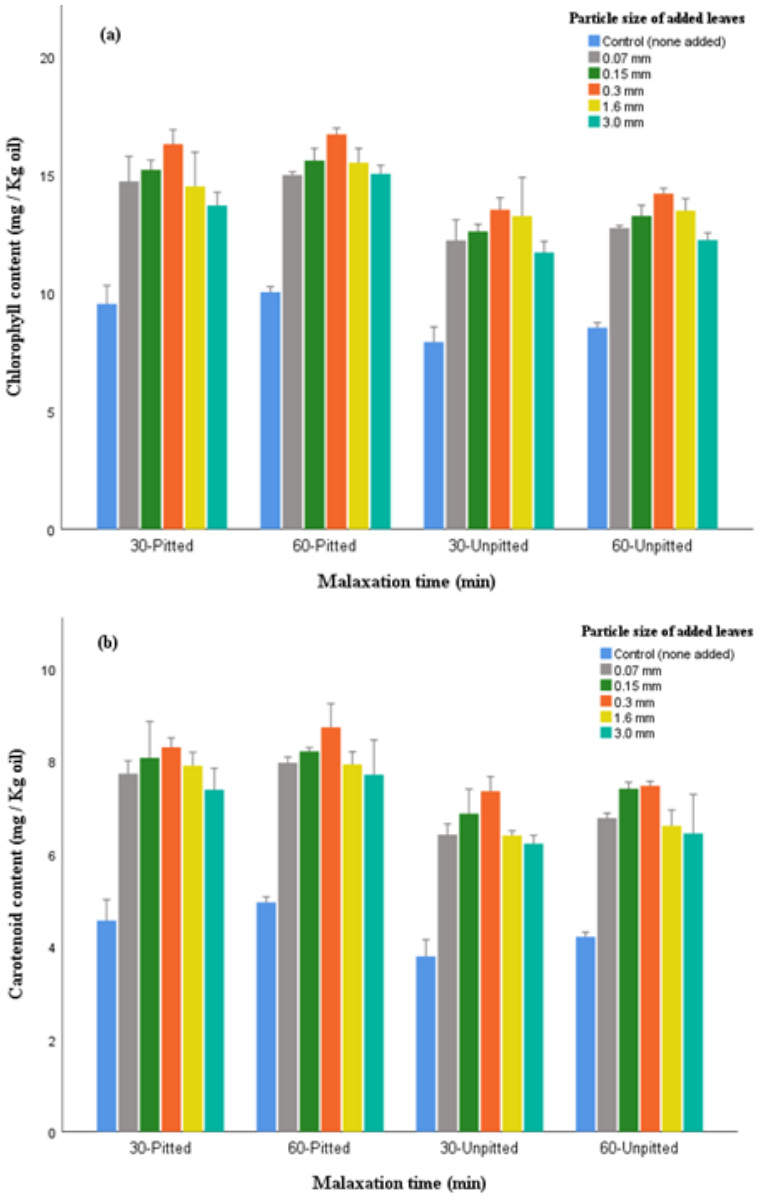


Figure 7. 5 Effect of particle size of added olive leaves on chlorophylls (a), and carotenoids (b) of the extracted olive oils (mg/ Kg oil) from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

Examining the durations of malaxations (in both pitted and unpitted), slight increase in pigments were found in oils obtained within longer malaxation. This relative changes may not justify the need for extending malaxation time, particularly when it was found that the shorter malaxation (30 min) surpassed the prolonged process in terms of peroxide and acidity levels (see **Figures 7.2** & **7.3**). Hence, the use of 0.3 mm leaf addition through a 30-pitted processing system can be considered suitable for pigment enhancement in olive oil.

7.3.2. Effect of particle size of added leaves on the content of total polyphenols and antioxidant capacity of oils

7.3.2.1. Total phenolic content (TPC)

In general, regardless of the factor of particle size, previous studies justified the favorable effects of adding leaves on TPC enhancements (Sonda et al. 2014; Safarzadeh Markhali et al. 2020; Tarchoune et al. 2019; Baccouri et al. 2022), though there is no consensus on their findings due to variations in percentages of added leaves (Ammar et al. 2017; Di Giovacchino et al. 1996; Mezghani et al. 2023; Novoselić et al. 2021), processing designs (Achat et al. 2012), along with others. The data from this section confirm the assumption that pitting, as a preferred method, can assist in the rise of TPC, as compared to the corresponding oils produced from unpitted olives. The TPC variations in all corresponding samples showed the same pattern across different malaxations (**Figure 7.6**). It was evident that the addition of 0.3 mm leaves exhibited the maximum values (the 30-pitted oil sample contained 368.01 mg/kg). The ranking of other fractions for TPC determined to be in descending order as follows: 0.15 mm > 0.07 mm > 1.6 mm > control > 3.0 mm. The mean differences between oils with 3.0 mm leaves and control (without leaves) were not significant.

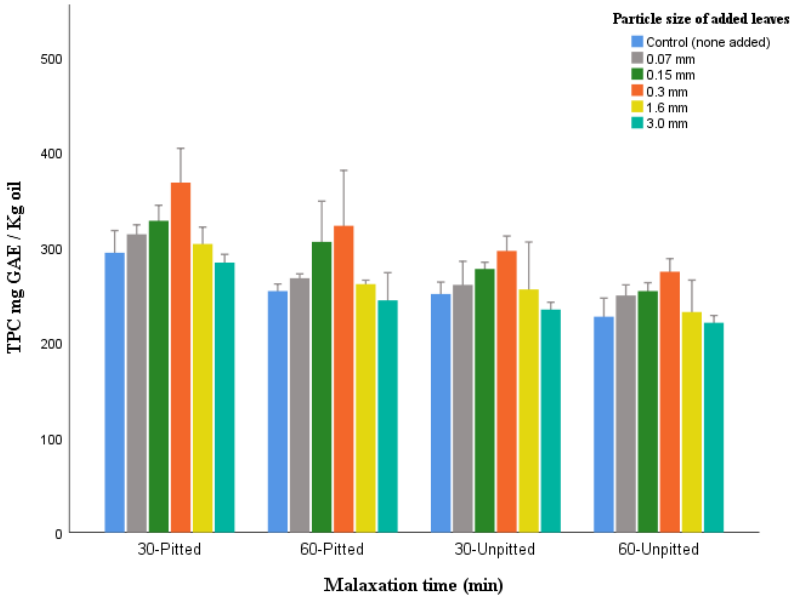


Figure 7. 6 Effect of particle size of added olive leaves on total phenolic content (TPC) of the extracted olive oils (mg GAE/Kg oil) from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min, respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

Results also validated the hypothesis that the extended malaxation is in part responsible for a significant drop in polyphenols. This can be due to the oxidation/catalyzing reactions of enzymes such as polyphenol oxidase (PPO). Therefore, the short-term malaxation (30 min) is preferred in this respect.

Likewise, pitting was found to be of a better choice as the TPC values were significantly higher in each pitted sample compared to the corresponding unpitted sample with the same malaxation time $p < 0.05$. It should also be pointed out that the favorable results of polyphenolic content in this study may be attributed to the time point of leaf addition, that was during the crushing step prior to malaxation which potentially assisted in ideal mixing of leaves with crushed olives, and hence eliminated the necessity of the use of the prolonged malaxation.

7.3.2.2. Trolox equivalent antioxidant capacity (TEAC)

Determination of antioxidant potential of oils with different size fractions of added leaves can be of value to ascertain whether the particle size of leaves has a significant role in this respect. The results here demonstrate that this is the case. In exception of 3.0 mm leaves, the inclusion of different fractions exhibited significant variations (in descending order of antiradical exertion: 0.3 mm, 0.15 mm, 0.07 mm, and 1.6 mm leaves). Relatively the same trends of variations were observed in all three types of TEAC assays (DPPH, FRAP, and ABTS) and the maximum levels belonged to 30-pitted oils with 0.3 mm leaves (1.51, 2.13, and 0.90 mM TE/kg oil detected by DPPH, FRAP, and ABTS, respectively).

The *in vitro* antioxidant assays partly reflect the presence and bio-functionality of polyphenols due to their strong antioxidant potential against reactivities of free radicals (the reactive oxygen species). In other words, the antioxidant activity potentially relies on the quantities of potent polyphenols. This was evident in our study as both TPC values and, correspondingly, antioxidant activities showed relatively similar trends of changes for each malaxation condition (**Figure 7.7**). Polyphenols indeed constitute a large proportion of phytonutrients in plant tissues. Their quantities, thus, play a potential role in minimizing activities of free radicals and maximizing oxidative stability of olive oil. In this context, the positive relations between total polyphenols and antioxidant capacity has been well justified in the literature (Achat et al. 2012; Ramos-Escudero et al. 2015; Öğütçü et al. 2008; Kiritsakis et al. 2010; Samaniego Sánchez et al. 2007; Pellegrini et al. 2001; Galvano et al. 2007; Gorinstein et al. 2003).

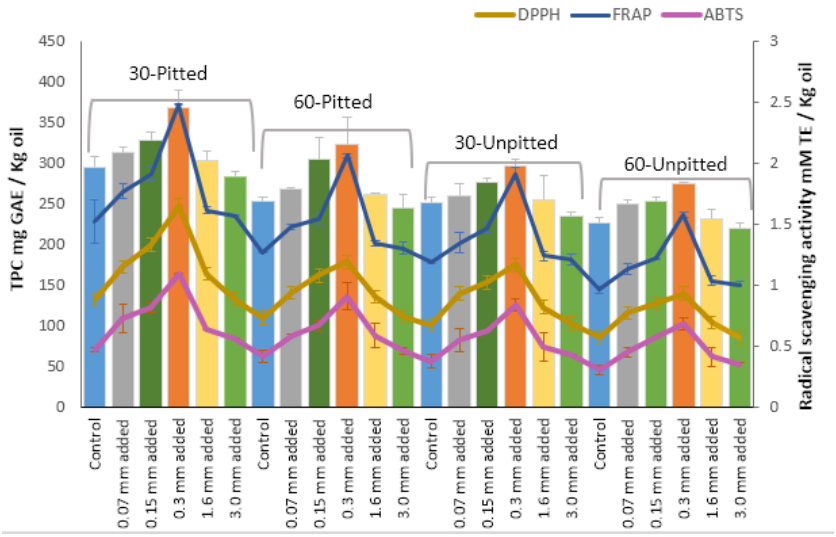


Figure 7. 7 Comparison of antiradical activities (detected by DPPH, FRAP, and ABTS) (mM TE/Kg), and their relations with total phenolic content (mg GAE/Kg) of the extracted oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

7.3.3. Effect of particle size of olive leaves on the recovery of target polyphenolic compounds

Quantitative analysis of the selected polyphenols (from polar fraction of olive oil samples) was examined by HPLC. The data were analyzed to explore the potential role of particle size of added leaves in phenolic concentrations of the oil samples.

7.3.3.1. Oleuropein content

As shown in **Figure 7.8**, the levels of oleuropein in oil samples with 0.3 mm leaves were significantly higher, particularly in 30-pitted samples (5.85 mg/oil). The control groups (oils without leaves) contained traces of oleuropein (0.29–0.89 mg/kg). This may be explained by that fact that oleuropein, that is abundantly found in the unprocessed olive fruits, is likely to be adversely influenced by degrading activities of the endogenous enzymes during processing. The degradation is more likely to occur during longer malaxation wherein the catalyzing active enzymes (particularly PPO) have the opportunity to exert oxidative effects. On the other hand, during shorter malaxation, the catalyzing actions of such enzymes can be suppressed/mitigated by a large numbers of intact/potent polyphenols, including oleuropein.

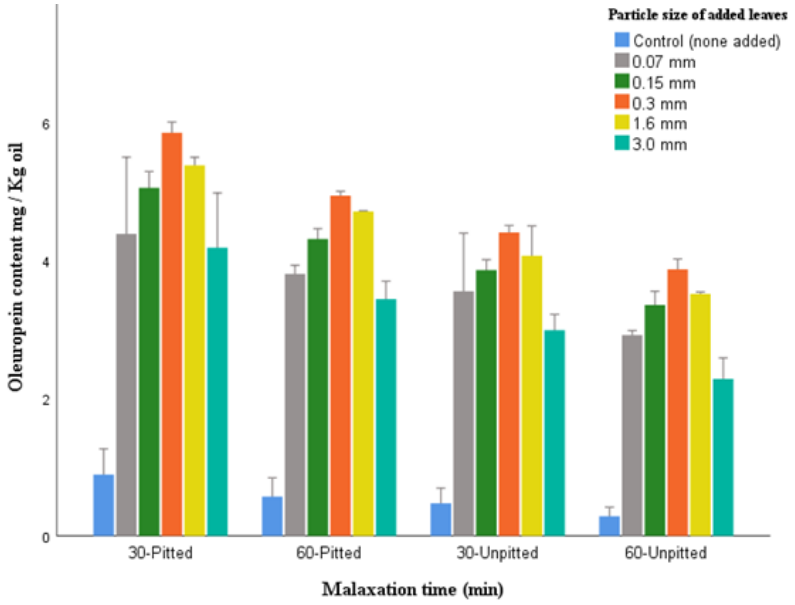


Figure 7. 8 Effect of particle size of added olive leaves on oleuropein content of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

7.3.3.2. Verbascoside content

As shown in **Figure 7.9**, the variations of verbascoside content across the oil samples followed the same pattern as those observed for oleuropein. The highest values (4.02 and 3.34 mg/kg) belonged to the oils of pitted olives with 0.3 mm leaves processed for 30 min and 60 min, respectively. Examining the pitting factor, results showed (**Figure 7.9**) that the addition of olive leaves enabled significantly higher yields of verbascoside in pitted samples (with leaves) compared to the corresponding ones in unpitted groups. This is in agreement with the research conducted previously (Servili et al. 2007).

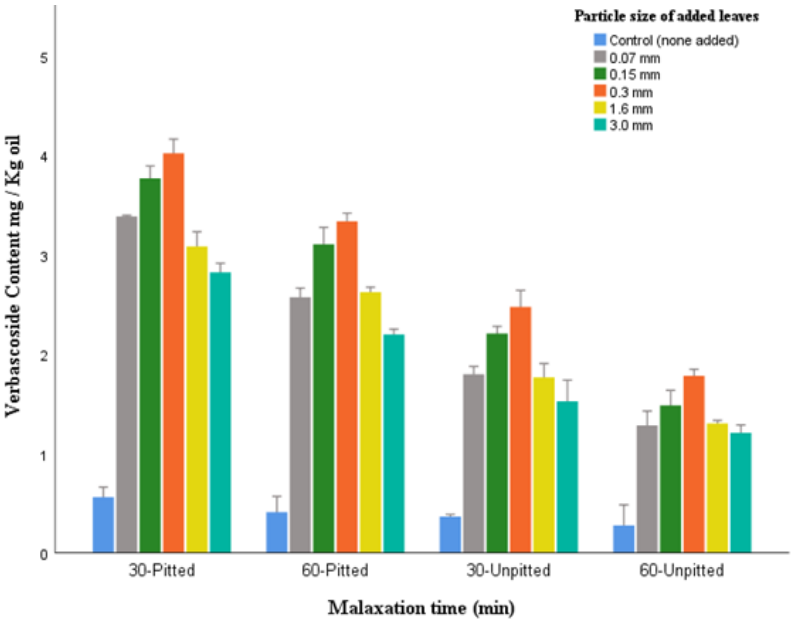


Figure 7. 9 Effect of particle size of added olive leaves on verbascoside content of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

Verbascoside, is characterized by a heterosidic ester of hydroxytyrosol and caffeic acid which makes it a distinctively valuable antioxidant due to its double di-phenolic structure (containing two phenolic hydroxyl groups; caffeic acid and hydroxytyrosol). This phenolic compound, while being largely available in olive fruits, is bound to be degraded or enzymatically hydrolyzed following the ripeness of fruits, storage, and processing; thus remaining in trace levels in olive oil. The results here confirm this postulation as the control samples contained minute amounts of this compound (0.28–0.56 mg/kg oil).

7.3.3.3. Hydroxytyrosol content

As shown in **Figure 7.10**, unlike the pattern of changes observed for oleuropein and verbascoside, the longer malaxation represented more of hydroxytyrosol in both pitted and unpitted samples. Within 60 min and 30 min malaxations respectively, the samples with 0.3 mm leaves contained 19.14 mg/kg and 18.05 mg/kg (in pitted samples) and 17.82 and 17.51 mg/kg (in unpitted samples). This may explain the partial hydrolysis of oleuropein (often by glycosidase and esterase enzymes) through extended malaxation, giving rise to the breakage of the ester bond and the liberation of hydroxytyrosol.

Moreover, compared to oleuropein and verbascoside, the content of hydroxytyrosol was much less affected by the enzymes of the olive stones present during malaxation, although the oils from pitted samples (for both malaxation times) yielded significantly greater amounts of hydroxytyrosol than those from unpitted ones.

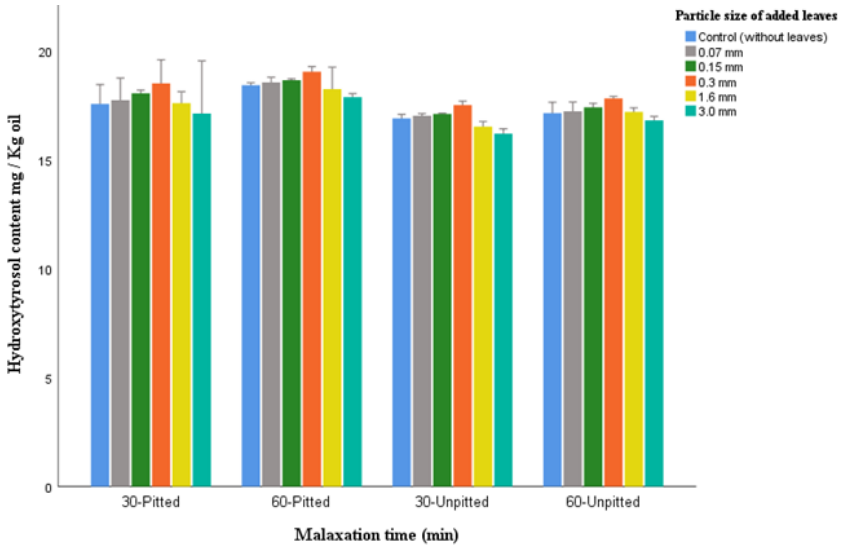


Figure 7. 10 Effect of particle size of added olive leaves on hydroxytyrosol content of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

7.3.3.4. Tyrosol content

The obtained data from tyrosol (**Figure 7.11**) showed a similar pattern, in respect of malaxation time, to that observed for hydroxytyrosol as the extended malaxation comparably enabled a rise in tyrosol content. The highest values belonged to the oils with 0.3 mm leaves (16.89 and 16.53 mg/kg, in 60-pitted and 30-pitted, respectively), and (16.12 and 15.83 mg/Kg, in 60-unpitted and 30-unpitted, respectively).

Compared to the majority of phenolic groups, tyrosol while having weaker antioxidant potential, is able to remain more stable, or become less affected by prolonged malaxation, hence a large numbers of tyrosol may readily be transferred from olive fruits to the resulting olive oil. This was the case in the present study as the control groups represented comparably a sizable proportion of tyrosol (16.24 mg/kg in 60-pitted, and 15.61 mg/kg in 30-pitted).

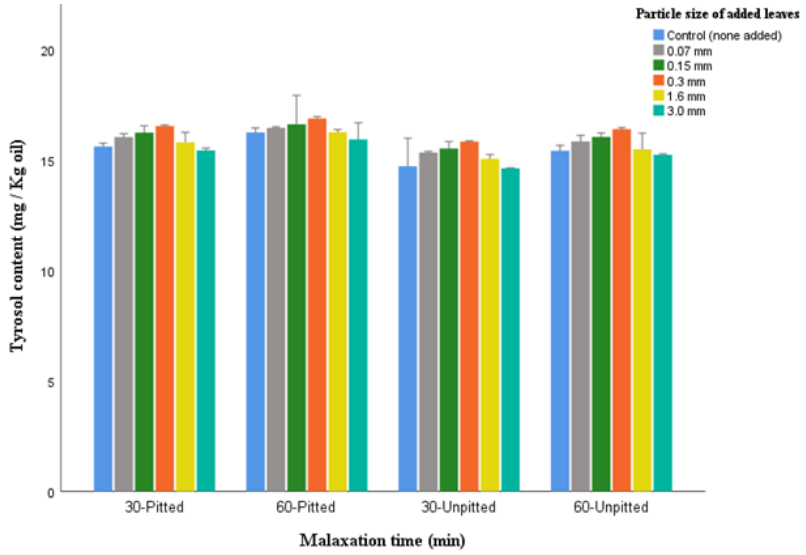


Figure 7. 11 Effect of particle size of added olive leaves on tyrosol content of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min, respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

7.3.3.5. Luteolin content

Irrespective of the factor of particle size, olive leaves when appropriately added during the extraction of olive oil, reportedly assist in intensification of flavonoid phenols including flavone luteolin in olive oil (in free or glycosylated form) (Ammar et al. 2017; Cuffaro et al. 2023; Sánchez de Medina et al. 2012). Examining the effect of particle size (**Figure 7.12**), it was evident that the oils with 0.3 mm leaves yielded maximum content of luteolin in all malaxation experiments (15.44 mg/kg and 14.92 mg/kg in 30 min pitted and 60 min unpitted, respectively). Among the leaf-added oils, those with large particles (3.0 mm), showed the lowest levels in all experiments (11.35–12.59 mg per kg oil). Also, insignificant variabilities were found between the control samples and the oils with 0.07 and 1.6 mm leaves ($p > 0.05$).

The luteolin proportions of non-enriched virgin olive oil tend to differ largely in previous studies, according to variations in cultivars, growing regions, and operating system. Fanali et al. (2018) examined different growing sites in Italy and reported: 12.34 mg/kg (for Sicily), 14.25 mg/kg (for Lazio), 13.82 mg/kg (for Tuscany), and 14.12 mg/kg (for Puglia). López-Yerena et al. (2021) through their investigation on the oil extraction methods, suggested that the use of lower crushing temperature (10 °C) and lower malaxation time (30 min) provide better conditions for the content of flavone constituents such as luteolin in olive oil.

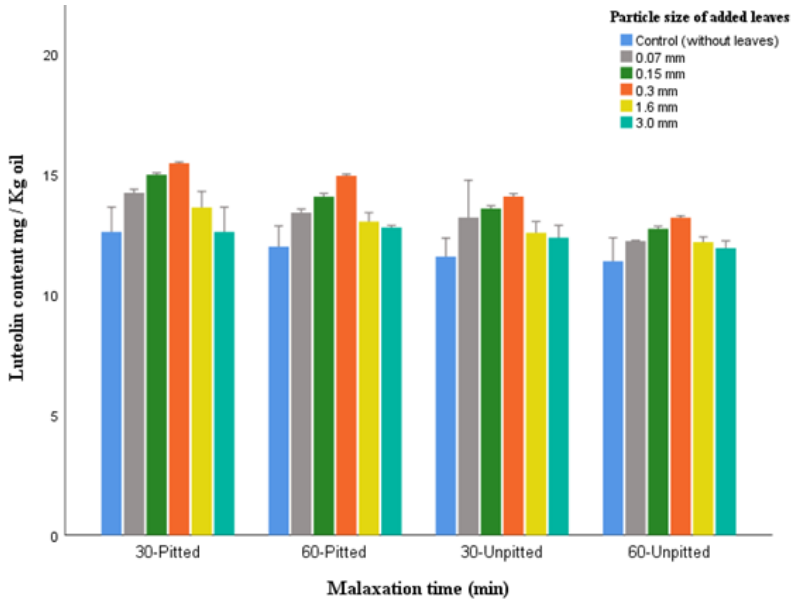


Figure 7. 12 Effect of particle size of added olive leaves on luteolin content of the extracted olive oils from pitted olives (30-pitted and 60-pitted, mixed for 30 and 60 min respectively), and unpitted olives (30-unpitted and 60-unpitted, mixed for 30 and 60 min, respectively).

7.3.3.6. Apigenin content

The highest value of apigenin (3.84 mg/kg) belonged to 0.3 mm leaf-added oils from pitted paste (30 min). As illustrated in **Figure 7.13**, the levels of apigenin were significantly higher in pitted samples compared to the corresponding oils in unpitted for the same malaxation time. Comparing the malaxation times, particularly in pitted groups, the shorter duration was more effective in the recovery of flavone apigenin. The particle size of 0.07 and 1.6 mm did not show significant effect on the content of apigenin for each malaxation group ($p > 0.05$).

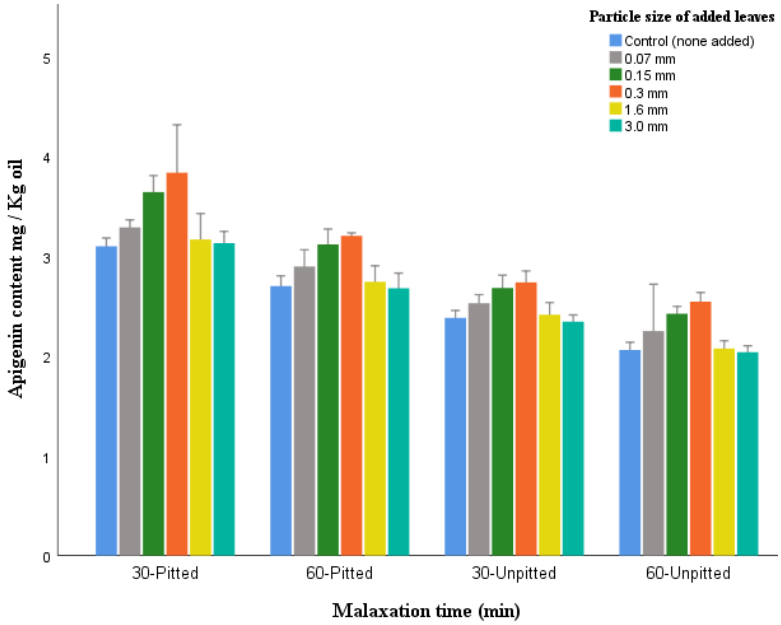


Figure 7. 13 Effect of particle size of added olive leaves on apigenin content of the extracted olive oils from pitted olives (30-pitted and 60-pitted mixed for 30 and 60 min, respectively), and unpitted olives (30-unpitted and 60-unpitted mixed for 30 and 60 min, respectively).

The data on apigenin content in virgin olive oil differs largely among studies based on the experimental conditions. A recent study reported that the addition of olive leaves of different proportions (0%, 1%, 2.5%, and 5%) during extraction of olive oil (Buža cultivar) resulted in 0.7, 0.8, 1.6, and 0.8 mg per kg oil, respectively (Novoselić et al. 2021). Another research addressed the factor of pitting through their experiment on five different cultivars. Based on their findings, the apigenin concentrations varied depending on different varieties as follows: (i) Picual cultivar: 4.55 mg/kg (unpitted) and 4.49 mg/kg (pitted), (ii) Koronakii cultivar: 2.30 mg/kg (unpitted) and 1.30 mg/kg (pitted), (iii) Arbiquene cultivar: 6.19 mg/kg (unpitted) and 7.12 mg/kg (pitted), (iv) Carotenia cultivar: 5.18 mg/kg (unpitted) and 4.50 mg/kg (pitted), and (v) Frantoi cultivar: 7.19 mg/kg (unpitted) and 7.00 mg/kg (pitted) (Amany Basuny et al. 2008). Also in the study of López-Yerena et al. (2021) the extra virgin olive oil (Corbella cultivar) when processed through a low-temperature crushing (10 °C) yielded more of apigenin content (2.85 mg/kg) compared to that obtained with higher temperature (1.43 mg/kg at 25 °C).

7.4. Conclusions

The present study demonstrated that size reduction of added olive leaves is among the key factors affecting the polyphenolic content of the resulting olive oil. Of particular interest was the addition of 0.3 mm leaves during crushing of pitted olives, prior to a 30 min malaxation, that favorably exhibited maximum effects on the enhancement of: (i) polyphenol concentrations, especially oleuropein and

verbascoside, (ii) antioxidant capacity, and (iii) oxidative stability, without adverse impact on the yield of the extracted oil. The data from this study may derive benefit from an in-depth research work based on olive leaf addition at various processing points of oil extraction considering a broader range of size fractions and their possible interactions with other major factors including olive cultivars. Beyond the extensive investigation of polyphenols and antioxidant potency, a need also exists to assess organoleptic quality of the leaf-added oils. In light of further information on the particle size reduction of olive leaves, the commercial entities may receive benefits of re-utilizing dry ground olive leaves sustainably in a range of food applications beyond olive oil production.

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CHAPTER 8

STABILITY OF TARGET POLYPHENOLS OF LEAF-ADDED VIRGIN OLIVE OIL UNDER DIFFERENT STORAGE CONDITIONS OVER TIME

This work has been submitted to the Royal Society of Chemistry (RSC) in the Journal of Sustainable Food Technology:

Safarzadeh Markhali, F., and Teixeira, J.A., 2023. Stability of Target Polyphenols of Leaf-Added Virgin Olive Oil under Different Storage Conditions over Time. *Sustainable Food Technology*.

Abstract

In this study the effect of storage conditions on polyphenolic content of virgin olive oils (VOO) enriched with 0.3 mm olive leaf powders was investigated compared to those without leaves. The main objective was to determine the magnitude of the impacts of oxygen and light exposures over storage durations on the concentrations of the major polar phenols. This is particularly important as the exposure of oxygen and light to the oil often occurs under domestic situations or even during manufacturing and commercial conditions that, consequently, may bring about a drastic depletion of antioxidants in the affected oil. Importantly, for polyphenols to be biologically active, they need to be available/unaffected in sufficient proportions all around the shelf-life of the oil. The findings from both groups of oil samples (with and without leaves) showed relatively similar trends of phenolic drops upon exposure to light and oxygen over time. However the leaf-added oils (particularly 30 min malaxation) contained significant proportions of oleuropein and verbascoside over six months; with final concentrations of 4.97 and 4.10 mg/kg oil (for oleuropein) and 3.42 and 2.83 mg/kg oil (for verbascoside) when exposed to light and oxygen, respectively. On the other hand, only trace levels of these compounds were found in leaf-free oils. Overall, the data support the role of appropriate particle size of leaf fractions (0.3 mm) in the oil as considerable levels of polyphenols, and correspondingly antiradical capacity, still remained having been exposed to oxygen and light over the course of time.

Keywords: Olive leaf powder, leaf-added olive oil, storage, polyphenols, oleuropein, verbascoside.

8.1. Introduction

Extra virgin olive oil (EVOO), compared to the majority of edible oils, generally has a less susceptibility to oxidation with a fairly long shelf-life (12–18 months) because it is relatively high in antioxidants (particularly polyphenols) and low in polyunsaturated fatty acids (Ayton et al. 2012; Morelló et al. 2004). Having said that, inadequate storage of EVOO, even over a short period of time, potentially renders the oil oxidized to the extent that it can lose its extra virgin attributes (Ayton et al. 2012). The oxidation reaction, beyond growing rancidity/off-flavor, may degrade/deplete the endogenous polyphenols to which the nutritional quality of EVOO highly depends on. The occurrence of oxidation is not uncommon particularly through (i) improper handling/storage conditions (e.g., exposure of oil to light and oxygen) throughout manufacturing, shipping, and marketing, and (ii) mishandling under domestic conditions where the unsealed bottles are often exposed to light and/or oxygen over time. The latter may occur routinely as consumers, although generally health conscious, may not be fully aware of the detailed instructions for keepability and soundness of the oils they are commonly using. In this respect, some exploratory studies dealing with stability of olive oil have postulated potential processing approaches to retain/upgrade the nutritional properties of virgin olive oil for longer shelf life. These include: (i) microemulsion of vitamin-C as a potential antioxidant for improving the stability of virgin olive oil (Osanloo et al. 2021), (ii) addition of fresh olive leaves (0–3%) in advance of extraction (Sevim et al. 2013), and (iii) upgrading the value addition of polyphenols in olive oil through their exposure to olive mill waste water fermented by L. *Lactobacillus plantarum* (Kachouri & Hamdi 2004).

Another example that has received substantial attention is the incorporation of olive leaves into the oil; considering that the polyphenols of olive leaves upon their successful transfer to the oil may potently and concentration-dependently enhance the quality and shelf life of the oil (Achat et al. 2012; Gutiérrez-Rosales et al. 1992; Mezghani et al. 2023; Tarchoune et al. 2019; Marx et al. 2022; Ammar et al. 2017; Sevim et al. 2013; Sanchez de Medina et al. 2011). In this respect, our recent study (Safarzadeh Markhali & Teixeira, 2023) compared a range of particle size fractions of olive leaves added to crushed olives before malaxation of olive paste. Importantly, it was found that the inclusion of dry ground olive leaves with 0.3 mm particle size, significantly enabled (i) maximum recovery of target polyphenols and antioxidant capacity, and (ii) minimum peroxide value and free acidity in the resulting virgin olive oil. In light of these findings, the present study aimed at examining the storage stability of olive oils with and without 0.3 mm olive leaf powders after exposure to severe surrounding conditions over time. The main focus was placed on understanding to what extent the content of polar phenols may be affected under various the selected storage conditions over the time points of the keeping oils.

8.2. Material and methods

Plant materials – olive leaves and olive fruits (Picual), supplied by "Center for Advanced Studies in Energy and Environment", University of Jaén, Campus of Las Lagunillas, Jaén, Spain. Sampling preparations were as follows: (i) *olive leaves* – after initial cleaning, washing, drying (37 °C, 48 h), were ground and size reduced to 0.3 mm particle fractions. The leaf powders (vacuum packed in polypropylene bags and refrigerated) were subjected to the mechanical processing system (see **Section 8.2.2**) within two weeks. (ii) Olive fruits, were manually cleaned to remove foreign objects/bruised olives, laid flat on food-grade/dry trays, refrigerated, and processed for oil extraction within two weeks.

Chemicals – the following reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA): (i) *analytical grade* – Folin–Ciocalteu, anhydrous gallic acid, anhydrous sodium carbonate (≥99%), hydrochloric acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride ($FeCl_3$), ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), hexane, methanol, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), potassium iodide, chloroform, and acetic acid, (ii) *HPLC grade* – phenolic standards (oleuropein, verbascoside, and hydroxytyrosol, tyrosol, luteolin, and apigenin), acetonitrile, and formic acid.

8.2.1. Experimental design

Preliminary findings – in our earlier study (Safarzadeh & Teixeira, 2023), through adding dry ground olive leaves prior to the malaxation, the effects of different particle size fractions of added olive leaves (the primary factor) and their interactions with malaxation time, and olive pitting in the extracted olive oil (the secondary factors) on phenolic recovery and oxidative quality of the resulting virgin olive oils were assessed. Since the results of the previous study provided important insights, it served as a basis in this model project to decide on selecting the independent variables – only the factors that showed statistically significant effect(s) on the respective dependent variables, were included in the factors of the present study. In this respect, the oil samples (from both malaxation times) with 0.3 mm were chosen. Also, pitting remained a benchmark constant in the current study as the oils samples from unpitted olives showed insignificant results in the preliminary findings.

Therefore in the current study the abovementioned factors were taken into account as well as the chosen independent variable of this study (stability condition/time) (**Table 8.1**) to assess the following dependent variables: (i) total phenolic content (TPC), (ii) target polar phenols consisting of oleuropein, verbascoside, hydroxytyrosol, tyrosol, luteolin, and apigenin (iii) antioxidant assays *in vitro*, and (iv) peroxide value and free acidity of the oil samples.

Table 8. 1 Specifications of independent variables designed for this study.

Factor of study	Parameters	Coded oil samples
Particle size of added leaves	▪ With addition (0.3 mm leaf fractions) ▪ Without addition (Control)	▪ Control-30 min ▪ Control-60 min ▪ Leaf-Added-30 min ▪ Leaf-Added-60 min
Malaxation time	▪ 30 min ▪ 60 min	
Storage condition of oil samples	▪ Without light/oxygen exposure (*oxygen/light protected*) ▪ Light exposure only ▪ Oxygen exposure only	

8.2.2. Mechanical processing – extraction of olive oil

The for the extraction of virgin olive oil was carried out using the same operation design applied in our earlier experiment (Safarzadeh Markhali & Teixeira, 2023). Briefly, the operations consisted of: (i) crushing the pitted olives wherein dry ground leaves (with 0.3 mm particle fractions) were added, (ii) Malaxation of the crushed olive paste (with leaves and without pits) under slow spinning for various times (30 and 60 min), and (iii) two-phase separation by centrifugation of the mixed olive paste was centrifuged at 3500 rpm for 5 min. The oil fraction was separated, weighed, bottled before being subjected to the storage trials descried below (**Section 8.2.3**). Also, the *control* samples were obtained accordingly through the same procedure with the exclusion of olive leaves. Temperature remained constant at 27 °C throughout the entire operation.

8.2.3. Storage of samples

Storage of oil samples – the stability of the extracted olive oil samples was analyzed over time points of a six month storage period under various atmospheric conditions described below. All samples were stored at room temperature (20 ± 5).

8.2.3.1. Storage of oils without exposure to light/oxygen

The falcon sterile tubes loaded with oil samples, nitrogen flushed, tightly closed with the caps and wrapped with aluminum foils prior to their storage in darkness.

8.2.3.2. Storage of oils with light exposure only

The falcon tubes (unwrapped with aluminum) containing oil samples nitrogen flushed, with caps on (screwed tightly) were stored in a room where the light (from sunlight and/or artificial source) was available over the course of six months.

8.2.3.3. Storage of oils with oxygen exposure only

The oil samples without nitrogen flush in falcon tubes (with aluminum foils wrapped around) were stored in dark leaving the lids loose to ensure exposure of oxygen while protecting them from atmospheric light.

8.2.4. Evaluation of polar phenolic fraction

8.2.4.1. Sample preparation – isolation of phenolic fraction from oil samples

The polar phenolic fraction was separated from the lipid fraction of the oil using liquid–liquid extraction method of Lozano-Castellón et al. (2021) with slight modifications as described in our previous report (Safarzadeh Markhali & Teixeira, 2023). Briefly, the oil (2 g) was mixed with hexane (2 mL) and methanol/water (4 mL), centrifuged at 3000 rpm for 5 min. The extracted polar fraction (methanol/water), after removing the solvent, was then dissolved in HPLC-grade methanol/water (50/50, v/v) and nitrogen flushed before the experiments described below. For HPLC assessments, the sample was initially passed through a 0.22 μm membrane filter.

8.2.4.2. Total phenolic content (TPC)

The colorimetric analysis of TPC using Folin–Ciocalteau was determined based on the method of Singleton and Rossi (1965) as described previously (Safarzadeh Markhali & Teixeira, 2023). The results were expressed as mg gallic acid equivalents/kg oil (mg GAE/kg).

8.2.4.3. Quantification of target phenolic compounds

The chromatographic separations of target polar phenols were carried out on a reverse-phase Aquity UPLC BEH C18 column (100 mm × 2.1 mm, with 1.7 μm particle size, Waters Corporation). The eluents were as follows: water/formic acid (99.9/0.1 *v/v*) as mobile phase (A), and acetonitrile (100%) as mobile phase (B). The gradient elution program was based on the method of Quero et al.(2022) with slight modifications as detailed previously (Safarzadeh Markhali & Teixeira, 2023). The temperature remained constant (40 °C) with a flow rate of 0.3 mL/min, and injection volume of 5 μL.

8.2.4.4. Antioxidant capacity

The *in vitro* antioxidant capacity of phenolic fractions of the oil samples was examined based on Trolox Equivalent Antioxidant Capacity (TEAC) via DPPH (Brand-Williams et al. 1995), FRAP (Benzie & Strain 1996), and ABTS (Re et al. 1999) radical scavenging methods as performed and described in our previous experiment (Safarzadeh Makrhali & Texeira, 2023). The absorbance readings [(515 nm) for

DPPH, (593 nm) for FRAP, and (734 nm) for ABTS] were calculated against Trolox standard curve and the results were expressed as mM Trolox equivalents/kg oil (mM TE per kg).

8.2.5. Evaluation of chemical quality of oil samples

The rancidity levels of oil samples over the storage conditions/time points were examined through peroxide value (PV) (for oxidation) and free fatty acids (FFA) (for lipolysis) using titration assays according to the official methods of American Oil Chemists' Society (AOCS, 1998) as performed and described previously (Safarzadeh Markhali & Teixeira, 2023). The PV results were reported as milliequivalents of active oxygen per kilogram oil (mEq. O_2/kg). The FFA results were reported as percentage of oleic acids (g /100 g, w/w).

8.2.6. Statistical analysis

The significant differences ($p < 0.05$) between the mean values (±SD) of all determinations were statistically assessed via Analysis of Variance (ANOVA) using SPSS software, version 27.0. The effect of independent variables on each dependent variable was analyzed through factorial ANOVA (two-way ANOVA). The multiple comparison interactions between independent variables: (i) oil groups *vs* storage times for the same malaxation time, and (ii) malaxation times *vs* storage times for the same oil group, were assessed using pairwise comparisons. The assumption of homogeneity of equal variance was assessed through the Levene's test (homogeneity of variance assumption was not violated when p-value was greater than 0.05). Each experiment was carried out in triplicates.

8.3. Results and discussion

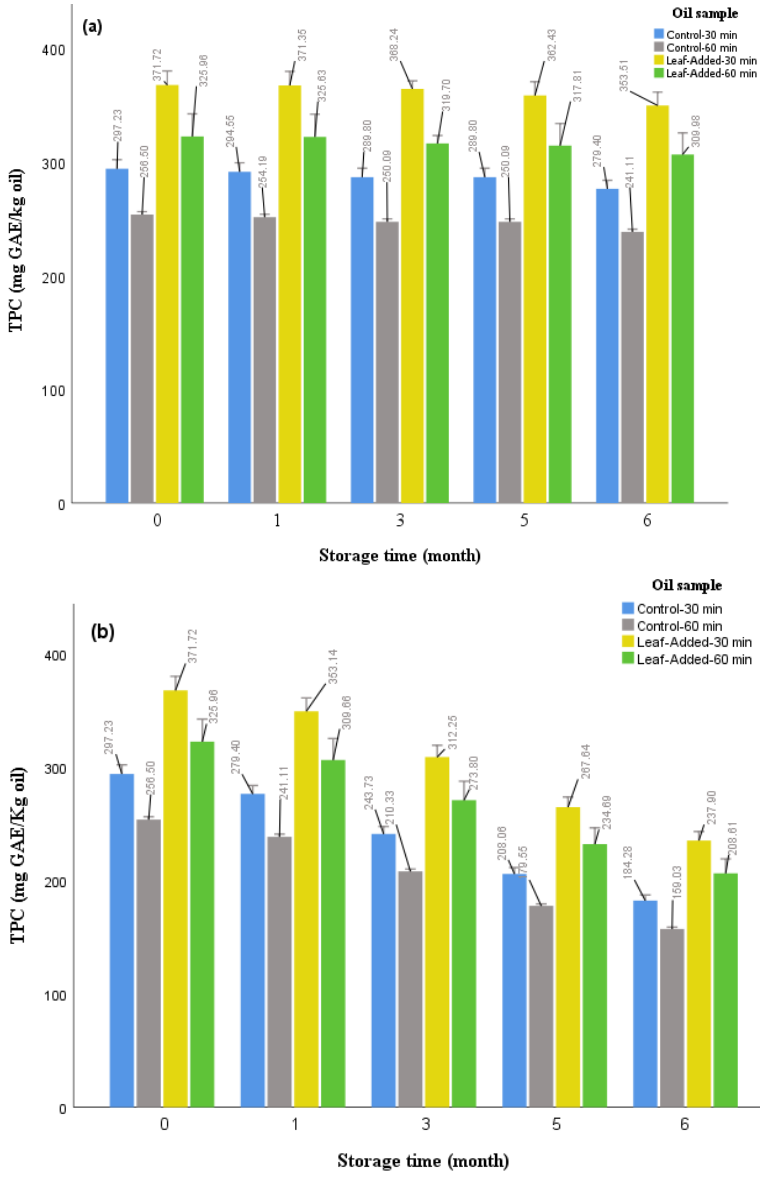
The aim of this study was to evaluate the storage stability of leaf-added virgin olive oil (with o.3 mm leaves) compared to that without leaves (*Control*). By referring to the preliminary findings from our earlier study (Safarzadeh Markhali & Teixeira, 2023) the independent variables were selected for the current study. In this regard, the oil samples (from both short and extended malaxations) with 0.3 mm were assessed for their storage stabilities in terms of polar target/total phenolic content, antioxidant capacity, and free acidity/peroxide values over time (month 0, month 1, month 3, month 5, and month 6) under a set of atmospheric conditions.

8.3.1. Effect of storage on phenolic content of leaf-added oils

8.3.1.1. Stability of total phenolic content (TPC)

The changes of TPC in oil samples under different storage conditions are shown in **Figure 8.1**. The data demonstrate that the TPC in all oil groups declined most rapidly at the exposure of light (up to

around 36%) and, slightly less sharply at the exposure of oxygen (up to 33%) upon a six month storage period. On the other hand, in the absence of light/oxygen exposure the total phenols remained more stable over the six month period of storage (with 2–5% decline) which explains the favorable effect of nitrogen flushing in the headspace as it enables replacement of oxygen/air with the inert gas, and thereby zero/minimum chance can be provided for the air/oxygen to bring about oxidation.



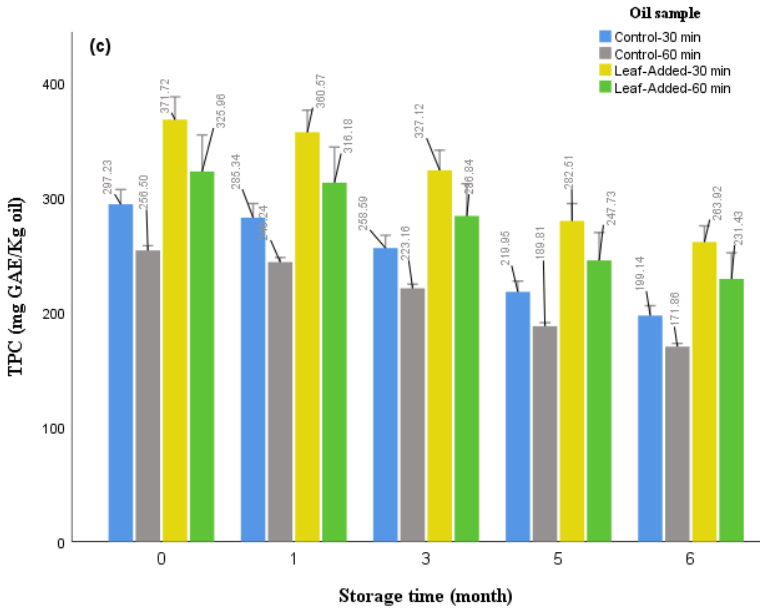


Figure 8. 1 Changes in total phenolic content of oil samples (mg GAE/Kg) stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values with standard deviation error bars.

Regardless of the variations in the experimental conditions, the detrimental impacts of storage conditions/duration on the reduction of polyphenols has been highlighted in several research studies (Ghanbari Shendi et al. 2020; Clodoveo et al. 2007; Baiano et al. 2009; Morelló et al. 2004). In terms of oxygen and light exposure, the data on the TPC depletions support those previously reported by other researchers that depending on their experiments polyphenols were highly (Okogeri & Tasioula-Margari, 2002, Gutiérrez & Fernandez, 2002; Luna et al. 2006).

During processing and storage, the atmospheric risk factors including oxygen and light, should not come into contact with the oil. Their exposures to the oil may cause autooxidation or photosensitized oxidation through which a significant drop in phenolic content may occur. As a result, the oil becomes less stable and nutritionally degraded. The ability to formulate a sustainable process design potentially assists in the rise of phenolic content at the initial stage of oil extraction. Therefore during the severe conditions of storage, sufficient amounts may still remain to exert bio-functional effects throughout the shelf-life of the oil.

Overall the data revealed that in all storage conditions, although the phenolic reductions across the oil groups followed the same pattern, the leaf-added oils with 30 min malaxation maintained the maximum phenolic concentrations over all time points for the same storage experiment. On the other hand, the oils without leaves (particularly with 60 min malaxation) maintained the lowest phenolic content over each time point for the same storage trial.

8.3.1.2. Comparison between polyphenolic content and antiradical activities

Polyphenols may dose-dependently (i) suppress/inhibit free radicals, and (ii) hinder oxidative/hydrolytic rancidity of the oil. Hence, they are potentially responsible for extending the shelf life and promoting the nutritional quality of the oil. To shed light on this matter, the TPC results were further compared with *in vitro* antiradical capacity detected by DPPH, FRAP, and ABTS (**Figure 8.2**).

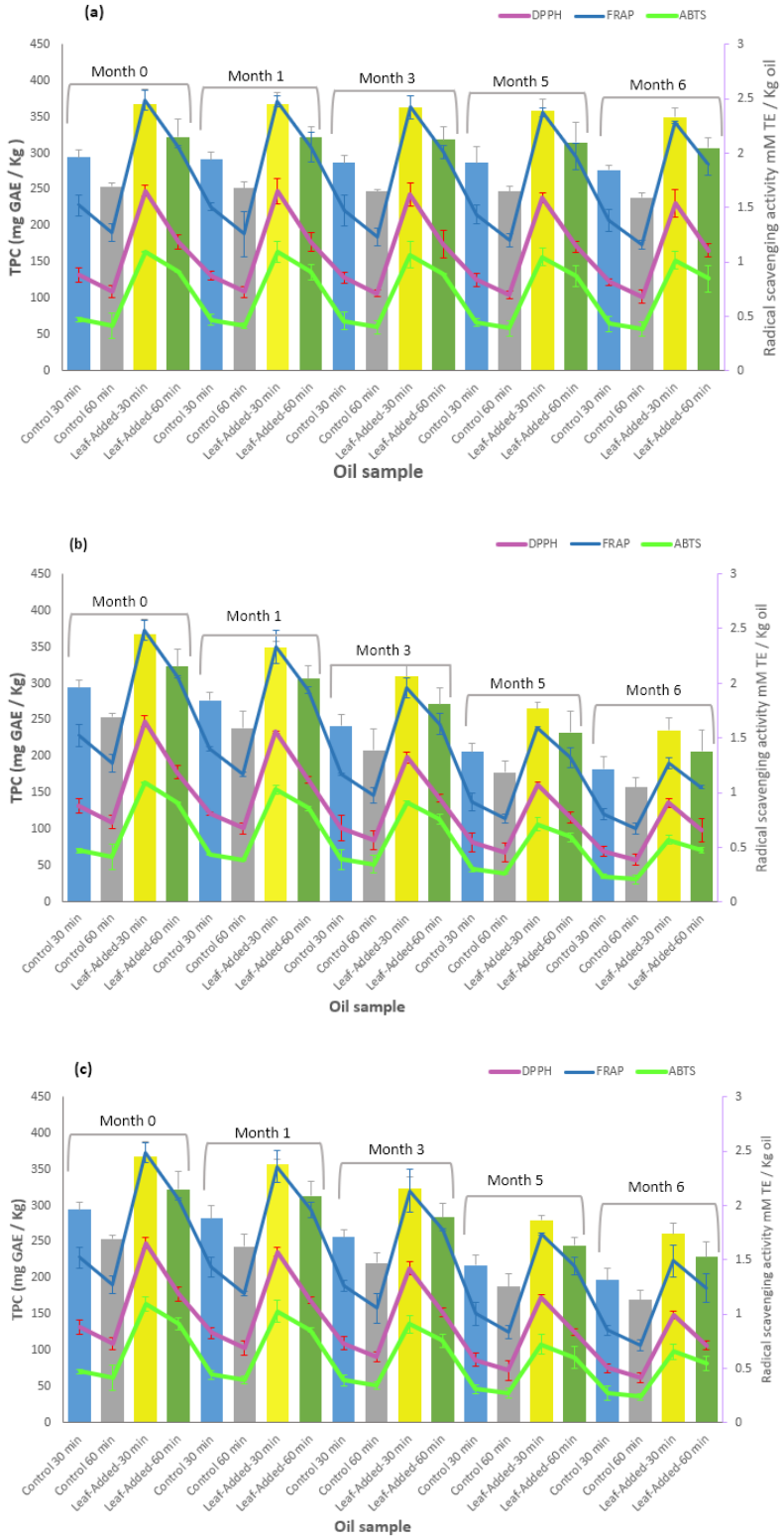
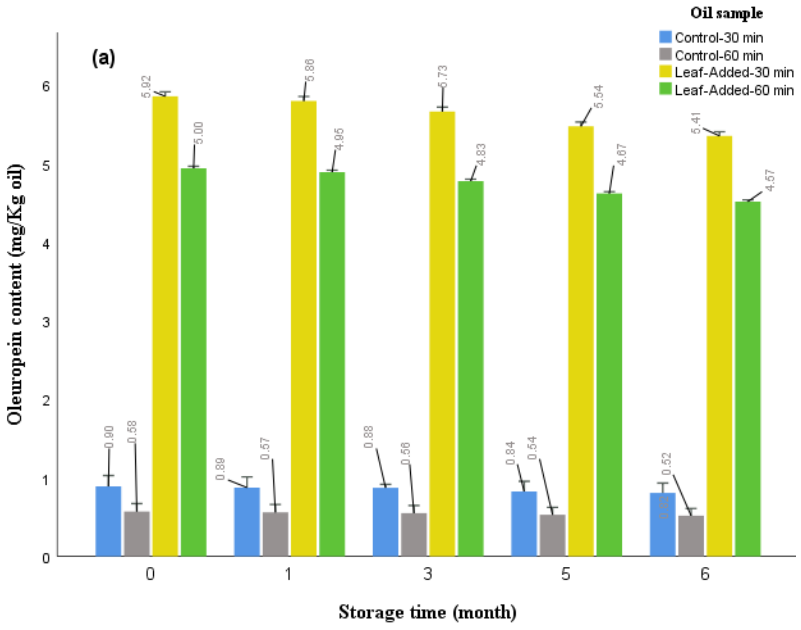


Figure 8. 2 Comparison of antiradical scavenging activities (detected by DPPH, FRAP, and ABTS) and total phenolic content (TPC) of oil samples stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The oil samples: with added leaves (0.3 mm particle size), and without leaves (Control). The values of 30 and 60 represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

As shown in **Figure 8.2**, it was found that, similar pattern of changes between antiradical activities were detected, for the same storage condition. In addition, the trends relatively corresponded with the changes of TPC for the same storage experiment. As seen in **Figure 8.2**, the leaf-added oil (30 min malaxation) that represented the highest degrees of TPC for each storage condition, comparably showed maximum antiradical activities, with the final antiradical values detected by FRAP, DPPH, and ABTS as follows: (i) 2.28, 1.58, and 1.02 mM TE/kg oil under no light/oxygen (ii) 1.27, 0.91, 0.57 mM TE/Kg oil under light exposure, and (iii) 1.49, 0.99, 0.66 mM TE/kg oil under oxygen exposure.

8.3.1.3. Stability of oleuropein

As demonstrated in **Figure 8.3**, oleuropein decreased with time under all storage conditions but the greatest loss occurred in the presence of oxygen (up to 30%) followed by those in light exposure (up to 15%). The concentrations remained more stable in light/oxygen protected, with slower drops at each storage time point (up to around 9% loss over the six months).



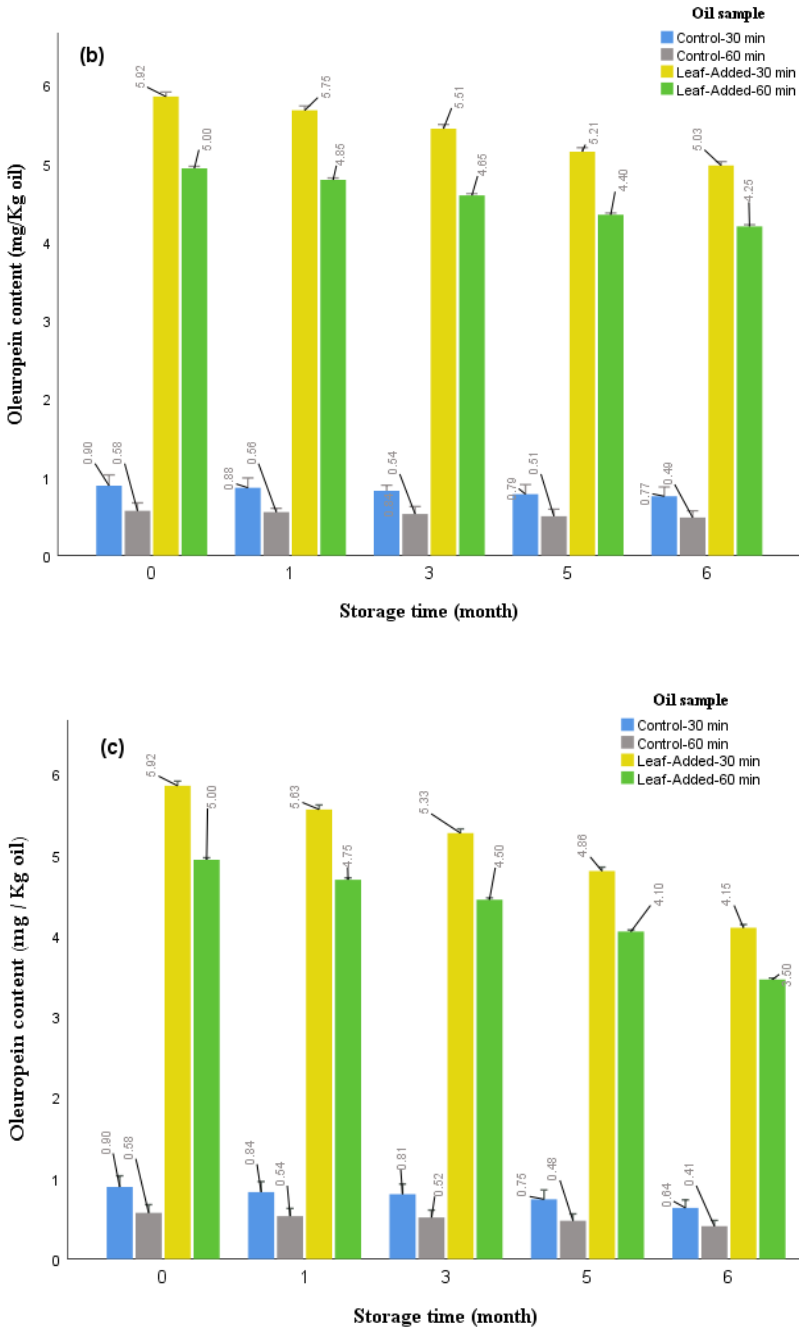


Figure 8. 3. Changes in oleuropein content of oil samples (mg/kg oil) stored over time points at (20 ± 5 °C) under: **(a)** no light/oxygen exposure; **(b)** light exposure only; **(c)** oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

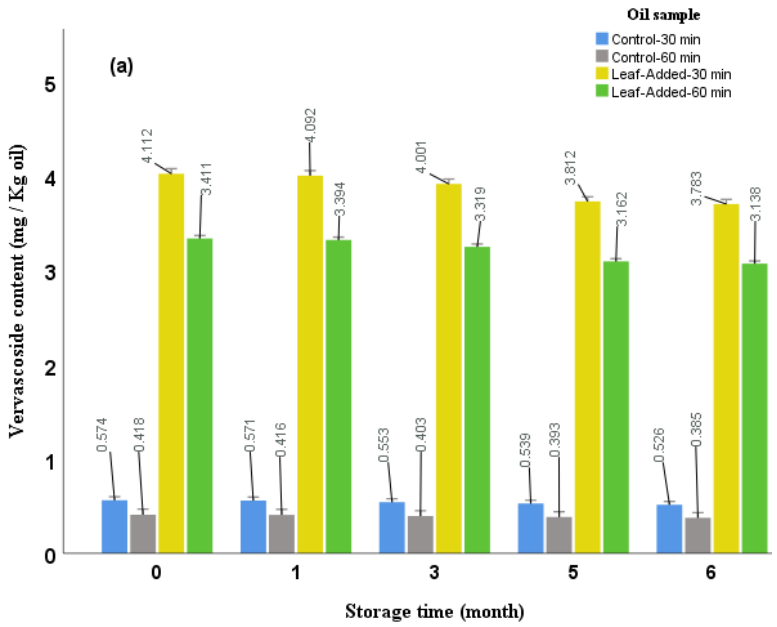
The storage condition/duration is among the major factors affecting the content of oleuropein which can potentially decompose in the course of: (i) oxidation reaction (Bounegru & Apetrei, 2022; De Leonardis et al. 2015) in the presence of atmospheric air/oxygen that promotes activities of oxidizing enzymes including polyphenol-oxidase (PPO) and peroxidase (POD) which can ultimately jeopardize the nutritional quality and stability of the oil, (ii) cleavage of ester bonds (Bounegru & Apetrei, 2022; De Leonardis et al. 2015), by the actions of enzymes such as esterase, with consequent formation of

oleuropein derivatives including hydroxytyrosol, and (iii) de-glycosylation (Bounegru & Apetrei, 2022) that generates oleuropein aglycone (a potential substrate for hydroxytyrosol formation) and glucose. In the case of oxidation that can cause a detrimental effect, the activities of oxidizing enzymes can be accelerated by the presence of oxygen which may be inevitably the case over the domestic storage of unsealed oil bottles particularly over the course of longer storage period.

Overall, the data suggest that the addition of 0.3 mm of leaf powders, particularly before shorter malaxation (30 min) favored greater content of oleuropein in the oil over time points; although, this compound in the leaf-added oils declined over the severe storage conditions/times, having being present in large numbers at the initial point (month 0), still remained in substantial levels over time (final values: 5.41–4.15 mg/kg oil)..

8.3.1.4. Stability of verbascoside

Similar to oleuropein changes, the leaf-added oils that yielded significantly large amounts of verbascoside at the original point (month 0), continued to represent the highest values over time points for the same storage experiment. As shown in **Figure 8.4**, the trends of changes in verbascoside content, over time points, showed relatively similar to those observed for oleuropein content, with oxygen exposure being the most detrimental factor (around 30%) followed by light exposure (around 15%).



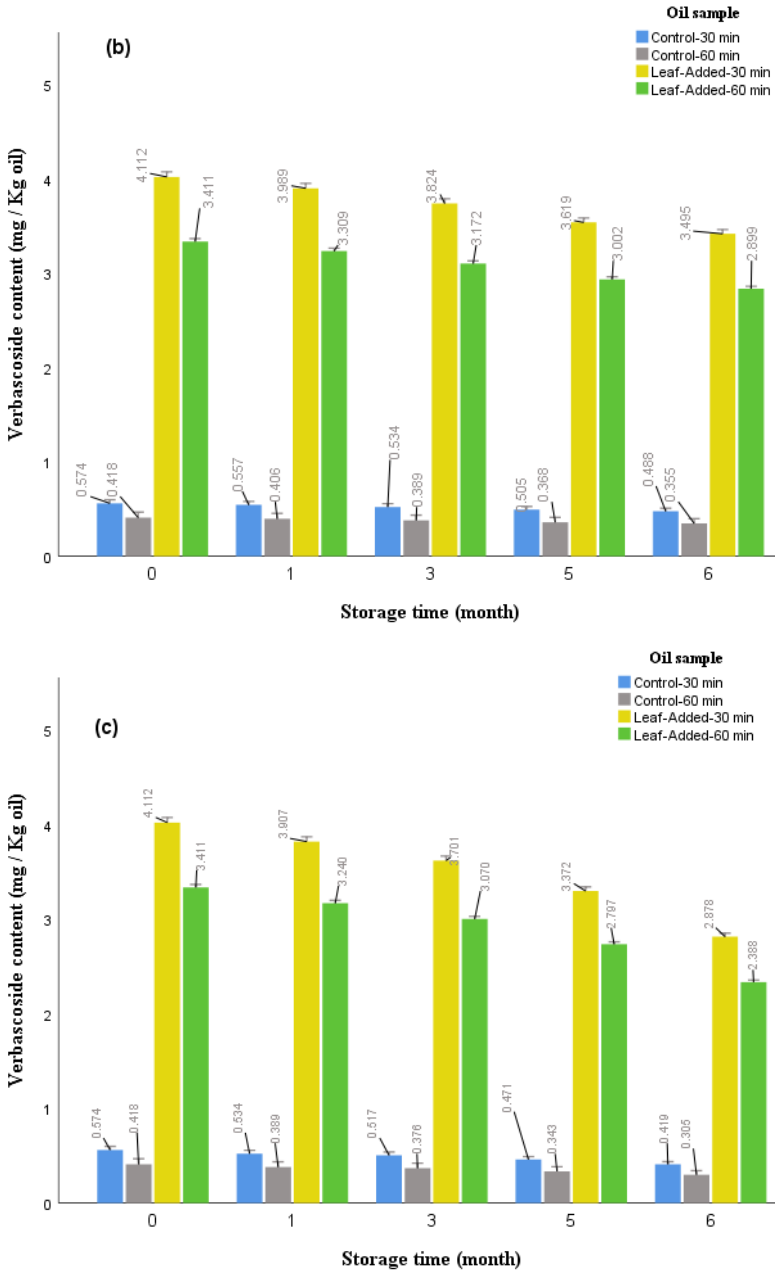


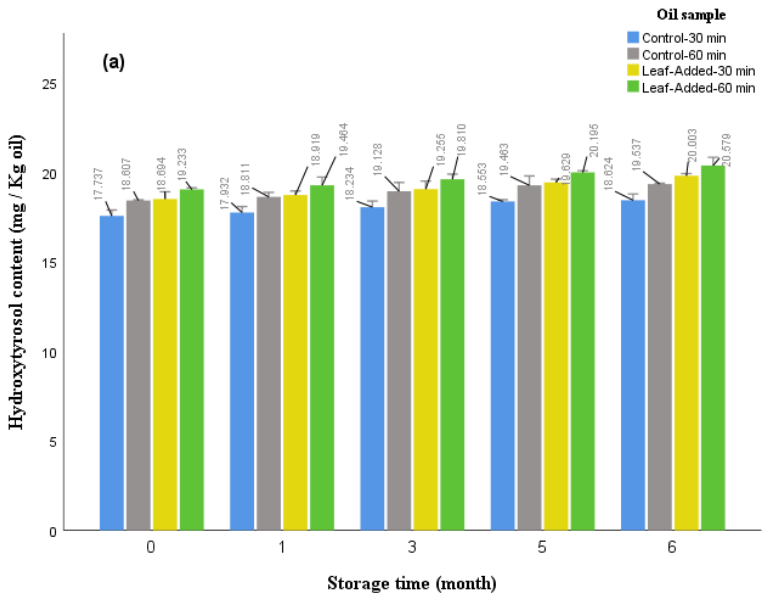
Figure 8. 4 Changes in verbascoside content of oil samples (mg/Kg oil) stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

Verbascoside, owing to its molecular structure with two di-phenolic groups, is among the powerful antioxidant compounds. However, this valuable phenolic compound is often found in a trance amount in a non-enriched olive oil while being present in non-processed olives and olive leaves. This issue was in part studied in our recent report (Safarzadeh Markhali & Teixeira, 2023) via comparing different particle size fractions of added olive leaves. It was found that the addition of 0.3 mm of leaves to the pitted olives during crushing, significantly assisted in the rise of verbascoside content in the resulting VOO. Following up this approach in the present study, it was found that this method of leaf addition offers additional

advantages in respect of verbascoside content upon storage. The impacts of oxygen and light, although unfavorably caused depletions, were not detrimental as the leaf added oils contained relatively significant amounts over the six month period (3.50 and 2.88 mg/kg, in the presence of light and oxygen respectively).

8.3.1.5. Stability of hydroxytyrosol

The stability of hydroxytyrosol under storage conditions over time points is illustrated in **Figure 8.5**. Unlike the changes observed for oleuropein and verbascoside, the content of hydroxytyrosol rose upon the six-month storage with oxygen exposure (around 15%), light exposure (around 9%), and no light/oxygen exposure (around 7%). This may suggest that the rise in hydroxytyrosol potentially resulted from the depletion of oleuropein; assuming that the biotransformation mechanisms favored the release of hydroxytyrosol which typically occurs through the breakage of the ester bonds of a potent oleuropein/oleuropein aglycone and possible formation of simple phenols including hydroxytyrosol. However, comparing the proportions of the loss in oleuropein and the gain in hydroxytyrosol, it appears that other mechanism of actions were potentially involved in oleuropein bio-degradations. Among which, is the occurrence of oxidation reactions by the increased activities of oxidizing enzymes under the given storage conditions/times. In other words, oleuropein, apart from being hydrolyzed to its derivatives, was potentially oxidized and bio-functionally disabled in the oil.



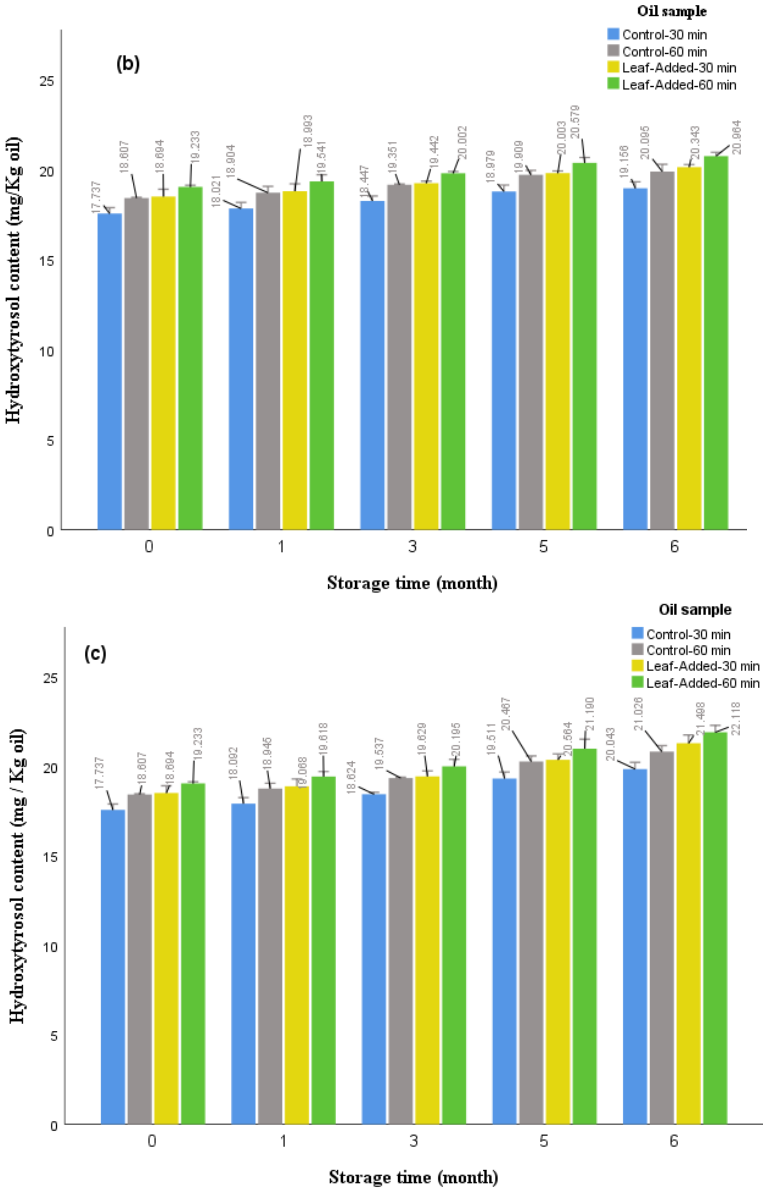


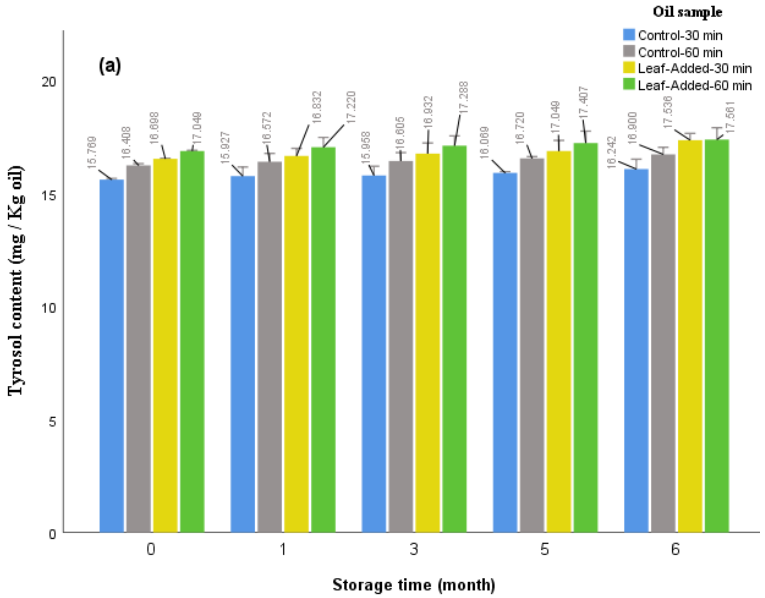
Figure 8. 5 Changes in hydroxytyrosol content of oil samples (mg/kg oil) stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

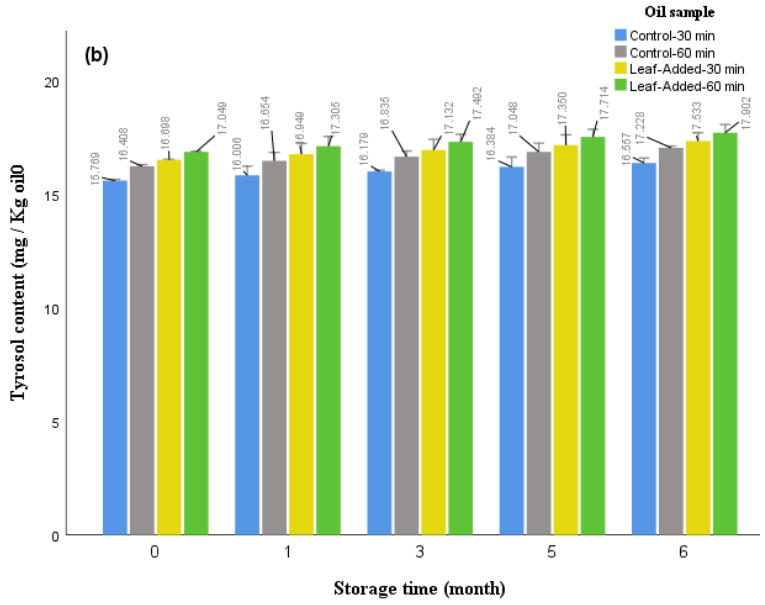
Overall the data demonstrate that the oils without olive leaves that initially contained significant hydroxytyrosol content (though not as large as leaf-added oils), comparatively maintained a sizable proportion over times for the same storage condition. That is to say, the storage conditions did not adversely affect the content of this compound, thus the need for the addition of leaves in VOO may not be crucially needed as long as the content of hydroxytyrosol is of particular interest.

8.3.1.6. Stability of tyrosol

Similar to the trend of changes observed for hydroxytyrosol, the content of tyrosol increased with the storage time, but proportionally to a lesser extent (**Figure 8.6**). It rose over the six month time point

by 8% (with oxygen exposure), 5% (with light exposure), and 3% (with no light/oxygen exposure). Tyrosol and hydroxytyrosol are both in the group of phenolic alcohols, and are structurally similar, only hydroxytyrosol has an extra hydroxyl group with substantially higher antioxidant potential.





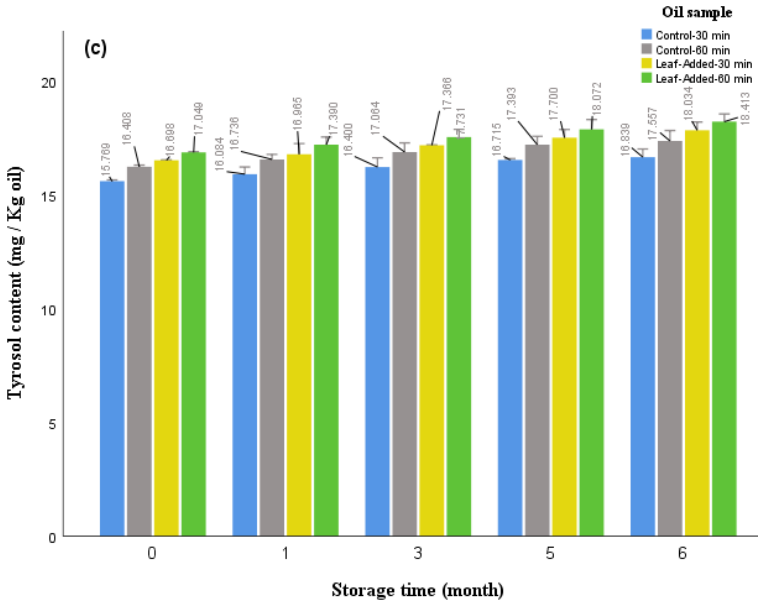
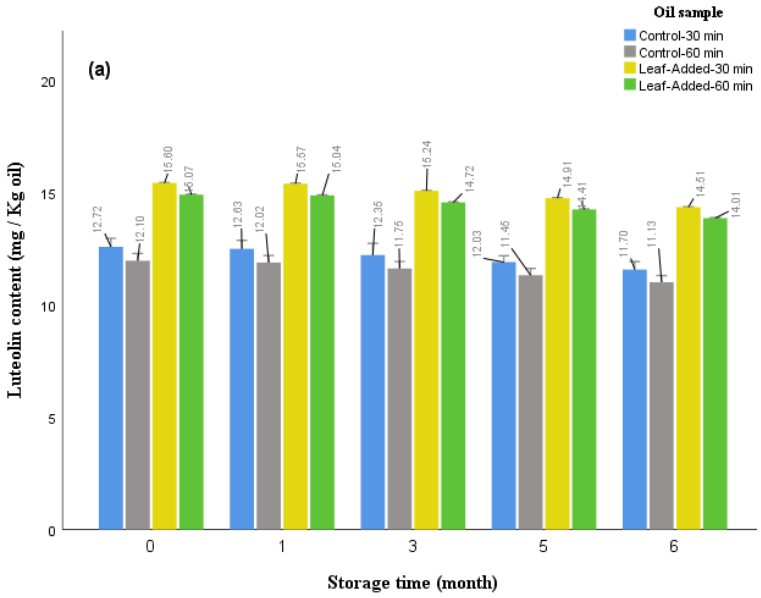


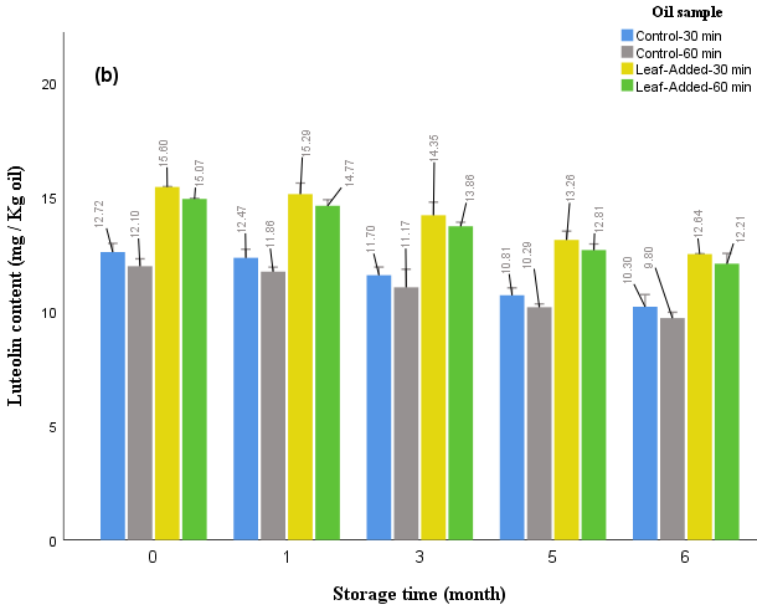
Figure 8. 6 Changes in tyrosol content of oil samples (mg/kg oil) stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

In general, compared to the other major polyphenols of olive leaves, tyrosol exerts less antioxidant power but it remains more stable/unaffected during the oil storage (Karković Marković et al. 2019; Safarzadeh Markhali et al. 2020). As mentioned earlier, the rise in hydroxytyrosol and the drop in oleuropein in the corresponding storage conditions/times may suggest that oleuropein, through its hydrolytic cleavage, served partly as a substrate for the production of hydroxytyrosol. Similar biotransformation potentially occurred here as tyrosol rather increased under corresponding storage conditions which may explain where it was possibly derived from.

8.3.1.7. Stability of luteolin

Luteolin, a flavonoid, is among the key flavone groups in olive oil with competing antioxidant potential. As shown in **Figure 8.7**, the presence of light, followed by the oxygen exposure, caused declines in luteolin content. The content of luteolin in all oil groups dropped by around 20% (with light exposure), and 16% (with oxygen exposure) over the course of six-month period. To suppress/diminish the adverse effects of light, it is recommended to bottle olive oils in opaque/tinted glass containers, though this is not strictly followed as some olive oil factories rather use transparent bottles. As seen in light/oxygen protected samples (**Figure 8.7a**), the use of inert gas (nitrogen) in the headspace of the tubes assisted in preserving more of luteolin over time points (around 7% loss over the six months).


(a)
Oil sample
Control-30 min
Control-60 min
Leaf-Added-30 min
Leaf-Added-60 min
Luteolin content (mg / Kg oil)
Storage time (month)
0
1
3
5
6
12.72
12.10
15.60
15.07
12.63
12.02
15.57
15.04
12.35
11.76
15.24
14.72
12.03
11.45
14.91
14.41
11.70
11.13
14.51
14.01


(b)
Oil sample
Control-30 min
Control-60 min
Leaf-Added-30 min
Leaf-Added-60 min
Luteolin content (mg / Kg oil)
Storage time (month)
0
1
3
5
6
12.72
12.10
15.60
15.07
12.47
11.88
15.29
14.77
11.70
11.17
14.35
13.88
10.81
10.29
13.26
12.81
10.30
9.80
12.64
12.21

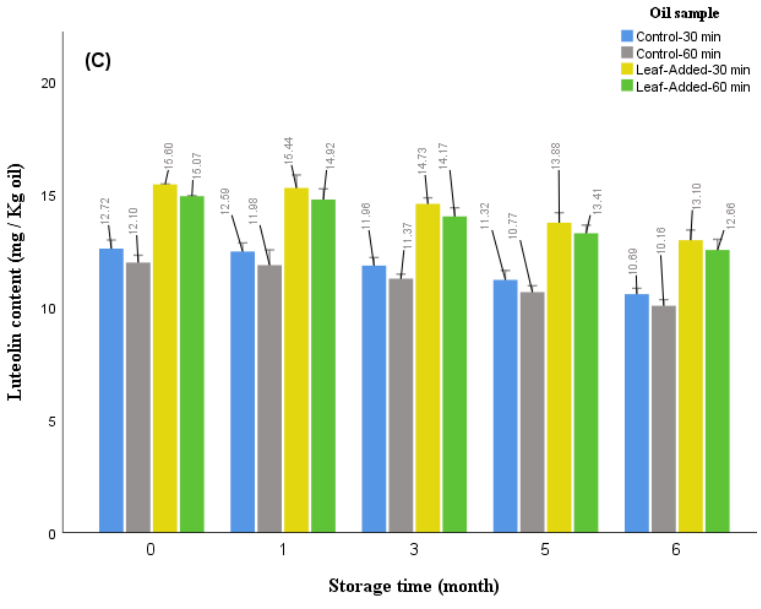
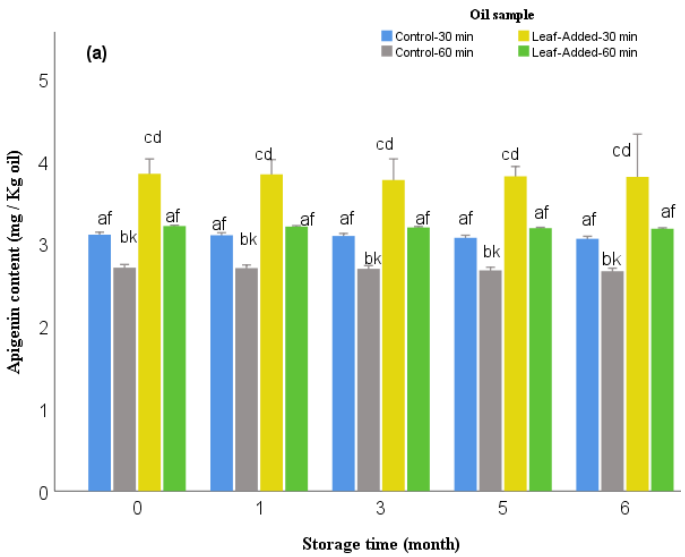

(C)
Oil sample
Control-30 min
Control-60 min
Leaf-Added-30 min
Leaf-Added-60 min
Luteolin content (mg / Kg oil)
Storage time (month)
0
1
3
5
6
12.72
12.10
15.60
15.07
12.59
11.98
15.44
14.92
11.96
11.37
14.73
14.17
11.32
10.77
13.88
13.41
10.69
10.18
13.10
12.66

Figure 8. 7 Changes in luteolin content of oil samples (mg/kg oil) stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

Overall, the addition of leaves to the oils (particularly with a 30 min malaxation) ideally enabled the preservation of luteolin content, with or without light/oxygen exposure, over six months (final range:15.60–12.64 mg/kg). Also, It should be noted that, the *Control* groups (without leaves), although proportionally suffered from light and oxygen exposures, preserved reasonable content of luteolin over the storage time. The final concentrations of *Control* groups (30 min malaxation) were 11.70 mg/kg (without light/oxygen exposure) 10.30 mg/kg (with light exposure), and 10.69 mg/kg (with oxygen exposure).

8.3.1.8. Stability of apigenin

Apigenin belongs to the same flavonoid sub-class (flavones) as luteolin does. The synergistic bio-functional effects of these two phenols have been well discussed in the literature (Jiang et al. 2021; Zhang et al. 2019; Visioli et al. 2002). As shown in **Figure 8.8**, except for the Control (30 min) having 11% reduction upon light exposure, the concentrations of apigenin remained relatively stable across the oil groups with slight reductions (around 3%) in the presence of light and oxygen over six months. The insignificant loss of apigenin content over time supports previous research wherein it was found that neither light nor oxygen affected the content of apigenin (2.4 mg/kg) over the course of one year storage period (Caipo et al. 2021).



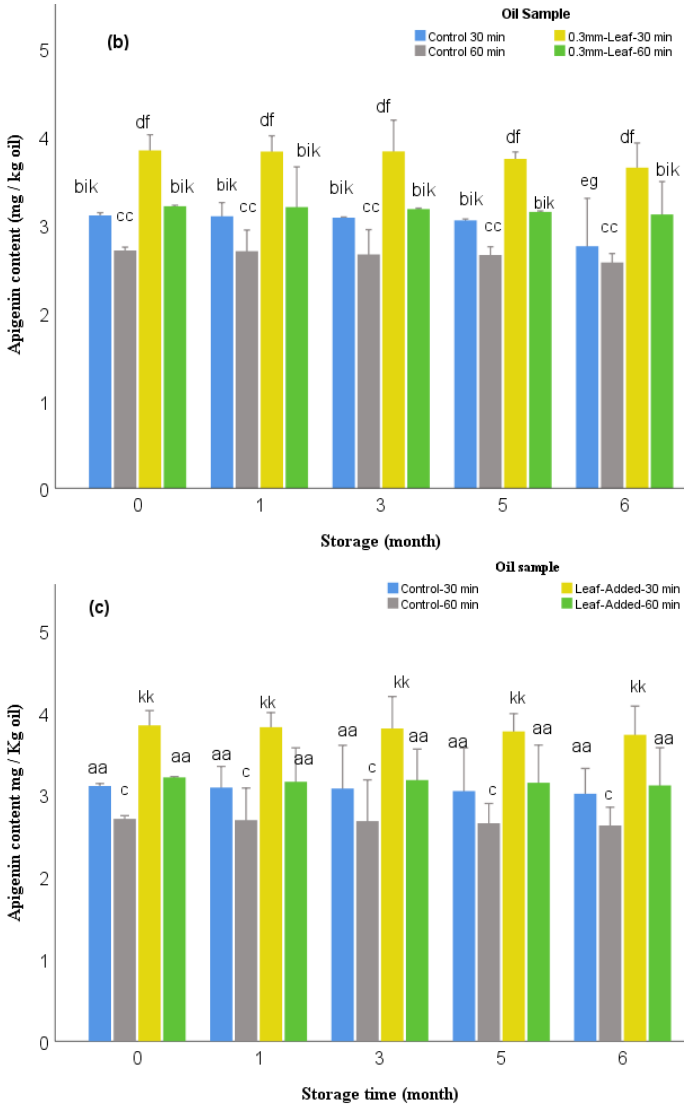


Figure 8. 8 Changes in apigenin content of oil samples (mg/kg oil) stored over time points at (20 ± 5 °C) under: **(a)** no light/oxygen exposure; **(b)** light exposure only; **(c)** oxygen exposure only. The 30 min and 60 min represent malaxation times. Different letters (a–k) above the bars indicate statistically significant differences between oil groups for the same storage condition over storage time points ($p < 0.05$). Data are shown as mean values ± standard deviation error bars.

Overall the data demonstrate that the content of apigenin for the same oil group depends significantly on the malaxation time as the magnitude of differences between malaxation times were significantly high across the storage times, for the same storage condition. In other words, the ability of 0.3 mm of added leaves to optimally keep the highest apigenin content throughout the storage rely in part on a shorter malaxation time (30 min) rather than the extended time.

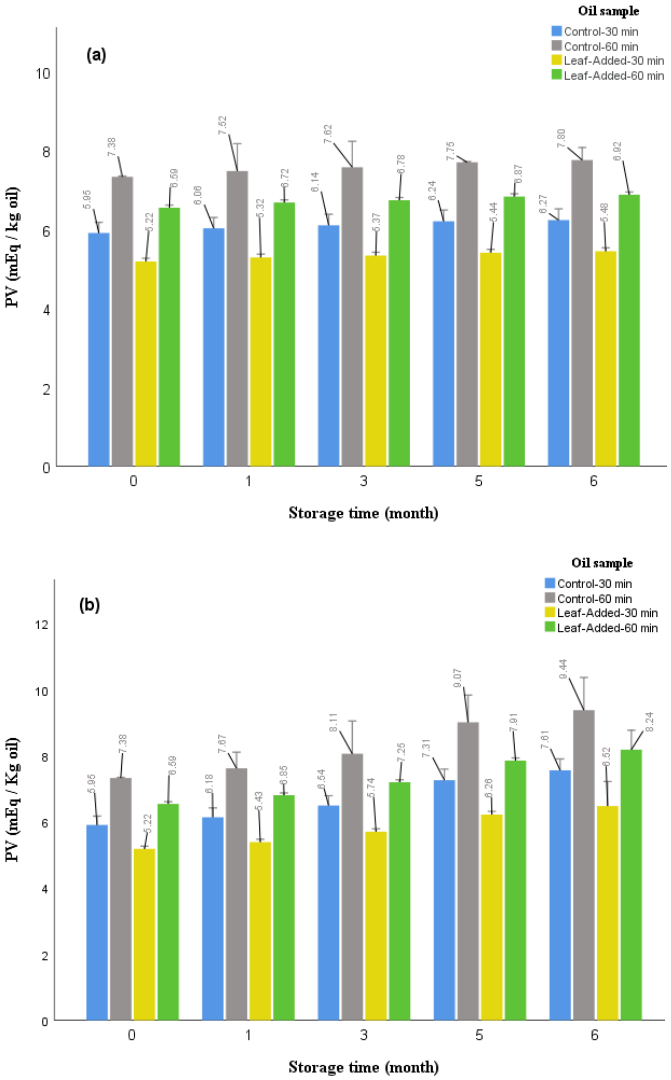
8.3.2. Effect of storage on chemical quality of leaf-added oils

In the preliminary study (Safarzadeh Markhali & Teixeira, 2023), the olive oils with 0.3 mm leaves favorably showed the lowest amounts of peroxide value and free acidity. In this model study, the peroxide and acidity levels were further assessed under the storage conditions/time points in order to determine

whether during storages the values stay below the maximum criteria for the commercial quality of EVOO, approved by the International Olive Council (IOC, 2019). This is particularly crucial as inappropriate storage/environmental conditions may disqualify the oil to be classified as EVOO.

8.3.2.1. Peroxide value

The occurrence and development of peroxide (the intermediate oxidation products) can continue, beyond the processing step, throughout packaging (bottling) and inadequate storage/ handling, particularly in the presence of oxygen, light, and elevated temperature. Regardless of the factor of leaf addition, the data in this study showed increased PV in the range of 20–23% (with oxygen exposure), 25–28% (with light exposure), and around 5% (light/oxygen protected) (**Figure 8.9**).



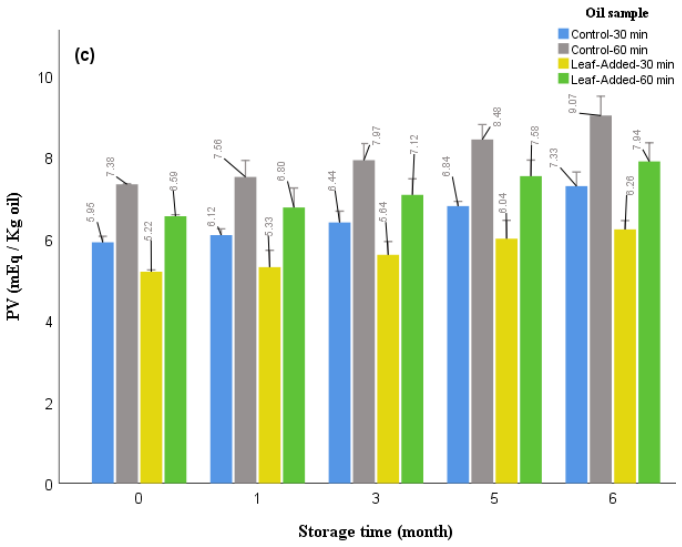


Figure 8. 9 Changes in peroxide value of oil samples (mEq O_2/kg) stored over time points at (20 ± 5 °C) under: **(a)** no light/oxygen exposure; **(b)** light exposure only; **(c)** oxygen exposure only. The 30 min and 60 min represent malaxation times. Data are expressed as mean values ± standard deviation error bars.

As mentioned earlier (**Section 8.3.1.1)**, the content of TPC dropped with time mostly under light and to a lesser extent under oxygen. Similar trends of decline were observed for peroxide values at corresponding time points. In other words, the leaf-added oils maintained the lowest PV over time points for the same storage condition. However, regardless of the factor of leaf addition, all groups of oil samples did not lose their extra virgin quality in terms of peroxide value as the obtained results lied below the maximum limit (20 mEq O_2/kg oil), designated for commercial standards of EVOO, under the examined storage conditions over the course of six-month period. Quantitatively, the leaf-added oil with 30 min malaxation, represented superior quality in terms of peroxide value, as it continued to have the lowest values over time points for the same storage experiment.

8.3.2.2. Free fatty acids

Free fatty acids (free acidity) can be generated in the course of the hydrolysis of triglycerides, typically by catalyzing effects of lipase enzymes. As shown in **Figure 8.10**, the acidity increased by around 18–20% with oxygen exposure over the six month storage period. Having said that, the values, including the *Control* groups, did not exceed the IOC standard criteria of free acidity designated for EVOO (0.8%). Above all, the leaf-added oils (from both malaxation times) contained the lowest free acidity (around 0.17% oleic acid) after six months with oxygen exposure. Taking a cue of this outcome, the addition of 0.3 mm leaves can be of preference for maintaining minimum free acidity of olive oil, particularly over the extended/inadequate storage conditions that is often the case during domestic uses.

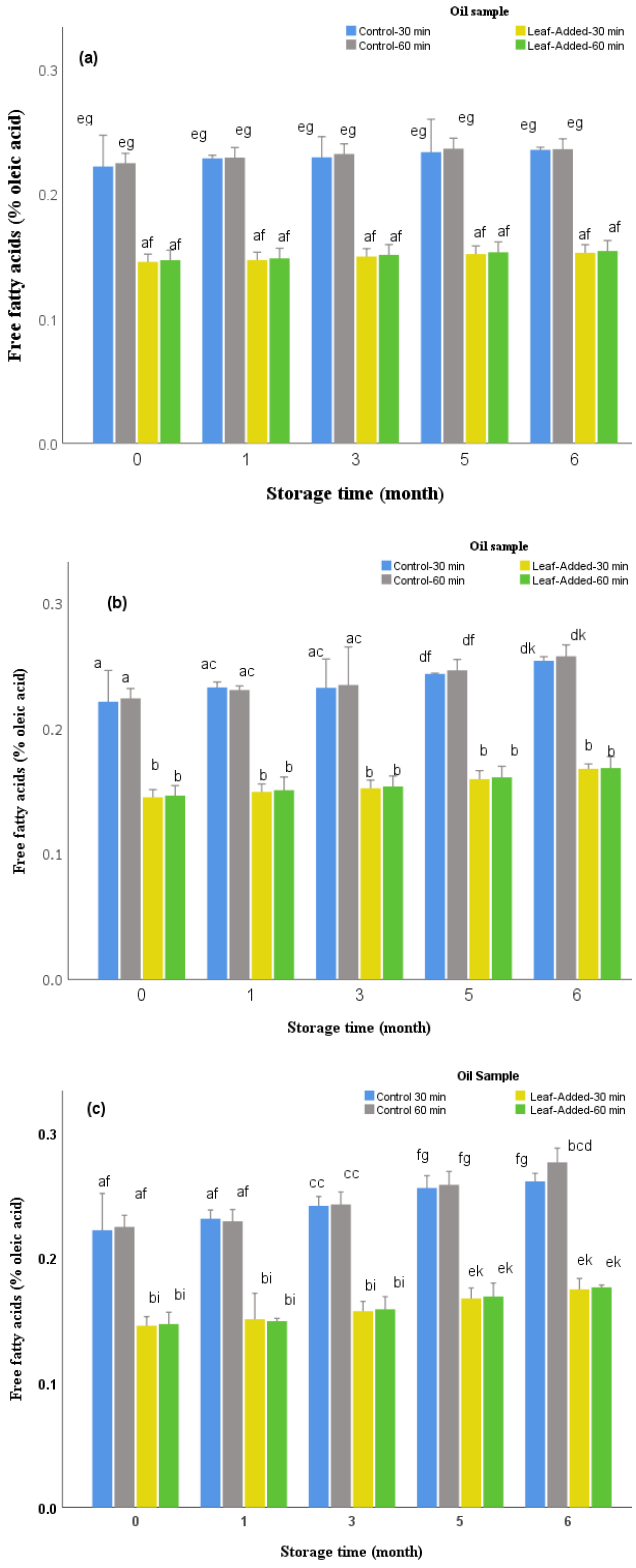


Figure 8. 10 Changes in free fatty acids of oil samples (% oleic acid) stored over time points at (20 ± 5 °C) under: (a) no light/oxygen exposure; (b) light exposure only; (c) oxygen exposure only. The 30 min and 60 min represent malaxation times. Different letters (a–k) above the bars indicate statistically significant differences between oil groups for the same storage condition over storage time points ($p < 0.05$). Data are shown as mean values ± standard deviation error bars.

8.4. Conclusions

In all oil groups (with/without leaves), similar rhythms of changes under corresponding storage conditions/time points were observed. Having said that, the leaf-added oils represented significantly higher content of total and target polar phenols over time points for the same storage experiment. This is attributed to the efficiency of the added leaves (being optimally size reduced) to raise the content of polyphenols at the preliminary stage of the extracted oil which, despite being suffered from storage conditions/times, represented significant phenolic content over a six month period. Moreover, the results also showed that in all groups of samples the peroxide and acidity values lied well below the standard criteria required for qualifying extra virgin olive oil with/without light or oxygen exposure over time.

To conclude, the data justify the significant role of 0.3 mm size fractions of olive leaf powders present in the oil (obtained by 30 min malaxation) as still substantial levels of polyphenols (and correspondingly antiradical values) remained after being exposed to oxygen and light over six months. If polyphenols were to decline exceedingly under the oxygen/light exposure, then they were unable to exert significant antiradical effects over time (the oils without leaves can partly justify this as the numbers of target/total polyphenols were significantly lower than the corresponding leaf-added oils over the storage times).

The outcome of this study provides information for potential researchers, when dealing with the keepability of specific/total polyphenols of virgin olive oil. Moreover, the data can be of value from the perspective of consumers as generally the inclination is often towards the assumption that EVOO sustain its supreme nutritional quality before the expiration date, rather than taking the factor of proper handling/storage into an equal consideration.

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Part III

Concluding Remarks

CHAPTER 9

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

9.1. General conclusions

After identification of the knowledge gaps existed on sustainable recovery of polyphenols of olive leaves, this research project addressed the following issues: *(i) possibilities for sustainable re-utilization of olive leaves through the processing system*– dealing with sustainable recovery of target/total polar phenols, ohmic heating as an emerging approach, compared to the conventional methods, was applied (considering a set of extraction temperatures and green solvent ratios) to obtain and assess the resulting olive leaf extracts and *(ii) efficient extractability of polyphenols from olive leaves through pre-processing system and possibilities for sustainable processes in olive oil industry*– following a novel investigation on the roles of pre-processing of olive leaves in extractability of bio-phenols (to validate the hypothesis that bio-phenols may be largely diffused upon preprocessing without being subjected to solvent/extraction process) an affordable re-utilization of target polyphenols through comparing different size fractions of olive leaf powders following their addition upon crushing of olives was examined. The particle size of leaf powders (primary factor) was taken into account along with the malaxation time and pitting/ of olives (secondary factors).

The most important findings of the first approach of study (ohmic extraction) are highlighted below:

- *The initial point of leaf extracts* – over all the data justify the role of ohmic heating as a value-added processing approach, over conventional heating system, for the recovery of polyphenols from olive leaves. Ohmic heating, compared to the conventional heating, proved significantly useful in:
  - ✓ The extraction yield (up to 34.53% at 75 °C using 80% ethanol)
  - ✓ The recovery of TPC at 55 °C (reached up to 42.53 mg GAE/g extract using 60% ethanol)
  - ✓ The antioxidant capacity (detected by FRAP, DPPH, and ABTS) with relatively similar trends of variations as those observed for total phenolic content
  - ✓ The recovery of target polar phenols particularly oleuropein (26.18 mg/g extract at 75 °C with 80% ethanol).
- *The stability of olive leaf extracts* – overall the data approve of the role of ohmic heating in the stability of leaf extracts, as oleuropein content, antioxidant capacity, and TPC of the extracts after being subjected to the storage trials over times, heating over times, and pH solutions, still remained significantly in the extracts.

- ✓ The results from the storage stability revealed that the temperature of –20 °C is preferrable for the stability of the extracts, while the storage at 25 °C was the least desirable condition particularly for oleuropein, TPC and antioxidant capacity.
- ✓ The data from the impact of the pH and thermal stability of leaf extracts showed that too acidic and/or too high temperature can result in hydroxytyrosol drops.
- ✓ The data also confirmed that the initial pH conditions of extracts (4.8–5.2) were relatively close to the optimum value (pH 5) determined for the protection of polar phenols; therefore the pH of the *OH* extracts may not need to be modified when the conservation of polyphenols is of interest.
- ✓ Heating of the extracts at higher temperature (110 °C) for the extended times (40 and 60 min) was detrimental to TPC, oleuropein content, and antioxidant capacity of the extracts. Ideally, temperature should not exceed 90 °C for more than 40 min.

The most significant findings of the second approach (different particle sizes of added olive leaves in virgin olive oil) are summarized below:

- ➢ ***Leaf-added virgin olive oils at the initial point–*** overall the results support the significant role of size reduction of the added olive leaf powders in polyphenolic content of the resulting olive oil.
  - ✓ The addition of 0.3 mm dry ground olive leaves during crushing of pitted olives, prior to a 30-min malaxation favorably exhibited significant effects on: (i) maximizing phenolic content [especially oleuropein (5.85 mg/kg oil) and verbascoside (4.02 mg/kg oil)], and antioxidant capacity (*in vitro*), and (ii) minimizing peroxide value and free acidity of the resulting virgin olive oil.
  - ✓ Overall, the factor of pitting and shorter malaxation both contributed to complementing the efficacy of the preferred particle size of the added leaves on the rise of polyphenols.
- ➢ ***Storage of leaf-added olive oils*** – overall the data support the significant role of 0.3 mm leaf powders present in the oil, particularly in respect of oleuropein as a sizable proportion remained after being affected by the severe storage conditions (oxygen and light) over the course of six month period (final data: 5.41–4.15 mg/kg oil).
  - ✓ Total phenolic content in all oil groups (with/without leaves) declined rapidly at the exposure of light (36%) and oxygen (33%) upon a six month storage period, while in the absence of light/oxygen the values remained more stable over the six month period of

storage (with 2–5% declines). Relatively, similar pattern of changes between antiradical activities were detected, for the same storage condition.

- ✓ the storage conditions did not adversely affect the content of hydroxytyrosol and tyrosol; i.e., if the content of such phenols is of particular interest, the inclusion of leaves in VOO loses its relevance. On the contrary, if the content of oleuropein or verbascoside is desired to be enhanced (and protected) in the oil, then such a strategy can be of value to serve the purpose.
- ✓ Regardless of the factor of leaf addition, the data in this study showed increased PV in the range of 20–23% (with oxygen exposure), 25–28% (with light exposure), and around 5% (light/oxygen protected). The acidity increased by around 30% (with oxygen), 15% (with light exposure), and 6% (with no light/oxygen exposure) over the six month storage period. However, the results support the applicability of leaf addition to the oil because both PV and acidity significantly showed the lowest levels after undergoing oxygen/light exposure over time.

9.2 Future perspectives

The current research, within the scopes of the objectives, responded to the following questions that have not been addressed in the literature before: (i) significant extractability of polyphenols is feasible through using ohmic heating, and (ii) significant re-utilization of target polyphenols of olive leaves for upgrading olive oil quality is viable through using appropriate size fractions of dry leaf powders (without undergoing solvent extraction process). Based on the information provided in this study, the following suggestions are given for potential future studies:

- ***Olive leaf extracts at the initial point***– the data from this study may partially serve as a benchmark for future/more extensive study in respect of the independent variables; i.e., by referring to the data presented in this study, the factors (independent variables) that showed statistically significant (e.g., ohmic temperatures of 55 and 75 °C) on the outcome variables (dependent variables) may be of consideration for an in-depth research using multiple factors to broaden the information, potentially through the following concepts:
  - ✓ Optimization of ohmic heating through an integrated research assessment considering multiple factors including cultivar and growing region of olive leaves and their effects on a diverse set of principal polyphenols (beyond those examined in this study) through instrumental analysis (in addition to HPLC) including NMR that potentially throw light on the applicability of analytical methods for better extraction selectivity.
  - ✓ Comparative study between ohmic heating and other emerging techniques such as MAE and UAE through which multiple factors may be considered in the future – beyond the extraction parameters (such as extraction temperature and time), other key factors including operating conditions and mechanical inputs and their potential interactions to influence the extractability of the desired bio-phenols from olive leaf tissues.
  - ✓ Although olive leaves, as natural plant ingredients, are regarded as generally safe products, the questions regarding potential toxicity of the extracted polyphenols from olive leaves may be answered comprehensively through an extensive *in vivo* study.
- ***Stability of olive leaf extracts*** – the findings of the storage/thermal/pH stability of the leaf extracts may be of value for future research (through broader parameters of the surrounding conditions of the extracts) to draw conclusions on the shelf-life of the extracts which can be used as a guideline throughout their potential value-additions across food, nutraceutical, and cosmetic applications. Likewise, for oleuropein to optimally exert antiradical effects, it needs to be present in sufficient proportions (and potent) in the extracts. Overall the optimum conditions assessed in

the current research were as follows: (i) pH of 5, (ii) heating not exceeding 90 °C (at this temperature not more than 20 min), and (iii) storage at –20 °C for a short time, These data may further benefit from the future research to ascertain optimal preservation of oleuropein of the *OH* extracts, possibly through using auxiliary preservation process such as encapsulation, to enable maximum keepability of such a valuable compound throughout its functionalization in potential applications.

- ***Leaf-added olive oils–*** the data from this study may draw up a basis for the future research work addressing optimization of olive leaf addition throughout the oil extraction considering a broader range of size fractions and their potential intercorrelations with other key factors such as olive cultivars, as well as drying operation (temperature/time).
  - ✓ For sustainable marketability, the data may also serve a benchmark for future study for optimization of leaf-added oils considering the factor of organoleptic assessments for presenting an enriched EVOO with an acceptable sensory attributes.
  - ✓ The findings may also partially lay down a basis for the addition of optimum particle fractions of leaf powders to other types of vegetable oils (such as corn oil or sunflower oil) or lower grades of olive oil (such as refined olive oil).
- ***Storage of leaf-added oils*** – this work presented evidence that addition of 0.3 mm dry ground olive leaves during olive crushing in advance a short-term malaxation is favorable to VOO during storage as considerable proportions of TPC, oleuropein and verbascose remained under oxygen/light exposure over six months. The data can be further followed up in the future to ascertain the stability of the leaf-added olive oils over longer exposures to oxygen and light, especially when the concentrations and/or biological activities of certain types of polyphenols are of interest.