# Utilising Olive Leaves to Enrich Olive Oil with Oleuropein Aglycone

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# Abstract

Olive oil (OO) is an excellent source of minor bioactive compounds, such as oleuropein aglycone, which is gaining increasing attention due to its biological properties and benefits on human health. The European Food Safety Authority (EFSA) have approved a health claim for OO if it contains 250 mg/kg of these desired bioactive compounds. However, the concentrations of bioactive compounds can vary between batches of OO and may contain bioactive compounds that are below the threshold which provides health benefits. Enrichment of OO is commonly employed to achieve a higher concentration of these bioactive compounds. Often a waste product such as olive leaves is used because it contains a significant amount of the oleuropein aglycone precursor, oleuropein glycoside (8-14% dry weight).

The present research aimed to analyse commercial extra virgin olive oils (EVOOs) in the New Zealand market, explore the effect of olive leaf drying on the composition of oleuropein in olive leaf tea (OLT) and the stability of OLT stored at different temperatures (−20, RT, 40 °C, 189 days). Lastly, an investigation was carried out to determine whether the oleuropein in OLT could be hydrolysed to oleuropein aglycone via an exogenous source of β-glucosidase (almond flour/meal) so that the resulting aglycone could be transferred into a low content OO producing an enriched marketable product with health benefits.

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was validated and was used to analyse target analytes in New Zealand commercial EVOOs (*n* = 9). There was a large variation in the concentration of target analytes (oleuropein, ligstroside, oleuropein aglycone, oleocanthal, oleacein, hydroxytyrosol, tyrosol and elenolic acid) and none of the oils analysed met the health claim (250 mg/kg). The content of oleuropein in OLT increased under different leaf drying conditions in the order fresh<RT<70 °C while other target analytes tended to increase with drying at RT. The period leaves were left to dry was also deemed a crucial factor in the concentrations obtained. A stability experiment of OLT

determined that the majority of target analytes were stable at −20 °C and RT, whereas precursors such as oleuropein and ligstroside degraded at 40 °C.

Almond flour/meal proved to be an effective source of exogenous β-glucosidase to hydrolyse oleuropein in OLT; however, the mass transfer of aglycone into the oil was complex. Changing the source of enzyme to almond milk resulted in an aglycone concentration of 6 mg/kg in the oil but this also resulted in oleuropein aglycone to be quantified in the aqueous portion of samples (the highest being 613 mg/kg). The presence of a hydrophobic compound in the aqueous portion of samples was unexpected. It was thought this result may be caused by solubilisation through thermodynamically stable micelle formation within the emulsion formed during enrichment experiments. Salting out the reaction resulted in the highest amount of aglycone (30 mg/kg) as this process likely disrupted micelle formation.

Since commercial EVOOs vary in concentration of analytes such as oleuropein aglycone, enriching an OO is desirable. OLT can be used as an effective source of oleuropein which can be hydrolysed via β-glucosidase present in almond flour/meal and milk. However, the mass transfer of the respective aglycone into olive oil to achieve an enriched product with a concentration at or above the health claim (250 mg/kg) was complex.

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# List of Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| BGM | Blood glucose meter |
| CE | Capillary electrophoresis |
| CRM | Certified reference material |
| cv. | Cultivar variety |
| DAD | Diode array detector |
| DMF | *N,N*-dimethylformamide |
| d.p | Decimal places |
| EC | End capped |
| EFSA | European Food Safety Authority |
| *et al.* | et alia |
| EU | European Union |
| EVOO | Extra virgin olive oil |
| GC-MS | Gas chromatography mass spectrometry |
| HPLC | High performance liquid chromatography |
| IOC | International Olive Council |
| IS | IonSpray Voltage |
| k | Rate constant |
| LC | Liquid chromatography |
| LC-MS | Liquid chromatography mass spectrometry |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LLE | Liquid-liquid extraction |
| LLME | Liquid-liquid microextraction |
| MeOH | Methanol |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometer |
| MS/MS | Tandem mass spectrometry |

| | |
|---|---|
| $N_2$ | Nitrogen gas |
| n/a | Not applicable |
| NaCl | Sodium chloride |
| N/D | No data |
| n.d. | No date |
| NMR | Nuclear magnetic resonance |
| LLE | Liquid-liquid extraction |
| LLME | Liquid-liquid micro extraction |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| *o* | *Ortho* |
| OA | Oleuropein aglycone |
| OLT | Olive leaf tea |
| OO | Olive oil |
| p.a. | Per annum |
| ppb | parts per billion |
| ppm | parts per million |
| psi | Pounds per square inch |
| QC | Quality control |
| LC-QqQ-MS/MS | Liquid chromatography triple quadrupole tandem mass spectrometry |
| $R^2$ | Coefficient of determination |
| RCF | Relative centrifugal force |
| ROO | Refined olive oil |
| RSD | Relative standard deviation |
| RT | Room temperature |
| SD | Standard deviation |
| SPE | Solid phase extraction |
| Std | Standard |
| THF | Tetrahydrofuran |

| | |
|---|---|
| UHPLC | Ultra-high performance liquid chromatography |
| UV | Ultraviolet |
| V | Volts |
| v | Velocity |
| *v*/*v* | Volume/volume |
| VOO | Virgin olive oil |
| vs. | Versus |
| *w*/*v* | Weight/volume |

# CHAPTER 1

# Introduction and Literature Review

## 1.1 Introduction

Olive oil (OO) is considered an excellent source of minor bioactive compounds which are associated with a range of health benefits. Oleuropein aglycone, one of the oil soluble bioactive compounds, is gaining increasing attention due to its biological properties.[1] The aglycone is derived from endogenous enzymatic hydrolysis (β-glucosidase) of oleuropein glycoside that exists in olive fruit during OO production.[1] However, the concentrations of bioactive compounds can vary between batches of OO and some batches may contain bioactive compounds that are below the threshold which provides health benefits. There is an opportunity to utilise oleuropein present in olive leaves, which are currently a waste product in the OO industry, to enhance the bioactive component of OO to produce a product with a high, consistent concentration of oleuropein aglycone. Olive leaves can be used to produce an olive leaf tea (OLT) that contains high amounts of oleuropein glycoside which can be hydrolysed to oleuropein aglycone via an exogenous source of β-glucosidase; one such source is almond flour/meal which is unexplored in literature. The resulting aglycone can be transferred into a low content OO producing a marketable product with health benefits, thus was investigated in this research for a company who wish to understand the possibility of OO enrichment for product development.

## 1.2 Olive history and symbolism

Archaeological excavations in the Mediterranean Basin have revealed the existence of olive plants dating back 20 million years.[2] Olive is thought to be one of the earliest fruits to be cultivated and this is considered to have begun during the Copper Age (6000 BC); there is also evidence of the development of techniques for oil extraction during this period.[3] Olive

plants and fruit have also been mentioned in the Koran, Hebrew and Christian Bibles and it is often regarded as the symbol of euphoria, purity, victory, peace and honour.[2]

## 1.3 Botanical characteristics of olive

The cultivated olive (*Olea europaea* L.) belongs to the family Oleaceae, genus *Olea*.[2] Oleaceae is a medium sized family comprising of approximately 25 genera which includes at least 600 species.[4]

***Botanical features***

The olive tree is a long-lived evergreen reaching up to a 1000 years of age as it is slow growing and tolerant to drought stress.[2] The tree can reach up to 15 m in height but is often shrubby (Figure 1.1 A).[3] Olive fruit, known as a drupe, is spherical or elliptic in shape and consists of an exocarp (skin) and mesocarp (flesh, edible portion), and the endocarp (pit).[2] The fruit is purplish black when completely ripe, however, some cultivars are green when ripe (Figure 1.1 B).[2] Olive fruit is of major agricultural importance in the Mediterranean region and a source of oil and canned olives.[3] The typical composition of olive fruit is water (50%), protein (1.6%), oil (22%), carbohydrate (19.1%), cellulose (5.8%), inorganic substances (1.5%) and phenolic compounds (1-3%). Other important compounds present in olive fruit are pectin, organic acids, and pigments.[5] Olive leaves are narrowly elliptic, green on the upper surface and whitish grey on the underside and are replaced at 2-3 year intervals during spring (Figure 1.1 C).[2,3] On average, the composition is water (49.8%), proteins (7.6%), lipids (1.1%), minerals (4.5%), and carbohydrates (37.1%).[6]

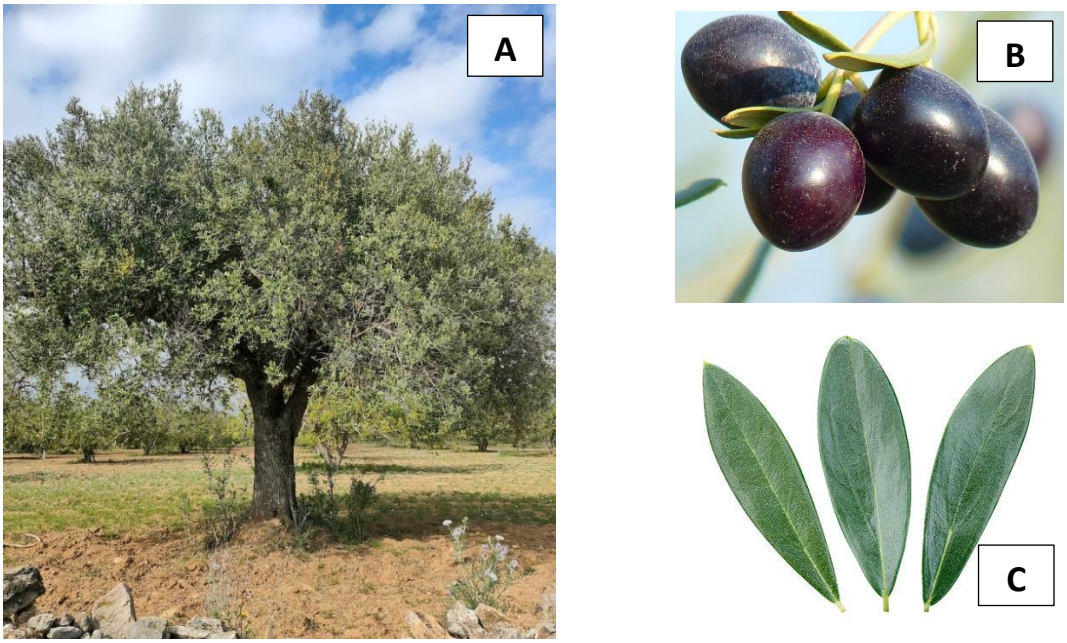



**Figure 1.1. Images of A) an olive tree,[7] B) olive fruit[8] and C) olive leaves.[9]**

***Olive cultivars***

The species *Olea europaea* L. includes more than 2600 cultivars, they are usually selected for their agronomic value such as larger fruits and higher oil content.[2,10] Due to the ideal growing conditions, the Mediterranean region accounts for 87% of the 11.5 million hectares dedicated to olive tree cultivation worldwide, 86.6% of which is devoted to OO supply.[11,12] The specific use of a given cultivar is determined by its oil content and size of drupes. Olive varieties with oil content less than 12% such as Ascolano, Calamata and Manzanillo are almost exclusively used for table olive production, while those with higher oil yields such as Hojiblanca, Verdial, Picual, Gemlik, Nychati Kalamon and Arauco are usually preferred for OO production.[5]

***Geographical location and growing conditions***

The olive tree is native to the Mediterranean region, the tropics, central Asia and various parts of Africa.[2] Cultivation of olive is feasible throughout this subtropical zone as these

areas have a mean annual temperature of 15-20 °C with a minimum of 4 °C and a maximum of 40 °C.[2] Varieties more tolerant to cold are present in the northern Mediterranean regions. However, dry summers and mild winters are the most appropriate climate conditions for the growth of olive trees.[2,4] In the last two decades there has been increasing international demand for OO and table olives. This has led to the expansion of olive cultivation in non-Mediterranean countries located in the subtropical and temperate regions such as the United States of America, Argentina, Chile, Peru, Mexico, South Africa, Australia, New Zealand and some Asian countries.[2,11]

## 1.4 Phenolic compounds (phenolics)

### 1.4.1 Phenolic compounds overview

Phenolic compounds (also known as phenolics) are naturally derived secondary plant metabolites which are biologically active (bioactive).[13,14] Phenolic compounds are produced by plants for protection against UV light, insects, viruses, and bacteria.[15] They are comprised of an aromatic or aliphatic ring bearing one or more hydroxyl group, forming a structure from simple phenolic compounds to complex-high molecular weight polymers.[13] These compounds are mainly derived from shikimate, phenylpropanoid, and pentose phosphate pathway in plants.[13]

Phenolic compounds are the largest group of phytochemicals and 8000 phenolic structures are currently known, making them highly diverse.[16] Phenolic compounds are categorised into several sub-groups and are classified by their source of origin, biological function, and chemical structure (Figure 1.2).[16]

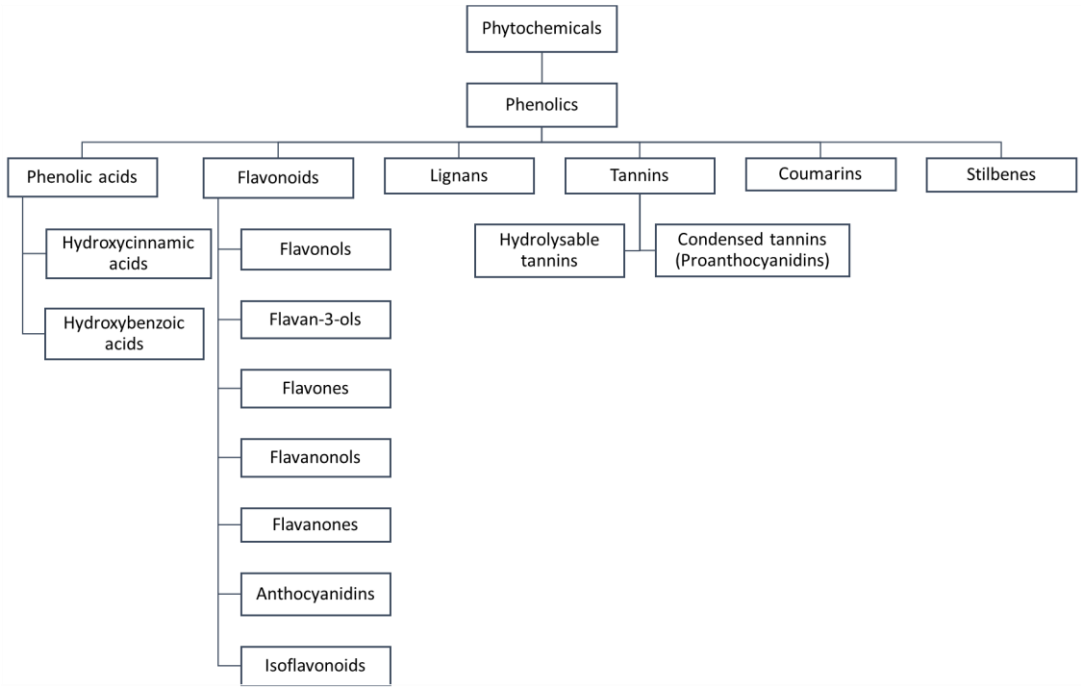



**Figure 1.2. The phytochemical family tree, with expansion of the phenolic compound category. Adapted from Shahidi *et al.* (2015)[17] and Martinez *et al.* (2017).[18]**

Structurally, despite their extreme variety, phenolic compounds possess a common carbon skeleton building block: the C6–C3 phenylpropanoid unit (Figure 1.3). The C and number represent the number of carbons in different units required to form the relevant structure as circled in Figure 1.3.

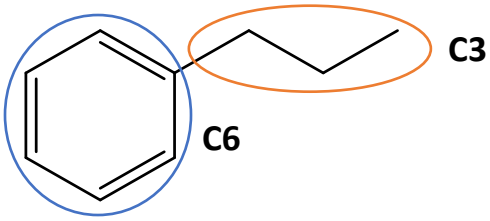



**Figure 1.3. C6–C3 phenylpropanoid unit.**

Biosynthesis by this pathway leads to a wide range of plant phenolics: phenolic acids (cinnamic acids (C6-C3) and benzoic acids (C6-C1)), flavonoids (C6-C3-C6), coumarins (C6-C3), stilbenes (C6–C2–C6), lignans (C6-C3–C3–C6), proanthocyanidins (condensed

tannins) [(C6-C3–C6)*n*], and lignins [(C6-C3)*n*] (Figure 1.4, the latter two are excluded due to complexity).[19,20] For proanthocyanidins and lignins the journal articles did not specify what *n* equals. The flavonoids, which are further subcategorised into flavonols, flavan-3-ols, flavones, isoflavones, flavanols, and anthocyanins, consist of two aromatic rings that are attached to an oxygen heterocycle.[13] There are phenolic acids (often referred to as simple phenols) which are subcategorised into hydroxycinnamic acids and hydroxybenzoic acids. Other groups include coumarins, lignans, stilbenes and lignins. Lastly tannins, which are further subcategorised into hydrolysable and condensed tannins.[21,22] Each group is classified based on of the number of carbons and hydroxyl groups as well as the type and the position of other substituents.[21,22]

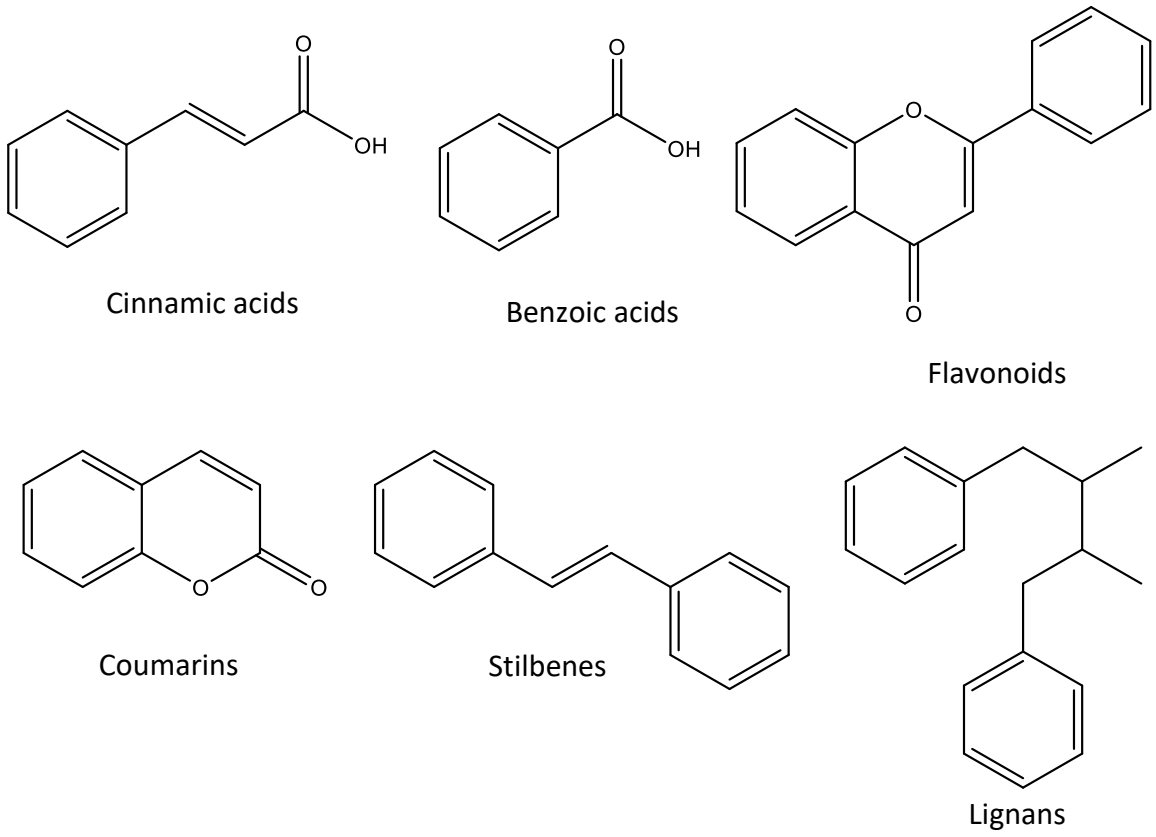



**Figure 1.4. Chemical structures of the different classes of phenolic compounds including phenolic acids (Cinnamic and benzoic acids), flavonoids, coumarins, stilbenes and lignans. Adapted from Chaiprasongsuk *et al.* (2022)[23] and Annunziata *et al.* (2020).[24]**

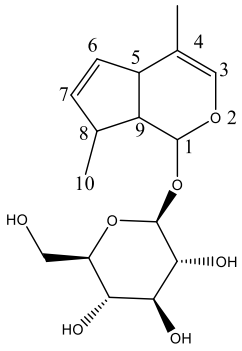
Phenolic compounds are present in bark, leaves, fruits, spices, berries, vegetables, roots, nuts, seeds and herbs. Hence, they can be transferred into processed foods of plant origin such as OO.[21] Soil composition, growing environment, cultivation technique and genetics are mainly responsible for the variation in phenolic compound concentrations observed in these food products.[13]

Phenolic compounds are becoming increasingly important because of their beneficial effects on human health. They are known to possess antioxidant, antimicrobial and anti-inflammatory properties. There are recent reports of remarkable effects on chronic diseases such as neurodegenerative and cardiovascular diseases as well as the prevention and treatment of cancer and inflammation.[13,22] Phenolics also have a wide range of applications as food supplements, pharmaceutical and cosmetic additives.[22]

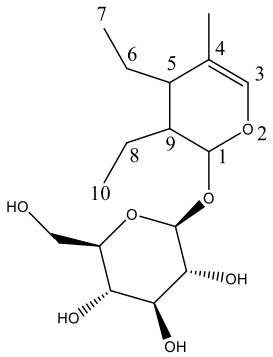
### 1.4.2 Phenolic compounds in olive oil and olive leaves

OO and olive leaves are an important part of this research as both components contain sources of bioactive compounds. OO bioactive compounds include phenolic alcohols (simple phenols), lignans, flavonoids, phenolic acids and the secoiridoid derivatives.[25] Olive leaves also contain a large variety of phenolic compounds and mainly consist of phenolic alcohols, flavonoids, and secoiridoids.[19] An important group of phenolic compounds in this research are the secoiridoids. Secoiridoids are a class of iridoids which are naturally active but are rare in plant species. Nevertheless, they are found in abundance in Oleaceae species, representing the majority of bioactive phenolic compounds in olive (*O. europaea*).[26,27] Iridoids are a large and still expanding class of cyclopentane pyran monoterpenes which are geraniol-derived monoterpenes.[28] The chemical structure results from a cyclopentane ring fused to a six-member heterocycle with an oxygen atom.[28] Iridoids have hemiacetal hydroxyl groups and because of the unstable nature of its $C_1$-OH group they often react with glucose to form glycosides.[28] According to the integrity of the cyclopentane unit, cleavage of the ring in iridoid glycosides produces secoiridoid glycosides (Figure 1.5).[16,21,28] Secoiridoid glycosides are characterised by a 10-carbon core skeleton in which the bond between C-7 and C-8 in the cyclopentene ring is cleaved.[29]

Numerous biological activities for secoiridoid glycosides have been reported, including antioxidant activity associated with tyrosol and hydroxytyrosol groups.[29] In olive, these glycosides are known as oleuropein and ligstroside. Oleuropein and its derivatives are the focus of this research as these compounds are largely responsible for properties associated with health benefits. The next two sections will describe in detail the synthetic pathways of these phenolic compounds present in the olive matrices of interest: OO and olive leaves.




**Iridoid glycosides**



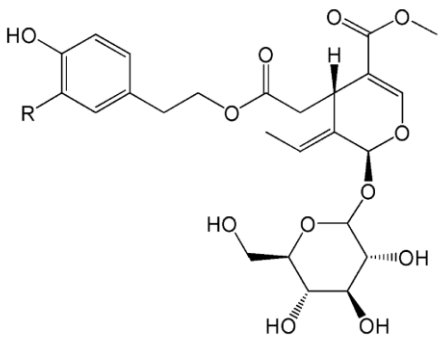

**Secoiridoids**

**Figure 1.5. The basic skeleton of iridoids and secoiridoids. Adapted from Wang *et al.* (2020).[28]**

### *1.4.2.1 Olive oil*

More than 30 different phenolic compounds have been identified in OO, these minor components with bioactive properties range from 1% to 2% of the total weight content of OO.[30] The compounds include phenolic acids (simple phenols) such as hydroxybenzoic, *p*-Coumaric, ferulic, gallic, syringic, vanillic, caffeic, *o*-coumaric and sinapic acids.[5,26,31] Other types of phenolics are lignans such as (+)-pinoresinol and (+)-1-acetoxypinoresinol, hydroxy-isocromans, and flavonoids.[17,19] Predominant flavonoids include luteolin-7-glucoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, rutin, apigenin-7-glucoside, quercetin-3-rhamnoside, and luteolin.[5,31] However, majority of the phenolic profile of OO is derived from the amount of the secoiridoid glycosides originally found in the tissues of olive fruit such as oleuropein and ligstroside. Oleuropein is an ester

consisting of hydroxytyrosol and elenolic acid and ligstroside is an ester consisting of tyrosol and elenolic acid, each containing a respective glucose molecule.[32] These glycosides are water soluble due to the glycosidic moiety increasing the hydrophilicity and polarity due to hydroxyl groups (Figure 1.6).[26,33,34]




R=OH Oleuropein

R=H Ligstroside

**Figure 1.6. Structure of the secoiridoids oleuropein and ligstroside present in *O. europaea* L.[31]**

Hydrolysis of oleuropein and ligstroside occurs during manufacture of OO due to endogenous hydrolytic enzymes such as β-glucosidase.[35-37] The secoiridoid derivatives, oleuropein aglycone and ligstroside aglycone are a result of this activity; hence they are the main phenolic compounds detected in extra virgin olive oils (EVOOs). Removal of the polar glucose moiety on the glycosides through hydrolysis results in the aglycones having significantly hydrophobic character making them soluble in oil (high phospholipid/water partition coefficient).[38,39] The aglycones can undergo further ester hydrolysis (esterase cleavage) to produce non-phenolic elenolic acid and phenolic alcohols hydroxytyrosol or tyrosol, Figure 1.7.[26,40] It has been suggested by Briante *et al.* (2000)[41] that the formation of tyrosol and hydroxytyrosol may also occur spontaneously. Oleacein and oleocanthal are also present in EVOO due to hydrolysis of the methyl esters in oleuropein and ligstroside aglycones, circled in Figure 1.7, by esterase.[42,43] The resulting carboxylic derivatives are unstable and are easily thermally decarboxylated.[44] This process, along with simultaneous opening of the secoiridoid ring leads to their formation.[34,45,46]

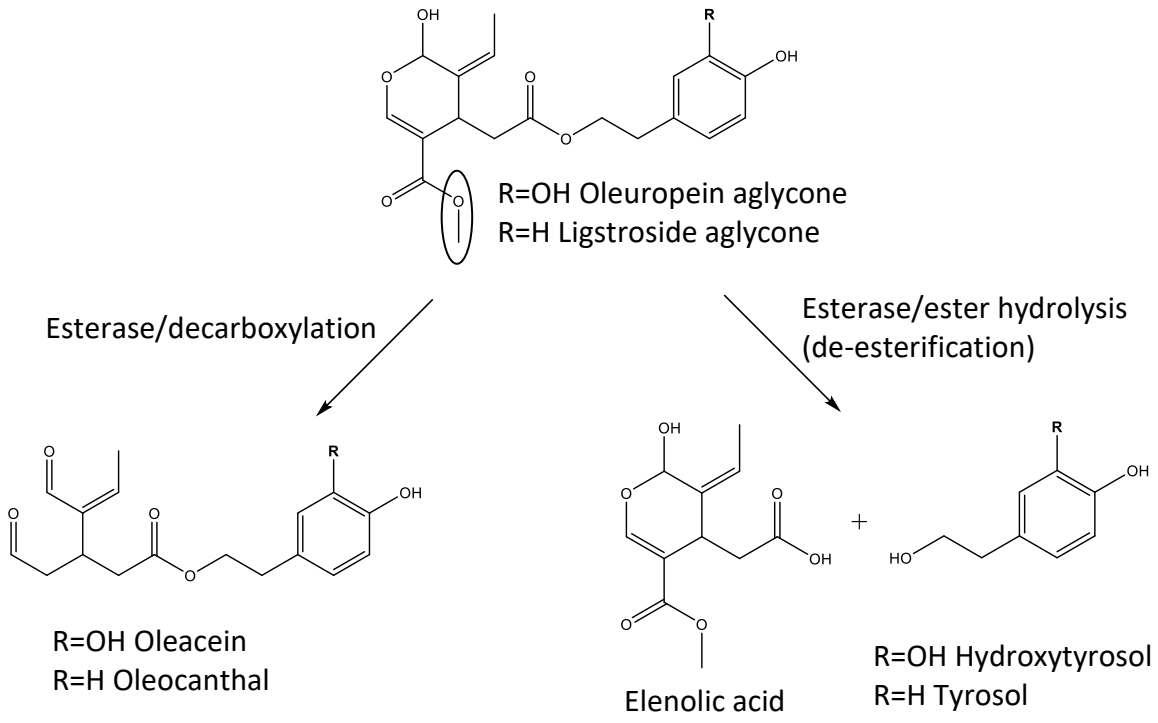



**Figure 1.7. Conversion of oleuropein aglycone and ligstroside aglycone to secoiridoid derivatives by endogenous enzymatic hydrolysis and decarboxylation. The methyl ester has been circled on the aglycone to show which group is hydrolysed prior to decarboxylation to produce oleacein and oleocanthal. Adapted from Johnson *et al.* (2018)[34] and Nikou *et al.* (2022).[45]**

### *1.4.2.2 Olive leaves*

Olive leaves contain a large variety of phenolic compounds and consist of simple phenols (phenylethanoids, hydroxybenzoic and hydroxycinnamic acids) flavonoids (flavones, flavanones, flavonols, 3-flavanols) and the most predominant group, secoiridoids.[19,47-49] Oleuropein and ligstroside glycoside are the most common secoiridoids in olive leaves and both have related chemical structures as shown in Figure 1.6, Section 1.4.2.1.[6,31] Out of the two compounds, oleuropein is the most abundant, around 8-14% dry leaf weight and can reach concentrations between 60-90 mg/g in dried leaves.[6,50] Oleuropein is responsible for the bitter taste of leaves, which together with the products of its enzymatic hydrolysis (e.g., oleuropein aglycone) have the greatest biological interest.[6] As a result of endogenous

enzymatic activity, hydroxytyrosol has also been widely described as a main component of simple phenols in olive leaves.[19,51-54]

## 1.5 Endogenous enzymes in olive

As mentioned in Section 1.4.2, many of the secoiridoid derivatives in olive are present due to the hydrolysis of oleuropein and ligstroside by endogenous enzymatic activity.

Olive constitutes a complex medium in which several endogenous enzymes such as pectinases, lipases, esterases, lipoxygenases, hydroperoxide lyases, β-glucosidases, peroxidases, and polyphenol oxidases operate.[55] Endogenous enzymes including β-glucosidase, esterase and oxidoreductase control the hydrolysis and the oxidation of secoiridoids such as oleuropein.[56] As discussed in Section 1.4.2.1, different compounds can be derived from oleuropein depending on the enzyme that is acting on the compound. β-glucosidase releases glucose from oleuropein and ligstroside forming principally oleuropein aglycone and ligstroside aglycone. Additionally, esterase hydrolyses the ester bonds of the aglycones, producing hydroxytyrosol, tyrosol and elenolic acid. The aglycones can also undergo esterase hydrolysis and decarboxylation to produce oleacein and oleocanthal.[57-59]

### 1.5.1 β-glucosidase

Many plants utilise activated chemical defences to fend off herbivore or pathogen attacks.[60] These small molecular weight compounds are stored as biologically inactive pro-toxins in the intact tissue and are enzymatically activated to form bioactive toxic compounds after tissue damage by an attacking herbivore or pathogen.[60] This two-component system avoids the problem of auto-toxicity, as these defensive molecules can be stored without negative effects to the plant.

A very common activation strategy is the enzymatic removal of a protecting glucose group by β-glucosidase. β-glucosidase is an enzyme that catalyses the hydrolysis of the β-glycosidic bond between two glycone residues or, in olive, between glucose and an aryl

or alkyl aglycone.[55,61] In olive, this endogenous enzyme (β-glucosidase) has a high affinity for oleuropein and ligstroside. During fruit ripening, or when any part of olive tissue is injured by pathogens, herbivores, or mechanical damage, the cellular structures are disrupted and β-glucosidase is able to access stored glycosides hydrolysing oleuropein and ligstroside glycosides to their aglycone forms (Figure 1.8).[31,34,55,61,62] The resulting aglycone is typically more reactive than the parent glycoside; its lower polarity facilitates diffusion through cell membranes and penetration into the herbivore cells where increased toxicity is often exerted when plant matter is consumed in large quantities.[60]

β-glucosidase is an important part of this research because it hydrolyses glycosidic precursors, liberating aglycone moieties (such as oleuropein aglycone) that provide desirable organoleptic properties to plant-derived foods (e.g., OO) and are endowed with different biological activities in preventing and or treating diseases.[63-65]

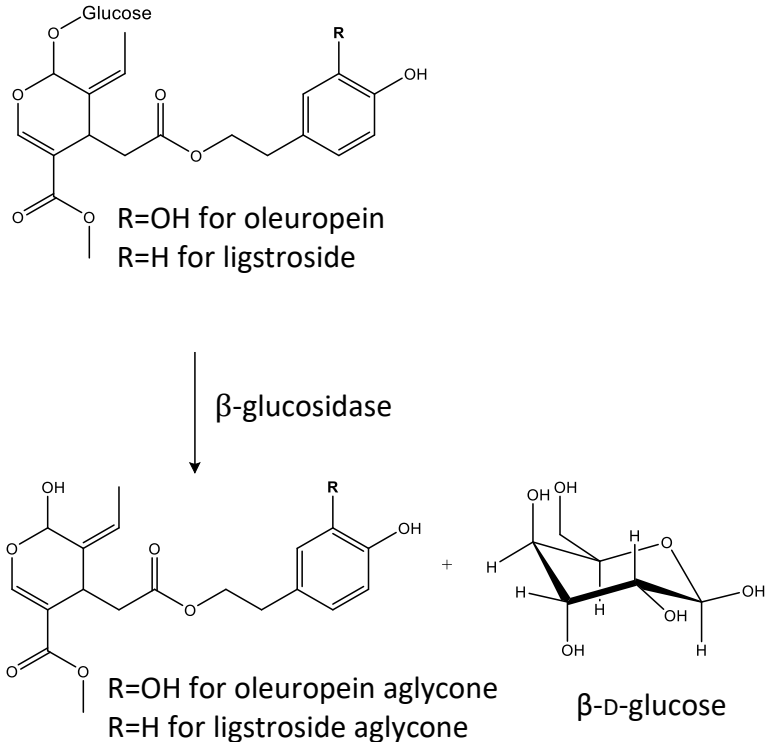



**Figure 1.8. Oleuropein and ligstroside glycoside hydrolysis via β-glucosidase to oleuropein and ligstroside aglycone. Adapted from Johnson *et al.* (2018).[66]**

### *1.5.1.1 Alternative sources of β-glucosidase*

When developing an enriched oil, there is one issue with relying on endogenous β-glucosidase for the production of the aglycone; there is minimal control over the hydrolysis of oleuropein to oleuropein aglycone with undesirable hydrolysis to hydroxytyrosol, tyrosol and elenolic acid. From a commercial perspective, if the amount of oleuropein hydrolysed can be monitored by the addition of an exogenous source of β-glucosidase, there would be better control of the reaction to obtain a known amount of oleuropein aglycone in an oil. Therefore, one aim of this research was to investigate an alternative source of β-glucosidase.

There are several exogenous sources of β-glucosidase in the literature used to hydrolyse oleuropein to oleuropein aglycone; these are summarised in Table 1.1. Most commonly β-glucosidase from sweet and bitter almonds (*Prunus amygdalus*), purchased from Merck have been used. Other sources include fungus, thermophilic archaeon, and bacterium.

**Table 1.1. Exogenous sources of β-glucosidase for the hydrolysis of oleuropein to oleuropein aglycone reported in the literature.**

| Enzymes | Source | References |
|---|---|---|
| Recombinant β-glucosidase Mtbgl3a | *Myceliophthora thermophila* | 67 |
| Recombinant β-glucosidase (EcSβgly) | *Sulfolobus solfataricus* | 41 |
| β-glucosidase | *Lactobacillus plantarumtype* strains | 68 |
| β-glucosidase | Almond | 40,56,69-75 |
| β-glucosidase | *Aspergillus niger* | 76 |
| β-glucosidase | *Streptomyces* | 40 |

Sweet and bitter almonds, part of the Rosaceae family, contain cyanogenic glycosides. Cyanogenic glycosides are sequestered in vacuoles and when plant tissue is disrupted (e.g., chewing, physical damage, etc.) damaged vacuoles allow the cyanogenic glycosides to come into contact with β-glucosidase (E.C. 3.2.1.117).[77] Hydrolysed cyanogenic glycosides provide the almond plant with chemical defence against herbivores and pathogens via generation

of hydrogen cyanide.[78,79] The content of cyanogenic glycosides is significantly higher in mature bitter almond seeds than in sweet seeds (between 200 and 1000-fold higher, respectively).[78]

β-glucosidase originating from almonds has been the subject of many kinetic studies compared to other glucosidases; it has been known for a long time, is easily isolated in large quantities from a readily available material, and is commercially available.[80] The use of β-glucosidase from sweet almonds was of particular interest in this research as it is derived from an edible product. Extracted and purified β-glucosidase from almonds is commercially available from Merck; however, it is costly, (1000 units for $2,540.00) and it is not suitable for use in edible products such as an enriched oil. In the literature, there are methods to extract β-glucosidase from almonds but mostly from bitter almonds which are not suitable for human consumption.[81] Extracting the β-glucosidase from sweet almond meal/flour is also possible but requires extraction, purification and utilises chemicals such as buffer solutions, polyvinyl pyrrolidone, ammonium sulfate and ammonium hydroxide.[81-84] In this research, the use of these chemicals would make the enriched oil inconsumable. Hence readily available unprocessed sweet almond flour/meal was investigated as a source of β-glucosidase to hydrolyse oleuropein as it has not been previously studied in the literature.

## 1.6 Olive Oil

OO is an important part of the research as there is currently large variation in the concentration of secoiridoid derivatives preset. As a result, there is an opportunity to investigate the enrichment of OO by transferring a high concentration of oleuropein aglycone from hydrolysed oleuropein in olive leaves via an exogenous enzyme.

### 1.6.1 Overview

The European Union (EU) is the world's biggest producer, consumer, and exporter of OO; Spain, Italy, and Greece account for approximately 66% of the global production. Production in these areas has tripled over the last three decades, reaching over 3 million tonnes of final

product yearly. This is mainly driven by changes in dietary patterns because of the success of the Mediterranean diet and its prevention of health-related issues. OO is considered the main source of fats in the Mediterranean diet because of its unique composition which includes healthy monounsaturated fatty acids and the presence of minor constituents as discussed in Section 1.4.2.1.[12,85]

Edible OOs are graded into six categories: EVOO, virgin olive oil (VOO), refined/light olive oil, mixed OO, refined olive-pomace/residue oil, and olive residue oil (also known as refined olive-pomace oil).[85,86] The flowchart below (Figure 1.9) describes how these oils are produced from the olive milling process. EVOO and VOO can be used for consumption straight after processing and contain the highest phenolic compound content compared to the other oils.[30] The third category, lampante, is only edible after a physio-chemical refining process and the product is called 'refined olive oil' (ROO). This can be combined with VOO to produce "olive oil". Pomace, the solid residue from the milling process, still contains a small amount of OO that is impossible to extract by mechanical means so is extracted with solvents resulting in 'refined olive-pomace oil' and 'olive residue oil'.[86]

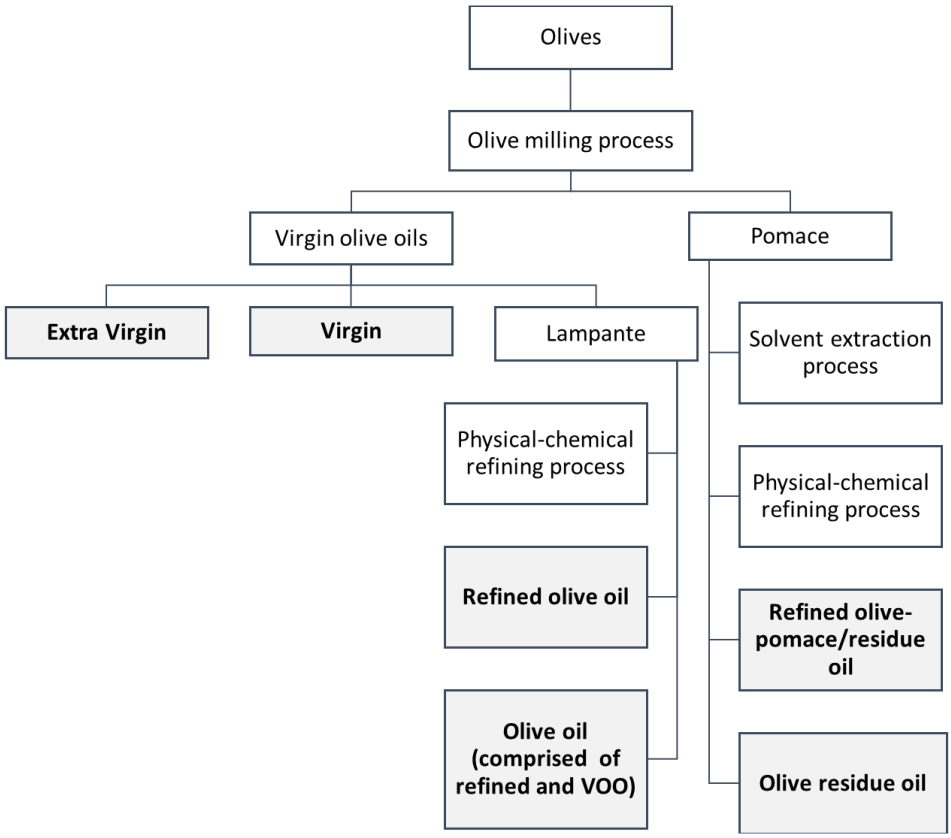



**Figure 1.9. The flow chart for different olive oils produced from the milling process. Adapted from Peri *et al.* (2014).[86]**

## 1.6.2 Extra virgin olive oil

EVOO has remarkable health properties, not only for its balanced composition of fatty acids but also for its content of minor components (1.5-2%) such as phenolic compounds which offer bioactive value.[87] The absence of any refining techniques during production results in the successful transfer of bioactive compounds from the olive fruit into the oil.[88] EVOO is the focus of this research and further details on the production, legal definition and composition are given below.

### *1.6.2.1 Production*

The first step in EVOO production is olive fruit harvesting, either through hand picking or mechanical processes.[86] Olive fruit cleaning is carried out in two steps; in the first separation

step, particulate foreign materials are removed by sifting, vibrating screens and air blowing. In the second washing step, olives are shaken into a washing basin and finally rinsed with clean water.[86]

The next important steps are the milling, pitting and malaxation. Milling is required to reduce the olives to a homogeneous paste by breaking the pits, skin, pulp cells and vacuoles which contain tiny droplets of oil. The main hydrophobic phenolics of EVOO, such as oleuropein aglycone, are formed during this step by the hydrolysis of oleuropein and ligstroside via endogenous β-glucosidase.[89] Any remaining glycosides persist in the water phase. The pastes from milling and pitting are then subjected to malaxation.[86]

Malaxation is where the olive paste is subjected to a slow, continuous kneading. This is an important step in EVOO production as complex physical and biochemical phenomena take place.[86] It also has critical effects on extraction yield as well as nutritional and sensory qualities.[86] The aim of malaxation is to improve oil separation in subsequent centrifugation steps.

The olive paste derived from the milling and malaxing operations is the material used in the centrifugal separation step.[86] The olive paste is a heterogeneous mixture of three phases. There is the 'insoluble solids phase' – this phase has the largest density as it consists of organic semisolid components and the woody fragments from the pit shells. There is the 'aqueous phase' which is not as dense as the solids phase and consists of water and water soluble components (salts, simple sugars, simple phenolics and glycosylated secoiridoids).[86] The least dense is the 'oil phase' which results in EVOO.[86,89]

### *1.6.2.2 Legal definition of EVOO*

For an oil to be in the EVOO category, certain criteria must be fulfilled: production should be exclusively by mechanical procedures, the oil must have a free fatty acidity content of <0.8%, a low peroxide index (<20 milliequivalents of active oxygen/kg oil according to the International Olive Council) and specific spectrophotometric values ($K_{232} \leq 2.5$, $K_{270} \leq 0.22$, $\Delta K \leq 0.01$).[90] These are indicators not only of quality but also authenticity.[90] The oil must also

pass a sensory test by a trained tasting panel in addition to chemical examination with the official methods.[25] Tasters work under fixed facilities with controlled temperature and moisture and rank the intensity of the sensations they experience on a scale from 0 to 10.[25]

### *1.6.2.3 Composition*

EVOO is mainly composed of triglycerides (97-99%) and minor compounds (1-3%) (discussed in Section 1.4.2.1), which are responsible for its biological properties and sensory attributes.[26] It has a high content of monounsaturated fatty acids (65-83%), especially oleic acid, and some polyunsaturated fatty acids such as linoleic acid.[26] Oleuropein and ligstroside aglycone account for approximately 90% of the minor compounds in EVOO;[26] however, the concentration of these two secoiridoid derivatives can vary. This is a result of an array of factors depending on both the intrinsic characteristics of the olive fruits and the technological conditions used during OO processing.[62] The content of the minor components in OO varies depending on the cultivar, climate (geographical origin), ripeness of the olives at harvesting, soil composition and the processing system employed to produce the OO.[30,91]

The existence of this variation is problematic. EVOOs in the New Zealand market are not tested for the presence of phenolic compounds and there have been no studies investigating commercial EVOOs in New Zealand. Studies have been carried out internationally, particularly in European countries. The concentration of some of the studied secoiridoid derivatives in European EVOOs with corresponding references are summarised in Table 1.2. The concentration of the secoiridoids present varies; for example, oleuropein aglycones concentration ranged from 117 to 620 mg/kg in a study by Różańska *et al.* (2020).[92] Whereas López-Huertas *et al*. (2021)[93] reported a low aglycone concentration of 22 mg/kg. Therefore, consumers who purchase EVOO expecting health benefits (discussed in Section 1.6.3) may not be consuming oil of suitable quality. Thus, the purpose of this research is to explore the possibility of enriching OO with compounds such as oleuropein aglycone so an oil can be marketed with a concentration that is within the range for relevant health benefits.

**Table 1.2. Summary of secoiridoid derivatives in EVOO samples categorised by country/region of origin and cultivar variety (cv., if applicable) analysed in the literature.**

| Origin/region and/or cv. | | Secoiridoid derivatives | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Oleuropein aglycone | Ligstroside aglycone | Oleacein | Oleocanthal | Hydroxytyrosol | Tyrosol | Ref |
| | | mg/kg | | | | | | |
| EU | Range | 219-737 | 21-143 | 22-177 | 0.3-5 | 38-208 | 22-91 | [92] |
| | Average | 378 | 46 | 105 | 2 | 78 | 52 | |
| Italy | Range | 211-692 | 21-137 | 23-259 | 0.3-15 | 48-145 | 39-124 | [92] |
| | Average | 435 | 68 | 137 | 5 | 84 | 60 | |
| Sardinia (Italy) | Range | 591-1105 | 111-288 | 155-272 | 4-9 | 39-70 | 19-42 | [92] |
| | Average | 778 | 165 | 213 | 6 | 55 | 30 | |
| Apulia (Italy) | Range | 343-1249 | 102-513 | 180-566 | <LOD-12 | 23-147 | 26-71 | [92] |
| | Average | 904 | 299 | 352 | 7.6 | 70 | 41 | |
| Liguria (Italy) | Range | 103-143 | 40-45 | 28-32 | 2-3 | 122-129 | 37-38 | [92] |
| | Average | 123 | 42 | 30 | 2.5 | 126 | 37.5 | |
| Sicily (Italy) | Range | 140-418 | 6-78 | 34-396 | 1.8-31 | 28-81 | 5.4-80 | [92] |
| | Average | 275 | 44 | 149 | 8 | 63 | 37 | |
| Spain (cv. Picual) | Average ± SD | 233 ± 135 | 270 ± 174 | 89.9 ± 68.3 | 119 ±72.5 | N/D[†] | N/D[†] | [94] |
| Spain (cv. Arbequina) | Average ± SD | 44.1 ± 74.1 | 32.4 ± 63.5 | 82.5 ± 83 | 88.9 ± 81.0 | N/D[†] | N/D[†] | [94] |
| Spain (cv. Hojiblanca) | Average ± SD | 115 ± 89.9 | 93.9 ± 96.9 | 75.0 ± 53.5 | 80.8 ± 692 | N/D[†] | N/D[†] | [94] |
| Spain (cv. Cornicabra) | Average ± SD | 110 ± 2 | 3.2 ± 0.1 | 16 ± 1 | 0.33 ± 0.03 | N/D[†] | 0.75 ± 0.04 | [93] |
| Spain (cv. Hojiblanca) | Average ± SD | 22 ± 1 | 0.52 ± 0.01 | 23.2 ± 0.3 | 0.323 ± 0.002 | 1.89 ± 0.01 | 0.59 ± 0.01 | [93] |
| Croatia | Average ± SD | 75.8 ± 2.8 | 10.5 ± 0.6 | 101 ± 3 | 54.3 ± 2.2 | 1.56 ± 0.18 | 3.28 ± 0.23 | [95] |
| Italy/Portugal/Croatia/ Spain | Average | 929 | 410 | 351.1 | 153.5 | 64.4 | 114.0 | [96] |
| | Range | 52-3482 | 18-1496 | 15.2-762.9 | <LOD-407.9 | 3.6-299.5 | 42.9-297.6 | |
| Greece (cv. Asprolia) | Average ± SD | 178 ± 79 | 98 ± 40 | 27.0 ± 17.6 | 39.5 ± 27.3 | ND[†] | ND[†] | [97] |
| Greece (cv. Ntopia) | Average ± SD | 26.5 ± 22.6 | 25.8 ± 16.6 | 78.4 ± 55.2 | 181.7 ± 64.7 | ND[†] | ND[†] | [97] |

*[†]N/D – no data: these analytes were not analysed in the particular study.*

### 1.6.3 Health benefits

As discussed in Section 1.6.2.3, olive oil contains 1-3% minor bioactive compounds. These naturally occurring phenolic compounds make OO a nutraceutical functional food, playing a critical metabolic role in the human organism.[25,92,98] The word nutraceutical is a combination of nutrition with pharmaceutical terms, and it defines a food (or part of it) that provides health benefits. As a result, OO has a range of health benefits which are often associated with the Mediterranean diet. This diet has been the subject of a large number of studies on its ability to prevent different chronic-degenerative diseases, suggesting an association between the alimentation of Mediterranean people and their low cardiovascular mortality.[21]

The positive health effects of a Mediterranean diet rich in EVOO have been reported to correlate with the antioxidant activity of phenolic compounds present therein that are capable of scavenging free radicals and reactive oxygen species.[99-101] Most researchers are in agreement that the health benefits of EVOO are mostly attributed to the hydrolysis products of oleuropein[102] (largely oleuropein aglycone, Section 1.4.2.1). These derivatives are known to possess biological activities which have been widely reported in literature; including antioxidant,[103,104] anti-inflammation,[105-107] anticancer,[108-110] anti-diabetic activities,[106,111] protection against neurodegenerative and coronary artery disease,[108,112-115] antimicrobial and antiviral effects,[108,116,117] promote the oxidative stability of lipids and decrease low-density lipoprotein cholesterol.[1,118] As these health benefits are largely associated with oleuropein aglycone, this analyte will be of particular interest in this research during enrichment experiments.

### 1.6.4 European Union (EU) regulation No. 432/2012

In 2011, the panel on Nutrition, Novel Foods, Food Allergens and the European Food Safety Authority (EFSA) concluded that there is evidence for a cause-and-effect relationship between the consumption of bioactive phenolic compounds in OO and the protection of low-density lipoprotein, i.e., cholesterol particles, from oxidative damage.[119,120] For this

reason, the EFSA has allowed the acknowledgement of health claims based on OO phenolic content. The list of health claims, the conditions and the restrictions of their use are reported in EU Regulation (European Commission Regulation EC No. 432/2012). This regulation permits the acknowledgement of the health claim for the benefits of the daily ingestion of OO rich in phenolic compounds, such as EVOO; "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress".[121] This claim is applicable for OOs containing at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complexes and tyrosol) per 20 g of olive oil or 250 mg/kg.[30,120,122,123] The specific compounds to which the EFSA refer to are hydroxytyrosol, tyrosol and other more complex high-molecular mass phenolics from which hydroxytyrosol and tyrosol can originate (hydroxytyrosol and tyrosol bound forms), namely, secoiridoids.[30,93,121]

This claim has a significant impact on the field of OO marketing and labelling.[122] The possibility of adopting a label with the health claim based on the content of bioactive phenolics is useful in effectively signalling both the "healthiest" and the "highest quality" EVOOs,[122] becoming a useful differentiation tool for the consumer to attribute a premium price to the best products.[30] While the health claim cannot be labelled on OOs in New Zealand, this research aimed to achieve an enriched OO with secoiridoid derivatives such as the aglycone to a content above 250 mg/kg because this would give certainty of health benefits to consumers.

### 1.6.5 Fraud

Fraudulent activities in the food industry is a global issue of increasing concern. It comprises of a wide range of malpractices, including adulteration (dilution, substitution, and unapproved enhancement), mislabelling, and smuggling, mainly for economic gain.[90] Due to its high economic value, unique sensory, compositional and nutritional characteristics, EVOO is considered at high risk of non-compliances and fraud.[124] Despite the rules provided by the International Olive Council and the EU, fraud and adulteration of EVOOs are common and represent one of the main problems in the EVOO industry.[125] The most commonly reported frauds in EVOO production are: (i) mixing EVOO with lower quality oils that are

cheaper to produce; (ii) mislabelling, for example, falsely claiming that refined olive oil is EVOO, or concealing the real place of origin; and (iii) price fraud, which consists of reducing the oil price to artificially lower the market value.[90] For example, Frankel *et al*. (2011)[125] reported many EVOO brands sold in California did not meet the international legal requirements to be classified as extra virgin. Selling adulterated or wrongly labelled OOs as EVOO not only causes an economic detriment to honest OO producers and traders, but also results in trade barriers and confusion among countries and consumers.[125] Therefore the quality of OO is governed by the International Olive Council together with the European Community and the Codex Alimentarius Commission.[90] Even with this governance of quality, fraudulent EVOO is prevalent.[124] Therefore, consumers are still being misled. This is of particular concern for New Zealand as 90% of the OO sold (30,000 tonnes) is imported from mainly European countries such as Spain, Greece and Italy.[126] These imported oils are seldom tested for quality or authenticity. As a result, a portion of the EVOOs on the commercial market may not be fresh, influencing the quality and amounts of bioactive compounds. Again, this provides a gap in the New Zealand market for an enriched OO that has concentrations above 250 mg/kg, giving consumers guaranteed health benefits.

## 1.7 Olive leaves

As discussed in Section 1.4.2.2, the major secoiridoid constituent in olive leaves is oleuropein.[127] Naturally, the content of phenolic compounds including oleuropein varies in olive leaves. The distribution and composition is dependent on pre- and post-harvest parameters including cultivar, geographical origin, maturity stage, climate, tree/leaf lifetime, as well as sampling/harvest time, drying, storage and processing factors.[19,118]

Olive leaves are considered an agricultural waste by-product generated during the processes of pruning olive trees, collection of olive fruits, and cleaning-blending steps in the extraction of OO.[128] About 25 kg of twigs and leaves per tree are accumulated annually by pruning olive trees, and a large amount (about 10% of fruit weight) is separated from the fruits during oil extraction.[129-132] However, leaves still contain considerable amounts of

phenolic compounds and have the highest level of oleuropein compared to the rest of the olive tree.[102] As a result, olive leaf extracts have been marketed as dietary products.[133] Commercial products in the form of herbal teas or food supplements are available all over the world, such as dried leaves, powders, extracts and capsules to exert health benefits on humans.[19,102,129,134] Most researchers are in agreement that the health benefits of olive leaf are mostly attributed to oleuropein, since hydrolysis products of this compound have significant health benefits (discussed in Section 1.6.3).

With large amounts of olive leaves going to waste each year, there is an underutilised source of oleuropein which can be extracted and hydrolysed to desired secoiridoid derivatives such as oleuropein aglycone and later transferred into OO. This research aims to show that leaves can be used as a source of oleuropein for enhancing OO.

### 1.7.1 Olive leaf extracts and teas

In this research, there was the requirement to produce an olive leaf extract containing oleuropein which could be later hydrolysed. Drying of the leaves is the most common commercial technique employed before the extraction of high added-value compounds from plant materials.[135] There are several advantages to drying leaves since fresh leaves contain free and bound water. Firstly, reduction of the water content prevents enzymatic and microbial activity that leads to a reduction in nutritional value.[6,135] Drying also improves stability and can result in a higher concentration of target analytes.[136] However, a disadvantage of drying leaves is the potential to affect the activity of bioactive compounds due to chemical and enzymatic degradation, and losses caused by volatisation and/or thermal decomposition.[136,137] Thus, the drying treatment is the most critical stage in the process because it will greatly affect the concentration of these bioactive compounds in the final product. Since bioactive compounds in olive leaves are very sensitive to environmental stresses,[118] post-harvest treatments such as drying operations are important to consider in this research. In the published literature there have been several studies on the effect of drying procedures on the nutritive value of olive leaves adopted for olive leaf extracts: air

drying, solar drying, oven drying, microwave drying, infrared drying and freeze drying.[6,138-141]

Extraction is the next critical step for both the isolation and exploitation of bioactive compounds in leaves; ideally, an extraction method should be quantitative, non-destructive, and time-saving; moreover, it should be selected on the basis of the desired ingredients, extract composition and purity one wishes to obtain.[118,128,142,143] In the literature there are several methods commonly employed for recovering phenolic compounds from olive leaves:[144] solid-liquid extraction (e.g., maceration, Soxhlet extraction),[145] microwave-assisted extraction,[146] ultrasound-assisted extraction,[146] supercritical fluid extraction,[147] percolation, distillation, heat reflux and pressurised liquid extraction.[148] These methods involve large proportions of solvent, the choice of which strongly influences the extraction yield. Moreover, agitation and/or high temperature maximise the diffusivity/mass transfer of the desired compounds from the matrix.[143] It should be noted that both thermal and nonthermal extractions are also time consuming, have a high energy or production cost, and possibly produce a lower-quality extract because of prolonged extraction time and the use of high temperatures.[144] With regards to solvent type, boiling water (which inactivates endogenous enzymes), methanol, ethanol, diethyl ether, ethyl acetate, chloroform, butanol or hexane are reported in the literature.[129] Extraction of olive leaves with 80% methanol was stated as more efficient for the recovery of phenolic compounds as it resulted in a 95% recovery yield.[129] However, it may cause unacceptable levels of toxic deposits in the extracts. For safety, water and ethanol are the most commonly used solvents.[129] Extraction by boiling water for 10-30 minutes has also been reported.[149,150] This type of extraction method is straightforward to operate and is a static process. Generally, shorter extraction times are required at higher temperatures.[129] In the literature, different ratios (sample/extracting solvent; *w*/*v*) from 5 to 100 have been studied, but a ratio between 10 and 50 was considered most suitable for extraction.[151]

This research does not require complete extraction of oleuropein so mild extraction conditions were focused on to avoid the use of toxic chemicals making the enriched oil

inedible. An olive leaf tea (OLT) was produced rather than an olive leaf extract, the latter being highly concentrated and requiring more aggressive extraction techniques with organic solvents (discussed above). On the contrary, OLT is generally less concentrated and made by steeping dried olive leaves in boiling water, similar to traditional herbal teas. The strength and potency of the tea is dependent on the number/mass of leaves, drying treatment of leaves, batch/origin of leaves, the temperature of steeping and how long the leaves are steeped in the water.

## 1.8 Enrichment of olive oil

As mentioned in Section 1.1, another aim of this research was to hydrolyse oleuropein present in OLT and transfer the resulting oleuropein aglycone into oil to obtain an enriched OO. In the food industry, this type of research is an active area where possibilities to exploit the high content of phenolics present in olive tree materials are being investigated to improve the health-related properties of oil.[152] Several authors[144,153-156] have looked at the enrichment of OO with olive phenolics from waste products such as leaves, pomace and wastewater, with olive pomace being the most common choice. Bioactive compounds from various herbs such as thyme, garlic, and spices such as chilli have also been investigated.[156-158] With regard to the enrichment step, there are three alternatives in the literature for oil enrichment with these valuable compounds from the olive tree: (1) liquid-liquid extraction,[159] in which the oil is put into contact with an alcoholic extract of phenols, and are transferred to the oily phase as a function of their distribution factor, removing the alcoholic phase by centrifugation; (2) solid-liquid extraction,[160] in which the purified phenolic extract is dried under appropriate conditions and the paste obtained is partially dissolved into the oil as a function of the solubility of the different paste components in the oily phase; and (3) a combination of these procedures, in which the alcoholic extract and the oil are put into contact and the two-phase system is subject to alcohol removal with a rotary evaporator.

This research focused on using olive leaves as they are an abundant by-product of the OO industry and can be used as a cheap source of phenolic compounds such as oleuropein which is present in high amounts (60–90 mg/g of dry matter).[50,161] In the literature, there are no studies that investigate the use of almond flour/meal as a source of β-glucosidase to hydrolyse oleuropein in OLT and later transfer the resulting oleuropein aglycone into oil. Thus, the potential to obtain an enriched OO through such methods was explored in this research.

## 1.9 Methods for determination of phenolic compounds in olive oil

Phenolic compounds in OO can be measured in two ways: determination of total content through a non-specific method, or quantitation of individual phenolic profiles. The total content methods, usually a spectrophotometric method (Folin-Ciocalteau),[162-165] are simple and relatively universal. However, for individual compound determination, there is great variation in the methods of analysis (discussed below). Each method has its own advantages and disadvantages. For the purposes of this research, a total phenolic method would not be suitable as it would provide minimal information on the increases and decreases of secoiridoids during the enrichment experiments. Hence, individual phenolic profile methods were of interest and are discussed below.

There are several important steps that are utilised in most methods for the determination and quantification of phenolic compounds in OO: isolation and extraction and instrument analysis. Isolation and extraction are a necessary step prior to successful analysis. Isolation of a specific fraction or target compound can be carried out using a variety of different procedures, filtration, concentration, centrifugation, clean up or a combination.[166] The aim of extraction in all instances is to prepare an extract uniformly enriched with all compounds of interest and be free from interfering matrix components.[166] Selection of an appropriate extraction procedure depends on the matrix (e.g., oil), chemical structure of components, interactions between matrix and target compounds and the concentration level required

for analysis.[166] Lastly, instrument analysis is required for separation, identification, and quantification of desired analytes.

A frequently used method for quantitative analysis of phenolics in OO is the method proposed by the International Olive Council (IOC) (IOC/T.20/Doc No. 29), developed by Agilent Technologies.[167] This method is based on a relative quantitation and uses a liquid−liquid extraction (LLE) to isolate phenolics in a hydroalcoholic phase that is analysed by liquid chromatography with photometric detection.[168] Tyrosol is used as a reference for relative quantitation which leads to underestimation of the absolute phenolic content.[94] Hence other methods in literature are usually adopted for analysis.

A range of methods found in literature are summarised in Table 1.3. Generalised concepts of these methods will be discussed in the following sections.

**Table 1.3. Phenolic compound extraction, instrument, and detection information from literature articles.**

| Oil type | Samples analysed | Extractant | Extraction method | Instrumentation | Stationary phase/mobile phase | Phenolic compounds* | Ref |
|---|---|---|---|---|---|---|---|
| EVOO | 18 | MeOH/water (80:20, *v*/*v*)<br>diethyl ether<br>ACN | LLE | GC-MS<br>HPLC-UV | C-18, 5 µm 250 x 4 mm<br>A-2% acetic acid (aq)<br>B-MeOH | 10 phenolic compounds<br>2 secoiridoid derivatives | [169] |
| VOO | 1 | MeOH/water (80:20, *v*/*v*; 60:40, *v*/*v*)<br>hexane | LLE | HPLC-DAD<br>NMR | C-18, 10 µm, 500 x 9.4 mm<br>A-0.2% acetic acid (aq)<br>B-MeOH | 4 phenolic compounds | [170] |
| EVOO | 4 | 2 mL MeOH/water (80:20, *v*/*v*)<br>2 mL hexane | SPE $C_8$ cartridge<br>LLE | HPLC-UV-DAD | C-18, 3 µm, 250 x 4.6 mm<br>A-0.5% phosphoric acid (aq)<br>B-ACN/MeOH (50:50, *v*/*v*) | 3 secoiridoid derivatives | [171] |
| ROO | 1 | 20 mL MeOH-water (80:20, *v*/*v*)<br>10 mL hexane<br>5 mL ACN | LLE | UPLC–MS/MS | C-18, 1.7 µm 100 x 2.1 mm<br>A-0.2% acetic acid (aq)<br>B-ACN | 12 phenolic compounds<br>10 secoiridoid derivatives | [172] |
| VOO | 3 | 1 mL MeOH/water (60:40, *v*/*v*)<br>1 mL hexane<br>1 mL ACN | LLE | LC–QTOF/QqQ MS/MS | C-18, 2.8 µm 50 x 2.0 mm<br>A-0.1% formic acid (aq)<br>B-0.1% formic acid in MeOH | 2 secoiridoid derivatives | [173] |
| EVOO | 6 | ACN:THF (1:1, *v*/*v*) | LLE | UHPLC-MS/MS | C-18, 2.8 µm 100 x 2.8 mm<br>A-Type 1 water<br>B-ACN | 6 secoiridoid derivatives | [174] |
| EVOO | 2 | MeOH-water (60:40, *v*/*v*)<br>15 mL hexane<br>30 mL ACN | LLE | UHPLC-MS/MS | C-18, 1.7 µm 100 x 2.1 mm<br>A-0.5% acetic acid (aq)<br>B-0.5% acetic acid in ACN | 30 phenolic compounds | [175] |
| EVOO | 4 | MeOH/water (80:20, *v*/*v*)<br>DMF | SPE $C_8$ cartridge<br>LLE | HPLC-UV<br>EC-UV | C-18, 5 µm 250 x 4.1 mm<br>HPLC: A-0.2% acetic acid (aq)<br>B-MeOH, EC: NA | 15 phenolic compounds | [176] |
| EVOO | 4 | LLE: 20 mL MeOH/water (80:20, *v*/*v*)<br>25 mL hexane<br>LLME: 2 mL MeOH/water (80:20, *v*/*v*) | LLE<br>LLME | HPLC-UV | C-18, 4 µm 300 x 3.9 mm<br>A-2% acetic acid in water/<br>B-2% acetic acid in MeOH | 10 phenolic compounds<br>3 secoiridoid derivatives | [177] |
| VOO | 1 | MeOH | LLE | LC-MS | C-18, 5 µm 250 x 4 mm<br>A-0.2 acetic acid (aq)<br>B-MeOH | 3 phenolic compounds<br>1 secoiridoid derivatives | [178] |

| Oli type | Samples analysed | Extractant | Extraction method | Instrumentation | Stationary phase/mobile phase | Phenolic compounds* | Ref |
|---|---|---|---|---|---|---|---|
| VOO | NA | 6 mL hexane<br>6 mL MeOH | SPE with diol-phase cartridge | HPLC<br>GC-MS | C-18, 5 µm 250 x 4 mm<br>A-3% acetic acid (aq)<br>B-ACN/MeOH (50:50, *v*/*v*) | 12 phenolic compounds<br>7 secoiridoid derivatives | [179] |
| VOO | 10 | SPE: 20 mL hexane<br>40 mL MeOH<br>LLE: MeOH/water (60:40, *v*/*v*) | SPE $C_{18}$ and $C_{18}$ ED cartridges<br>LLE | GC-MS | n/a | 7 phenolic compounds<br>1 secoiridoid derivatives | [180] |
| NA | NA | 5 mL MeOH/water (80:20, *v*/*v*) | LLE | HPLC-DAD | C-18, 5 µm 250 x 4.6 mm<br>A-0.2% phosphoric acid (aq)<br>B-MeOH, C-ACN | 10 phenolic compounds<br>3 secoiridoid derivatives | [168] |
| VOO | 23 | MeOH-water (60:40, *v*/*v*)<br>15 mL hexane | LLE<br>SPE $C_{18}$ and diol-phase cartridge | HPLC-DAD | C-18, 5 µm 250 x 4 mm<br>A-0.1% formic acid (aq)<br>B-MeOH/ACN (1:1, v/v) | 2 phenolic compounds<br>7 secoiridoid derivatives | [181] |
| EVOO | 12 | 10 mL MeOH-water (60:40, *v*/*v*)<br>5 mL hexane | LLE | LC-DAD-ESI-MS/MS | C-18, 5 µm 250 x 4.6 mm<br>A-1% formic acid (aq)<br>B-ACN/solvent A (60:40, *v*/*v*) | 7 phenolic compounds<br>6 secoiridoid derivatives | [182] |
| EVOO | 5 | 60 mL hexane<br>40 mL MeOH | SPE with Diol-cartridge | CE and HPLC-TOF-MS | C-18, 3.5 µm 100 x 2.1 mm<br>A-0.1% acetic acid (aq)<br>B- ACN | 10 phenolic compounds<br>7 secoiridoid derivatives | [183] |
| EVOO | 16 | 20 mL MeOH/water (80:20, *v*/*v*)<br>5 mL hexane | LLE | GC-MS | n/a | 16 phenolic compounds<br>7 secoiridoid derivatives | [184] |
| VOO | NA | SPE: 6 mL hexane, 10 mL MeOH/water (20:80, *v*/*v*)<br>LLE: 1 mL hexane, 2 mL MeOH | SPEs with diol phase cartridge<br>LLE | HPLC-DAD and LC-MS | C-18, 5 µm 250 x 4.6 mm and C-18, 1.8 µm 100 x 2.1 mm<br>A-0.1% formic acid (aq)<br>B-0.1% formic acid in ACN | 4 phenolic compounds<br>2 secoiridoid derivatives | [185] |
| EVOO | 10 | Hexane<br>ACN<br>MeOH | LLE | HPLC-UV | C-18, 5 µm 250 x 4.6 mm<br>A-2% acetic acid (aq)<br>B-MeOH | 1 secoiridoid derivatives | [186] |
| EVOO | 30 | MeOH-water (60:40, *v*/*v*)<br>2 mL hexane | LLE | HPLC-DAD-ESI-MS | C-18, 2.7 µm 150 x 2.1 mm<br>A-0.1% formic acid (aq)<br>B-0.1 formic acid in MeOH/ACN (1:1, *v*/*v*) | 4 phenolic compounds<br>10 secoiridoid derivatives | [165] |

*n/a – not applicable; *analysis of phenolic compounds have been separated into "phenolic compounds" which includes flavonoids, phenolic acids, lignans etc, and the "secoiridoid derivatives" as these were of interest in this research.*

### 1.9.1 Isolation and extraction

In the literature isolation and extraction of the phenolic compounds from OO utilise two basic extraction techniques, liquid-liquid extraction (LLE), including liquid-liquid micro extraction (LLME), or solid-phase extraction (SPE). Various isolation systems have been proposed by different authors depending on the aim of the particular study.[187] The procedures not only vary in solvents and/or solid-phase cartridges used but also the mass of the sample and volume of solvent used for analysis.[187] These factors directly influence the cost, greenness, and speed of extraction.

A LLE is a process through which the analytes are transferred from a solution into an immiscible solvent through mixing. For OO, LLE procedures use methanol or methanol (MeOH)/water mixtures (the most common solvents).[169-172] Montedoro *et al.* (1992)[170] evaluated the combination of methanol and water at different percentages for extraction of simple and hydrolysable phenolic compounds in OO. The best solvent was MeOH/water (80:20, *v*/*v*). In some studies, addition of tensioactive substances to a hydroalcoholic solution (e.g., Tween 20 at 2% *v*/*w*) increased the effectiveness of the phenolic extraction.[188,189] Other studies have reported that a MeOH/water solution (60:40, *v*/*v*) is the best proportion for extracting these kinds of compounds. Pirisi *et al.* (2000)[171] demonstrated an increase in the recovery efficiency of phenolics by reducing the percentage of MeOH to 60%. Thus, several authors have used these conditions for the extraction of phenolic compounds in OO.[5,51,190,191] Some studies have examined the effectiveness of other solvent systems such as acetonitrile (ACN), ethanol and tetrahydrofuran (THF).[173-175] Brenes *et al.* (2000)[176] evaluated the use of ethanol/water (80:20, *v*/*v*), (THF)/water (50:50, *v*/*v*), ACN, water at 5 °C, and *N,N*-dimethylformamide (DMF). The results showed that none of the solvents assayed were more efficient than MeOH/water (80:20, *v*/*v*). Luque-Muñoz *et al.* (2019)[174] investigated 1:1 ACN/dry THF but no recovery data were provided. In most LLE methods, after the extraction step, a clean-up of the residue by solvent partition is needed. Several solvents have been used to remove the fat phase (petroleum ether, chloroform, hexane), but the most commonly used solvent

is hexane.[165,179,181,186,187,192] Some methods do not clean up the extract via solvent partition where the upper methanolic phase is usually filtered and analysed.[169,177,178] Pizarro *et al.* (2013)[177] used a LLME to extract OO using methanol/water (80:20, *v*/*v*) before filtration (0.45 µm) and dilution, achieving greater than 80% recoveries.

SPE has also become a popular isolation method for OO phenolic compounds. These compounds are separated from the oil matrix due to their affinities for a specific sorbent. Several optimised procedures have been reported indicating the advantages of different solid phase sorbents. Some of the sorbents that seem most suitable for this purpose are alkyl-silica resins, such as C8 or C18. Pirisi *et al.* (2000)[171] proposed the application of C8 cartridges to recover the phenolic fraction from OO. Several studies used C18 cartridges for recovering OO phenolics, and obtained better recovery efficiencies than for C8.[193] Even though working with reverse-phase using C18 phase could seem less suitable for the isolation of polar components from a nonpolar matrix than normal-phase SPE, several authors have chosen C18 cartridges. Liberatore *et al.* (2001)[180] evaluated the advantages of the SPE C18 cartridge with suppression of residual silanol groups (C18 EC, end-capped) against an SPE C18 cartridge with free silanol groups. The latter seemed to improve the release mechanism, increasing the recovery. Other experimental approaches involve the use of an acidified eluent.[38,187] Indeed, pH values of the mobile phase affect the partitioning behaviour during extraction, and it has been established that C18 cartridges could generally be recommended for simple phenolics. Nevertheless, these procedures have shown poor recovery for secoiridoid derivatives. Therefore, researchers have pointed to other solid phases as alternative approaches. Mateos *et al.* (2001)[179] worked with amino-phase and diol-bond phase SPE cartridges, achieving recoveries of >90% for all major olive phenolic compounds. This was also corroborated by Bendini *et al.* (2003)[194] who achieved the highest recoveries (92.1 ± 6.4%) with the diol-phase cartridge compared to C8, modified C8 and C18 cartridges. Afterward, Bendini *et al.* (2003)[194] compared the results obtained by SPE approaches with the LLE procedure previously described.[171] The LLE approach exhibited significantly higher recovery of all phenolics compared to SPE procedures. This is in agreement with another study[181] in which the experimental work demonstrated that the

application of a LLE led to significantly better recoveries of total phenolic compounds (93%) than the C18-phase cartridge. As a result, LLEs are generally more popular methods for the isolation and extraction of phenolic compounds in OO.

### 1.9.2 Instrumentation and detection methods

Liquid chromatography (LC) is the predominant technique used for analysis of phenolic compounds in OO; generally high-performance liquid chromatography (HPLC) and Ultra HPLC (UHPLC) are used. Most studies use reverse-phase columns (stationary phase) to separate individual components, consisting of a nonpolar octadecylsilane (C18) bonded phase with preferable lengths, diameters and particle sizes of 100 to 250 mm, 2-4.6 mm and 1.8-5 µm, respectively.[122,168,174,175,177,181] Shorter and narrower columns with small particle sizes are preferred to obtain better resolution and reduce the time of analysis. Recent studies exclusively use gradient elution mode. This highlights the complexity of the phenolic profile that is not well separated by isocratic elution mode. However, one study reported isocratic elution using a solvent composition of water (solvent A, 30%) and ACN (solvent B, 70%).[174] Concerning the mobile phases, there is a wide range of possibilities; however, binary systems consisting of water and a less-polar solvent (MeOH, ACN) are the most common mobile phases. Usually, acetic acid, formic acid, or phosphoric acid are added to the aqueous phase to maintain a low pH and avoid phenolic dissociation.

HPLC coupled with ultraviolet (UV) detectors (photodiode array detector, DAD), mass spectrometers (MS) and nuclear magnetic resonance (NMR) detectors have all been used as tools in identification, quantification and structural characterisation of phenolic compounds in OO.[185] In the past, the most common detectors coupled to the HPLC (or UHPLC) for OO phenolic compound quantification were UV detectors (DAD).[171,179,188] While UV detectors are cheap and easy to use, there are several disadvantages including poor sensitivity, especially for analytes in low quantities or with weak UV absorption, and difficulty in identification of peaks that have similar UV spectra or that co-elute.[195,196] As a result, LC systems coupled to MS or MS/MS (tandem mass spectrometry) detectors are now more common and have been used in various methods reported in the literature (Table

1.3).[34,173,175,182,197,198] This detection system has high sensitivity and specificity, a quick analysis time, capability to determine the molecular weight and provide structural information. These aspects are ideal since compounds in OO are often found at trace amounts.[199] Older articles[163] make use of atmospheric-pressure chemical ionisation, whereas more recent works use electrospray ionisation (ESI), mainly in negative ion mode due to phenolic compounds being acidic and loss of a proton during the ionisation generates the negatively charged ions, resulting in greater sensitivity.[123,198,200] Different mass analysers such as ion trap, time-of-flight (TOF) and single or triple quadrupole (QqQ) have been used.

NMR has been previously reported, but it is the least common detection method.[201,202] This is due to difficulties relating spectra obtained from multicomponent mixtures such as OO to compounds preset. Strong signal overlap, dynamic range problems, diversity of intensities due to various concentrations of the food constituents, and the lack of scalar coupling (J coupling) information between different moieties lead to ambiguous or incomplete assignments, thus hindering detection even with the use of multidimensional NMR.[200]

Another instrument used for analysis is capillary electrophoresis (CE) coupled to various detectors (MS, UV).[183,203,204] However, there are only a few publications reporting this technique. This is due to some disadvantages of CE, such as poor reproducibility, difficulties with the MS coupling, long capillaries that give long analysis time, refrigeration of capillary, and the need for sheath liquid addition to use ESI.[166,183]

Separation via Gas chromatography (GC)/MS has also been used but the limited volatility of many phenolic compounds in OO has restricted its use. However, the analysis of the phenolic compounds may be accomplished with derivatisation. The drawback of an additional step to the sample preparation and the appearance of different chemical species because of incomplete derivatisation means the number of publications using this technique to analyse phenolic compounds in OO is minimal.[166,169,184]

## 1.10 Research aims

OO is an excellent source of minor bioactive compounds such as oleuropein aglycone which is associated with a range of health benefits. Currently, the concentration of bioactive compounds can vary between batches of OO and some batches may contain bioactive compounds that are below the threshold which provides health benefits. As a result, this project was carried out for a company who wish to enhance OO so it can be later developed into a marketable product. Therefore, the research largely focused on investigating the possibility to enrich OO with higher amounts of oleuropein aglycone. A concentration above 250 mg/kg was the target as this would give certainty of health benefits to consumers. To achieve this goal several different experiments were carried out, the three overarching aims of these experiments are described below.

**Aim 1:** Determine the concentration of target analytes; oleuropein, ligstroside, oleuropein aglycone, oleacein, oleocanthal, hydroxytyrosol, tyrosol and elenolic acid in commercial EVOOs on the New Zealand market. These results were compared to the concentration of analytes transferred into OO during the research. The association between purchase price and oils meeting health claim level was also explored to gain an understanding of EVOOs in the New Zealand market.

**Aim 2:** Investigate the effect of olive leaf drying conditions on the concentration of oleuropein and other target analytes in the resulting OLT for the purpose of commercial production. The stability of target analytes in the OLT was also examined as there is minimal stability data present in the literature.

**Aim 3:** Investigate the use of almond flour/meal as an exogenous source of enzyme (β-glucosidase) to hydrolyse oleuropein present in OLT as this is unexplored in literature. The research will then investigate the potential to transfer the resulting oleuropein aglycone into a low content OO to achieve a concentration over 250 mg/kg. Thus, producing an enriched marketable oil with health benefits.

## 1.11 Thesis outline

The thesis has been divided into the following chapters to achieve the overall aims of the research:

**Chapter 2** details the experimental methodology used for this research.

Method optimisation and validation of the LC-MS/MS method used to quantify target analytes in this research (oleuropein, ligstroside, oleuropein aglycone, oleacein, oleocanthal, hydroxytyrosol, tyrosol and elenolic acid) in oil and OLT are described in **Chapter 3**.

Commercial EVOOs in the New Zealand market (*n* = 9) were analysed for the target analytes to understand the range of concentrations present in samples due to the lack of literature. Whether or not they met the health claim and if this had any correlation to their cost was also examined. The concentration of analytes in commercial oils were also compared to the concentration obtained in the enriched oils that were produced during the research. The effect that drying olive leaves had on the concentrations of oleuropein and other target analytes in OLT was examined. A long-term storage experiment (−20, 25, and 40 °C, 189 days) of OLT was also carried out to test the stability of target analytes over time in an aqueous matrix. These experiments are discussed in **Chapter 4**.

The effectiveness of almond flour/meal as an exogenous source of β-glucosidase to hydrolyse the oleuropein present in OLT and the experiments undertaken to investigate the potential of transferring the resulting oleuropein aglycone into low phenolic content oil are discussed in **Chapter 5**.

The main conclusions of the research are summarised in **Chapter 6**, along with recommendations for future research on this topic.

# CHAPTER 2

# Methods and Materials

## 2.1 Samples

### 2.1.1 Olive oil

EVOOs (*n* = 5) were purchased from local supermarkets in the Hamilton region, New Zealand. EVOOs were also supplied via two external companies (Olive Black *n* = 1 and The Olive Press *n* = 3). For the method validation, refined OOs were purchased (*n* = 2) because they had a lower phenolic content making them suitable for use in method validation recovery studies. All oils were subsampled into 250 mL clear glass containers or 20 mL amber vials and stored in a dark and dry cupboard until use. The details of the oils purchased are summarised in Table 2.1.

**Table 2.1. Brands and supplier of EVOO and OO used in this research. n.d. denotes no date.**

| Brand | Supplier | Country of origin | Purchase date |
|---|---|---|---|
| **EVOO** | | | |
| Red Island | Supermarket | Australia | 19/07/22 |
| The Village Press, First Press | Supermarket | New Zealand, Australia, Spain | 19/07/22 |
| Oleo Martos | Supermarket | Spain | 19/07/22 |
| Macro Organic | Supermarket | Spain | 19/07/22 |
| Matapiro | Supermarket | New Zealand and Australia | 19/07/22 |
| Olive Black | Olive Black | New Zealand | 2020 Harvest |
| Frantoio | The Olive Press | New Zealand | n.d. |
| Picual | The Olive Press | New Zealand | n.d. |
| Picholine | The Olive Press | New Zealand | n.d. |
| **OO** | | | |
| Pams, extra light | Supermarket | Not specified | 17/11/22 |
| Lupi, extra mild | Supermarket | Italy | 17/11/22 |

### 2.1.2 Olive leaves

Olive leaves were kindly donated. These were collected from properties in Waitao, Tauranga and Claudelands, Hamilton when required. The species of olive leaves picked were unknown. They were either used fresh or left to dry in an incubator at room temperature (RT, 24 °C) until constant weight before being placed into a brown paper bag and stored in a dry, dark cupboard until use (within 48 hours).

## 2.2 Solvents, standards, reagents, and consumables

### 2.2.1 Solvents

Type 1 water (distilled and deionised) was obtained using an ELGA LabWater PURELAB® Flex water purification system (18.2 MΩcm resistivity). Distilled water was made in-house through a distillation apparatus at the University of Waikato. Methanol (MeOH, Optima™ LC-MS grade >99.99%), acetonitrile (ACN, HPLC grade ≥99.99%) and formic acid (Optima™ LC-MS grade ≥99.0%) were obtained from Fischer Chemical™, part of Thermo Fischer Scientific. Acetic acid (glacial, *ReagentPlus*®, ≥99,0%) was obtained from Sigma Aldrich. Acetone (HPLC grade ≥99.5%) was obtained from J.T Baker™.

### 2.2.2 Standards and reagents

Oleuropein (≥80.0%) was obtained from Sigma Chemicals and tyrosol (98.5%) was obtained from Aldrich Chemicals. Elenolic acid (>90.0%), oleacein (90.0%), oleocanthal (95.0%), hydroxytyrosol (98%) and oleuropein aglycone (95.0%) were from Toronto Research Chemicals (Canada). Ligstroside (97.0%) was obtained from Biosynth. D-(+)-glucose (≥99.5%) was obtained from Sigma Aldrich. Sodium chloride (NaCl) was obtained from LabServ (Thermo Fischer Scientific). Almond flour ($n$ = 2; purchased in September 2022 and the 18$^{th}$ of November 2022) and whole raw almonds (purchased 24$^{th}$ of February 2023) were obtained from local supermarkets in New Zealand.

### 2.2.3 Consumables

Eppendorf centrifuge tubes (2 mL) were used for oil extraction, the combined extracts were placed into 15 mL polypropylene tubes to be mixed before analysis. Amber HPLC vials (2 mL) with 9 mm caps and silicone/PTFE septa were used for the analysis of oil and OLT samples. Glass inserts for 2 mL vials were used for analysis when samples were less than 250 µL. OLT samples that contained precipitate were filtered prior to analysis using nylon membrane filters (13 mm, 0.20 µm and 17 mm, 0.45 µm; Agilent Technologies) and sterile disposable syringes (1 mL). To analyse the glucose concentration, i·sens CareSens™ PRO testing strips were used in a CareSens™ Dual blood glucose meter (BGM). To make almond milk, Schleicher and Schuell filter paper 604 (55 mm) was used to separate the almond meal from the almond milk.

## 2.3 General Methods

### 2.3.1 Apparatus temperature management

Water baths, boiling water, ovens and freezers were used in this research for either sample experimentation or stability trials. To control the temperature of the water baths and boiling water, a mercury thermometer was used, the temperature was held consistent within ± 5 °C. The temperature control of the ovens and freezers housed in the commercial laboratory were constantly monitored by a Thermo Scientific™ Smart-Vue™ Sensor; this equipment had automatic and continuous data logging, so freezers were kept within −10 and −30 °C. The sensors were calibrated every 6 months. Samples that could not be analysed within the same day were stored in a walk-in chiller, this was also monitored with a Thermo Scientific™ Smart-Vue™ Sensor, keeping the temperature within 2-8 °C.

### 2.3.2 Glassware cleaning

Labware items used at the university to make almond milk and OLT were washed thoroughly with detergent and water. The glassware was rinsed three times with Type 1 water, then oven dried (100 °C). At the commercial laboratory, glassware was soaked

overnight (16 hours) in Decon 90 (labware detergent); the glassware was then rinsed three times with Type 1 water and rinsed twice with MeOH, then left to dry on a drying rack.

### 2.3.3 Statistical analysis and graphical image generation

Analysis of data and graphical visualisation were carried out using Microsoft Office Excel (Version 2303) and Minitab® (Version 21.4.1), while flow diagrams were carried out using Microsoft Office PowerPoint (Version 2303). For statistical calculation, samples that had an analyte concentration less than the limit of detection (<LOD) were treated as a missing value for summary statistics (e.g., averages). Chemical structures, diagrams and reaction pathways were produced using PerkinElmer ChemDraw (Version 21.00).

## 2.4 Triple Quadrupole Liquid Chromatography Tandem Mass Spectrometry

The Triple Quadrupole Liquid Chromatography Tandem Mass Spectrometry (LC-QqQ-MS/MS) system used for both oil and OLT analysis was a ExionLC™ high-performance liquid chromatography (HPLC)/ultra-high-performance liquid chromatography (UHPLC) system with a ExionLC™ AD Multiplate Autosampler and a SCIEX Triple Quad™ 6500 tandem mass spectrometer. The instrumentation was controlled by Analyst® software (Version 1.7.3). MultiQuant™ software (version 3.0.3) was used for the quantitative and qualitative workflow analysis.

## 2.5 Analysis and quantification of phenolic compounds in oil by LC-QqQ-MS/MS

### 2.5.1 Reagents and standards

The extraction solvent (MeOH/water, 80:20 *v*/*v*) was prepared by mixing MeOH (80 mL) and Type 1 water (20 mL) in a measuring cylinder. Primary stock standards of the following quantified analytes were accurately weighed (if possible) into an amber vial, 100% MeOH

(standard weight dependant) was then pipetted into the vial with a target concentration of 1000 mg/L (actual concentration is presented in parenthesis): oleuropein aglycone (7034 mg/L), oleocanthal (950 mg/L), oleacein (900 mg/L), hydroxytyrosol (859 mg/L), tyrosol (923 mg/L), oleuropein (605 mg/L), ligstroside (970 mg/L) and elenolic acid (900 mg/L). With the oleuropein aglycone standard having such a high concentration, a secondary standard with a concentration of 999 mg/L was made from the stock standard and was used in subsequent standard preparation.

Due to the inhibitory high cost of some of the standards, <1 mg was purchased making it difficult to obtain an accurate weight. For oleuropein aglycone, the standard was prepared by mass difference; the vial containing the compound from the manufacturer was weighed, the compound was dissolved in 100% MeOH (1 mL), the solvent + compound was transferred to a new weighed vial and the residual solvent was evaporated from the manufacturer's vial. The weight of compound was determined by comparing the weight of the manufacturers vial after evaporation to the weight before the addition of MeOH. The compound weight was then subtracted off the weight of solvent + compound that was determined in the new weighed vial. This gave the amount of MeOH in grams which was converted back to litres based on density, providing a concentration in mg/L. For elenolic acid (0.5 mg), oleacein (1 mg), ligstroside (1 mg) and oleocanthal (0.5 mg) an equivalent amount of solvent (0.5 mL, or 1 mL) was added. It is noted that this will not be quantitative if the amount supplied was not exactly what was stated on the vial. Preparation by mass difference was considered; however, the original vial the compound was in (from the manufacturer) was too heavy for the microbalance. After standards had been prepared, an easier mass difference method which did not require the manufacturer's vial to be weighed on a microbalance was suggested for future reference; the compound was dissolved in MeOH, transferred to a new weighed vial, the solvent was evaporated, and the same vial was re-weighed to determine the amount of compound. A specific volume of MeOH was added to determine a concentration in mg/L. Moreover, if standards had not already been purchased, it would have also been possible to pay extra and receive an accurate weight from the manufacturer, allowing for precise standard preparation when using extremely

small masses. All standards were stored in the freezer (−25 °C) until use and given an expiry date 2 years from preparation.

With a large range of concentrations required for the calibration curve (0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 mg/L) an initial mixed standard (10 mg/L, 2 mL) was made from the stock standards (approximately 1000 mg/L) and 100% MeOH. Three lower concentrated mixed standards (5.0, 1.0 0.5 mg/L, 2 mL) were then made from the initial mixed standard (10 mg/L) and 100% MeOH. This was carried out by aliquoting from the mixed standard for each concentration, not by serial dilution. These four mixed standards were used to achieve the concentrations required for the working standards of the calibration curve (Table 2.2). Before the 10 mg/L standard was consumed, a new mixed standard (10 mg/L) was prepared and deemed suitable for use if the concentration was within ± 10% of the old mixed standard.

**Table 2.2. The concentration of the four mixed standards (Std) used to make working standards for the calibration curve.**

| Mixed Std | Concentration of mixed Std (mg/L) | Working Standard (mg/L) |
|---|---|---|
| Mixed Std 1 | 10.0 | 1, 0.5, 0.2 |
| Mixed Std 2 | 5.0 | 0.05, 0.1 |
| Mixed Std 3 | 1.0 | 0.02 |
| Mixed Std 4 | 0.5 | 0.005, 0.01 |

## 2.5.2 Sample preparation

The oil sample preparation method was based on the method by Pizarro *et al*. (2013)[177] with modifications. For details on method optimisation and validation of this modified method see Chapter 3, Section 3.1.2.

Olive oil (0.100 ± 0.003 g) was weighed into an Eppendorf tube (2 mL). MeOH/water (80:20 *v*/*v*, 2 mL) was added. The mixture was vortexed (1 min), then centrifuged (5 min, 11,300 RCF). This procedure was repeated 3 times in total, each time 1.8 mL of supernatant was taken from the tube and placed into a 15 mL centrifuge tube. Once the triplicate

extraction was complete, the 15 mL tube was vortexed (30 seconds). A sample (1 mL) was taken for analysis in an amber HPLC vial (1.5 mL). If samples could not be analysed within the same day, they were left in a chiller (4 °C) and analysed within 48 hours.

#### *2.5.2.1 Recovery checks*

During method validation, the accuracy of the above method was tested by carrying out spike recovery checks. OO samples were spiked with the 10 mg/L mixed standard to achieve low and high spiked samples (Table 2.3). For the high spike (50 µg/L), 10 mg/L mixed stock standard (30 µL) was added to Pams or Lupi OO (0.100 ± 0.004 g); for the low spike (20 µg/L), 12 µL of the same mixed standard was added. Spiked samples were vortexed (1 min), then left on the bench (1 hour) for the spiked compounds to interact with the matrix. The extraction procedure above was then carried out. Details on method validation recoveries can be found in Chapter 3, Section 3.2.3.

**Table 2.3. Amount spiked and theoretical spike concentration detected on the instrument during method validation.**

| Spike | Amount (µL) of 10 µg/L Mixed Std spiked | Theoretical spike concentration (µg/L) |
|---|---|---|
| Low spike | 12 | 20 |
| High spike | 30 | 50 |

### 2.5.3 Instrument method

Compound analysis was performed on a ExionLC™ HPLC system interfaced with a SCIEX Triple Quad™ 6500 mass spectrometer (see Section 2.4 for details) with electrospray ionisation (ESI) via Turbo Spray IonDrive. The HPLC was equipped with a binary pump with an integrated vacuum degasser, an autosampler, and a thermostated column compartment.

Compounds were separated using a Poroshell 120 EC-$C_{18}$ column (2.7 µm, 2.1 x 50 mm, Agilent Technologies, USA) fitted with a Phenomenex KrudKatcher™ Ultra. The column compartment was kept at 50 °C, the flow rate was 0.6 mL/min, the injection volume was

2 µL and the sample cooler was kept at 15 °C. The mobile phase consisted of a gradient program using 0.1% acetic acid in Type 1 water (A) and 100% MeOH (B) as follows: 0 min, 10% B; 0.5 min 10% B; 3 min, 95% B; 4 min, 95% B; 4.10 min 10% B; with a final time of 5 minutes. The needle wash solution used between injections was MeOH/Type 1 water (50:50, *v*/*v*).

The retention times for the target analytes are summarised in Table 2.4. Oleuropein aglycone had several unresolved peaks corresponding to isomers and quantification was carried out using the sum of the four peaks. Elenolic acid was a broad peak and peak shape often varied with sample type. Therefore, quantification was carried out using the sum of elenolic acid peaks between 1.6 and 3.5 minutes (regardless of peak shape) to be consistent when reporting results.

**Table 2.4. Retention times for target analytes in this research.**

| Analyte | Retention time (min) |
|---|---|
| Oleuropein | 2.98 |
| Oleocanthal | 3.13 |
| Oleacein | 2.79 |
| Hydroxytyrosol | 0.63 |
| Tyrosol | 1.04 |
| Oleuropein aglycone | 3.40, 3.47, 3.60, 3.79 |
| Ligstroside | 3.24 |
| Elenolic acid | 1.6-3.5 |

The mass spectrometer (MS) was operated in ESI negative ion mode using multiple reaction monitoring (MRM), with a retention time window of 4.003 minutes and a target scan time of 0.2502 seconds. The following conditions were used: IonSpray Voltage, −4500 V; ion source gas 1, 70 psi; ion source gas 2, 60 psi; curtain gas, 25 psi; collision gas, 8 psi; dwell time, 5 milliseconds and a flow rate and drying gas (nitrogen gas, $N_2$) temperature of 10 L/min and 450 °C, respectively. The precursor ion, two most intense MRM transitions (quantifier and qualifier) and optimised MS parameters are summarised in Table 2.5. Details on the optimisation are discussed in Chapter 3, Section 3.1.1.

**Table 2.5. The MRM transitions and optimised MS parameters for the determination of olive oil and OLT target analytes. Product ion transitions used for quantification are shown in bold text.**

| Analyte | Precursor ion (*m/z*) | Product ion (*m/z*) | DP[1] (V) | EP[2] (V) | CE[3] (V) | CXP[4] (V) |
|---|---|---|---|---|---|---|
| Oleuropein | 539 | -307 | −101 | −6 | −31 | −15 |
| | | **−275** | −124 | | −28 | −14 |
| Oleocanthal | 303 | **−179** | −45 | −6 | −10 | −9 |
| | | −165 | | | −14 | −7 |
| Oleacein | 319 | **−195** | −60 | −12 | −16 | −16 |
| | | −183 | | | −10 | −10 |
| Hydroxytyrosol | 153 | **−123** | −60 | −14 | −19 | −14 |
| | | −122 | | | −28 | −12 |
| Tyrosol | 137 | **−119** | −19 | −19 | −20 | −13 |
| | | −106 | | | −21 | −14 |
| Oleuropein aglycone | 377 | **−307** | −58 | −12 | −15 | −9 |
| | | −275 | | | −17 | −8 |
| Ligstroside | 523 | **−361** | −100 | −8 | −20 | −11 |
| | | −291 | | | −31 | −9 |
| Elenolic acid | 241 | **−127** | −30 | −10 | −16 | −15 |
| | | −95 | | | −14 | −11 |

*[1]DP: delustering potential (V); [2]EP: entrance potential (V); [3]CE: collision energy (V); [4]CXP: collision cell exit potential (V).*

## 2.5.4 Data workup

To determine the concentration of analytes in the oil samples, the following steps were taken. Initially, the data from the instrument was processed via MultiQuant™ software. This outputs "in vial" results in µg/L (i.e., raw results), calculations were then carried out to determine "in sample" concentration (i.e., final concentration).

The final concentration (µg/kg) was determined based on Equation 2.1.

$$Concentration\ (\mu g/kg) = \frac{Extraction\ vol\ (mL) \times Dilution\ factor \times "In\ vial"\ (\mu g/L)}{Sample\ weight\ (g)} \quad \textbf{Equation 2.1}$$

Results were then reported in mg/kg using Equation 2.2.

$$Concentration\ (mg/kg) = \frac{Concentration(\mu g/kg)}{1000}$$ **Equation 2.2**

## 2.6 Olive leaf tea methods

### 2.6.1 Moisture content of leaves

Olive leaves (10 g) were weighed in triplicate, then dried at RT until constant weight. The difference in weight was used to calculate the moisture content.

### 2.6.2 Extraction method

To prepare leaves for use, leaves were hand picked off the olive branches; any leaves that were torn on removal were omitted due to the release of endogenous enzymes. Leaves were dried to consistent weight at room temperature (20-25 °C), initially this was done on the bench in the laboratory, but leaves would often pick up moisture from the air, so the method was changed, and leaves were dried in an incubator (24 °C). To investigate how leaf drying temperature affected the concentration of oleuropein in OLT, two drying experiments were undertaken (Chapter 4, Section 4.3.2) using fresh (without any drying procedure), dried at RT and oven dried at 70 °C olive leaves.

The OLT was made using leaves and distilled water in a 1:10 ratio. Olive leaves (50-100 g) were placed in a nylon mesh (100 µm) filter sock, and suspended in a beaker on a hot plate. Boiling water (amount dependent on the weight of leaves) was poured onto the leaves. The leaves were pushed and mixed with a glass mixing rod; tin foil was placed over the top of the beaker to prevent heat and moisture loss. The water was brought back to boiling (100 ± 5 °C), the leaves were steeped (30 min) while maintaining a temperature of 100 °C, with mixing every 5 minutes. The tea was taken off the heat and left to cool (10 min). The filter bag was hung above the beaker via a clamp allowing excess tea to drip back into the beaker (15 min). The filter sock was then squeezed above the beaker to acquire any further tea. Once cooled, the tea was transferred to a glass bottle and stored (−25 °C) until use.

#### *2.6.2.1 Recovery checks*

During validation of target analytes in the OLT (an aqueous medium), accuracy was carried out through spike recovery checks. Using Equation 2.3, Type 1 water (2 mL) was spiked with 16 uL of a mixed standard (10 mg/L) to give a raw result concentration of 80 µg/L. The samples were vortexed (1 min) to allow for sufficient mixing of the analytes in the aqueous matrix. All samples were left to interact with the water matrix (1 hour). A subsample (1 mL) was pipetted into an amber vial and analysed. Details on method validation for aqueous samples can be found in Chapter 3, Section 3.3.1.

$$Volume\ required\ (V_1) = \frac{C_2 V_2}{C_1} = \frac{80\ \mu g/L \times 2000\ uL}{10000\ \mu g/L} = 16\ \mu L \qquad \textbf{Equation 2.3}$$

Where: $V_1$ and $C_1$ is initial volume and concentration and $V_2$ and $C_2$ is final.

### 2.6.3 Analysis of OLT

Due to suppression effects (Chapter 3, Section 3.3.3) multiple dilutions with Type 1 water were carried out for each sample (up to 4). The number of dilutions required was dependent on the concentration of target analytes in different OLT samples analysed ranging between 20 and 20,000-fold dilutions. The same instrument method used for oils was also used for the analysis of OLT (Section 2.5.3).

### 2.6.4 Data workup

To determine the concentration of analytes in the OLT, the following steps were taken. The data from the instrument was processed via MultiQuant™ software. This output raw results in µg/L and calculations were undertaken to the determine final concentration of the sample in mg/kg. An example calculation can be seen in Equation 2.4; assuming 1 L of water equals 1000 g of water. The concentrations reported for OLT in this research were the average of the dilutions that had analyte concentrations consistent within ± 10% of dilutions undertaken.

$$Concentration\ \left(\frac{mg}{kg}\right) = \left(\frac{raw\ concentration\left(\frac{\mu g}{L}\right) \times dilution\ factor}{1000}\right) \times \left(\frac{volume\ (mL)}{weight\ (g)}\right)$$ **Equation 2.4**

Where volume is the amount of distilled water leaves were steeped in and weight is the mass of leaves weighed into the filter sock.

It should be noted that the volume of water leaves were steeped in may have been slightly lower than the amount measured out for steeping due to small evaporation losses, dry leaves absorbing water and water getting trapped in leaves during draining. All practical measures were taken to avoid water loss. However, for calculations, it was assumed the initial amount of distilled water measured out was the same amount the leaves were steeped in. This may result in slight discrepancies in the concentration but the precise concentration of target analytes in the tea was not the focus of this research. The main requirement of the tea was to provide high levels of oleuropein to understand its hydrolysis in further experiments.

### 2.6.5 pH determination

The pH of OLT was determined using a calibrated Hanna Edge pH meter. Triplicate measurements were taken for all OLTs. The values determined are presented in Section 2.7.1.

## 2.7 Olive leaf tea stability trials

An OLT was subsampled in duplicate and stored at three different temperatures: −25, RT and 40 °C for 189 days to explore compound stability. An initial storage trial was set up; however, after three days in storage, the instrument needed the injector replaced and was out of commission for one month. This meant there was no data for the initial storage

period and the degradation that took place in that first month. Hence a second trial was set up, under the same conditions to obtain data points for the first month during storage.

### 2.7.1 Batch ID for olive leaf teas

OLTs used in the research were distinguished by a batch ID using the date the teas were made. Each batch of tea had a different concentration of compounds. The initial concentration of target analytes and pH values of the OLTs used are summarised in Table 2.6. In experiments undertaken in this research, the selected OLT was re-analysed in duplicate as a control sample.

**Table 2.6. The initial concentration of target analytes in batches* of OLT. Results are presented as the average of dilutions undertaken ± standard deviation (SD).**

| Analyte | OLT3-2-23 | OLT15-2-23 | OLT21-4-23 | OLT12-5-23 | OLT31-5-23 |
|---|---|---|---|---|---|
| Oleuropein | 373 ± 6 | 3668 ± 29 | 1744 ± 8 | 695 ± 44 | 9159 ± 323 |
| Ligstroside | 77 ± 2 | 663 ± 8 | 906 ± 5 | 271 ± 9 | 2399 ± 45 |
| Oleuropein aglycone | 14 ± 1 | 17 ± 0.1 | 5 ± 0.1 | 28 ± 1 | 53 ± 6 |
| Oleacein | 196 ± 3 | 11 ± 0.3 | <LOD | 115 ± 16 | <LOD |
| Oleocanthal | 16 | 1 | <LOD | 32 ± 0.4 | <LOD |
| Hydroxytyrosol | 1034 ± 37 | 842 ± 11 | 781 ± 18 | 1632 ± 79 | 4507 ± 154 |
| Tyrosol | 32 ± 1 | 31 ± 3 | 11 ± 0.2 | 60 ± 3 | 184 |
| Elenolic acid | 547 ± 5 | 123 ± 4 | 197 ± 10 | 376 ± 27 | 826 ± 72 |
| **pH** | 4.9 ± 0.005 | 5.1 ± 0.005 | 5.0 ± 0.05 | 4.6 ± 0.03 | 5.2 ± 0.04 |

**Batch ID contains the date OLT was created.*

## 2.8 Blood glucose meter

A blood glucose meter (BGM) was used to measure glucose formed in OLT as a result of oleuropein and ligstroside hydrolysis with β-glucosidase.

### 2.8.1 Reagents and standards

A stock standard of D-(+)-glucose (400.05 mM = mmol/L) was prepared by weighing 1.4485 g of glucose (resulting in 1.4413 g of glucose when adjusted for 99.5% purity of the glucose standard) into a volumetric flask (20 mL) with distilled water, following Equation 2.5.

$$Mass\ (g)\ = \frac{400\ mM \times 20\ mL \times 180.156\ g/mol}{1{,}000{,}000} = \frac{1.4412\ g}{0.995} = 1.4485\ g$$

**Equation 2.5**

Working standards were made at concentrations of 1, 2, 4, 5, 8, 12, 14 mM with Type 1 water (final volume 1 mL). An example calculation for the 4 mM secondary standard using $C_1V_1 = C_2V_2$ can be seen in Equation 2.6. All standards were stored in the chiller (4 °C) and given an expiry date of 1 year from preparation.

$$Volume\ required\ (V_1) = \frac{C_2V_2}{C_1} = \frac{4\ mM \times 1000\ uL}{400.00\ mM} = 10\ \mu L$$

**Equation 2.6**

Where $V_1$ and $C_1$ is initial volume and concentration and $V_2$ and $C_2$ is final.

### 2.8.2 BGM Calibration

The glucose response using a BGM is different in the OLT aqueous matrix compared to in blood. The working standards were used to calibrate the meter before analysis of OLT samples. Calibration coefficients were obtained by comparing the glucose concentration to the value measured by the BGM. The use of a BGM is further discussed in Chapter 3, Section 3.4.

### 2.8.3 Analysis and data workup

A CareSens™ Dual BGM was used to measure the glucose concentration (mmol/L) in OLT samples. A CareSens™ PRO testing strip was placed into the meter and dipped into a 10 µL sub-sample of the tea, the value was recorded and the testing strip was then discarded. Three readings were taken for each sample to determine an average, SD, and %RSD value,

with %RSDs <5% deemed acceptable for reporting. The background glucose present in the OLT was subtracted off the final readings; teas that had been diluted also had a dilution factor applied. This final concentration was then calculated using the calibration coefficient from the calibration curve.

## 2.9 Almond meal/milk preparation

### 2.9.1 Almond meal

Several whole raw almonds (10-20) were blended to a fine powder via a Nutribullet 2.0 1200W (2 min). The resulting meal was stored in an airtight tube until use.

### 2.9.2 Almond milk

Almond milk was made during this research to determine if it could be an effective source of the enzyme β-glucosidase. The almond meal had an amount of distilled water added in a 1:5 ratio; the mix was gently shaken on the orbital shaker (15 min). The resulting liquid was filtered via a Büchner funnel with a Schleicher and Schuell filter paper 604 (55 mm). The resulting almond milk was stored in the freezer (−25 °C) until use in experiments and given an expiry date of 6 months from preparation.

### 2.9.3 Glucose readings

To confirm that the almond flour/meal and almond milk did not contain glucose, triplicate readings were carried out. Almond flour/meal (500 mg) was mixed with water (1 mL). Both solutions resulted in a “Lo” response from the meter which refers to a concentration below 1.1 mmol/L.

## 2.10 Final methods for enrichment experiments

The final methods used in Chapter 5 are described below.

## 2.10.1 Inactivation of β-glucosidase

To halt the enzymatic reactions, a 1:1 ratio of subsampled OLT and 100% MeOH were combined and chilled until analysis (2-8 °C, up to 24 hours). On the day of analysis, samples were brought to room temperature, centrifuged (5 min, 11,300 RCF) and prepared using methods described in Section 2.6.3.

## 2.10.2 Enzymatic experiments using almond flour/meal and almond milk

Almond flour or meal (25 ± 0.5 or 100 ± 2 mg) per 1 mL of OLT were vortexed (1 min). Samples were placed in an Eppendorf ThermoMixer® C at 60 °C for the quickest hydrolysis of oleuropein or 40 °C to preserve the formation of oleuropein aglycone (800 rpm, 10 min). The amount of almond flour/meal varied depending on the concentration of oleuropein in tea; OLT3 2-23 (1 mL) required 25 mg and OLT15-2-23 (1 mL) required 100 mg to get at least 50% hydrolysis of oleuropein at 60 °C within 30 minutes. In other hydrolysis experiments, almond milk (300 µL) and OLT15-2-23 (700 µL) were combined and vortexed (30 seconds), then placed in the Eppendorf ThermoMixer® C at the same temperature and time described in the above almond meal/flour experiments. After conversion took place both sources of enzyme were inactivated following methods described in Section 2.10.1.

### *2.10.2.1 Addition of oil*

For enrichment experiments, OO (506 mg = 550 µL, based on the density of OO) was added using a positive displacement pipette into OLT (milk: 700 µL or flour: 1 mL) with almond milk (300 µL) or almond flour/meal (25-100 mg) and was vortexed till an emulsion formed (2 min). Samples were placed in an Eppendorf ThermoMixer® C (800 rpm, 10 min, 60 °C for the quickest hydrolysis of oleuropein or 40 °C to preserve the formation of oleuropein aglycone). The sample was then vortexed (10 seconds) and centrifuged (20 min, 11,300 RCF). Oil was sub-sampled to the correct extraction amount (100 mg) and a sub-sample of the aqueous portion (100 µL) was taken if possible and inactivated as described in Section 2.10.1. Oils underwent extraction as described in Section 2.5.2. However, the

amount measured in control oils were subtracted from the concentration measured in enriched oils.

#### *2.10.2.2 Filtration*

Samples were prepared as described in Section 2.10.2, using almond milk, OLT and mixed for 10 minutes at 40 °C. The enzyme was then deactivated via the method in Section 2.10.1. The resulting mixture was filtered through two different membrane syringe filters with pore sizes of 0.20 and 0.45 µm (diameters of 13 and 17 mm respectively). The filtrates were prepared and analysed as described in Section 2.6.3.

#### *2.10.2.3 Addition of NaCl and glucose*

An experiment described in Section 2.10.2.1 had NaCl and glucose (0.5 ± 0.001 g and 1 ± 0.01 g, respectively) added after the samples were mixed on the Eppendorf ThermoMixer® C and vortexed (10 seconds). The saturated solution of NaCl and/or glucose was mixed on a vortex (2 min) then centrifuged (20 min, 11,300 RCF). A sub-sample of oil was taken and prepared via methods described in Section 2.5.2.

***Water activity***

The water activity was also determined prior to utilising NaCl and glucose to confirm the sample would still have a suitable water activity to facilitate enzymatic activity. Almond milk (300 µL) was combined with OLT (700 µL), and NaCl (0.5 g) was added. This was repeated for glucose (1 g). The samples were analysed using a Novasina LabMaster-$a_w$ neo water activity device. Four readings were taken for each sample to determine a mean and standard deviation.

### 2.10.3 Hydrolysis of oleuropein with almond milk at different temperatures

Almond milk (300 µL) and OLT (700 µL) were placed in a tube (2 mL), the sample was vortexed (30 seconds) and placed in an Eppendorf ThermoMixer® C (30, 40, 50, 60 °C; 800 rpm, 120 min). Subsamples were taken at 5, 10, 20, 40, 60, 80, 100 and 120 minutes,

inactivated as described in Section 2.10.1, centrifuged (5 min, 11,300 RCF) and prepared using the method described in Section 2.6.3.

### 2.10.4 Stability of oleuropein aglycone in 50% MeOH olive leaf tea

Almond milk (300 µL) and OLT (700 µL) were placed in a tube (2 mL), the sample was vortexed (30 seconds) and placed in an Eppendorf ThermoMixer® C (40 °C; 800 rpm, 10 min). The reaction was inactivated using the methods in Section 2.10.1. A sub-sample was taken, and the tube was placed back in the mixer (30, 40, 60 °C; 800 rpm, 300 min), with subsampling taking place at 30, 60, 120, 180, 240, and 300 minutes. Samples were centrifuged (5 min, 7830 RCF) and prepared using the method described in Section 2.6.3.

## 2.11 Quality control

For the analysis of oil and OLT samples various steps were taken to check the quality of results. For every batch of samples analysed, quality control (QC) measures were taken. Each batch run had a set of calibration standards analysed at the beginning and end of the sequence to check for instrument drift. Randomly selected individual standards were analysed every 15-20 samples for batches with greater than 20 samples. Blank samples of extraction solvent were analysed to check for contamination that may have occurred during sample preparation as well as check for carryover during analysis. Diluted OLT samples were analysed from most dilute to most concentrated, with blank samples ($n$ = 3) run after each set of dilutions; this helped to prevent excessive carryover. Some samples were prepared and analysed in duplicate to make sure the instrument was performing accurately with a percentage difference of 10% between duplicates deemed acceptable.

# CHAPTER 3

# Method Optimisation and Validation

The methods used in this research to quantify oleuropein, ligstroside, oleuropein aglycone, oleacein, oleocanthal, hydroxytyrosol, tyrosol and elenolic acid in OO and OLT were validated prior to their utilisation. Methods chosen from literature were modified to suit the current research. Therefore, validation was essential to ensure the reliability of the analytical data. This chapter details these procedures.

## 3.1 Optimisation of olive oil extraction method

### 3.1.1 Instrument optimisation

Instrument optimisation was the priority in optimising a method for the extraction and analysis of target analytes in OO. Several types of instrumentation have been used in literature for the analysis of OO, most commonly LC-MS, see Chapter 1, Section 1.9.2 for a detailed discussion on these methods. In this research, a liquid chromatography triple quadrupole tandem mass spectrometer (LC-QqQ-MS/MS) was used. The instrument was optimised using multiple reaction monitoring (MRM) in negative ESI ion mode. $[M-H]^-$ (single charged) ions were selected as precursor ions for all target analytes; no adducts were selected as precursor ions. Characterisation and optimisation were carried out by direct infusion of each target analyte via a syringe pump (7 µL/min) into the ESI mass spectrometer using a 10 µg/L stock solution mixed with mobile phase to increase ionisation efficiency. Q1 scans (survey scan) were run in a specific range based on the mass-to-charge ratio (*m/z*) of precursor ions ($[M-H]^-$) to identify fragmentation patterns taking place under different collision energies. The mass ranges were selected based on results from the literature and then further optimised for the instrument used in this research. The optimal declustering potential voltage to maximise formation of $[M-H]^-$ ions (precursor or parent ion), optimal collision energies, entrance potential and collision cell exit potential that gave the two most sensitive MRM transitions (two product ions also known as daughter ions or Q3 masses) were obtained for each analyte. The most sensitive and intense transitions were used for quantitative analysis and

referred to as the 'quantifier" transitions, while the second *m/z* (the "qualifier transition) was employed in the identification step as confirmation or to be used in the case of interferences. For the optimised MS/MS conditions see Chapter 2, Section 2.5.3.

LC conditions were optimised to achieve resolution within 4 minutes for the eight target analytes. To begin with, mobile phases and column types from literature[34,173,174] were identified (Chapter 1, Section 1.9.2). Most reported literature used Type 1 water (mobile phase A), and acetonitrile (ACN) or MeOH with either acetic acid or formic acid in varying percentages as mobile phase B. All reported methods used a reverse phase $C_{18}$ column, with one study opting to use isocratic elution while the rest used gradient elution. A mixed standard of target analytes was used to optimise the LC conditions. An Agilent Infinity Lab Poroshell 120 EC-$C_{18}$ column (2.7 µm, 2.1 x 50 mm, Agilent Technologies, USA) fitted with a Phenomenex KrudKatcher™ Ultra was used.

Different concentrations of formic and acetic acid (0.01, 0.1 and 0.2%) were trialled in Type 1 water and organic (ACN or MeOH) mobile phases via gradient elution. The use of formic acid resulted in the disappearance of tyrosol from the chromatograms so acetic acid was chosen. Obtaining a good peak shape for elenolic acid was difficult. The use of 0.1% acetic acid in Type 1 water and 100% ACN resulted in a narrow peak for elenolic acid (Figure 3.1). However, these conditions resulted in a poor peak shape and separation of oleocanthal and oleacein (Figure 3.2). 95% ACN as mobile phase B was tested but this did not improve the peak shape.

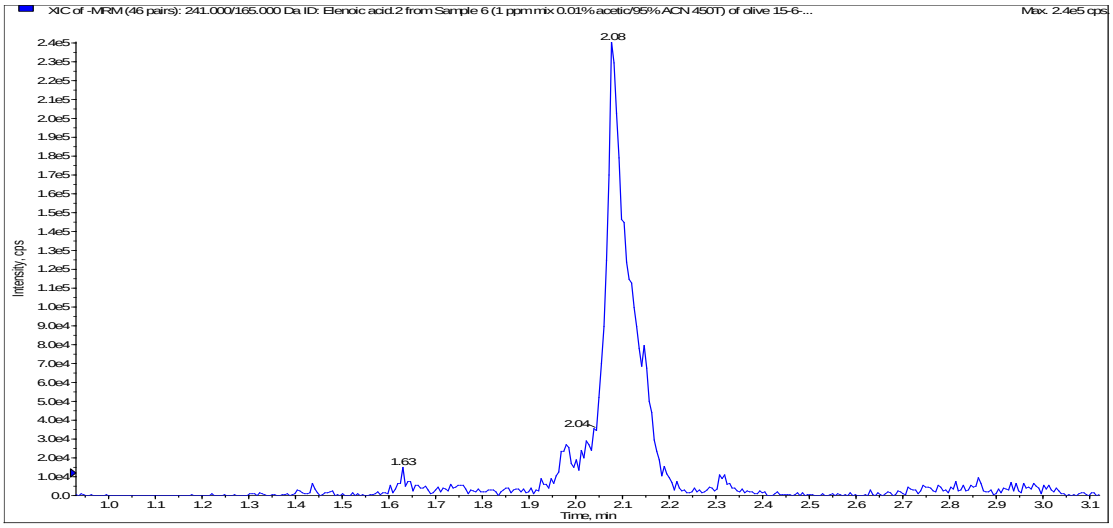



**Figure 3.1. Chromatogram showing the peak for elenolic acid, RT 2.08 minutes, using 0.1% acetic acid in both mobile phases A (Type 1 water) and B (100% ACN).**

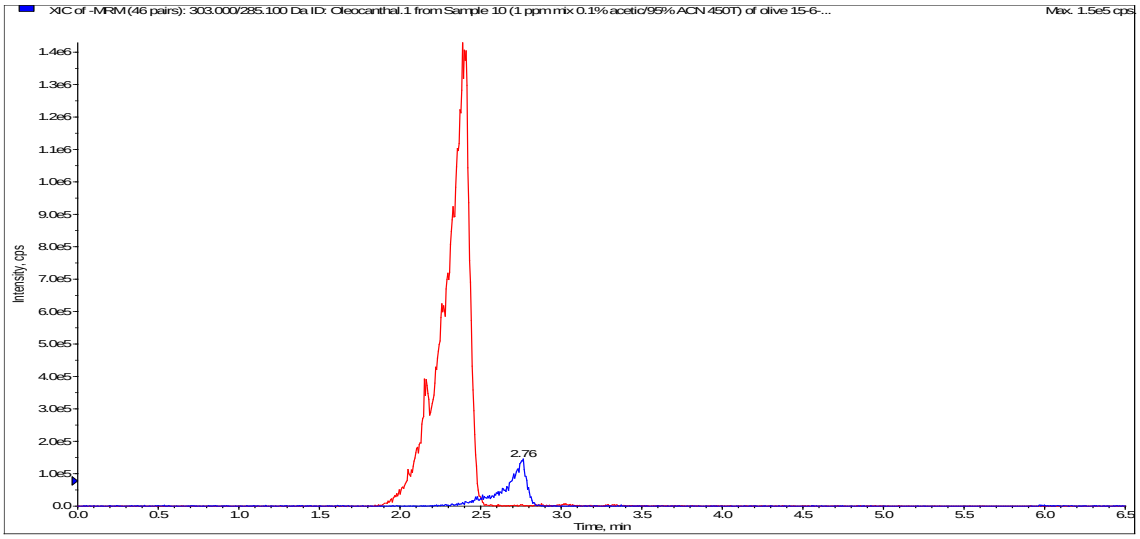



**Figure 3.2. Poor peak shape and separation for oleacein (RT 2.40 minutes, red peak) and oleocanthal (RT 2.76 minutes, blue peak) using mobile phases A (0.1% acetic acid in Type 1 water) and B (100% ACN).**

Due to more target analytes (oleacein and oleocanthal) having a poorer peak shape using 100% or 95% ACN as mobile phase B, the organic solvent was changed to 100% MeOH. This mobile phase along with Type 1 water were optimised by varying the amount of acetic acid (0.1 and 0.01%) added. The amount of acetic acid was initially reduced to 0.01% but this still resulted in a poor peak shape of elenolic acid so was

increased to 0.1% and this slightly improved the shape. The removal of acetic acid (0.1%) from the mobile phase B (100% MeOH) was investigated. This resulted in a much better peak shape for both oleocanthal and oleacein (Figure 3.3) compared to using ACN.

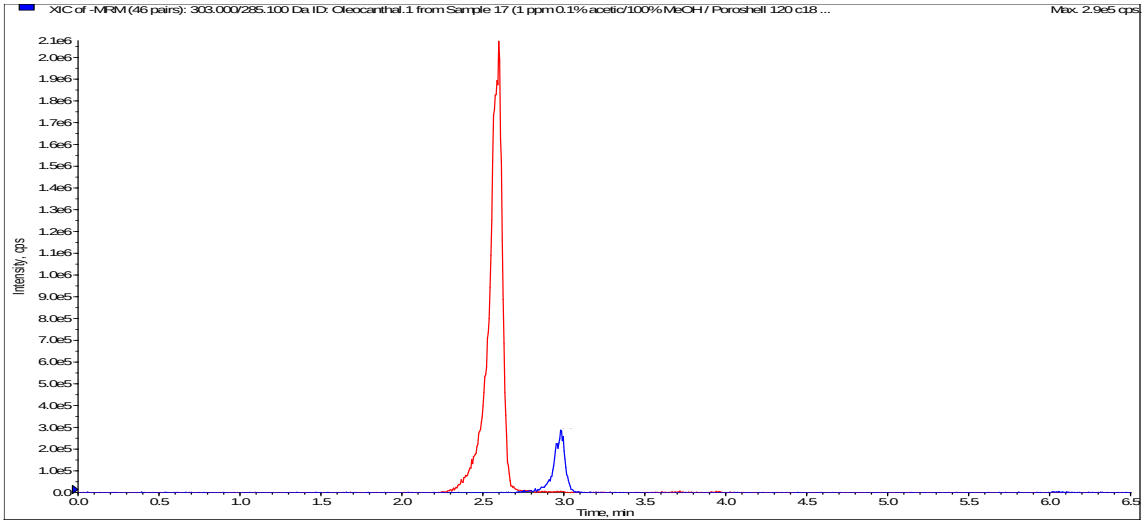



**Figure 3.3. Better resolution and peak shape for oleacein (RT 2.51 minutes, red peak) and oleocanthal (RT 2.98 minutes, blue peak) using mobile phases A (0.1% acetic acid in Type 1 water) and B (100% MeOH).**

The removal of acetic acid in mobile phase B also resulted in a narrower peak shape and better intensity for the other target analytes except for elenolic acid (still very broad) so acetic acid (0.1%) was only added into mobile phase A (Type 1 water). The resulting narrower peaks are shown in Figure 3.4 (A: 0.1% acetic acid in Type 1 water, and B: 100% MeOH) where wider peaks are prevalent using acetic acid in both mobile phases, as shown in Figure 3.5.

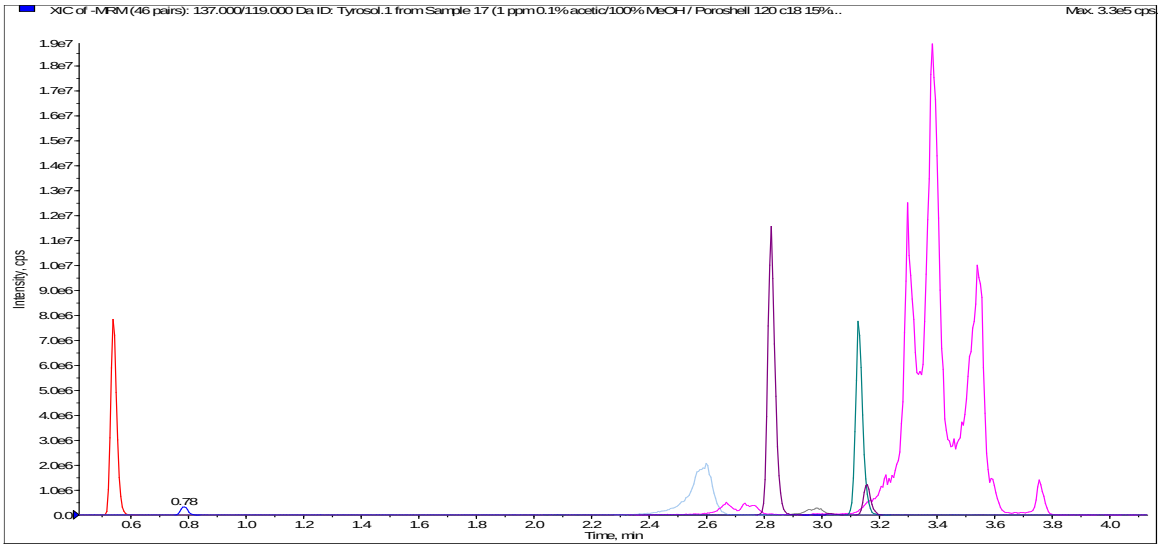



**Figure 3.4. A chromatogram showing the presence of narrower peaks for target analytes using mobile phases A (0.1% acetic acid in Type 1 water) and B (100% MeOH).**

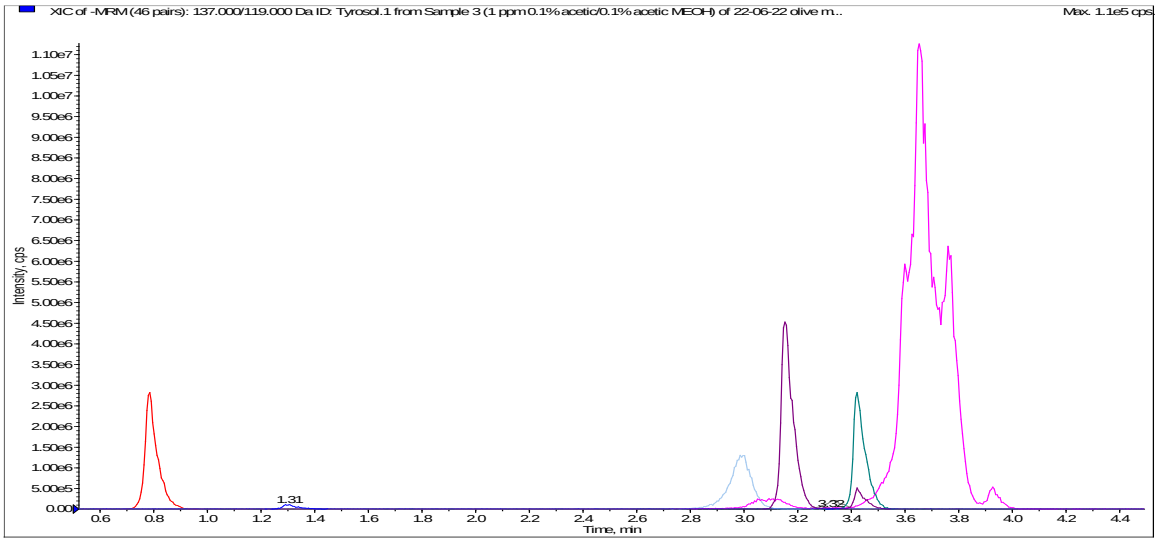



**Figure 3.5. A chromatogram showing the presence of broader peaks for target analytes using 0.1% acetic acid in mobile phases A (Type 1 water) and B (100% MeOH).**

Overall, optimal ionisation, peak resolution and shape were achieved using a mobile phase composition of 0.1% acetic acid in Type 1 water (A) and 100 %MeOH (B). Elenolic acid did not form a sharp peak and was very broad with this choice of mobile phase. To obtain a desirable peak shape, the starting solvent composition needed to have at least 50% MeOH (Figure 3.6). However, it is not good practice to start the solvent gradient

with a high organic phase and it caused very poor separation of all other analytes. Generally, a starting gradient will use a 'weak' elution solvent (low organic, high aqueous) at the start of the chromatographic run, where proportions of 'strong' eluting solvent (organic) is increased over the course of the separation. This is necessary as it allows compounds to be retained on the column and elute more slowly giving better separation.[205] Therefore, it was a compromise to leave elenolic acid broad (but still quantifiable) to maintain good separation and peak shape for all other analytes.

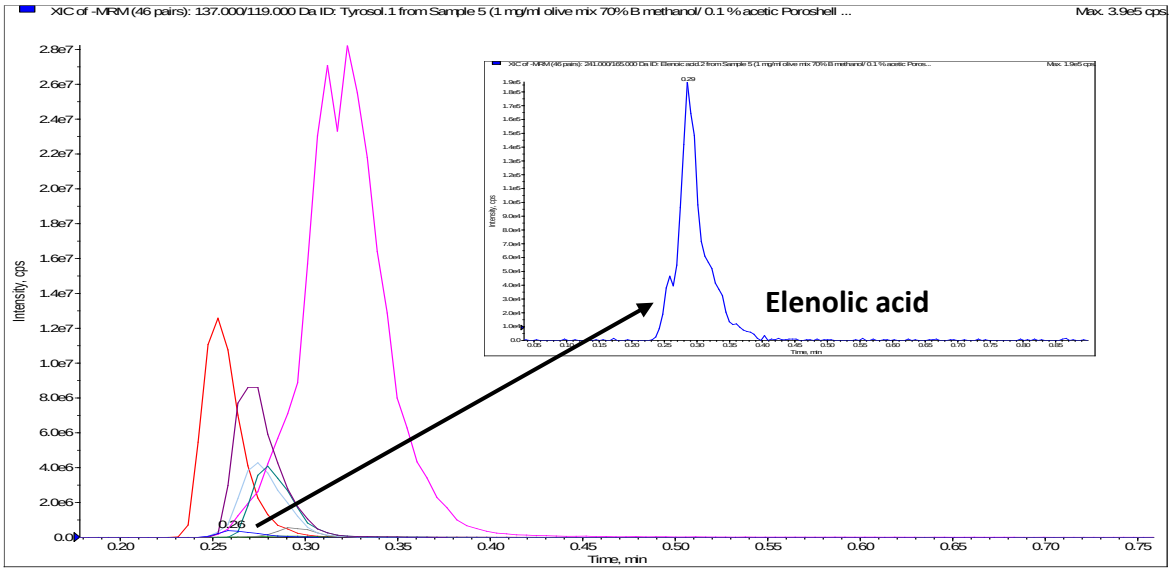



**Figure 3.6. Chromatogram showing improved peak shape for elenolic acid (see inset for zoom in of elenolic acid in this chromatogram) using a 70% MeOH gradient, but resulting in poor resolution of all other target analytes.**

In the chromatograms, oleuropein aglycone showed three peaks, with one peak unresolved (Figure 3.7). Johnson *et al*. (2017)[34] stated that oleuropein aglycone can undergo keto-enol isomerisation in aqueous solvents which can result in the presence of isomers, which in this research, correspond to the unresolved peaks. Therefore, the quantitation of oleuropein aglycone was not isomer specific as the peak area from the three peaks were taken for quantification. For the purposes of this research, this was considered acceptable.

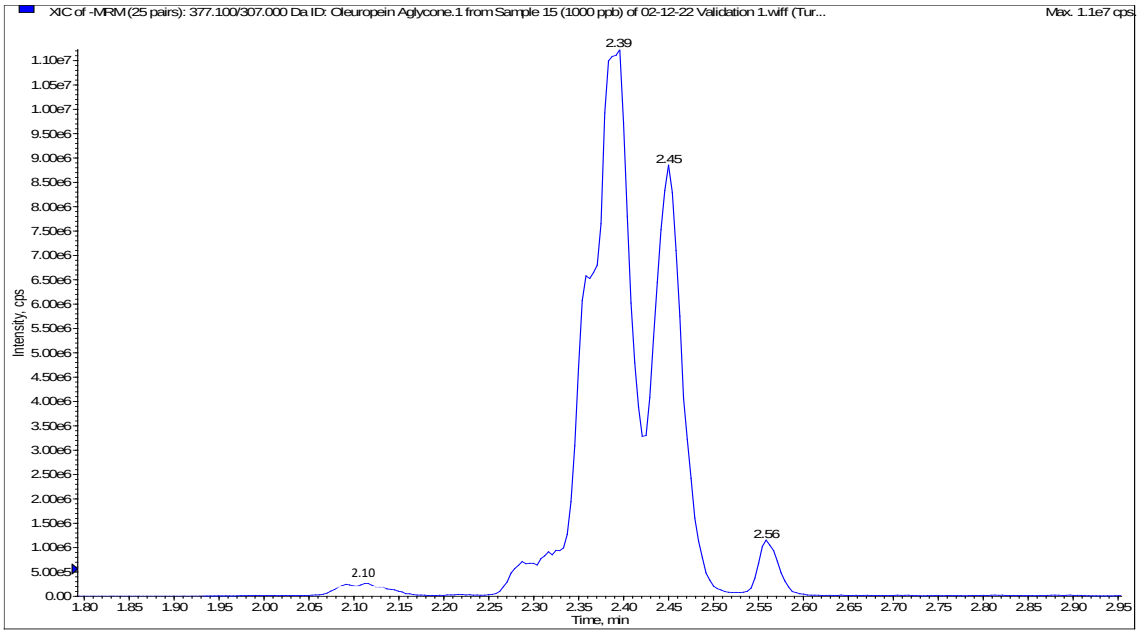



**Figure 3.7. Chromatogram for oleuropein aglycone with three peaks, including the unresolved peak (RT: 2.10, 2.39, 2.45, 2.56 min). Quantitation was performed using total peak area.**

There was also an issue of some analytes carrying over into blank samples, this was mainly prevalent in OLT samples due to their high concentration of the target analytes. Three blanks (80% MeOH) were run between each set of dilutions for OLT samples to reduce the chance of the carryover increasing the concentration of analytes in the following samples. The rinse solution during the initial method optimisation was also increased from 10% to 50% MeOH. Analytes present in blanks had to be <1.5 µg/L, otherwise samples were re-run with more blanks between them.

### 3.1.2 Extraction optimisation

As mentioned in Chapter 1, Section 1.9.1, liquid-liquid extractions (LLE) are one of the most common extraction techniques used for OO.[166] There are a variety of extraction solvents used; most methods use MeOH/water with 80% MeOH/water considered the most effective.[166,168,169,172,177,187,188] Several extraction procedures carried out two, three or four consecutive extractions as this greatly improved the phenolic recovery efficiency.[166,176,188] Since the final method will be used in a commercial laboratory, simplistic methods were focused on. Hence, a method that provided high recoveries,

used minimal solvent, was low cost and quick was selected to be optimised, then validated.

The International Olive Council has a current method for the detection and quantification of phenolic compounds in OOs via HPLC. Since this method has been approved by the International Olive Council, it was compared to other extraction methods to test its effectiveness for use in this research. In the specified method, OO (2 g) is extracted with methanol/water (80:20, *v*/*v*, 5 mL), vortexed (1 min), and sonicated (15 min) to help extract the compounds out of the oil.[167] The sample is centrifuged (5292 RCF, 25 min), then filtered (0.45 µm, 13 mm) and analysed.[167] In this research, experiments were carried out using sonication as stated in the method. The sonication was then replaced by implementing two consecutive extractions to compare effectiveness. Samples that were consecutively extracted twice ($n = 2$) resulted in a higher extracted amount of target analytes compared to sonication with percentage differences for target analytes ranging between 4 ± 0.8% (tyrosol) and 9 ± 2% (oleocanthal). With sonication not as effective and the issue of the ultrasonic bath being used frequently in the commercial laboratory, other methods were investigated for use.

As discussed, some methods in the literature use hexane to dissolve and remove the fat phase after a LLE with MeOH/water. Hexane is not miscible with water so must be discarded during the extraction and may result in loss of non-polar analytes such as oleuropein aglycone. To avoid this and the use of extra solvent, acetone was trialled. A pilot extraction was set up to determine if acetone could extract a higher quantity of each compound from the oil. The trial was performed on two different oils to observe any trends or patterns. OOs were dissolved in an amount of acetone until the oil was emulsified. For a 500 mg sample size, 2 mL of acetone was required. Once emulsified via hand shaking, MeOH/water (75:25, *v*/*v*, 8 mL) was added, resulting in a total extraction volume of 10 mL, this kept the organic content to 80% (6 mL MeOH + 2 mL acetone = 8 mL/10 mL = 80%). Based on pilot trial data ($n = 1$), acetone extracted a slightly higher quantity of each compound for one of the oils, with a 6.78% increase for oleocanthal, a 3.82% increase for tyrosol and a 3.14% increase for oleuropein aglycone. The increased quantity extracted for the rest of the analytes was <1.13%. Regarding results from the other oil, there was a decrease in the total quantity extracted for each analyte using

acetone when compared to using (80:20 *v*/*v*, 10 mL) MeOH, where 1.77% less oleocanthal and 3.18% less hydroxytyrosol were extracted. With variation between the two oils, no obvious increase in the quantity of each analyte extracted based on $n = 1$ where there is likely measurement error, and the higher cost of acetone compared to MeOH; a triplicate analysis trial was not deemed necessary and MeOH was focused on.

An extraction method by Pizarro *et al*. (2013)[177] used a liquid-liquid microextraction (LLME) which only required one extraction solvent; methanol/water (80:20 *v/v*), used a vortex for mixing, did not require a long extraction process such as sonication and produced recoveries >84%. The sample size in the published method was larger (0.5 g) than the requirements of this research so it was reduced (0.1 g), other method parameters were not scaled down accordingly but were altered (discussed below). The reduction in sample size did not affect the quantification of analytes due to the sensitivity of the instrumentation used in this research. In the published method, methanol/water (80:20 *v/v*, 1 mL) was added to the oil sample, vortexed and centrifuged (11,300 RCF, 5 minutes) to obtain an extract. The original oil was reextracted a further two times by the same process, the three portions of extract were combined to make a single extract. However, the methanolic phase extracted still required a dilution step in a 10 mL volumetric flask. Therefore, the volume of solvent was increased, and volumes of 1.6 mL and 2 mL were briefly investigated in this research. The percentage of MeOH (80% vs. 60%) and the number of consecutive extractions (more than three) were also briefly examined. Due to time constraints of using the instrument in the busy season at the commercial laboratory, triplicate analysis was not carried out.

Using MeOH/water (80:20, *v*/*v*) and the larger volume (2 mL) during extractions resulted in a higher concentration of each target analyte extracted (Table 3.1), with a percentage increase for all analytes after the first extraction compared to 1.6 mL. This was also true after three consecutive extractions. This was likely due to the solvent being less saturated at a higher volume, thus more analytes could be extracted out of the oil and move into solvent. Thus, 2 mL of the extraction solvent was used in future extractions.

**Table 3.1. Amount of OO target analytes extracted using 1.6 mL versus 2 mL MeOH/water (80:20, *v*/*v*) and the % increase for a 2 mL extraction.**

| | 1.6 mL extraction | 2 mL extraction | |
|---|---|---|---|
| **Analyte** | **Amount extracted after first extraction (mg/kg) (*n* = 1)** | | **% increase** |
| Oleuropein | <LOD | <LOD | N/D* |
| Ligstroside | <LOD | <LOD | N/D* |
| Oleuropein aglycone | 10.83 | 12.00 | 10.80 |
| Oleacein | 23.27 | 26.71 | 14.78 |
| Oleocanthal | 40.98 | 44.76 | 9.22 |
| Hydroxytyrosol | 17.88 | 18.16 | 1.57 |
| Tyrosol | 7.99 | 8.34 | 4.38 |
| Elenolic acid | 91.62 | 93.51 | 2.06 |

**N/D – no data: due to values being <LOD no percentage increase could be determined.*

As discussed earlier, some methods in the literature changed the percentage of water in the extraction solvent with one method stating that MeOH/water (60:40 *v*/*v*) was more effective than (80:20 *v*/*v*).[171] This was briefly investigated in the current research. However, MeOH/water (80:20 *v*/*v*) extracted a higher quantity of each analyte after the first extraction compared to a 60:40 *v*/*v* ratio. The increase ranged from 1.55% (hydroxytyrosol) to 13.74% (oleacein). As a result, the chosen solvent was 80% MeOH.

Using MeOH/water (80:20, *v*/*v*, 2 mL), more than three consecutive extractions were also investigated to determine if this would be suitable to extract majority of the analytes from the oil, up to seven consecutive extractions were trialled (Table 3.2). Based on the results, three extractions were deemed suitable since further extractions did not increase the amount extracted and would only add time to the procedure, use more solvent and increase the potential for method error. Pizarro *et al.* (2013)[177] also reported similar outcomes. It is interesting to note that this study also trialled three other methods using hexane, MeOH/water (60:40 *v/v*) and sonication with the same amount of solvent used in the LLME. However, in each instance the recoveries were lower.

**Table 3.2. The concentration of target analytes extracted after each of the seven consecutive extractions using MeOH/water (80:20, *v*/*v*, 2 mL), with majority of the analyte being extracted in the first three extractions.**

| Analyte | Number of consecutive extractions | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | mg/kg | | | | | | |
| Oleuropein | <LOD | | | | | | |
| Ligstroside | <LOD | | | | | | |
| Oleuropein aglycone | 12.0 | 0.24 | 0.28 | <LOD | <LOD | <LOD | <LOD |
| Oleacein | 26.7 | 1.3 | 1.3 | 0.4 | 0.4 | 0.2 | 0.2 |
| Oleocanthal | 44.8 | 1.4 | 1.5 | 0.5 | 0.4 | 0.3 | 0.3 |
| Hydroxytyrosol | 18.2 | 0.1 | 0.1 | <LOD | <LOD | <LOD | <LOD |
| Tyrosol | 8.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Elenolic acid | 93.5 | 1.5 | 1.7 | 0.5 | 0.4 | 0.3 | 0.3 |

The chosen LLME by Pizarro *et al*. (2013)[177] with modifications of decreased sample size and increased extraction volumes extracted the majority of the target analytes within the first several extractions. This method covered the desirable aspects such as being quick, easy, and efficient, hence it was selected to be validated.

## 3.2 Method validation of olive oil extraction method

Method validation is an important process to determine if an analytical method is acceptable for its intended purpose; it also determines the quantitative performance of the method.[206] To carry out method validation, various parameters must be analysed. The parameters that were examined during this validation were linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision (repeatability).

To determine these parameters, analysis of samples using the optimised method (Chapter 2, Section 2.5.2) was conducted over three different days to account for inter-day variability. On each day, triplicate samples of two low-grade olive oils – Lupi extra Mild and Pams Extra Light OO – were analysed with three treatments (endogenous/non-spiked) and spiked with a high (50 µg/L) and low (20 µg/L) spike. Over the course of the validation this gave a total of 9 replicates for each treatment.

### 3.2.1 Linearity

For analysis, the working range of a calibration curve should consist of five to eight points, use linear regression and encompass the expected analyte concentration in the sample types analysed, i.e., from 0 to 200% of the theoretical content to reduce the necessity of dilution.[207] For the samples analysed in this research (OO and OLT), meeting the latter requirement is difficult as phenolic content is affected by geographical region, climate conditions, harvest time, and various stress factors resulting in a variety of concentrations.[128] For example, Różańska *et al*. (2020)[92] reported concentrations of oleuropein aglycone in oil ranging between 102.6–1248.9 mg/kg. Moreover, the concentration of oleuropein in leaves can reach between 6 and 9% dry weight.[153,197,208,209] As a result, oleuropein content in aqueous extracts is also variable with up to 45,330 mg/kg being reported by Martínez *et al.* (2021).[210] Based on the variation in concentrations reported in the literature, it is not feasible to have the working range this high as this would saturate the instrument detector due to its sensitivity. Initially, a calibration curve was made from 10 to 1000 µg/L (0.6 to 60 mg/kg for oil samples), this was then reduced to include 5 µg/L (0.3 mg/kg) as some of the target analytes had good intensity and lack of interference at this concentration.

A quadratic regression model was used for the calibration curve in this research. Non-linear calibration curves are very commonly observed in LC-MS/MS assays.[211,212] The severity of non-linearity can vary when analysing the same analyte on different days/times or on a different system.[213] This phenomenon has several explanations such as saturation at a high concentration during ionisation, formation of dimer/multimer/cluster ions, charge competition and detector saturation.[213] In most cases, a quadratic regression model is used as it can help to extend the dynamic range of the standard curve. A broader standard curve range is favoured since it can significantly save time and labour by avoiding the need for sample dilution.[212,213] However, caution is often advised due to quantitative bias. If the model is extended to the point where the top of the calibration curve is flat, often at very high concentrations, the calibration curve can become parabolic, affecting the accuracy of the upper limit of quantification.[213] In most cases, linearity can be improved with the use of a stable-isotope-labelled internal standard as it has identical chemical and physical properties to

the target analytes. This allows a stable-isotope-labelled internal standard to compensate for variability during extractions, chromatographic separation, MS ionisation and ion suppression from the matrix.[212] Hence an assay with greater accuracy, precision and generally better linearity can be obtained.[212] Unfortunately, purchasing stable-isotope-labelled internal standards for each analyte of interest (eight in total) was unachievable in this research due to their high cost.

In this research, the upper limit of the calibration curve was 1000 µg/L. Using calibration standards higher than this resulted in the curve becoming parabolic. On most occasions, for most analytes, the calibration curve looked linear and did not become parabolic when using a quadric regression model. Therefore, 1000 µg/L was considered an appropriate top standard as it should encompass the majority of the expected concentrations in this research. All target analytes in OLT were always above this range (1000 µg/L), with concentrations for oleuropein ranging from approximately 21,000 to 922,684 ug/L (373 to 9158 mg/kg). Thus, multiple dilutions of a sample were required. The correlation coefficient (R) for the quadratic calibration curves for each analyte over the course of validation was <0.998, showing strong correlation with little deviation.

### 3.2.2 Limit of detection and limit of quantification

There are various ways to determine the LOD and LOQ and there are several different terms used to define them.[214-218] In general, the LOD is the lowest concentration of analyte that can be detected and reliably distinguished from zero (or noise level of the system) but not necessarily quantified with confidence.[214-216] The LOQ is the lowest concentration of analyte that can be determined quantitatively with an acceptable level of precision and accuracy in the desired sample matrix.[214-216] In this research, the LOD and LOQ were calculated as 3 and 10 times the standard deviation respectively[215,218] of either Pams or Lupi endogenous or low spiked (20 µg/L) samples across 3 days ($n$ = 9). This helps to reflect the levels that can be accurately quantified in a sample matrix and considers the intra-sample variability.

The LOD and LOQ are presented as raw results (µg/L), which is the concentration reported in the analysed sample and the final concentration (mg/kg), which is the concentration reported taking into consideration the sample weight (Table 3.3). The

majority of the detection and quantification limits are based on endogenous Pams oil data as compounds in this sample type had the lowest detectable analyte concentrations above the lowest calibration standard. For hydroxytyrosol and tyrosol, the Pams low spike was used as endogenous concentrations were below the lowest calibration standard so may not be accurate for the purposes of determining these limits. Oleuropein, ligstroside and elenolic acid used Lupi oil data as these had lower values compared to Pams oil data. Most of the LOD values fall below the lowest calibration standard, this is not true for the LOQ values which are all greater than the lowest calibration standards (5 µg/L).

**Table 3.3. Raw result (µg/L) and final concentration (mg/kg) LOD and LOQ for the target analytes.**

| Analyte | LOD | | LOQ | | Data used |
|---|---|---|---|---|---|
| | µg/L | mg/kg | µg/L | mg/kg | |
| Oleuropein | 1.82 | 0.14 | 6.07 | 0.47 | Lupi low spike |
| Ligstroside | 1.48 | 0.13 | 4.92 | 0.43 | Lupi low spike |
| Oleuropein aglycone | 2.17 | 0.10 | 7.22 | 0.34 | Pams endogenous |
| Oleacein | 0.99 | 0.04 | 3.31 | 0.13 | Pams endogenous |
| Oleocanthal | 3.67 | 0.16 | 12.24 | 0.53 | Pams endogenous |
| Hydroxytyrosol | 2.64 | 0.16 | 8.81 | 0.55 | Pams low spike |
| Tyrosol | 5.16 | 0.31 | 17.20 | 1.03 | Pams low spike |
| Elenolic acid | 5.78 | 0.35 | 19.26 | 1.18 | Lupi endogenous |

It is difficult to directly compare the LOD and LOQ results obtained in this research to literature because most published work does not investigate the same analytes and if they do, it is in a different matrix e.g., olive fruit. One study[174] that analysed oleacein, oleocanthal, oleuropein aglycone and hydroxytyrosol in OO had LOD values of 1.0, 4, 0.3 and 1.0 µg/L and LOQ values of 3.3, 12, 0.9 and 3.5 µg/L, respectively. The LOD and LOQ for oleacein and oleocanthal are similar to the results obtained in this research. However, this current research obtained higher values for oleuropein aglycone and hydroxytyrosol. It is difficult to determine why the LOD and LOQ reported in the study are lower for these analytes. Firstly, the instrumentation used in their research was different (Waters Acquity UPLC™ and Waters Xevo TQS tandem quadrupole mass spectrometer) and may have better sensitivity resulting in lower values. The injection

volume was also larger (10 µL) compared to this research (2 µL). Johnson *et al.* (2018)[34] analysed the same analytes as this research, albeit in a different matrix (olive fruit). The study reported lower LOQ values except for oleacein (Table 3.4). The LOQ values could be lower in their work due to the use of a different instrument; Agilent 1290 Infinity ultrahigh-pressure liquid chromatography system (UHPLC) interfaced to a 6460 QqQ mass spectrometer with ESI via Jet Stream technology. This instrumentation may be more sensitive compared to the instrumentation used in this research. The LOQ results obtained in this current research are much lower than what is expected to be present in the samples analysed. Therefore, the method LOQ is acceptable for the purposes of this research.

**Table 3.4. The LOQ values obtained in this research versus Johnson *et al.* (2018).[34]**

| | LOQ (mg/kg) | |
|---|---|---|
| **Analyte** | **Obtained in this research** | **Obtained by Johnson *et al*.[34]** |
| Oleuropein | 0.47 | 0.28 |
| Ligstroside | 0.43 | n/a◊ |
| Oleuropein aglycone | 0.34 | 0.24 |
| Oleacein | 0.13 | 0.32 |
| Oleocanthal | 0.53 | 0.16 |
| Hydroxytyrosol | 0.55 | 0.04 |
| Tyrosol | 1.03 | 0.44 |
| Elenolic acid | 1.18 | n/a◊ |

◊*n/a – not applicable: these analytes were not analysed in the study.*

### 3.2.3 Accuracy

Certified reference materials (CRM) to determine accuracy are available for OO; however, they do not contain desired analytes. Therefore, the accuracy was determined via spike recovery based on raw results (µg/L). Determining accuracy like this can be an issue as it does not consider if compounds are matrix-bound, and spiking would not be able to reflect this. However, spiking does help determine if there is loss of analyte and indicate if suppression/enhancement on the MS/MS is occurring.

EVOO contains high levels of the target analytes and spiking at high concentrations would have been costly due to the price of standards. Thus, it was determined that oils with lower levels would need to be sourced to carry out the validation successfully. In

order to keep the matrix similar when spiking, a mild/light refined OO was used as concentrations of target analytes were notably reduced compared to commercial EVOO purchased (Table 3.5).[87]

**Table 3.5. A comparison of target analyte concentrations in EVOO and OO, showing that refined OO has lower, more suitable concentrations to spike above.**

| Analyte | EVOO | Refined OO | |
|---|---|---|---|
| | Picholine | Pams | Lupi |
| | | mg/kg | |
| Oleuropein | <LOD | <LOD | <LOD |
| Ligstroside | <LOD | <LOD | <LOD |
| Oleuropein aglycone | 88.26 | 1.11 | 1.23 |
| Oleacein | 22.82 | 0.56 | 3.08 |
| Oleocanthal | 24.72 | 2.05 | 11.47 |
| Hydroxytyrosol | 7.15 | <LOD | <LOD |
| Tyrosol | 1.46 | <LOD | <LOD |
| Elenolic acid | 64.59 | 2.26 | 1.66 |

A low and high spike were used to help cover the upper and lower range of concentrations present in the oils. Mixed stock standards were used for the low and high spike; see Chapter 2, Section 2.5.2.1 for details on spiking. The concentration to be spiked was decided based on the total extraction volume (6 mL), this allowed the raw result to be either 50 or 20 µg/L higher than the endogenous oil concentration. Unfortunately, the volumes spiked into the oil were not the same. Ideally, the same volume should have been added so variation in the matrix is held constant, however, the differing amounts added (12 and 30 µL) made a negligible difference to the total volume (0.2 and 0.6% difference) and did not affect the matrix during analysis. The resulting analyte concentrations from the endogenous and spiked samples for Pams and Lupi OO are summarised in Table 3.6 and Table 3.7, respectively. Samples that had values lower than the LOQ but higher than the LOD were included in the tables as they were used in calculations. The recoveries were calculated following Equation 3.1.

$$\%\ Recovery = \frac{A_s - A_{us}}{S} \times 100 \qquad \textbf{Equation 3.1}$$

Where: $A_s$ = Measured concentration of spiked sample, $A_{us}$ = Measured concentration of unspiked sample, S = Concentration of spike

Ideally, recoveries should fall within 90-110% of the expected concentration.[219] However, for some purposes where accuracy is not crucial or values are close to LOQ, 80-120% is deemed acceptable. As shown in Table 3.6 (Pams OO) and Table 3.7 (Lupi OO), all mean recoveries are within the acceptable range. The standard deviation for oleocanthal in the Lupi low spike data is very large (± 27.5 µg/L), however, this is due to the endogenous concentration in the sample being large (196.86 µg/L) and the spike only being low (20 µg/L). It is interesting to note, that the method by Pizarro *et al*. (2013),[177] which was modified for this research, had recoveries that were slightly lower for oleuropein (85.0%), tyrosol (84%) and hydroxytyrosol (83.6%); the study did not analyse any other target analytes from this research. The lower recoveries could relate to the oil they used and how their samples were spiked. In their methodology, oils were refined phenolics-free, which were not accessible for this project. The oil (30 g) was spiked with individual stock solutions of analytes (40 µg/g) via MeOH in a round bottom flask and left to interact (24 hours) under nitrogen, MeOH was rotary evaporated off. This longer interaction may have allowed analytes to bind more thoroughly to the matrix, lowering recoveries. Alternatively, it is possible the modifications made to this method during optimisation (increasing the extraction volume and reducing sample size) extracted more target analytes, resulting in higher recoveries. However, spiking a larger amount and evaporating off the excess MeOH would be useful to consider in future studies. Overall, all recoveries are within the ideal range (97-110%) making this method suitable for the analysis of the target analytes.

**Table 3.6. The endogenous, high and low spike sample concentrations (µg/L) and spike recoveries for Pams OO reported as raw results. Results are presented as the average ± SD (*n* = 9).**

| Analyte | Pams OO (µg/L) | | | % Recovery | |
|---|---|---|---|---|---|
| | Endogenous concentration | Concentration after a low spike◊ | Concentration after a high spike◊ | Low Spike | High spike |
| Oleuropein | <LOD | 19.4 ± 0.6 | 51.4 ± 4.8 | 97 ± 3 | 103 ± 7 |
| Ligstroside | <LOD | 20.6 ± 0.5 | 53.7 ± 5.2 | 103 ± 2 | 107 ± 8 |
| Oleuropein aglycone | 19.6 ± 0.7 | 39.7 ± 0.6 | 72.2 ± 3.8 | 100 ± 1 | 105 ± 5 |
| Oleacein | 10.0 ± 0.3 | 32.0 ± 0.5 | 65.2 ± 5.6 | 110 ± 3 | 110 ± 8 |
| Oleocanthal | 34.3 ± 1.2 | 54.6 ± 1.6 | 85.2 ± 5.6 | 102 ± 7 | 102 ± 5 |
| Hydroxytyrosol | 4.3* ± 0.5 | 24.6 ± 0.8 | 55.7 ± 5.4 | 102 ± 1 | 103 ± 5 |
| Tyrosol | <LOD | 23.7 ± 1.7 | 55.8 ± 5.2 | 110 ± 1 | 106 ± 6 |
| Elenolic acid | 38.1 ± 1.6 | 59.3 ± 2.2 | 91.5 ± 6.2 | 106 ± 7 | 107 ± 13 |

**Denotes concentrations >LOD and <LOQ; ◊Low and high spike concentrations were 20 and 50 µg/L, respectively.*

**Table 3.7. The endogenous, high and low spike sample concentrations (µg/L) and spike recoveries for Lupi OO reported as raw results. Results are presented as the average ± SD (*n* = 9).**

| Analyte | Lupi OO (µg/L) | | | % Recovery | |
|---|---|---|---|---|---|
| | Endogenous concentration | Concentration after a low spike◊ | Concentration after a high spike◊ | low Spike | high spike |
| Oleuropein | <LOD | 19.4 ± 0.6 | 52.1 ± 1.6 | 97 ± 3 | 104 ± 3 |
| Ligstroside | <LOD | 19.9 ± 0.5 | 53.5 ± 1.8 | 100 ± 2 | 107 ± 3 |
| Oleuropein aglycone | 21.9 ± 1.0 | 42.9 ± 0.8 | 75.3 ± 1.8 | 105 ± 2 | 107 ± 4 |
| Oleacein | 56.9 ± 1.8 | 78.6 ± 1.8 | 110.1 ± 2.4 | 109 ± 5 | 106 ± 5 |
| Oleocanthal | 196.9 ± 7.2 | 216.7 ± 4.5 | 245.7 ± 4.0 | 104 ± 28 | 100 ± 11 |
| Hydroxytyrosol | 4.1* ± 0.4 | 24.6 ± 0.8 | 57.8 ± 3.1 | 102 ± 2 | 107 ± 5 |
| Tyrosol | <LOD | 24.0 ± 2.0 | 55.9 ± 3.5 | 108 ± 6 | 107 ± 3 |
| Elenolic acid | 30.4 ± 1.9 | 52.1 ± 2.3 | 85.3 ± 4.6 | 108 ± 4 | 110 ± 7 |

**Denotes concentrations >LOD and <LOQ; ◊Low and high spike concentrations were 20 and 50 µg/L, respectively.*

### 3.2.4 Precision

Intra- and inter-day precision was examined for the oil extraction method. Precision was determined as the percentage relative standard deviation (%RSD) of the final concentration (mg/kg) as this considers the differences in oil sample weight during validation. Results below the quantification limit were excluded. The intra-day precision was determined for each of the triplicate sample results for endogenous, low, and high spike treatments within one day of validation ($n$ = 3). Since there were three different concentration treatments applied (spiked and endogenous) to two samples (Pams and Lupi OO), eighteen separate intra-day %RSDs were generated for each analyte. The inter-day precision was calculated as the %RSD over the three validation days ($n$ = 9) for each different treatment. This resulted in six %RSDs for each analyte over the course of the validation. The intra- and inter-day precision ranges, separated out by treatments, for the Pams and Lupi oil, are summarised in Table 3.8 and Table 3.9, respectively.

There is some variation among the intra- and inter-day precision, all values apart from one fall within the acceptable threshold range of 10% RSD.[219] The analytes that have lower %RSDs are mostly from the spiked sample data as well as the samples with higher endogenous concentrations. This is due to spiking resulting in higher levels being detected by the instrument making it further away from the LOQ compared to endogenous samples. The Pams %RSDs, in general, are much higher than the Lupi %RSDs and are nearly outside the acceptable threshold. For Pams intra-day high spiked (50 µg/L) sample, hydroxytyrosol is just outside this threshold (11.43%). This is likely due to the poor sensitivity of hydroxytyrosol and it being quite close to the LOQ. Often when an analyte is close to the LOQ, a %RSD of 30% is deemed acceptable. On the contrary, Lupi samples had higher endogenous concentrations, so are further from the LOQ, as a result, they have lower %RSDs compared to Pams oil samples. The intra- and inter-day %RSDs obtained in this research were slightly higher compared to the %RSDs achieved in the study this method is adapted from (Pizarro *et al.* 2013).[177] The results they attained are expressed as intra-day then inter-day as follows: oleuropein (3.2% and 4.8%), hydroxytyrosol (3.7% and 4.7%) and

tyrosol (2.4% and 3.0%). The results in their study could be lower as they may have used data that was further away from the LOQ. Overall, the results in this research are still a good indication that the method is reproducible over time, at varying concentrations of target analytes.

**Table 3.8. Pams intra- and inter-day %RSDs for endogenous, low and high spike samples. Intra-day results (*n* = 3) are presented as the range of %RSDs from each validation day. The inter-day precision is presented as %RSD over three days (*n* = 9).**

| Analyte | Pams OO intra-day precision (%RSD, *n* = 3) | | |
|---|---|---|---|
| | **Endogenous** | **Low spike** | **High spike** |
| Oleuropein | n/a[◊] | 2.01-2.91 | 2.92-8.61 |
| Ligstroside | n/a[◊] | 0.45-2.50 | 1.71-6.84 |
| Oleuropein aglycone | 0.47-2.20 | 0.86-1.16 | 1.68-4.38 |
| Oleacein | 1.33-2.89 | 0.86-1.72 | 1.12-8.81 |
| Oleocanthal | 0.37-3.45 | 1.20-1.65 | 1.47-7.60 |
| Hydroxytyrosol | n/a[◊] | 1.99-3.84 | 1.19-11.43 |
| Tyrosol | n/a[◊] | 2.17-7.10 | 2.97-6.65 |
| Elenolic acid | 0.10-2.99 | 1.94-3.93 | 1.28-4.09 |
| | **Inter-day precision (%RSD, *n* = 9)** | | |
| Oleuropein | n/a[◊] | 3.44 | 9.46 |
| Ligstroside | n/a[◊] | 2.93 | 9.96 |
| Oleuropein aglycone | 2.95 | 1.09 | 5.29 |
| Oleocanthal | 2.60 | 2.50 | 6.30 |
| Oleacein | 2.25 | 2.30 | 8.83 |
| Hydroxytyrosol | n/a[◊] | 3.70 | 9.62 |
| Tyrosol | n/a[◊] | 7.24 | 9.38 |
| Elenolic acid | 4.90 | 3.62 | 6.82 |

[◊]*n/a – not applicable: values were <LOD so no %RSDs could be determined.*

**Table 3.9. Lupi intra- and inter-day %RSDs for endogenous, low spike and higher spike samples. Intra-day results (*n* = 3) are presented as the range of %RSDs from each validation day. The inter-day precision is presented as %RSD over three days (*n* = 9).**

| Analyte | Lupi OO intra-day precision (%RSD, *n* = 3) | | |
|---|---|---|---|
| | **Endogenous** | **Low spike** | **High spike** |
| Oleuropein | n/a[◊] | 0.86-1.52 | 1.70-2.61 |
| Ligstroside | n/a[◊] | 0.71-1.80 | 0.67-1.99 |
| Oleuropein aglycone | 1.11-3.69 | 1.05-1.62 | 1.07-2.07 |
| Oleacein | 0.34-1.97 | 0.24-1.83 | 0.18-0.95 |
| Oleocanthal | 1.06-2.40 | 0.62-1.59 | 1.37-1.93 |
| Hydroxytyrosol | n/a[◊] | 1.83-2.62 | 1.33-2.91 |
| Tyrosol | n/a[◊] | 2.53-5.33 | 0.37-4.87 |
| Elenolic acid | 0.50-6.57 | 1.36-5.47 | 0.18-3.94 |
| | **Inter-day precision (%RSD, *n* = 9)** | | |
| Oleuropein | n/a[◊] | 4.02 | 3.25 |
| Ligstroside | n/a[◊] | 3.57 | 3.24 |
| Oleuropein aglycone | 3.72 | 1.62 | 2.24 |
| Oleacein | 2.41 | 1.57 | 2.31 |
| Oleocanthal | 3.00 | 1.30 | 1.81 |
| Hydroxytyrosol | n/a[◊] | 3.24 | 5.32 |
| Tyrosol | n/a[◊] | 8.02 | 6.36 |
| Elenolic acid | 6.47 | 4.44 | 5.30 |

[◊]*n/a – not applicable: values were <LOD so no %RSDs could be determined.*

### 3.2.5 Stability

Due to the analyses occurring on the LC-MS/MS in a commercial laboratory, the instrument was not always available when required so the stability of compounds in extracted oil samples was tested. After validation 2 data was analysed (Date: 6-12-2022), one of the Lupi-50 spike samples was re-capped, placed in a chiller (2-8 °C) and re-analysed 12 days later during validation 3 (Date: 18-12-2022). The resulting values and percentage differences are summarised in Table 3.10. All percentage differences except for oleuropein are below 5% suggesting that the choice of extraction solvent, the compounds and the amber glass vials allowed the extracted analytes to be stable for up to 12 days. However, oleuropein had a difference of 7% where the concentration decreased after 12 days. This is likely due to the polar glucose moiety attached to the oleuropein, which makes it insoluble

in oil.[50,220,221] Since oleuropein is not naturally present in oil, its lack of stability in this matrix was not an issue for the purposes of this research.

**Table 3.10. The concentration of target analytes (µg/L) in a Lupi high spike sample over two validation days stored at 2-8 °C to determine the stability of analytes in oil samples over time.**

| Analyte | Lupi high spike[◊] instrument data (µg/L) | | Percentage difference (%) |
|---|---|---|---|
| | Analysis date: 6-12-22 | Analysis date: 18-12-22 | |
| Oleuropein | 51.54 | 47.6 | 7.6 |
| Oleocanthal | 245 | 243 | 1.0 |
| Oleacein | 108 | 113 | 4.1 |
| Hydroxytyrosol | 56.7 | 54.9 | 3.2 |
| Tyrosol | 54.3 | 55.6 | 2.4 |
| Oleuropein aglycone | 74.7 | 73.3 | 1.9 |
| Ligstroside | 53.2 | 51.7 | 2.9 |
| Elenolic acid | 85.0 | 82.1 | 3.4 |

[◊]*The high spike concentration was 50 µg/L.*

## 3.3 Analysis of aqueous olive leaf tea samples

In this research, OLT (aqueous based samples) required analysis, so parameters including accuracy and precision in an aqueous matrix were investigated during validation. Type 1 water was selected as the matrix to be spiked and analysed alongside non-spiked/blank Type 1 water samples. Ideally, an OLT free of phenolic compounds would have been used as the matrix but this was not possible as the tea had very high concentrations of the target analytes. As a result, it would have been unfeasible to spike amounts high enough to carry out spike recovery analysis. Each treatment was carried out in triplicate over three validation days, resulting in a total of nine replicates. Two-fold dilutions were also carried out to check for consistency of pipetting and check for any changes when samples were diluted, reducing the likelihood of issues occurring during the analysis of OLT samples.

### 3.3.1 Accuracy and precision

As with the oil extraction method, the accuracy of aqueous samples was determined via a spike recovery. Type 1 water was spiked to give a raw result of 80 µg/L, see Chapter 2, Section 2.6.2.1 for details on the spiking method. The recoveries were calculated based on the recovery calculation in Equation 3.1, Section 3.2.3. The recoveries for target analytes in aqueous samples fell within the acceptable range of 80-120%[219] (Table 3.11). It should be noted that recovery of oleuropein aglycone is close to the unacceptable range (<80%). This is due to oleuropein aglycone being hydrophobic and far less polar than oleuropein glycoside due to the lack of the polar glucose moiety.[50,220,221] The aglycone is likely adhering to glassware or precipitating out of solution. All diluted samples were accurate and the percentage difference between the spiked samples and dilutions were within 10% for all analytes except for oleuropein aglycone, which fell between 10 and 20%. This again relates to the solubility of oleuropein aglycone discussed above.

**Table 3.11. Spike recoveries, intra-day and inter-day precision ranges for the target analytes using 80 µg/L spiked aqueous data. Spike results are presented as the average ± SD (*n* = 9). Intra-day results are presented as the range of %RSDs from each validation day (*n* = 3). Inter-day results are presented as %RSD over three validation days (*n* = 9).**

| Analyte | Spike recovery (%) (*n* = 9) | Intra-day precision (%RSD, *n* = 3) | Inter-day precision (%RSD, *n* = 9) |
|---|---|---|---|
| Oleuropein | 91 ± 4 | 0.53-2.94 | 4.95 |
| Ligstroside | 93 ± 2 | 1.78-5.43 | 3.73 |
| Oleuropein aglycone | 80 ± 11 | 3.26-4.45 | 13.21 |
| Oleacein | 99 ± 7 | 0.38-3.25 | 7.21 |
| Oleocanthal | 94 ± 4 | 1.41-2.36 | 6.06 |
| Hydroxytyrosol | 105 ± 4 | 1.89-5.25 | 5.97 |
| Tyrosol | 98 ± 2 | 1.20-2.46 | 3.25 |
| Elenolic acid | 112 ± 9 | 2.52-7.18 | 9.33 |

There were two levels of precision examined for the aqueous method: intra- and inter-day precision. Both precisions were determined as %RSD of the instrument concentration (µg/L) of spiked (80 µg/L) samples. The intra-day precision was determined by triplicate sample results within one day of validation (*n* = 3) for each validation day. The inter-day precision

was calculated as the %RSD over the three validation days (*n* = 9). The intra- and inter-day precision of the aqueous samples is summarised in Table 3.11. All values apart from the inter-day precision of oleuropein aglycone (13.21%) fall within the acceptable threshold range of 10%.[219] Oleuropein aglycones %RSD falling outside the threshold is not unexpected and likely relates to the aglycones hydrophobic nature.[220] Therefore, the %RSDs are a good indication that the method is reproducible for target analytes in aqueous OLT samples over time.

### 3.3.2 Stability

To determine the stability of analytes in the aqueous samples, one spiked aqueous sample from validation 2 (Date: 6-12-2022) was re-capped and left in the chiller (2-8 °C), then re-analysed 12 days later during validation 3 (18-12-22). Five out of the eight analytes were stable as they still had acceptable spike recoveries (80-120%), Table 3.12. Hydroxytyrosol and elenolic acid were above the acceptable threshold; this could be a result of degradation of their respective precursors during storage. Oleuropein aglycone had the worst recovery (35%), again this is due to the aglycone being hydrophobic.[220] Due to the lack of stability in aqueous samples, diluted OLT samples were never analysed more than one day after preparation.

**Table 3.12. Target analyte recoveries for spiked (80 µg/L) Type 1 water samples analysed after 12 days stored at 2-8 °C.**

| Analyte | Spike recovery (%) (*n* = 1) |
|---|---|
| Oleuropein | 88 |
| Ligstroside | 94 |
| Oleuropein aglycone | 35 |
| Oleacein | 99 |
| Oleocanthal | 99 |
| Hydroxytyrosol | 138 |
| Tyrosol | 108 |
| Elenolic acid | 159 |

### 3.3.3 Suppression effects

During the analysis of OLT, it was discovered that highly concentrated samples resulted in suppression effects when analysed by MS/MS. At low dilution levels, for highly concentrated analytes, the instrument would suppress the response. To investigate the extent of suppression, a range of dilutions, spikes and altering the injection volume from 2 µL were carried out. Varying injection volume (0.5 µL = 400 x dilution, 1 µL =200 x dilution, 1.5 µL = 133.33 x dilution) affected the instrument response for hydroxytyrosol and ligstroside (Figure 3.8). Larger injection volumes resulted in a lower reported concentration suggesting that analytes in concentrated samples (less dilute) were being suppressed. Similar trends were observed for the other target analytes. Thus, all aqueous samples analysed had several dilutions carried out until a plateau was observed, Figure 3.9. In this research, the concentration of analytes in OLT are reported as the average of several dilutions that have analyte concentrations consistent within ± 10% of dilutions undertaken.

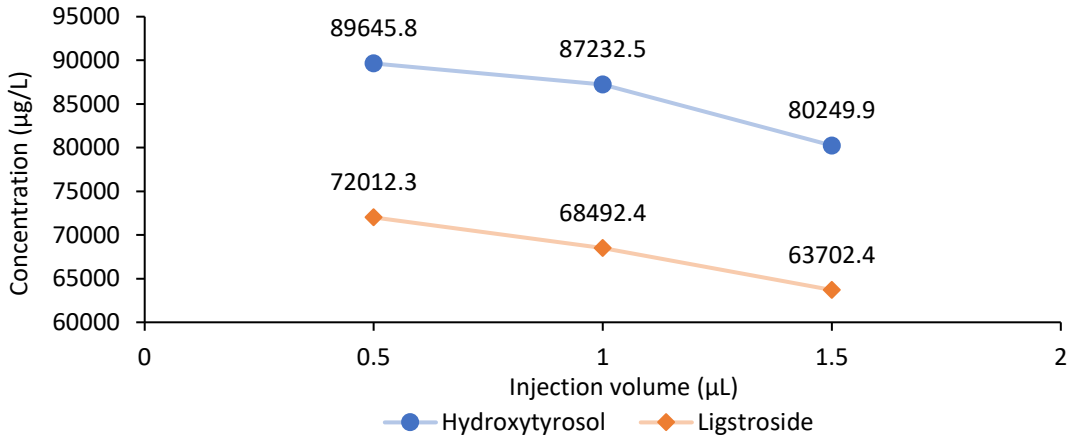



**Figure 3.8. Instrument concentration (µg/L) of hydroxytyrosol and ligstroside at varying injection volumes to simulate different dilution levels to confirm the effect of suppression of target analytes.**

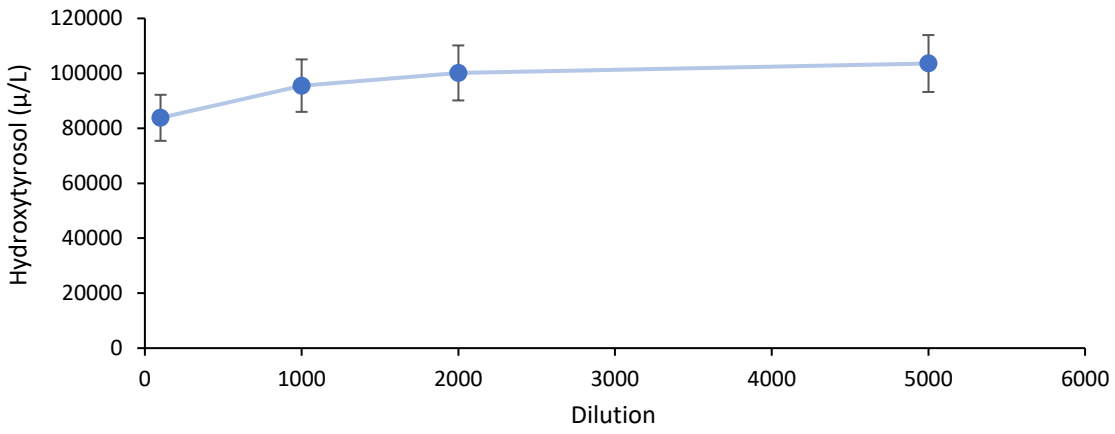



**Figure 3.9. Instrument concentration (µg/L) of hydroxytyrosol at different dilution factors (100, 1000, 2000, 5000) showing the plateau in concentration required to quantify analyte concentration and confirm suppression was not affecting results. Error bars represent ± 10% of the concentration measured.**

## 3.4 Glucose Analysis

The hydrolysis of oleuropein glycoside to oleuropein aglycone removes one glucose molecule (the same is true for ligstroside glycoside), hence a cheaper alternative to confirm the hydrolysis via the formation of glucose in OLT was carried out using a diabetes blood glucose meter (BGM). Several studies in the literature have used a BGM for various types of research such as measuring sucrose in beverages,[222] hydrolysis of sucrose to lactose[223] and non-catalysed and enzyme-catalysed mutarotation of glucose.[224] The use of a BGM is inexpensive, requires a small sample volume, and is efficient compared to other conventional methods such as HPLC which require a method to be developed, larger sample sizes, sample preparation and does not provide results instantly.[223,225] The BGM used in this research required a minimum sample size of 0.5 µL, provided data within 5 seconds and had a measurement range of 1.1 to 33.3 mmol/L in blood.

To use a BGM for glucose measurements in aqueous samples like OLT it must be calibrated with an aqueous solution to find the relationship of glucose concentration to detector response. This is because a blood matrix is very different and the direct glucose reading measured would not be accurate due to differences in viscosity and composition. Therefore,

several glucose standards (0 to 14 mmol/L or mM) were made up in distilled water following methods described in Chapter 2, Section 2.8.1.

The first BGM purchased was a i-SENS CareSens™ N POP which required CareSens™ N testing strips. The initial use of N POP BGM did not provide good linearity (Figure 3.10). It should be noted that calibration curves are generally plotted with the independent variables on the x-axis,[226] e.g., the concentration of glucose standards (mmol/L). However, in the calibration curves plotted below, the opposite is true, where the independent variable is plotted on the y-axis (glucose standards, mmol/L). The calibration curves were also plotted this way in previously reported research.[223,224] This also simplified calculations with the easy substitution of the BGM reading (x variable) in the equation displayed on the graph. The calibration curve began to plateau above 12 mmol/L. This was not acceptable for this research; hence another meter was purchased.

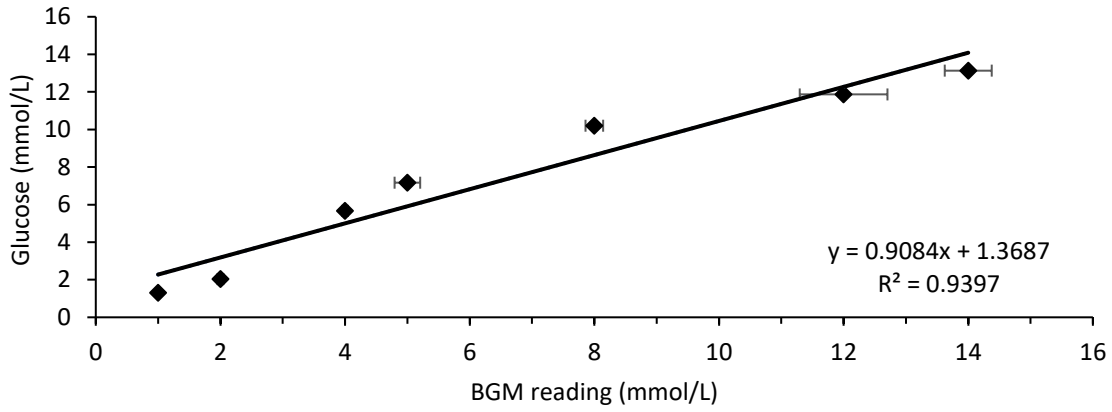



**Figure 3.10. Relationship between glucose concentration (mmol/L) versus BGM reading for the CareSens™ N POP meter. Results are presented as the average ± SD (*n* = 3). The independent variables (glucose standards, mmol/L) have been intentionally plotted on the y-axis.**

The second BGM purchased was produced by the same company (The CareSens™ Dual). This meter was more expensive as it had Bluetooth® functionality and it required different

testing strips (CareSens™ PRO). This meter had good linearity with an $R^2$ of >0.997 and was still linear at higher concentrations compared to the N pop (Figure 3.11).

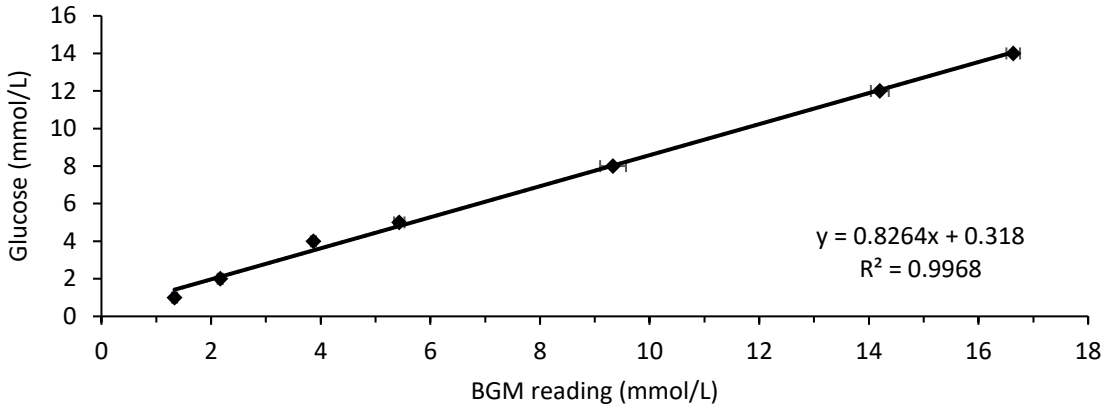



**Figure 3.11. Relationship between glucose concentration (mmol/L) versus BGM reading for the CareSens™ Dual meter. Results are presented as the average ± SD (*n* = 3). The independent variables (glucose standards, mmol/L) have been intentionally plotted on the y-axis.**

The %RSDs for the N POP BGM were more varied compared to the Dual. All %RSDs for the Dual BGM were under 3.54% (Table 3.13), hence it was chosen to monitor glucose formation in this research. It was also determined that standards higher than 14 mmol/L gave unreproducible results, greater variation and significant plateauing of the curve. As a result, the working range of the calibration curve in aqueous samples were made up from 1 to 14 mmol/L.

**Table 3.13. The %RSDs and average %RSD ± SD (*n* = 3) highlighted in bold text for each glucose standard using the CareSens™ N POP versus the Dual BGM.**

| Glucose Std (mmol/L) | %RSD | |
|---|---|---|
| | **CareSens™ N POP** | **CareSens™ Dual** |
| 1 | 0.00 | 3.54 |
| 2 | 2.32 | 2.18 |
| 4 | 0.83 | 1.22 |
| 5 | 2.87 | 1.74 |
| 8 | 1.39 | 2.53 |
| 12 | 5.93 | 1.15 |
| 14 | 2.87 | 0.75 |
| **Average %RSD** | **2.32 ± 1.78** | **1.87 ± 0.89** |

## 3.5 Summary of method optimisation and validation

Overall, the method based on that by Pizarro *et al.* (2013)[177] was optimised and successfully validated having met all of the required validation parameters. Moreover, the BGM selected also had an acceptable $R^2$ and %RSDs. Therefore, all methods discussed are fit for purpose to quantify the target analytes from OO, OLT and measure the formation of glucose in the research undertaken.

# CHAPTER 4

# Investigation of Secoiridoids in Olive Oil and Olive Leaf Tea

## 4.1 Introduction

This chapter will discuss the analyses of commercial EVOOs (*n* = 9); the content of target analytes in commercially available oils has not been extensively studied in New Zealand and serves as a comparison to the content measured in enriched oils. Additionally, there is currently minimal literature detailing the production of OLT. Therefore, the influence of drying methods on oleuropein content in OLT and the stability of analytes in OLT under different storage temperatures (−20 °C, RT and 40 °C, 189 days) were also examined.

## 4.2 Commercial extra virgin olive oils

### 4.2.1 Overview

In New Zealand and around the world, labelling of commercial EVOOs do not state the quantity of compounds attributed to health benefits; including the target analytes in this research: oleuropein, ligstroside, oleuropein aglycone, oleocanthal, oleacein, hydroxytyrosol, tyrosol and elenolic acid. As discussed in literature (Chapter 1, Section 1.6.4), there is a health claim (EU Reg. 432/2012) approved by the EFSA which states 'olive oil polyphenols contribute to the protection of blood lipids from oxidative stress'.[121] The claim may be used for OO that contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein aglycone and tyrosol) per 20 g of olive oil (equivalent to 250 mg/kg).[93,121,227]

Therefore, it was important to determine the concentration of target analytes in commercial EVOOs so they could be compared to concentrations transferred into oil during the research (discussed in Chapter 5). The aim was to achieve a content above 250 mg/kg

so an enriched oil could be marketed with a concentration that is within the range for relevant health benefits.

### 4.2.2 Target analytes

The concentration of individual target analytes, total simple phenolics/non-phenolic (sum of hydroxytyrosol, tyrosol and non-phenolic elenolic acid), total complex phenolics (sum of oleuropein aglycone, oleacein and oleocanthal), total phenolics (sum of oleuropein aglycone, oleacein, oleocanthal, hydroxytyrosol and tyrosol) and the ratio between total simple/non-phenolic and total complex phenolics in the EVOOs analysed are summarised in Table 4.1. Overall, the results were varied. The most abundant phenolic compounds in descending order were oleuropein aglycone, oleocanthal and oleacein, with a mean content of 34.6, 20.4 and 15.3 mg/kg, respectively. This is in agreement with previous studies by García-Rodríguez *et al*. (2017),[228] Karkoula *et al*. (2014)[229] and two separate studies by Miho *et al*. (2018)[230] and (2021).[231] Where Miho *et al*. (2018)[230] reported that oleocanthal had a higher concentration compared to oleacein. However, all mentioned studies reported much higher concentrations of these phenolic compounds; 449 mg/kg being the highest.[44] Generally these analytes (complex phenolics) will have a higher content in oils.[44] As discussed in the literature (Chapter 1, Section 1.4.2.1), during OO production, the two secoiridoid glycosides (oleuropein and ligstroside) are hydrolysed by β-glucosidase forming oil soluble oleuropein aglycone and ligstroside aglycone, which are hydrolysed via esterase and decarboxylated to produce oleocanthal and oleacein. Five out of the nine oils (Oleo Martos, Red Island, Matapiro, The Olive Press – Picual and Picholine) analysed in this research had a higher amount of these complex phenolics.

For the EVOOs that were analysed, the ranges for hydroxytyrosol and tyrosol were similar, 2.8-17.0 mg/kg and 2.9-17.1 mg/kg, respectively. In a study by Caporaso *et al*. (2015),[232] hydroxytyrosol and tyrosol had a similar concentration range; 3.1-20.2 mg/kg and 2.1-25.6 mg/kg, respectively. In this research, the range of complex phenolics (oleuropein aglycone, oleocanthal and oleacein) varied more; 9.8-97.3, 5.9-45.2 and 0.3-24.6 mg/kg, respectively. This trend was also evident in other studies.[230,231]

In this research, EVOO made from individual cultivar varieties had the highest (cv. Picual and Picholine) and lowest (cv. Frantoio) content of oleuropein aglycone. A study by López-Huertas *et al*. (2021)[93] analysing OOs from different olive fruit varieties at various stages of ripening in Spain determined that the Picual variety contained higher amounts of oleuropein aglycone compared to the Frantoio variety. In contrast, Miho *et al*. (2021)[231] reported that the Frantoio variety had a higher concentration of complex phenolics compared to the Picual variety in Spanish made OOs. As discussed in the literature, these discrepancies could result from agronomic effects and differences in OO production. Overall, in this research, The Olive Press – Picual and Picholine variety EVOOs had the highest total phenolic content with concentrations of 126 and 154 mg/kg respectively, whereas Frantoio had the lowest (34 mg/kg).

Elenolic acid, a non-phenolic compound, varied among the oils analysed. The Olive Press - Frantoio and Macro Organic had the highest content (125 and 103 mg/kg, respectively) and The Village Press had the lowest (22 mg/kg). Oleuropein and ligstroside content were <LOD in all samples which was not unexpected as both of these analytes are significantly polar due to the presence of the glucose moiety and favour the aqueous fraction during production of OO. Low concentrations of oleuropein (<1.33 mg/kg) have been reported by Bayram *et al*. (2012)[162] analysing phenolic compounds in 55 OOs. The study did not specify individual LODs but the method LOD for all compounds (0.03-1.7 µg/L) was lower than the present study (1.82 µg/L, oleuropein).

The differences in concentration between the commercial oils analysed could be influenced by processing, batch, as well as the age of the oils which is unknown. During OO storage, phenolic compounds such as the aglycones can undergo further degradation (mainly hydrolysis) leading to an increase in concentration of tyrosol, hydroxytyrosol as well as elenolic acid (simple phenolics/non-phenolic).[170,232] This trend was observed for seven out of nine oils (Oleo Martos, Red Island, Macro Organic, Village Press, The Olive Press – Frantoio and Olive Black) analysed in this research. Conversely, The Olive Press – Picual and Picholine had a higher oleuropein aglycone content compared to the total simple

phenolics/non-phenolic analytes. It has been reported[233] that the ratio between total simple phenolics/non-phenolic and total complex phenolic compounds could be considered an index of oil freshness. This suggests that EVOOs from The Olive Press – Frantoio, Olive Black and Macro Organic may be older with ratios of 8.4, 1.6 and 1.4, respectively. Unusually, the other two oils from The Olive Press (cv. Picual and Picholine) had much lower ratios of 0.7 and 0.6, respectively. However, as widely reported in literature, phenolic concentration in OOs can vary depending on the olive cultivar, agronomic practices, degree of fruit ripening, conditions of processing (type of olive mill, malaxation, etc.) and oil storage.[232] The variability in the studies may also exist due to differences in analytical methodology, such as detection technique, quantitation strategy as well as extraction methods.

**Table 4.1. Individual concentration, total contents and ratio of target analytes in commercial EVOOs purchased from the New Zealand market. Individual concentration results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | Brands of commercial EVOOs analysed | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Oleo Martos** | **Red Island** | **Matapiro** | **Macro Organic** | **The Village Press** | **The Olive Press - Frantoio** | **The Olive Press - Picual** | **The Olive Press - Picholine** | **Olive Black** |
| | **mg/kg** | | | | | | | | |
| Oleuropein | <LOD* | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Ligstroside | <LOD* | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD | <LOD |
| Oleuropein aglycone | 14.9 ± 0.1 | 24.6 ± 0.3 | 15.97 ± 0.04 | 35.7 ± 1.8 | 9.8 ± 0.2 | 10.8 ± 0.2 | 97.3 ± 3.5 | 91.6 ± 3.3 | 10.5 ± 0.5 |
| Oleacein | 13.8 ± 0.2 | 31.7 ± 1.9 | 14.3 ± 0.2 | 24.6 ± 1.3 | 8.8 ± 0.4 | 0.3 ± 0.2 | 7.1 ± 0.2 | 23.8 ± 1.0 | 13.0 ± 0.2 |
| Oleocanthal | 16.3 ± 0.3 | 45.2 ± 1.6 | 21.2 ± 0.1 | 37.5 ± 0.5 | 12.3 ± 0.4 | 5.9 ± 0.1 | 10.1 ± 0.2 | 24.9 ± 0.2 | 10.5 ± 0.1 |
| Hydroxytyrosol | 2.8 ± 0.1 | 6.2 ± 0.4 | 5.8 ± 0.4 | 17.0 ± 0.5 | 4.45 ± 0.01 | 8.6 ± 0.1 | 5.9 ± 0.3 | 6.9 ± 0.3 | 3.59 ± 0.04 |
| Tyrosol | 2.9 ± 0.3 | 6.0 ± 1.2 | 5.9 ± 0.1 | 17.1 ± 1.0 | 4.2 ± 0.3 | 8.8 ± 1.0 | 5.7 ± 0.5 | 6.6 ± 0.1 | 3.4 ± 0.7 |
| Elenolic acid | 25.1 ± 1.5 | 43.8 ± 1.9 | 31.6 ± 4.6 | 103.0 ± 7.5 | 22.3 ± 0.4 | 125.0 ± 1.3 | 69.5 ± 1.8 | 65.3 ± 0.7 | 48.5 ± 3.5 |
| **Total contents** | **mg/kg** | | | | | | | | |
| Total simple phenolics[◊] | 30.9 | 56.1 | 43.2 | 137.2 | 31.0 | 142.5 | 81.0 | 78.8 | 55.5 |
| Total complex phenolics | 44.9 | 101.5 | 51.5 | 97.8 | 30.9 | 17.0 | 114.5 | 140.3 | 34.1 |
| Total phenolics | 50.7 | 113.8 | 63.1 | 132 | 39.5 | 34.4 | 126.1 | 153.7 | 41.1 |
| **Ratio of simple to complex** | 0.7 | 0.6 | 0.8 | 1.4 | 1.0 | 8.4 | 0.7 | 0.6 | 1.6 |

**The LOD for oleuropein and ligstroside were 1.82 and 1.48 mg/kg respectively; [◊]Includes elenolic acid which is non-phenolic.*

### 4.2.3 Health claim, cost, origin, and purchase year

The association between the sum of derivatives in oil required to meet the health claim (EU Reg. 432/2012, 250 mg/kg) and cost per 100 mL, as well as origin and purchase year were also explored to gain an understanding of the EVOOs in the New Zealand market (Table 4.2). The specific phenolic compounds to which the EFSA refer to in the health claim are hydroxytyrosol, tyrosol and complex high-molecular mass polyphenols from which hydroxytyrosol and tyrosol can originate, namely, secoiridoids.[93,121] There is currently no clarification by the EFSA on what secoiridoid 'derivatives' are included when determining the health claim. Generally, it includes the oleuropein complex (oleuropein aglycone)[93] but other studies have mentioned that the derivatives also include ligstroside aglycone, oleocanthal and oleacein.[94,234] Therefore, for this research the sum of hydroxytyrosol, tyrosol, oleuropein aglycone, oleocanthal and oleacein was used to assess whether or not a commercial oil met the health claim. Ligstroside aglycone and elenolic acid were not included as ligstroside aglycone was not quantified and elenolic acid is not a phenolic compound.

**Table 4.2. The sum of derivatives used to determine the health claim (250 mg/kg), cost per 100 mL (highest and lowest cost are shown in bold text), origin and year of purchase for commercial EVOOs (*n* = 9) analysed in this research.**

| Commercial Oil | Variables of interest to consumers | | | |
|---|---|---|---|---|
| | Sum of derivatives in EVOO[†] | Cost ($) per 100 mL[◊] | Origin | Year of purchase* |
| The Olive Press - Frantoio | 34.44 | 5.95 | New Zealand | n.d. |
| The Village Press | 39.5 | 3.00 | New Zealand | 2022 |
| Olive Black | 41.08 | **6.90** | New Zealand | 2020* |
| Oleo Martos | 50.66 | **0.99** | Spain | 2022 |
| Matapiro | 63.11 | 3.40 | New Zealand and Australia | 2022 |
| Red Island | 113.77 | 2.80 | Australia | 2022 |
| The Olive Press - Picual | 126.12 | 5.95 | New Zealand | n.d. |
| Macro Organic | 131.96 | 1.60 | Spain | 2022 |
| The Olive Press - Picholine | 153.72 | 5.95 | New Zealand | n.d. |

*[†]Sum of target phenolic analytes for different EVOO analysed (n = 2, presented as an average); [◊]Prices as of September 2023; *The year of purchase with an Asterix refers to harvest year; n.d. – no date available.*

There was a large range of results for the summed analytes (34-154 mg/kg); these results indicate that none of the commercial EVOOs analysed would meet the health claim stated by EU Reg. 432/201. The Olive Press - Picholine had the highest sum (154 mg/kg) and was the closest to meeting the claim (250 mg/kg). It should be noted that ligstroside aglycone was not included as it was not quantified, which may have resulted in a higher summed concentration. In the literature, its concentration has been in the hundreds[174,235] but this can be influenced by geographic location, cultivar and OO processing etc. These results were not unexpected. Ninety percent of the OO sold in New Zealand (30,000 tonne p.a.) are imported from mainly European countries such as Spain, Greece and Italy.[126] These imported OOs are seldom tested for quality or authenticity. As a result, a portion of EVOOs on the commercial market are not guaranteed to be fresh which will influence the quality. There is currently a gap in the New Zealand market for an enriched oil that meets health claim concentration (250 mg/kg).[126] In the literature, oils below and above the health claim concentration have been reported. However, due to ambiguity around the compounds that should be summed, comparisons with literature should be carried out with caution. A study by Caporaso *et al*. (2013)[232] analysed 32 OOs from the Southern Italian market; less than 10% of the oils analysed had a suitable phenolic content for the application of the health claim.[232,236] This study did not state which derivatives were summed to determine the values. Conversely, Martínez-Navarro *et al.* (2020)[94] analysed the phenolic content in EVOO samples (*n* = 1239) from different cultivars and growing seasons in Spain. They detected that over 76.9% of the samples had a concentration above 250 mg/kg. Therefore, a high proportion of analysed samples had a phenolic concentration suitable in meeting the health claim with the recommended daily consumption. These results give some indication that the phenolic concentration in bottled EVOOs from the retail market in New Zealand and overseas is varied. This variation supports the necessity of this research; to investigate the transfer of compounds such as oleuropein aglycone to obtain an enriched OO that can be marketed with a concentration that is within the range for health benefits.

Trends between the sum of phenolic derivatives, cost per 100 mL, and origin of the oils was also explored (Table 4.2). As this research was carried out for a company who wants to

enhance OO and develop a new product it was important to understand what consumers are currently paying for an oil without information on compounds associated with health benefits. The cost per 100 mL of oil showed variation, which seemed to be influenced by the origin; local oils (New Zealand) were more expensive. EVOOs from Australia were the next most expensive, oils of Spanish origin were the cheapest. The high costs associated with New Zealand and Australian EVOOs is likely related to the small amount produced compared to countries in the EU; New Zealand and Australia produce 300 and 30,000 tonnes p.a., respectively[126] compared with Spain, who produce 1.3 million tonnes p.a.[237,238] By comparing the sum of derivatives to the cost per 100 mL, generally, EVOOs with a lower content had a higher price, Figure 4.1. The Village Press EVOO had the third highest cost ($3/100 mL) but the second lowest phenolic content (40 mg/kg). This company is one of New Zealand's largest oil producers and is on a mission to "show New Zealand what premium EVOO really tastes like".[239] In contrast, the cheapest oil (Oleo Martos, $0.99/100 mL) had a higher sum of derivatives (51 mg/kg).

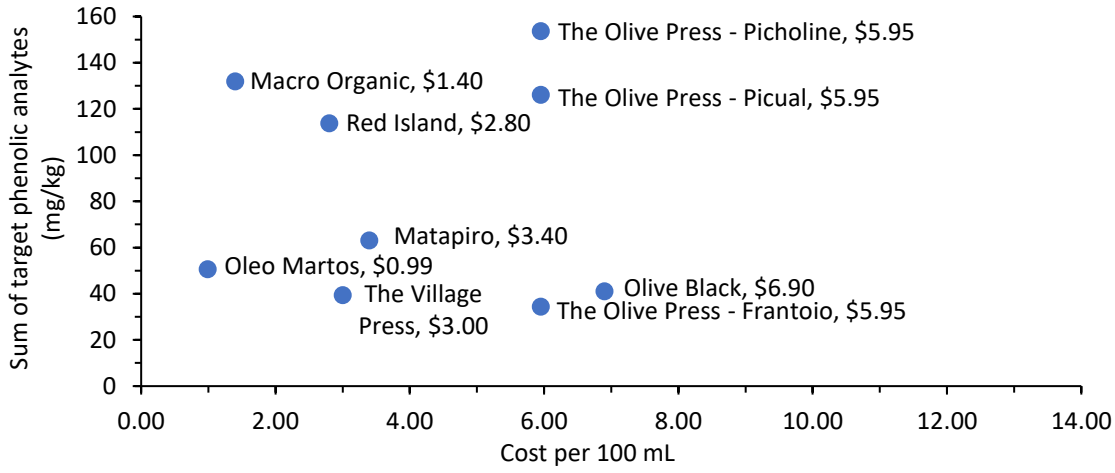



**Figure 4.1. The sum of target phenolic analytes versus cost per 100 mL for commercial EVOOs.**

Researchers' findings indicate that consumers base the quality and authenticity of OO on many other factors such as labels specifying "organic" production, brand name, packaging and selling price.[240-242] Bearing these habits in mind, the consumer may consider The Village

Press oil to be of "superior" quality due to branding and a higher price. However, in some cases, the unbranded cheaper oils analysed in this research had a higher phenolic content such as Macro Organic (132 mg/kg, $1.40/100 mL). It is also important to note that the second most expensive oil ($5.95/100 mL) had a considerable amount of phenolic compounds (154 mg/kg, The Olive Press – Picholine). A study by Caporaso *et al*. (2015)[232] also reported variation when investigating cost and phenolic concentration for oils purchased from Southern Italian supermarkets; however, the highest phenolic content oils were the most expensive which is not evident in this research.

Lastly, it was also important to discuss the length of time some oils had spent in storage since being purchased as this may have affected the sum of phenolic derivatives. During storage, EVOO usually suffers degradation leading to an inferior quality level.[243] This degradation is unavoidable and starts immediately after the OO extraction when oil is exposed to oxygen, heat and light. The lipid fraction in OO is most susceptible to oxidation (auto-oxidation). This occurs even in the absence of light, following a free radical mechanism where the absorption of oxygen results in the formation of hydroperoxides through the interaction of fatty acids with triplet and singlet oxygen.[244-247] These hydroperoxides are susceptible to further oxidation or degradation into secondary reaction products such as aldehydes, ketones, acids, and alcohols.[244,248-250] A second type of oxidation can also occur if OO is exposed to light. Photosensitised oxidation of lipids (photo-oxidation) occurs through the action of natural photosensitisers such as chlorophyll which can react with triplet oxygen to form excited state singlet oxygen.[244] The highly unstable and reactive singlet oxygen reacts with the unsaturated fatty acids, leading to the formation of the undesirable hydroperoxides.[251] As a consequence, storage and packing conditions of OO become of primary importance[245,246] as oxidation often leads to rancidity and deterioration in nutritional and biological quality,[248,249,251] appearance and flavour.[189,249,252,253] Hence, EVOO shelf-life is usually 12 months. These factors likely influenced the data for the EVOO from Olive Black and one from The Olive Press (cv. Frantoio) which are both marketed as premium New Zealand brands. These oils had the highest costs (>$5.95/100 mL) and the second and third lowest sums of phenolic target

analytes (under 40 mg/kg, Table 4.2). The production date (2020) for the Olive Black EVOO exceeded an age of 1.5 years (18 months) and The Olive Press – Frantoio had an unknown purchase date. The storage conditions for both oils were also unknown prior to being utilised in this research. These factors likely explain the low phenolic content of these oils and should not be judged in any of the above discussion. It is particularly interesting that under the same conditions, the other two EVOOs from The Olive Press (cv. Picual and Picholine) had the highest concentrations of target analytes which may be related to differences in variety.[244,253]

### 4.2.4 Summary of commercial EVOO analysis

Results from the analysis of commercial EVOOs (*n* = 9) in the New Zealand market suggest phenolic concentrations are variable and there are no guarantees that a known brand or a higher purchase price will result in an EVOO that contains high levels of target analytes suitable to meet the EU health claim (250 mg/kg). There is a gap in the New Zealand market providing an opportunity for the development of an enriched OO. Thus, this thesis will investigate the potential to increase the content of bioactive compounds such as the aglycone to levels above 250 mg/kg to give certainty of health benefits to consumers.

## 4.3 Olive leaf tea

OLT is one means of providing a high concentration of oleuropein which could be enzymatically converted to oil soluble oleuropein aglycone and transferred into an OO. During the research, several batches of tea were made using a 1:10 ratio of dry leaf weight to water. Water was used in steeping of the leaves as it is nontoxic. Producing a tea that contained a sufficient concentration of oleuropein was the focus of this research, rather than obtaining a complete extraction from the leaves. An investigation was carried out to determine how the concentration of oleuropein in the OLT could be varied by drying leaves at different temperatures (fresh, RT and 70 °C) and is discussed below.

### 4.3.1 Moisture content

Olive leaves contain around 50% moisture.[137,254] Determination of the moisture content was carried out based on the method specified in Chapter 2, Section 2.6.1 and ranged between 52 and 56% (average of 55%). Boudhrioua *et al*. (2009)[138] reported higher values with a range of 46-50%. Another study[255] reported an average moisture content of 50.7 ± 0.8% and Cabrera-Gomez *et al*. (1992)[256] reported a moisture content of 50%. Therefore, while slightly higher, results obtained in this research are similar to those reported in the literature.

### 4.3.2 The effect of olive leaf drying on the composition of OLT

This section discusses the drying experiments carried out using two different batches of olive leaves. Initially, two batches of OLT were made using one batch of leaves; one half was used fresh (without drying) and the remaining half was dried at RT. Later in the research, temperatures above RT were of interest so a further two bathes of OLT were made using another batch of leaves; one half dried at RT and the other oven dried at 70 °C. Therefore, the results are laid out in two different tables. It should also be noted that hydrophobic oleuropein aglycone was quantified in the OLTs (<22 mg/kg), this was unexpected. A possible explanation was that the additional matrix components from extracted olive leaf material made the aqueous OLT more favourable for the aglycone.

Table 4.3 summarises the concentration of each analyte in OLT prepared using leaves dried at RT versus the use of fresh leaves. For comparison, a wet weight equivalent of the leaves was determined by factoring in the percentage of water lost during drying using Equation 4.1. All discussions compare the results using wet weight equivalent of the dried leaves versus wet weight of fresh leaves.

$$Wet\ weight\ equivalent = \frac{Dry\ weight\ (g)}{\left(1 - \frac{weight\ of\ H_2O\ lost\ (g)}{100}\right)} \qquad \textbf{Equation 4.1}$$

Based on the results, there is more oleuropein glycoside present in the OLT made with leaves dried at RT, compared to fresh leaves (2056 vs. 1635 mg/kg). There were also increases in the concentrations of hydroxytyrosol (484 vs. 96 mg/kg) and elenolic acid (69 vs. 24 mg/kg), with slightly higher concentrations of oleacein, tyrosol and oleuropein aglycone.

**Table 4.3. The effect of using dry versus fresh leaves on the concentration of target analytes extracted into the OLT. Leaves were collected on the same day from the same tree. Results are presented as the average of dilutions undertaken ± SD.**

| Analyte | Method of drying | | | Percentage difference (%) |
|---|---|---|---|---|
| | RT Dry weight | RT Wet weight equivalent | Fresh Wet weight | Dried vs. fresh leaves |
| | mg/kg | | | |
| Oleuropein | 3667.5 ± 28.7 | 2055.9 ± 16.1 | 1634.9 | 23 |
| Oleuropein aglycone | 17.3 ± 0.14 | 9.7 ± 0.1 | 4.7 ± 0.2 | 69 |
| Oleacein | 11.5 ± 0.3 | 6.4 ± 0.2 | <LOD | N/D* |
| Oleocanthal | <LOD | <LOD | <LOD | N/D* |
| Ligstroside | 663.0 ± 7.9 | 371.7 ± 4.4 | 257.3 ± 16.3 | 36 |
| Hydroxytyrosol | 841.7 ± 11.4 | 483.8 ± 12.2 | 95.8 ± 1.7 | 134 |
| Tyrosol | 30.6 ± 2.6 | 17.2 ± 1.5 | 1.4 ± 0.04 | 170 |
| Elenolic acid | 122.7 ± 3.6 | 68.8 ± 2.0 | 23.7 ± 3.0 | 97 |

**N/D – no data: due to some values being <LOD no percentage difference could be determined.*

These results corroborate with the majority of published work that has investigated total phenolic content in a slightly different matrix; olive leaf extracts. Boudhriouna *et al*. (2008)[138] measured total phenolic content in fresh, blanched and infrared dried (40, 50, 60, 70 °C) olive leaves. The results stated that the phenolic content in dried leaves (blanched or not) remained higher than those measured in fresh leaves. Another study by Ghomari *et al*. (2019)[257] also reported similar results where the total phenolic concentration in dried leaves (40 °C) was higher compared to fresh leaves. Kamran *et al*. (2015)[255] obtained a similar result when comparing fresh versus air dried olive leaves (25 °C). Even though all studies discussed total phenolics, which was not measured in this research, it is evident fresh leaves result in a reduction in the bioactive compounds in olive leaves. This observation is likely attributed to the fact that drying destroys the cell walls present in leaves and facilitates the liberation of the phenolic compounds. Two studies have also reported that fresh leaves will have a lower oleuropein content due to the action of the endogenous enzyme β-glucosidase.[139,141]

Aside from olive leaves, increases in total phenolics was observed by Chang *et al.* (2006)[258] for two varieties of tomatoes that were air-dried. This trend was observed for total phenolic content in fresh and dried herbal materials.[259] A study investigating the drying methods of phenolic compounds in stinging nettle also reported dried samples having a higher phenolic content compared to fresh.[260] These results reveal that a leaf drying process tends to enhance the functional values of resulting products (OLT).[138] However, a study by Australian researchers[257] reported a higher concentration of oleuropein and hydroxytyrosol in fresh olive leaf extracts during analysis of phytochemicals in commercial extracts. There are several variables that could explain this; they classified the extracts as fresh or dry based on the label provided by the manufacturer which may be inaccurate. The methods used to make the olive leaf extracts were also not stated. In most cases, dry leaves tend to produce higher phenolic concentrations.

The results of the second experiment using a different batch of leaves dried at RT and oven dried at 70 °C are summarised in Table 4.4. OLT made using leaves dried at 70 °C resulted

in a higher amount of oleuropein and ligstroside compared to leaves dried at RT. However, concentrations of elenolic acid, oleuropein aglycone, tyrosol, oleocanthal and oleacein were higher in the tea utilising leaves dried at RT.

**Table 4.4. The effect of using leaves oven dried at 70 °C versus RT on the concentration of target analytes extracted into OLT. Leaves were collected on the same day from the same tree. Results are presented as the average of dilutions undertaken ± SD.**

| Analyte | Drying temperature | | Percentage difference (%) |
|---|---|---|---|
| | 70 °C (dry weight, mg/kg) | RT (dry weight, mg/kg) | Dried at 70 °C vs. RT |
| Oleuropein | 1744 ±7.7 | 1167 ± 10.3 | 40 |
| Ligstroside | 906 ± 4.8 | 325 ± 2.6 | 94 |
| Oleuropein aglycone | 4.9 ± 0.1 | 22.1 ± 0.9 | 127 |
| Oleacein | <LOD | 76.2 ± 0.5 | N/D* |
| Oleocanthal | <LOD | 6.3 | N/D* |
| Hydroxytyrosol | 781 ± 18.0 | 1890 ± 10.6 | 83 |
| Tyrosol | 10.7 ± 0.2 | 36.3 | 109 |
| Elenolic acid | 198 ± 9.6 | 268.8 ± 0.5 | 31 |

**N/D – no data: due to some values being <LOD no percentage difference could be determined.*

A study by Al-Rimawi *et al*. (2015)[140] who studied the effect of oleuropein content in dried leaf extracts had opposing results to this research. Drying olive leaves at an ambient temperature (25 °C) resulted in a higher recovery of oleuropein than at a temperature of 50 °C. However, caution should be considered when interpreting these results because the methods used to prepare the leaves likely released the endogenous enzyme; dried leaves were ground to a powder and left in a brown paper bag for an unspecified amount of time. This may have activated the endogenous enzyme β-glucosidase and hydrolysed oleuropein to oleuropein aglycone which can undergo further hydrolysis to hydroxytyrosol and elenolic acid. Two further studies reported a higher concentration of phenolic compounds in leaves dried at RT. Cör Andrejč *et al*. (2022)[139] air dried leaves from two different cultivars at RT for 10 days (in the dark) and 105 °C for 90 minutes. The content of oleuropein decreased

when drying at 105 °C (707 mg/kg) versus room temperature (3522 mg/kg). Similar results were obtained for ligstroside (RT, 80 mg/kg vs. 105 °C, 18 mg/kg). Afaneh *et al*. (2015)[140] reported that drying leaves at RT was more favourable in preserving oleuropein content compared to the leaves dried at 50 °C. In another study by Hata *et al*. (2004)[261] dried leaves at varying temperatures ranging from 25 °C to 105 °C. In the results, drying leaves at RT (25 °C) resulted in a higher concentration of oleuropein compared to drying at 70 °C. Interestingly, drying at a temperature >80 °C resulted in an increase in oleuropein concentration. This study suggested that oleuropein is trapped in compartments based on mechanisms understood in the privet tree, the same family as olive (Oleaceae). Heat stress at higher temperatures (between 65 and 80 °C) results in the compartments being broken, where oleuropein can be degraded through endogenous enzymes. Temperatures >80 °C still destroy these compartments, however, the higher temperature would deactivate the endogenous enzymes. Şahin *et al*. (2018)[135] investigated different drying temperature and time (50, 80, and 100 °C at times of 270, 75, and 45 minutes, respectively) on oleuropein concentration. They reported an increase in the quantity of oleuropein at a higher temperature for a shorter period of oven drying. Similar results were achieved in two separate studies investigating total phenolic and oleuropein content albeit longer drying times; RT-10 days, 60 °C-48 hours, 105 °C-3 hours[255] and RT, 60, 130 °C (24 hours).[262] These results have been attributed to the high temperatures denaturing the enzymes such as β-glucosidase, minimising the degradation of oleuropein.[262,263]

In all instances, the results from literature contradict the results obtained in this experiment, where drying at 70 °C obtained more oleuropein and ligstroside compared to drying at RT. In future, it would be beneficial to conduct an experiment at a drying temperature >100 °C. In this experiment, achieving higher oleuropein content at 70 °C could be explained by the period leaves spent drying. Leaves oven dried at 70 °C were left for 3 days while leaves dried at RT were left for 14 days. Drying at RT for a long period of time may have resulted in endogenous β-glucosidase having more time to convert oleuropein and ligstroside to their respective aglycones. This would also explain why the concentration of oleuropein aglycone is higher in the tea prepared with leaves dried at RT. It has been

previously suggested that exposure to atmospheric oxygen for a longer period of drying time may reduce the concentration of phenolic compounds via nonenzymatic oxidation.[135,264]

In both drying experiments (dried at RT vs. fresh and oven dried at 70 °C vs. dried at RT), OLT made with leaves dried at RT resulted in an increased concentration of hydroxytyrosol, tyrosol and elenolic acid. Native hydroxytyrosol, tyrosol and elenolic acid are rarely in their free form, and their formation requires enzymatic ester hydrolysis. Hydrolysis of oleuropein aglycone forms hydroxytyrosol and hydrolysis of ligstroside aglycone forms tyrosol, while elenolic acid is a hydrolysis product of both analytes.[265] In both experiments, the length of drying at RT (>5 days) was likely enough time for the formed aglycones to be further enzymatically hydrolysed into their respective derivatives: hydroxytyrosol, tyrosol and elenolic acid.[265] Cör Andrejč *et al*. (2022)[139] also reported a higher concentration of hydroxytyrosol in extracts with leaves dried at RT (814 mg/kg) compared to 105 °C (25 mg/kg). Hydroxytyrosol and tyrosol had the largest percentage difference between the two temperature conditions for each experiment (see right hand column of Table 4.3 and Table 4.4) where drying at RT increased their concentrations. The largest difference between drying conditions was observed for tyrosol, with the first (RT vs. fresh) and the second (70 °C vs. RT) experiment resulting in a 170% and 109% difference, respectively. In this research, a higher concentration of oleacein and oleocanthal was obtained in the OLT made with leaves dried at RT, where leaves used fresh, and oven dried at 70 °C resulted in concentrations <LOD. As discussed in Chapter 1, Section 1.4.2.1, these analytes are formed as a result of enzymatic (esterase) hydrolysis of the methyl esters on oleuropein aglycone and ligstroside aglycone followed by decarboxylation.[43,65] Therefore, the longer periods dried at RT likely gave the endogenous enzymes time to convert oleuropein and ligstroside aglycone to these derivatives.

The differences achieved in the second drying experiment (RT drying vs. oven drying at 70 °C) compared to literature suggest more thorough investigations need to be undertaken to understand the effect of time and temperature on the phenolic content during olive leaf

drying. It is evident there are multiple factors that affect the phenolic content of OLT and extracts. It has been argued by Kamran *et al*. (2015)[255] that changes in phenolic content are both plant and tissue specific and the assumption that one drying method suits all plants or all tissue from the same plant should be renounced.[255]

### 4.3.3 Summary of drying conditions on the composition of OLT

The content of oleuropein in OLT made from two different batches of leaves increased under different drying conditions in the order fresh<RT<70°C while other target analytes tended to increase with drying at RT. Results from the second experiment (leaves dried at RT vs. oven dried at 70 °C) were in contrast to the literature but this was likely related to the time left drying. Further experiments would need to be carried out to optimise this step; however, a preliminary understanding of the important considerations when preparing OLT have been established.

## 4.4 Stability of analytes in olive leaf tea

The stability of oleuropein and other target analytes in OLT was important to investigate as it would determine if new batches of OLT would need to be made frequently when preparing for the enrichment of OOs commercially. There is also ambiguity on oleuropein stability in the literature and many of the target analytes (such as ligstroside, oleocanthal, oleacein, oleuropein aglycone and elenolic acid) have not been studied extensively. During preparation of OLT (Chapter 2, Section 2.6.2), the leaves were boiled for an extensive period of time (30 min) theoretically inactivating the endogenous enzymes which would prevent the hydrolysis of oleuropein.[261] One batch of OLT3-2-23 (oleuropein content of 343 mg/kg) was stored at various temperatures (−20, RT and 40 °C) and periodically analysed over time (up to 189 days). Unfortunately, there was a 29-day gap between the first and second analysis point. Hence, a second trial (89 days) was set up to obtain more data points within the first month of storage and was used when required to confirm patterns observed in the first trial. The following results and discussion primarily use data from storage trial 1; the text will specify if trial 2 data is used.

Analysis of analytes in this section was carried out using the average of the duplicate sample results. In most instances the error (± SD) between measurements is small so error bars are not visible for some samples; an example of the variation between duplicates for oleuropein is shown in Figure 4.2. Therefore, error bars have been excluded from the plots in the following section. Stability plots with error bars for the rest of the target analytes have been included in Appendix A.

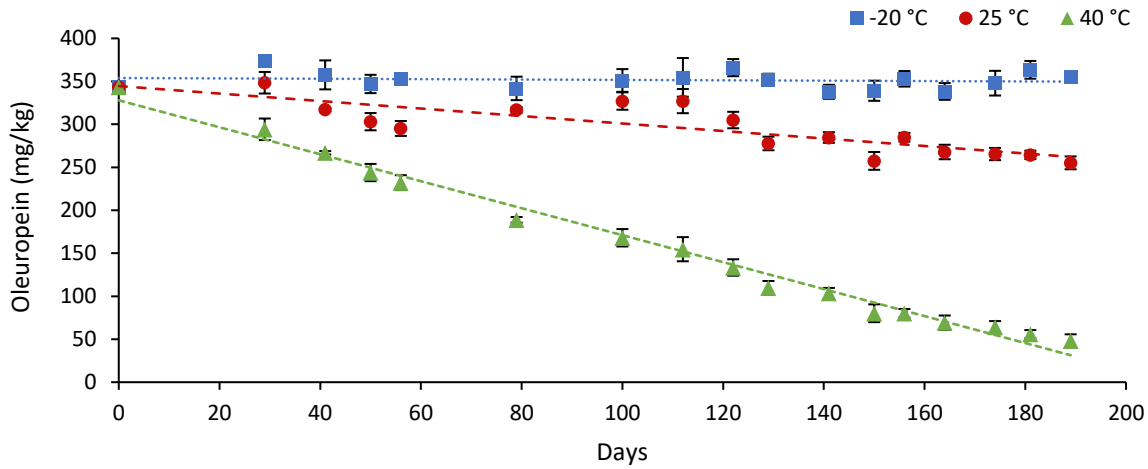



**Figure 4.2. Concentration of oleuropein versus time for stability samples stored at −20 °C (blue square), 25 °C (RT, red circle) and 40 °C (green triangle) for 189 days. The error bars are set to the SD of duplicate samples thus results are presented as the average ± SD (*n* = 2).**

### 4.4.1 Oleuropein and ligstroside

The stability of oleuropein and ligstroside was comparable under different temperature storage conditions, Figure 4.3. The initial concentration of oleuropein was 343 ± 6 mg/kg and remained stable when stored at −20 °C with an average of 351 ± 10 mg/kg after 189 days. The concentration of oleuropein in OLT stored at RT and 40 °C decreased over time, with a larger decrease at higher temperatures. At RT, the concentration reduced from 343 to 255 mg/kg, a 26% decrease, after 189 days. At 40 °C, oleuropein decreased 29% within 50 days and was reduced to 56 mg/kg after 189 days (84% decrease).

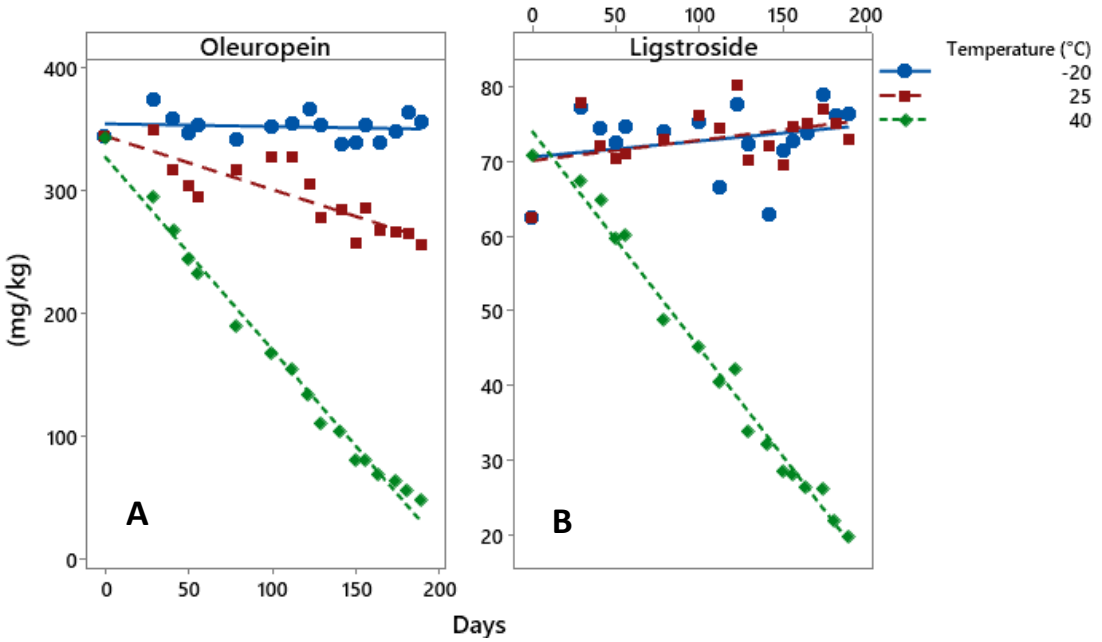



**Figure 4.3. Concentration of A) oleuropein and B) ligstroside versus time for stability samples stored at −20 °C (blue circle), 25 °C (RT, red square) and 40 °C (green diamond) for 189 days. Results are presented as the average (*n* = 2).**

With the inactivation of endogenous enzymes, oleuropein likely underwent degradation at 40 °C in the forms of oxidation, cleavage of covalent bonds or active groups undergoing side reactions such as ring closer and polymerisation.[266] These stability results contrast the literature. A study by Malik *et al*. (2008)[262] investigated the stability of oleuropein content at RT. There was a decline in oleuropein after 7 days in storage. Martínez-Navarro *et al*. (2021)[210] tested the stability of oleuropein in an aqueous extract at six temperatures, two of which were used in this research (RT and 40 °C). Their stability trials ran for 60 days and samples stored at 40 °C decreased from 300 mg/L to 50 mg/L (83% decrease) within 40 days of storage, this trend was also observed for samples stored at RT. This is a much larger percentage decrease compared to the results obtained in this research. The enhanced stability of oleuropein in this research could relate to its preparation. In the first study discussed, leaves were covered in boiling water for 10 minutes. In the second study, the method dried leaves, made them into a powder and extracted them with distilled water in a domestic microwave for 30 seconds. These methods are significantly shorter and the latter is more intense than the method used in this research (leaves steeped in boiling water

for 30 minutes). The differences in preparation could possibly contribute to the discrepancies in oleuropein stability. Interestingly, a study by Ahmad-Qasem *et al*. (2016)[136] investigated the effect of leaf preparation (fresh, freeze dried and hot air drying at 120 °C) on the stability of extracts extracted with ethanol/water (80:20 *v*/*v*). The leaf extracts from different preparations were stored at 4 °C for 28 days and extracts subjected to the same drying conditions were placed in different storage temperatures (4, 25 and 35 °C, 28 days). In both instances, they reported no significant decrease appreciated for oleuropein regardless of how the raw material was processed or stored. However, the period extracts were left in storage may not have been long enough to observe any changes in oleuropein concentration. In this research, analysis of samples on day 29 showed similar results with a small decrease in oleuropein content at 40 °C (Figure 4.4). However, longer storage times (>29 days) at elevated temperatures (40 °C) did result in a decrease in oleuropein content. In future, it would be beneficial to extend a storage trial with OLTs prepared differently to better understand how this may alter analyte stability.

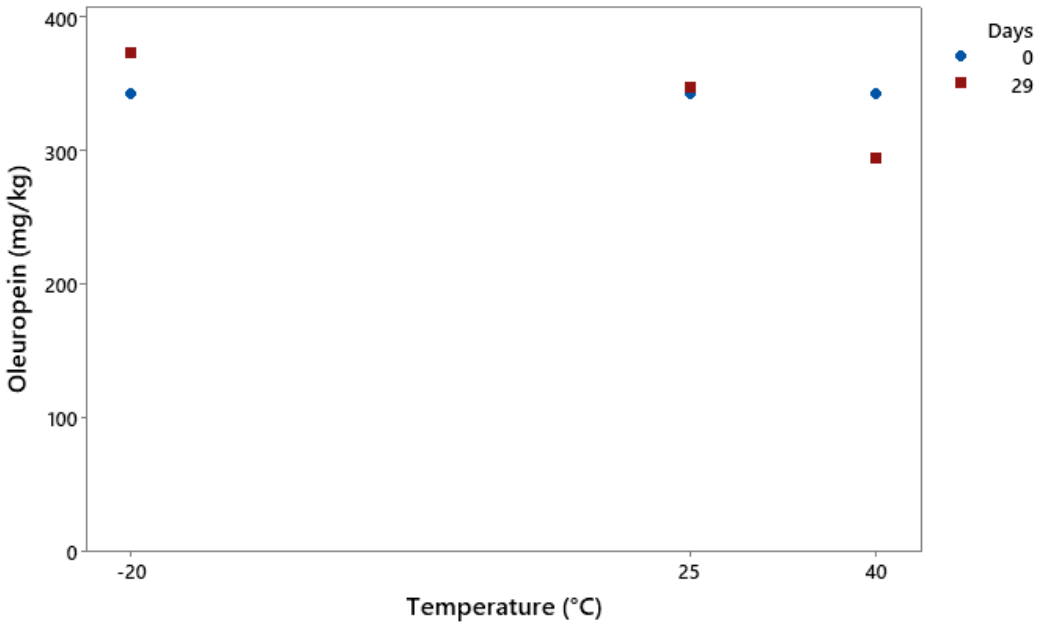



**Figure 4.4. Content of oleuropein (mg/kg) in OLT stored at −20, RT and 40 °C for 29 days. Results are presented as the average (*n* = 2).**

To determine the reaction order for oleuropein degradation at 40 °C, zero-order ([oleuropein] vs. time), first-order (ln[oleuropein] vs. time), and second-order (1/[oleuropein]) plots were created (Figure 4.5).

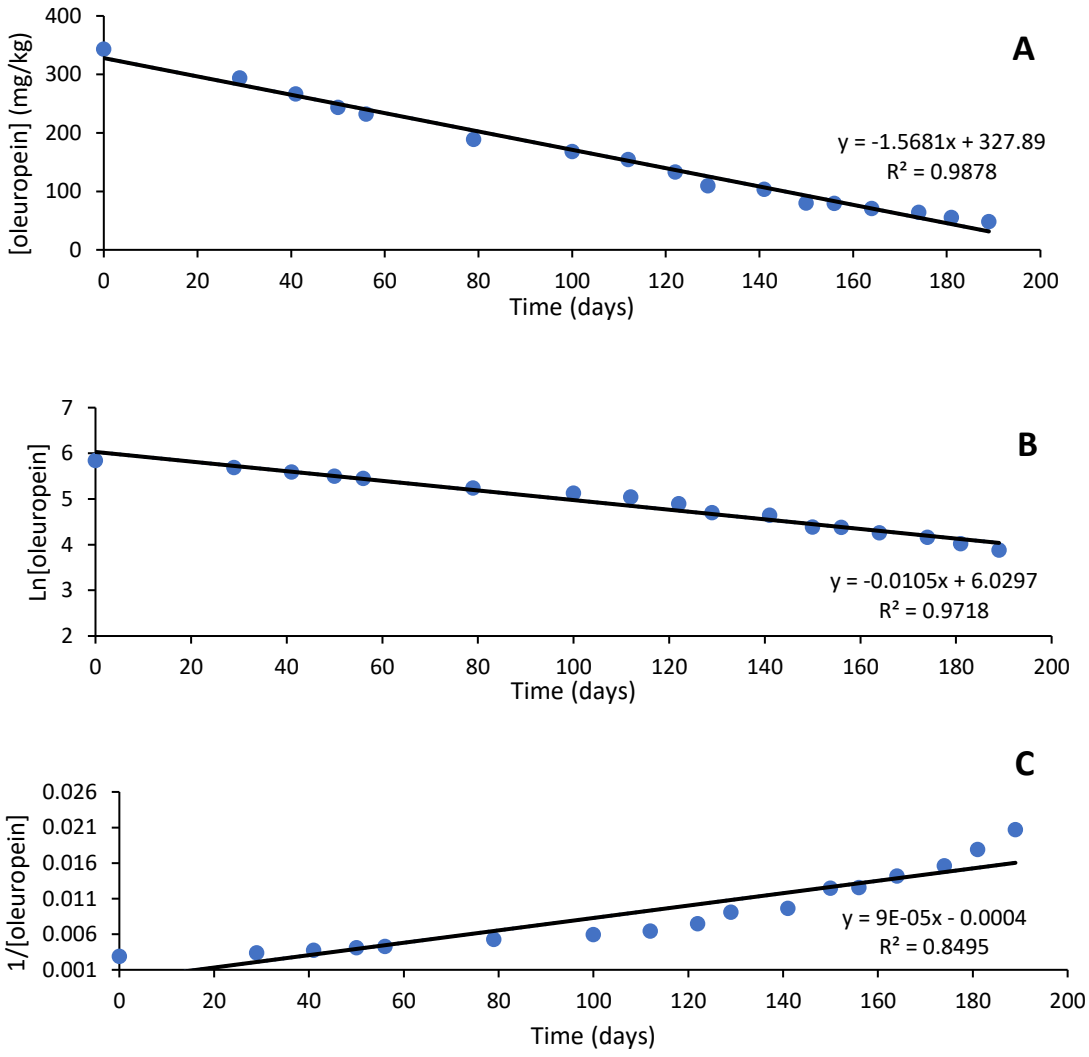



**Figure 4.5. Reaction order plots for oleuropein in OLT stored at 40 °C. The zero-order (A. [oleuropein] (mg/kg) vs. time), first-order (B. ln[oleuropein] vs. time) and second-order (C. 1/[oleuropein] vs. time) plots with first and second-order plots deviating from linearity.**

A plot that describes a particular order properly will be linear when the data is fitted. Oleuropein content behaved by adjusting to a zero-order kinetic model at 40 °C and

deviated from linearity for the first-order and second-order plots. Oleuropein fitting a zero-order kinetic model was unexpected. A zero-order reaction rate is constant over time and independent of reactant concentration; however, the half-life ($t_{1/2}$) depends on the initial concentration as it is the time required for the concentration to decrease by half. For a first-order reaction, the reaction rate linearly depends on the concentration of one reactant; however, the half-life is constant throughout the reaction. By these definitions, it is unlikely the degradation of oleuropein is zero-order and is more likely to be first-order where the rate is dependent on only one reactant (oleuropein). If the stability trial had continued for more days, it is likely that linearity for the first-order plot would have occurred. It has been previously reported in the literature that thermal degradation of oleuropein in olive leaf extracts fits a first-order kinetic model.[210,267,268] Because of this ambiguity, calculating a half-live for zero-order degradation was not carried out. Malik *et al*. (2008)[262] carried out a kinetic study for the degradation of oleuropein at 40 °C and RT. They reported short first-order kinetic half-lives of 10 and 13 days, respectively.

As mentioned, the stability of ligstroside behaved in a similar way to oleuropein, likely due to similarities in structure, with ligstroside containing a hydrogen instead of a hydroxyl group. At 40 °C, the initial concentration reduced from 71 to 23 mg/kg (68% decrease) after 189 days. Contrasting oleuropein stability at RT, ligstroside tended to remain stable at RT and −20 °C with an average concentration of 73 ± 4 mg/kg and 73 ± 5 mg/kg, respectively after 189 days. The decreased stability of oleuropein at RT is likely related to its structure. Oleuropein is an *ortho*-diphenolic compound, these compounds are deemed more susceptible to chemical oxidation. The disappearance of ligstroside from OLT at a storage temperature of 40 °C also showed linearity for the zero-order plot (Figure 4.6); however, as with oleuropein, zero-order degradation was unlikely. Thus, no half-lives were calculated.

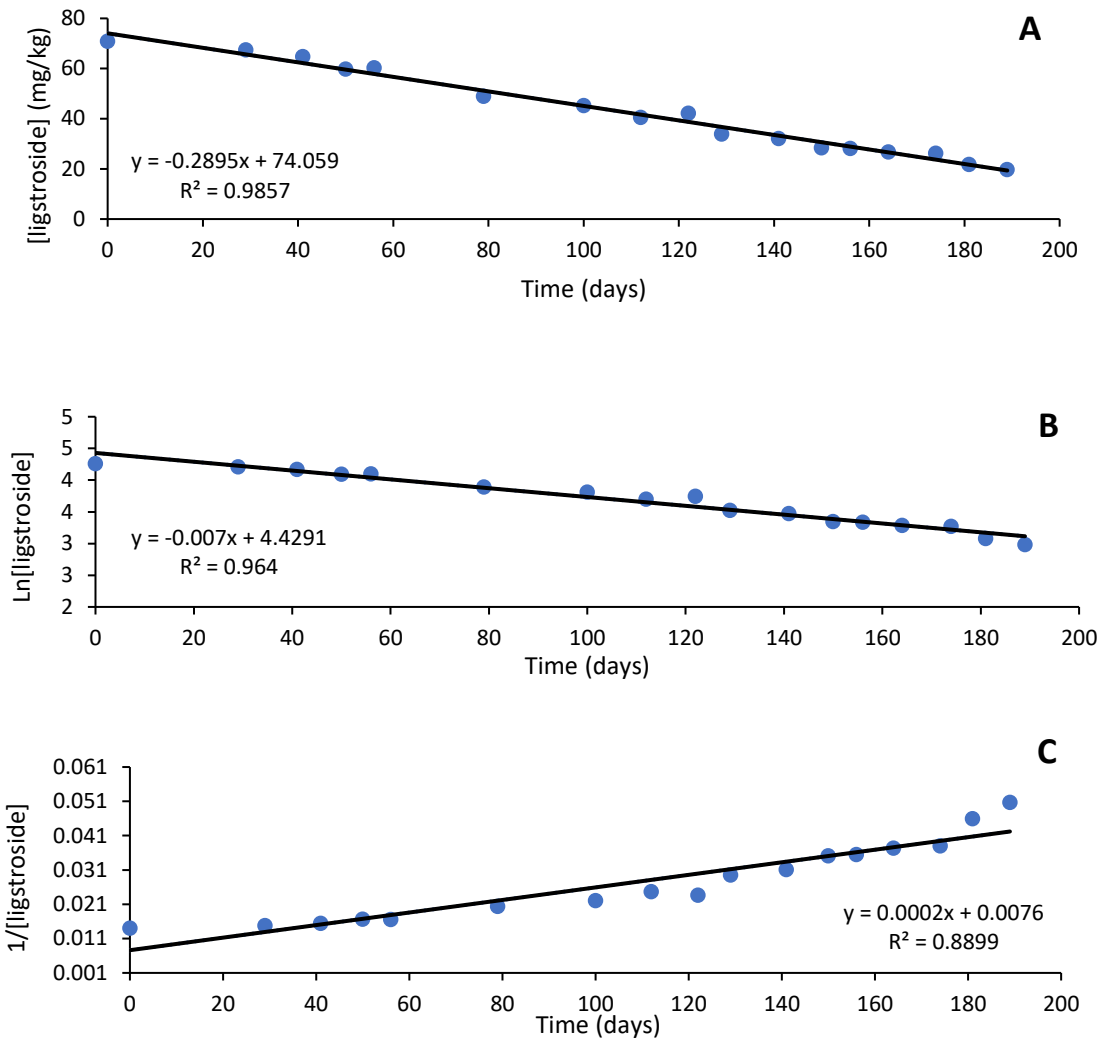



**Figure 4.6. Reaction order plots for ligstroside in OLT stored at 40 °C. The zero-order (A. [ligstroside] (mg/kg) vs. time), first-order (B. ln[ligstroside] vs. time) and second-order (C. 1/[ligstroside] vs. time) with first and second-order plots deviating from linearity.**

### 4.4.2 Oleuropein aglycone

Oleuropein aglycone is formed from the degradation of oleuropein. There is no literature on oleuropein aglycone stability in aqueous samples as it is a hydrophobic compound. However, it was measured in stability samples in this research; this could be a result of the OLT containing other components from olive leaf material which altered the teas chemical composition. The initial concentration of oleuropein aglycone in OLT was 14 mg/kg. At

−20 °C, there was no obvious change in the concentration with an average of 14 ± 2 mg/kg for 189 days (Figure 4.7). This was expected as its precursor, oleuropein, remained stable at −20 °C. At RT, the concentration of oleuropein aglycone reduced from 14 mg/kg to 7 mg/kg, a 50% decrease after 189 days. At 40 °C, there was an increase to 26 mg/kg on day 79, then a reduction to 13 mg/kg on day 189. As mentioned in Section 4.4.1, oleuropein underwent thermal degradation at 40 °C which would have resulted in the initial increase in oleuropein aglycone. After day 79, it is likely oleuropein aglycone degraded into its respective derivatives; oleacein, hydroxytyrosol or elenolic acid. Oleacein and elenolic acid did show respective increases but hydroxytyrosol did not (discussed in Section 4.4.4).

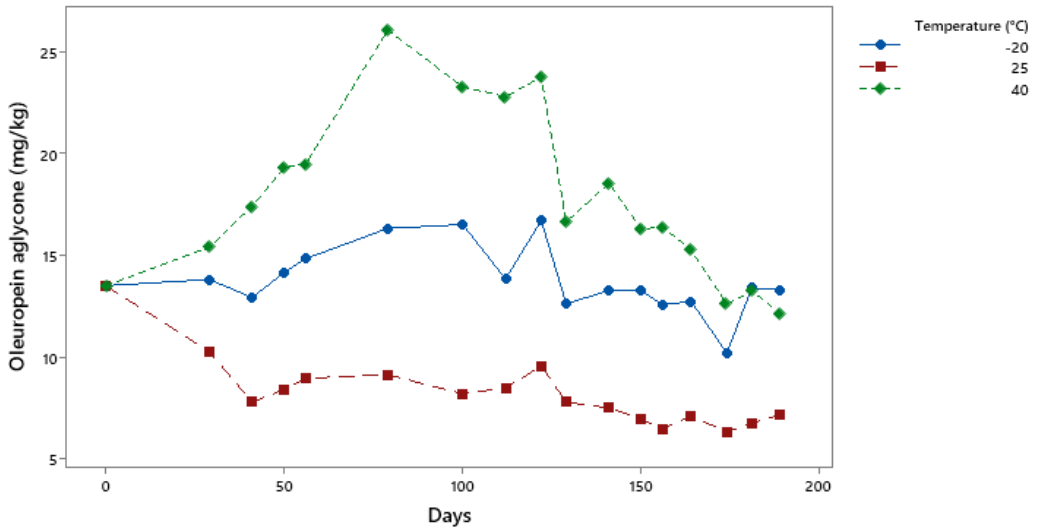



**Figure 4.7. Oleuropein aglycone concentration versus time for stability samples stored at −20 °C (blue circle), 25 °C (RT, red square) and 40 °C (green diamond) for 189 days. Results are presented as the average (*n* = 2).**

### 4.4.3 Oleocanthal and oleacein

At the three storage temperatures, the stability of oleocanthal (produced from ligstroside aglycone) and oleacein (produced from oleuropein aglycone) behaved similarly, Figure 4.8. This was likely due to similarities in structure, with oleocanthal containing a hydrogen instead of a hydroxyl group. At −20 °C, the initial concentration of oleacein was reduced from 221 mg/kg to 158 mg/kg, a 29% decrease after 189 days. At RT, the concentration of

oleacein remained stable with an average of 232 ± 15 mg/kg for 189 days. With regards to oleocanthal, the initial concentration was 16 mg/kg. At −20 °C and RT, the concentration of oleocanthal remained stable with averages of 15 ± 1 mg/kg and 18 ± 2 mg/kg, respectively after 189 days. Treatment at 40 °C resulted in an increase in the concentration of oleocanthal from 16 to 24 mg/kg and oleacein from 221 to 356 mg/kg until day 29 (Figure 4.8 C). Since trial 1 had no further data points between day 1 and day 29, trial 2 data (Figure 4.8 D) was used to confirm if this increase in concentration was legitimate. Corroborating trial 1, trial 2 data also had an increase in concentration around day 30. In trial 1, the large increase in concentration for oleocanthal and oleacein was followed by a reduction in concentration to 6 and 52 mg/kg, respectively.

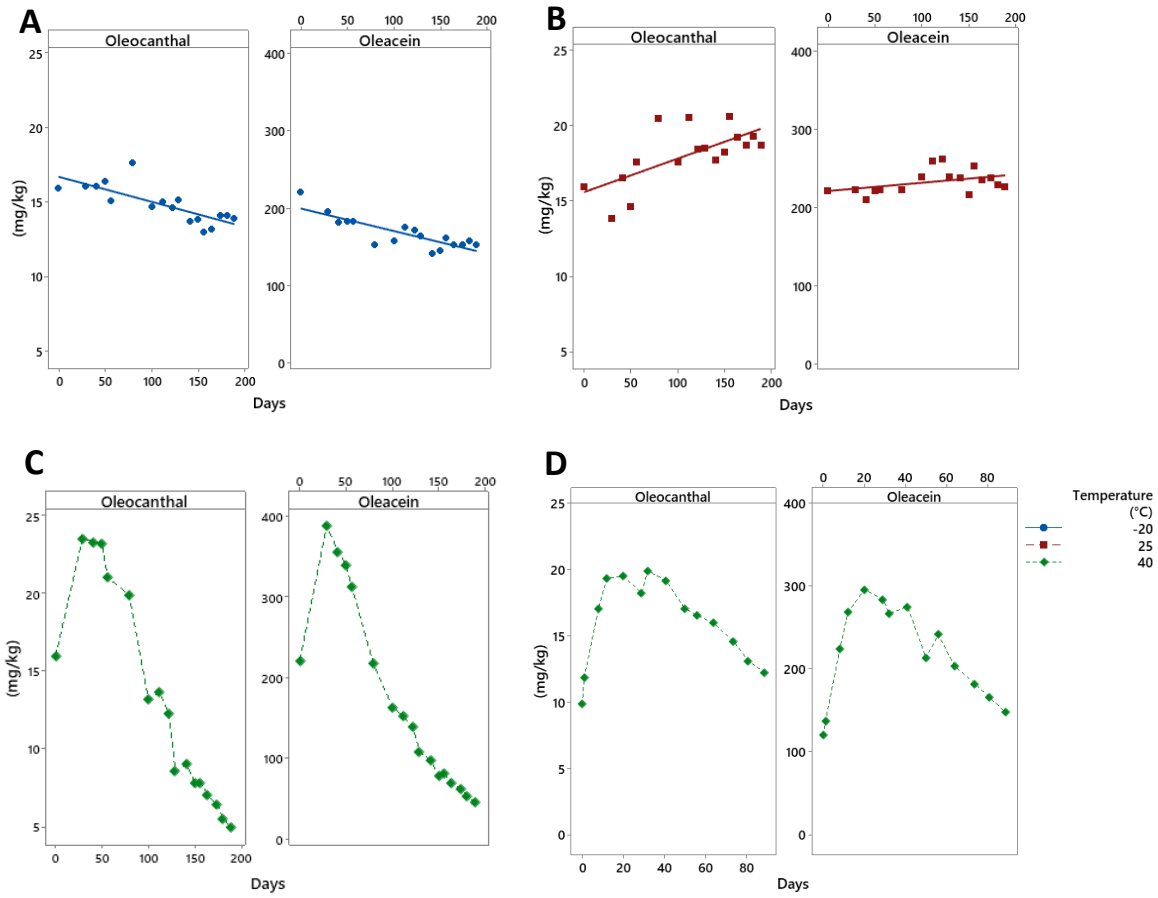



**Figure 4.8. The concentration of oleocanthal and oleacein versus time for stability samples stored at A) −20 °C (blue circles). B) 25 °C (RT, red squares). C) 40 °C (green diamonds). D) Trial 2, 40 °C (green diamonds). Results are presented as the average (*n* = 2).**

There is no research reported in the literature on the degradation of oleocanthal and oleacein in OLT, albeit in OO. Mousavi *et al.* (2021)[269] analysed phenolic compounds in OO at different storage temperatures (−18 °C, 4 °C and ambient). They concluded that oleacein experienced severe decay at −20 °C and oleocanthal remained stable which was also observed in this research. The degradation of oleuropein and ligstroside (Section 4.4.1) and oleuropein aglycone (Section 4.4.2) at 40 °C likely resulted in the initial spike in concentration for oleocanthal and oleacein with stability waning after day 29 at 40 °C. Similar trends during malaxing have been reported by Diamantakos *et al.* (2020)[44] where an increase followed by a decrease were observed. This was especially true for oleacein; an *ortho*-diphenolic compound deemed more susceptible to enzymatic and chemical oxidation. In oil, oxidation products of oleocanthal and oleacein (oleocanthalic and oleaceinic acid) have been determined.[123,270] These oxidation products increase with reducing concentration of oleocanthal and oleacein.[123,270] Measuring the OLT for these respective oxidation products should be investigated in future work.

### 4.4.4 Hydroxytyrosol and tyrosol

The stability of hydroxytyrosol and tyrosol behaved differently (Figure 4.9). Hydroxytyrosol is produced from ester hydrolysis of oleuropein aglycone. It had an initial concentration of 981 mg/kg and remained stable at −20 °C with an average concentration of 1003 ± 66 mg/kg for 189 days. At RT and 40 °C, hydroxytyrosol content in OLT was reduced from 981 mg/kg to 754 and 767 mg/kg (decreases of 23% and 22%), respectively after 189 days. These results contrast some studies in literature; Martínez-Navarro *et al*. (2021)[210] reported oleuropein degradation generated other compounds such as hydroxytyrosol in the first week of storage at RT. This was corroborated by Feng *et al*. (2021)[271] who observed an increase in the concentration of hydroxytyrosol in olive leaf methanol-water extracts in the first week of storage (25 °C) then noted a decrease after two weeks. A study by Ramírez *et al.* (2016)[37] contradicted the above studies and experienced similar trends to this research, although a different matrix; olive fruit in brine solution stored at 10, 22, and 40 °C. They observed a decrease in hydroxytyrosol concentration after 1.5 months (46 days) when

incubated at 40 °C, suggesting that the degradation or oxidation of hydroxytyrosol occurred at this high temperature.[37]

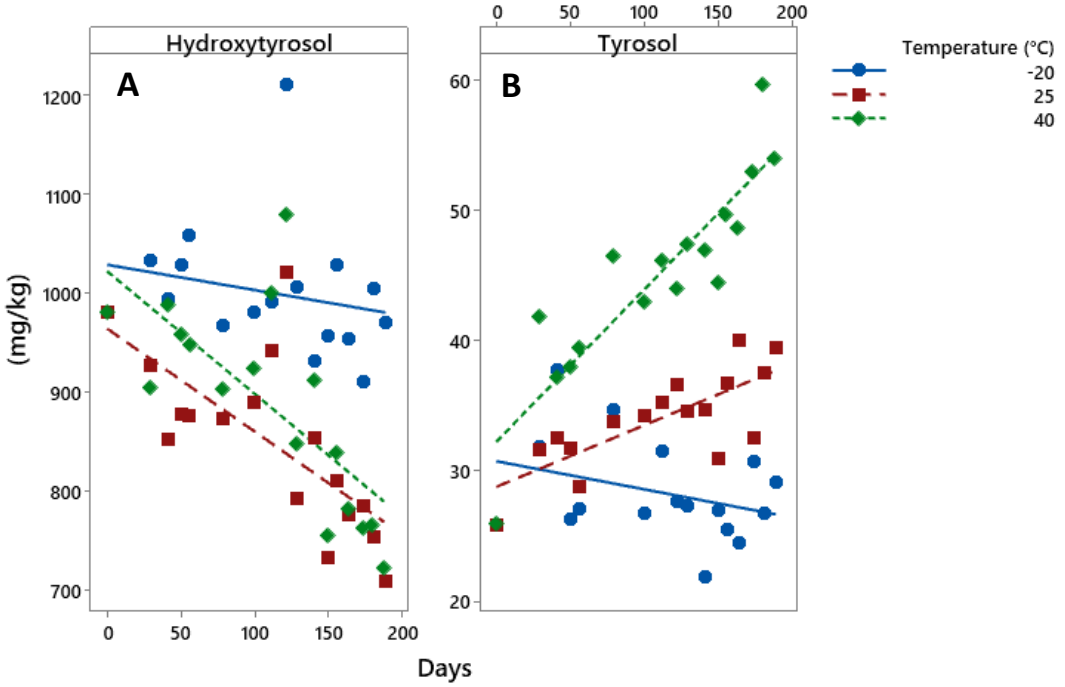



**Figure 4.9. The concentration of A) hydroxytyrosol and B) tyrosol versus time for stability samples stored at −20 °C (blue circle), 25 °C (RT, red square) and 40 °C (green diamond) for 189 days. Results are presented as the average (*n* = 2).**

Tyrosol, which is produced from ester hydrolysis of ligstroside aglycone, displayed similar behaviour at −20 °C. It had an initial concentration of 26 mg/kg and remained stable at −20 °C with an average of 28 ± 4 mg/kg for 189 days in storage. The concentration of tyrosol at RT and 40 °C increased over time, with a larger increase at higher temperatures; the content increased from 26 mg/kg to 38 and 59 mg/kg (increases of 45% and 130%), respectively after 189 days. Ligstroside degraded quickly at 40 °C (Section 4.4.1), thermal degradation of the formed ligstroside aglycone (which was not analysed in this research) would have increased the concentration of tyrosol where it then remained stable. Tyrosol is known to be very stable compared to other phenolic compounds because it is less likely to be subjected to auto-oxidation.[272]

The patterns observed between hydroxytyrosol and tyrosol in this research are corroborated by Yalcin *et al.* (2018).[273] This study tested the oxidative stability of both analytes in OO (different matrix). Tyrosol was more stable against oxidation at elevated temperatures (60 °C) compared to hydroxytyrosol.[273] They suggested hydroxytyrosol provided a high oxidative stability to OO at 60 °C, while tyrosol has less antioxidant effects. Gómez-Alonso *et al.* (2002)[274] reported that the high antioxidant activity of hydroxytyrosol favours its degradation and disappearance. This was also reported in another study where hydroxytyrosol was reduced during accelerated storage (60 °C) while tyrosol showed high stability against oxidation.[275]

### 4.4.5 Elenolic acid

Elenolic acid is a non-phenolic derivative of both oleuropein aglycone and ligstroside aglycone. The initial concentration of elenolic acid was 475 mg/kg. At −20 °C, the concentration remained stable with an average of 461 ± 40 mg/kg for 189 days (Figure 4.10). At RT, the concentration decreased from 475 mg/kg to 246 mg/kg until day 79, where the data began to plateau, averaging 273 ± 18 mg/kg between day 79 and 189. At 40 °C, the content of elenolic acid increased from 475 mg/kg to 611 mg/kg (29% increase) after treatment for 189 days. This likely occurred because oleuropein aglycone (precursor) underwent thermal degradation after day 79 at 40 °C. As a result, the concentration of elenolic acid began to increase at this same period of time. Therefore, there are larger quantities of elenolic acid at this temperature (40 °C) compared to −20 °C and RT where oleuropein aglycone remained stable and decreased, respectively.

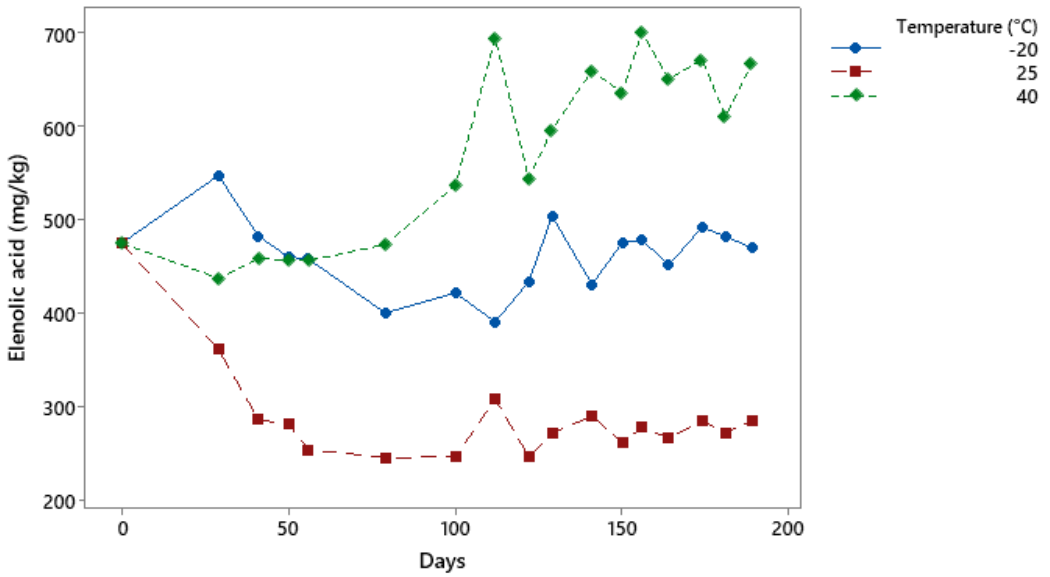



**Figure 4.10. The concentration of elenolic acid versus time for stability samples stored at −20 °C (blue circle), 25 °C (RT, red square) and 40 °C (green diamond) for 189 days. Results are presented as the average (*n* = 2).**

### 4.4.6 Summary of OLT stability trials

There is currently little work in the literature on the stability of target analytes in OLT. Results of the stability trial at different temperatures (−20, RT, 40 °C, 189 days) have determined that the majority of target analytes in OLT were stable at −20 °C and RT, with precursors such as oleuropein and ligstroside degrading at 40 °C. Consequently, this resulted in increases of their respective derivatives, where unstable derivatives such as oleocanthal and oleacein further degraded. These results are useful as it allows producers of OLT to understand how long teas can be stored without influencing the concentration of target analytes.

# CHAPTER 5

# Enrichment of Olive Oil with Oleuropein Aglycone using Olive Leaf Tea

## 5.1 Introduction

This chapter explores the experiments undertaken to investigate the use of almond flour/meal as an enzymatic source of β-glucosidase to hydrolyse water-soluble oleuropein glycoside present in OLT to oil soluble oleuropein aglycone to later transfer into OOs (refined OO and EVOO). To understand the results discussed, Figure 5.1 has been generated to show the solubility of target analytes in this research. Oleuropein, a glycoside, is water soluble (hydrophilic) due to the presence of a glucose moiety with hydroxyl groups, this increases the structures polarity.[33,34] The polarity is further enhanced as the aglycone moiety has additional polar groups; two carbonyl groups and additional hydroxyl groups.[34] The removal of the polar glucose moiety through hydrolysis results in an oil soluble, hydrophobic compound; oleuropein aglycone.[38,39]

Originally, the aim of this project was to enrich OOs with oleuropein aglycone to levels at or above the EU health claim (250 mg/kg) to aid the development of a marketable product for a company. This was going to be done by investigating the use of almond flour/meal as a source of β-glucosidase to hydrolyse oleuropein in OLT; the resulting aglycone could then be transferred into OO. While initial investigations proved almond flour/meal was an effective source of enzyme (to be discussed), the transfer of oleuropein aglycone into oil was discovered to be complex and there is little currently known about this process. Therefore, the aims of this research were altered; experiments were carried out to understand why the combined matrix of OLT, oil and almond resulted in minimal mass transfer of oil soluble aglycone into oil.

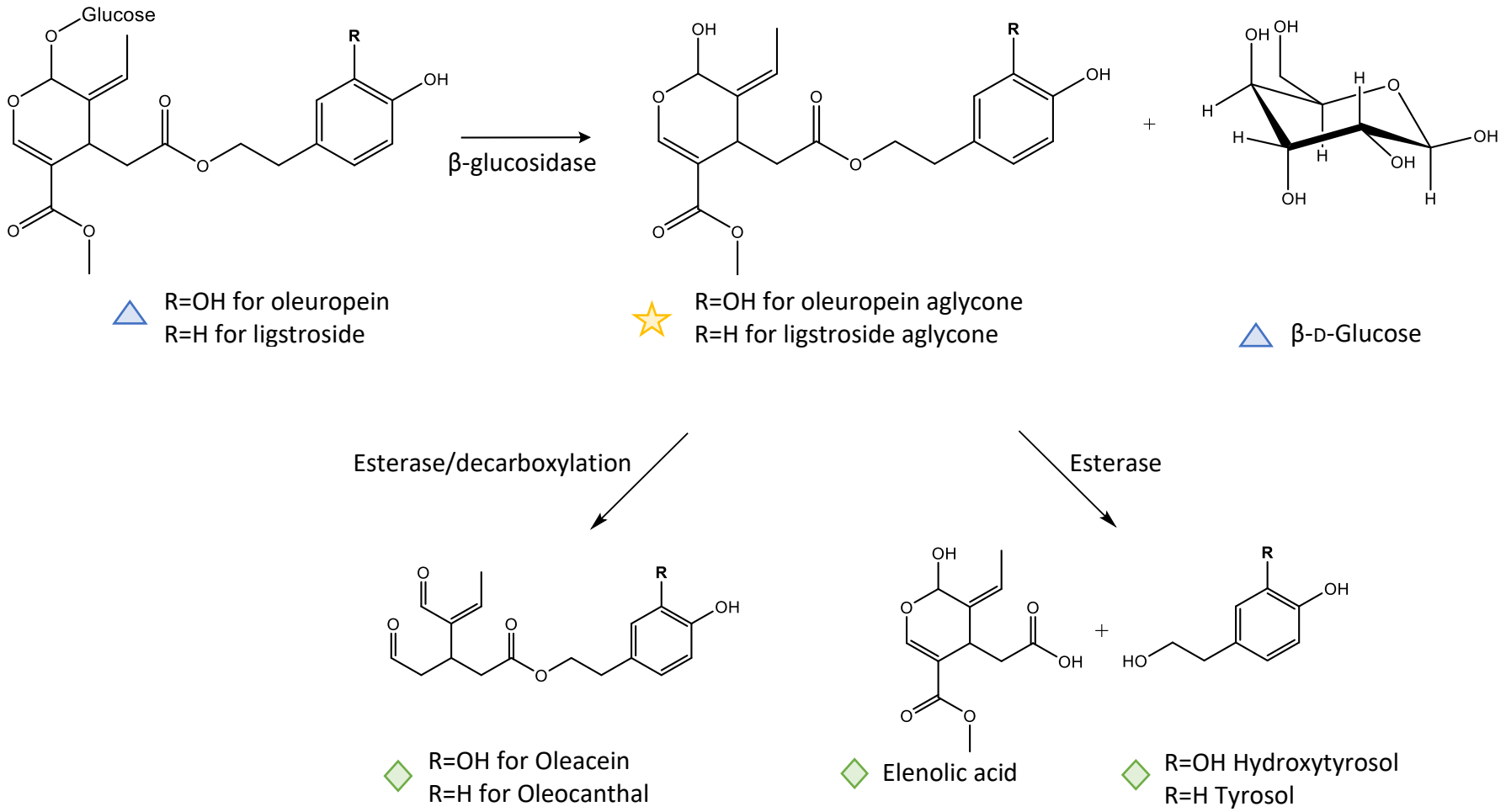



**Figure 5.1. Hydrolysis pathway of olive phenolics initially via β-glucosidase. The blue triangle denotes analytes that are water soluble, the yellow star denotes oil soluble analytes and the green diamonds denote analytes that are both oil and water soluble.**

## 5.2 Almond flour/meal as a source of β-glucosidase

It was important to establish the viability of β-glucosidase from almond flour (blanched and skinned almonds) and almond meal (not blanched or skinned) to hydrolyse oleuropein present in OLT as it is currently unexplored in literature. As discussed in Chapter 1, Section 1.5.1, there are a variety of endogenous and exogenous sources of β-glucosidase. Olive leaves are an endogenous source of β-glucosidase; however, there is minimal control over the hydrolysis of oleuropein to oleuropein aglycone which is commercially undesirable. Alternatively, the addition of an exogenous source of β-glucosidase such as almond would provide more control over the reaction to obtain a known amount of oleuropein aglycone which could be transferred into an oil at a later time. As discussed in Chapter 1, Section 1.5.1.1, there are several sources of exogenous β-glucosidase that can successfully hydrolyse oleuropein, most commonly from almonds. Extracted and purified β-glucosidase from almonds is commercially available from Merck; however, it is costly (1000 units for $2,540.00) and not suitable for use in edible products. In the literature, there are methods to extract β-glucosidase from almonds but most extractions use bitter almonds which are not suitable for human consumption.[81] Extracting the β-glucosidase from raw sweet almond meal/flour is also possible but requires purification and utilises chemicals; buffer solutions, polyvinyl pyrrolidone, ammonium sulfate and ammonium hydroxide.[81-84] In this research, the use of such chemicals would make the enriched oil inconsumable. Hence readily available unprocessed sweet almond flour/meal and almond milk were investigated as a source of β-glucosidase to hydrolyse oleuropein. This has not been previously studied in the literature.

This section will discuss experiments undertaken to confirm the viability of almond flour/meal as a source of β-glucosidase, methods to inactive the enzyme and explore how the age of almond flour/meal used may result in differing levels of enzymatic activity.

### 5.2.1 Hydrolysis of oleuropein with almond flour/meal

Almond flour was investigated as a source of β-glucosidase to hydrolyse oleuropein in OLT. It was unspecified whether almond flour purchased from local supermarkets would contain active β-glucosidase. Since ligstroside is a glycoside and naturally present in OLT, it was also used as an indicator for successful hydrolysis. The literature reports a variety of optimum working temperatures for β-glucosidase to hydrolyse glycosides.[276-279] Some are relatively thermostable, withstanding temperatures of 60 °C.[276] However, a temperature of 40 °C was chosen as this is often considered the optimum temperature in the literature.[277-279]

OLT15-2-23 and OLT3-2-23 were used as source of oleuropein and ligstroside, experiments undertaken in this section followed methods described in Chapter 2, Section 2.10.2, using a temperature of 40 °C with a longer incubation period of 30 minutes and 12 hours (in later experiments method parameters where modified based on results achieved). For OLT3-2-23, the amount of almond flour added was scaled up 4x as it was undetermined how much β-glucosidase was present. Table 5.1 summarises results for two different incubation periods (30 minutes and 12 hours). Almond flour (100 mg) had enough β-glucosidase activity to hydrolyse the oleuropein and ligstroside present in OLT3-2-23 and OLT15-2-23 (1 mL) at 40 °C after 30 minutes. OLT3-2-23 had a lower amount of oleuropein prior to hydrolysis (342 mg/kg) so the amount of almond flour was reduced to 25 mg and almost complete conversion was achieved overnight (oleuropein decrease of 320 mg/kg). Thus, this amount of almond flour was used in subsequent experiments. Since OLT15-2-23 had more oleuropein (3668 mg/kg), the amount of almond flour was kept at 100 mg which resulted in a decrease of 3627 mg/kg overnight (12 hours). From a commercial perspective, using a minimum quantity of almond flour/meal is important to reduce the cost of production. A further reduction in the amount of almond flour could be investigated as part of future experimental development. However, for the purposes of this research, a suitable decrease in oleuropein was observed with 25 and 100 mg of almond flour.

**Table 5.1. Change in oleuropein and ligstroside concentration in OLT3-2-23 and OLT15-2-23 using almond flour (25 and 100 mg) as a source of β-glucosidase at 40 °C, after 30 minutes and 12 hours. Results are presented as the average of the duplicate samples ± SD (*n* = 2).**

| | Control OLT | | 30 minutes, 40 °C | | 12 hours, 40 °C | |
|---|---|---|---|---|---|---|
| **Analyte** | **OLT3-2-23** | **OLT15-2-23** | **OLT3-2-23** | **OLT15-2-23** | **OLT3-2-23** | **OLT15-2-23** |
| | | | **100 mg** | **100 mg** | **25 mg** | **100 mg** |
| | **mg/kg** | | **Δ mg/kg** | | | |
| Oleuropein | 342 ± 6 | 3668 ± 29 | −207 ± 8 | −1714 ± 4 | −320.0 ± 0.4 | −3627.1 ± 0.3 |
| Ligstroside | 64 ± 9 | 585 ± 26 | −44 ± 2 | −315 ± 16 | −59.5 ± 0.1 | <LOD |

## 5.2.2 Methods to inactivate β-glucosidase

With the hydrolysis of oleuropein successful, it was important to establish a way to halt the conversion from β-glucosidase during experiments. There were several options: heating the enzyme at a high temperature (100 °C) to denature it, adding in solvent such as MeOH or freezing. Freezing often slows the enzyme down but does not inactivate it and when heated back to a desired temperature, normal enzymatic activity resumes so was not investigated further.[255,280]

***Heat inactivation***

Preliminary results (*n* = 1) of heat inactivation are summarised in Table 5.2. There is little difference between the decrease in concentrations for samples that were not heat treated (356 mg/kg) versus heat treated (359 mg/kg). This is likely due to the inadequate submersion period at 100 °C. There were also issues with this form of inactivation; there was an immense pressure build up in the vessel which caused the lid to open resulting in sample loss, it was also difficult to determine if the internal temperature of the vessel had surpassed 100 °C. Therefore, solvent inactivation of β-glucosidase was investigated.

**Table 5.2. Change in target analyte concentration using heat inactivation for β-glucosidase. Results are presented as the average of dilutions undertaken ± SD (*n* = 1). Some samples have no error due to one dilution result being used.**

| Analyte | OLT (1mL) and almond flour (100 mg) | | |
|---|---|---|---|
| | OLT control | Addition of enzyme – not heat treated | Addition of enzyme – heat treated |
| | mg/kg | Δ mg/kg | |
| Oleuropein | 373 ± 6 | −356 | −359 |
| Ligstroside | <LOD | <LOD | <LOD |
| Oleuropein aglycone | 13.5 ± 2 | −2.4 ± 1.4 | −4.2 ± 1.4 |
| Oleacein | 197 ± 3 | −183 ± 3 | −187 ± 4 |
| Oleocanthal | 14. ± 4 | <LOD | <LOD |
| Hydroxytyrosol | 1039 | −27 | −107 |
| Tyrosol | 30.4 | −2 ± 1 | −5.4 ± 0.9 |
| Elenolic acid | 509.8 | −464 | −465 |

***Solvent inactivation***

Results from inactivation with 100% MeOH are summarised in Table 5.3. The concentration of oleuropein in the control OLT (no addition of enzyme or MeOH) compared to the sample that was inactivated with a 1:1 ratio of MeOH/OLT had a percentage difference of 4%. The small percentage difference suggests MeOH can effectively inactivate β-glucosidase as the concentration of oleuropein did not decrease in the latter sample. To confirm MeOH can effectively inactivate the enzyme, one sample was not inactivated and had an oleuropein decrease of 322 mg/kg. Other target analytes, excluding oleocanthal (<LOD), oleacein and elenolic acid had percentage differences of <10% between control and inactivated samples. Oleacein and elenolic acid showed a large percentage difference (>100%); these analytes were likely affected by the addition of the almond flour, possibly destabilising them. However, the β-glucosidase present in almond flour can be successfully inactivated with addition of MeOH. The final method used throughout the research is described in Chapter 2, Section 2.10.1.

**Table 5.3. Change in target analyte concentration in inactivated and not inactivated with MeOH samples and the percentage difference between the control and samples inactivated with MeOH. Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | OLT control-OLT3-2-23 | Inactivated with MeOH | Not inactivated | Percentage difference (%) |
|---|---|---|---|---|
| | mg/kg | Δ mg/kg | | |
| Oleuropein | 342 ± 5 | −13 ± 9 | −322 ± 2 | 4 |
| Ligstroside | 64 ± 9 | −5 ± 8 | <LOD | 8 |
| Oleuropein aglycone | 12.8 ± 0.9 | −0.2 ± 0.1 | −4.3 ± 0.2 | 2 |
| Oleacein | 150 ± 5 | −135 ± 1 | −142.3 ± 0.3 | 164 |
| Oleocanthal | 10.3 ± 0.3 | <LOD | <LOD | N/D* |
| Hydroxytyrosol | 949 ± 6 | 64 ± 51 | −42 ± 38 | 7 |
| Tyrosol | 27.7 ± 1.8 | −2.1± 0.5 | −4.1 ± 0.2 | 8 |
| Elenolic acid | 472 ± 6 | −330 ± 9 | −400 ± 4 | 107 |

**N/D – no data: due to some values being <LOD no percentage difference could be determined.*

### 5.2.3 Comparison of enzymatic activity in almond

The hydrolysis rate of oleuropein was compared between almond flour with different purchase dates and a laboratory made almond meal. Commercially, a higher enzymatic activity would facilitate a reduction in the amount of almond required, decreasing the cost and time taken to carry out the reaction. There are two varieties of almond power available to purchase: almond flour and almond meal. Almond flour is made from blanched and skinned almonds while almond meal is produced from raw almonds without blanching. This experiment compared two almond flours that were purchased in September and November 2022 and one almond meal that was made in-house (April 2023). The LC-QqQ-MS/MS results suggest that the age and type of powder affects the hydrolysis rate of oleuropein in OLT, Figure 5.2 (A). The reaction using the almond meal (April 2023) resulted in rapid hydrolysis of oleuropein (−2904 mg/kg) after 30 minutes. Almond flour purchased in November 2022 resulted in a decrease of 2125 mg/kg and almond flour with the earliest purchase date (September 2022) had the slowest conversion (−648 mg/kg). The glucose

that is produced from hydrolysis of oleuropein and ligstroside was measured with a BGM for qualitative confirmation, Figure 5.2 (B). The use of almond meal (April 2023) resulted in the highest concentration of glucose (3.8 mmol/L) while almond flour with the earliest purchase date (September 2022) resulted in the lowest (1.3 mmol/L). This was in agreeance with the results obtained by LC-QqQ-MS/MS, suggesting age and type of almond power is an important factor to consider when commercialising the hydrolysis of oleuropein.

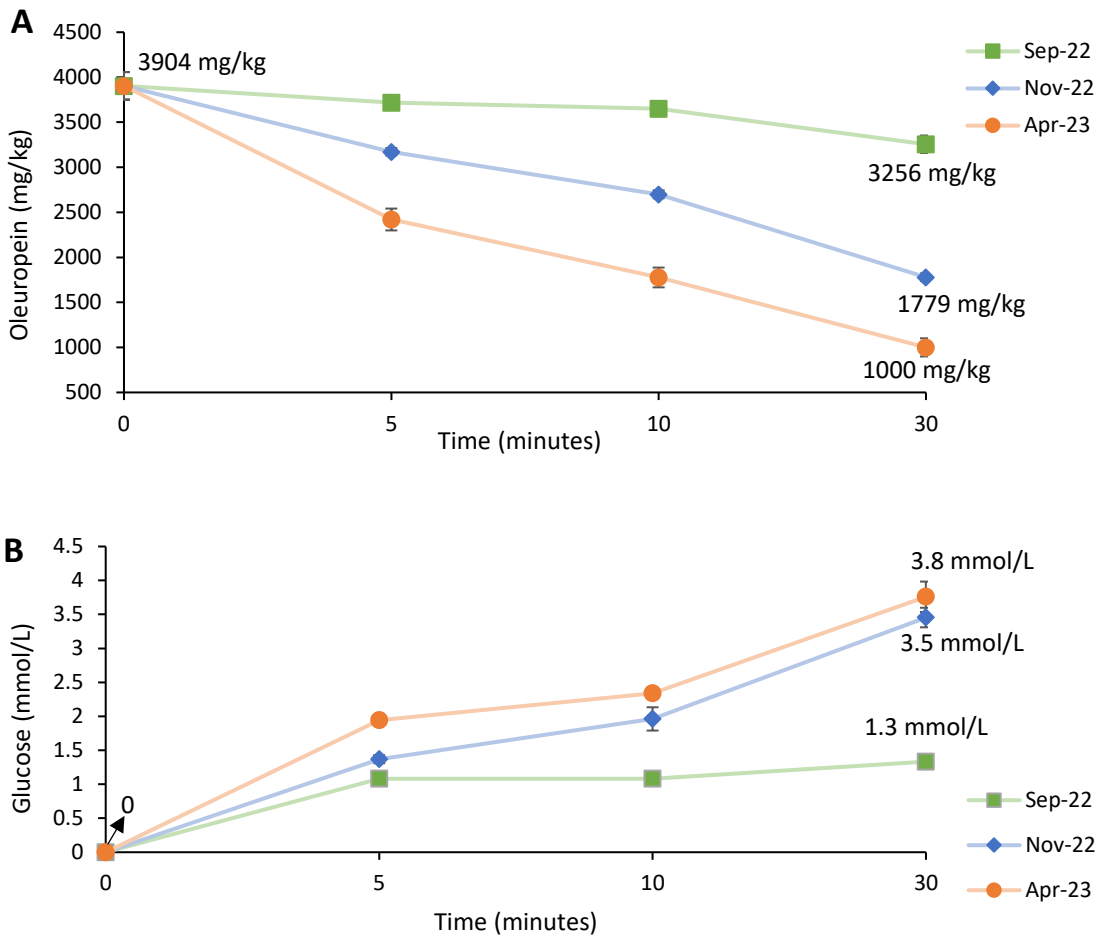



**Figure 5.2. A) initial and final concentration of oleuropein after hydrolysis by almond powders (flour vs meal) with different purchase dates at 60 °C. Results are presented as the average of duplicate samples ± SD (*n* = 2). B) Glucose concentration versus time using different almond powders. Results are presented as the average ± SD (*n* = 3). Some error bars (± SD) are too small to be visible.**

The variation in activity between the powders is likely related to their processing. Both almond flour and meal start with freshly picked raw almonds that are steam pasteurised, a process that is required by law in the USA, Canada and Mexico.[281] The origin of the almond flour and raw almonds (used to make meal) purchased in this project were unknown; in future it would be interesting to conduct experiments using almonds of known origin. Almonds are then passed through a series of dryers to cool them and reduce the moisture content.[281] For almond flour, the almonds are blanched (dipped in a water bath) for several minutes to remove the brown skins (pellicle) before milling into flour.[281] Blanching usually takes place at a temperature above 100 °C for 2-5 minutes.[282,283] Blanching the almonds at these temperatures may deactivate the β-glucosidase present and explain why the use of almond flour resulted in a lower conversion rate of oleuropein. Generally, most enzymes have an optimal activity temperature between 30 and 40 °C; above these temperatures they tend to denature and lose activity.[284] Conversely, almonds used in almond meal have not been blanched, leaving the skins intact. These skins act to protect the nutrients in the seed kernel from oxidation and microbial action until germination.[285,286] With no blanching and protection from the skin, β-glucosidase may be preserved as reflected by the results.

As discussed, almond meal (April 2023) and almond flour (September 2022) resulted in the highest and lowest concentration of glucose respectively. Based on Equation 5.1, oleuropein and ligstroside are hydrolysed to oleuropein aglycone, ligstroside aglycone and glucose in a 1:1 stoichiometric ratio.

Oleuropein: $H_2O + C_{25}H_{32}O_{13} \rightarrow C_6H_{12}O_6 + C_{19}H_{22}O_8$

Ligstroside: $H_2O + C_{25}H_{32}O_{12} \rightarrow C_6H_{12}O_6 + C_{19}H_{22}O_7$

**Equation 5.1**

Therefore, the theoretical amount of glucose formed from different almond powders could be calculated using the quantities of oleuropein and ligstroside that were hydrolysed and quantified by LC-QqQ-MS/MS. An example calculation for oleuropein is in Equation 5.2; assuming 1 kg of water equals 1 L of water.

$$[Glcuose]\ (mmol/L) =$$

$$\frac{initial\ oleurpoein\ (mg) - final\ oleuropein(mg)}{1000} = olueropien\ (g)$$

$$= \frac{Olueropein\ (g)}{M(oleuropein)g/mol} = moles\ of\ oleuropein\ hydrolysed \qquad \textbf{Equation 5.2}$$

Assuming moles of oleuropein hydrolysed is equal to moles of glucose formed, thus:

$$[Glucose](mmol/L) = (moles\ of\ oleuropein \times 1\ L) \times 1000$$

The theoretical amount of glucose and actual concentration measured (mmol/L) are presented in Table 5.4.

**Table 5.4. Theoretical and actual glucose concentrations (mmol/L) for different almond flour and meal used.**

| Time (min) | Glucose concentration (mmol/L) for different almond powders used | | | | | |
|---|---|---|---|---|---|---|
| | Theoretical | | | Actual | | |
| | April 2023[◊] | November 2022[†] | September 2022[†] | April 2023[◊] | November 2022[†] | September 2022[†] |
| 5 | 3.2 | 1.6 | 0.4 | 2.0 | 1.4 | 1.1 |
| 10 | 4.7 | 2.7 | 0.6 | 2.3 | 2 | 1.1 |
| 30 | 6.3 | 4.7 | 1.4 | 3.8 | 3.5 | 1.3 |

*[◊]Almond meal; [†]Almond flour.*

The results suggest that the actual glucose concentration can deviate from the theoretical value. Aside from the first two actual readings taken for almond flour (purchased in September 2022), the difference between the theoretical glucose value and actual readings varied with the theoretical values tending to be larger than the measured. Readings taken at 30 minutes had the largest percentage differences between the theoretical and actual value, ranging between 50% and 27%. The BGM user manual had a list of 32 interferences that may reduce the value recorded in blood samples. If the same interferences can occur using the BGM for aqueous samples, it could explain why the actual readings were lower. Interferences listed that could be present in the tea included fructose, galactose, maltose,

mannose and xylose. Mannitol (derived from mannose) and glucose are primary photosynthetic products and together with xylose, fructose and galactose are the predominant sugars in olive leaves.[287,288] This is a plausible explanation; however, the OLT should be tested in future to confirm their presence. While readings taken with the BGM were lower than the theoretical value, the difference between the theoretical and actual reading would be relative to each other. As a result, the BGM could still be used to qualitatively confirm successful hydrolysis of oleuropein in subsequent experiments.

## 5.3 Investigating the enrichment of oleuropein aglycone into olive oil

In the previous section, hydrolysis of oleuropein with almond flour/meal as a source of β-glucosidase was successful. Theoretically, this should produce oleuropein aglycone. Thus, this section explores the research aim investigation of the resulting aglycones transfer into an OO. It will discuss initial enrichment experiments using OLT with different concentrations of oleuropein, a different source of enzyme (almond milk), increased oil volume and level of emulsification. These aspects were investigated as the mass transfer of aglycone was a complex system and behaved unexpectedly.

### 5.3.1 Initial investigation

As discussed in Chapter 1, Section 1.8, several authors have looked at the enrichment of OO with bioactive olive phenolics from the waste products such as olive leaves, pomace, and wastewater, with olive pomace being the most common. At present, there are no studies investigating the use of almond flour/meal as a source of β-glucosidase to hydrolyse oleuropein present in OLT to oleuropein aglycone for enrichment purposes. Initial enrichment experiments used a low phenolic content oil (e.g., Pams) to easily measure the increase in phenolics after mass transfer had occurred. The experimental set up followed the methods described in Chapter 2, Section 2.10.2.1, with the use of OLT3-2-23, almond flour and were left in an incubator for 30 minutes, with vortex mixing every 5 minutes for 30 seconds (the final method described in Chapter 2 had an incubation time of 10 minutes

as this was determined to be more favourable in later experiments, Section 5.4.2). The results are summarised in Table 5.5. Firstly, the hydrolysis of oleuropein in OLT to its respective aglycone occurred (−322 mg/kg). Conversely, the concentration of oleuropein aglycone measured in the oil after enrichment was small (0.3 mg/kg), with a higher concentration measured in the aqueous portion of the sample (9 mg/kg). This was unforeseen as the hydrolysis of oleuropein results in the removal of the polar glucose moiety making oleuropein aglycone hydrophobic.[38,39] This was problematic for achieving the research aims. It was hypothesised that an enriched product could be developed due to the aglycones higher oil solubility. Thus, the direction of the project changed, and investigations were focused around trying to understand the mass transfer of oleuropein aglycone into OO by altering various experimental parameters (discussed below).

**Table 5.5. Change in target analyte concentration in OLT and oil before and after enzymatic hydrolysis using almond flour. Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | OLT3-2-23 | | Oil | |
|---|---|---|---|---|
| | Control | After conversion | Control | After conversion |
| | mg/kg | Δ mg/kg | mg/kg | Δ mg/kg |
| Oleuropein | 341.4 ± 5.8 | −322 ± 2 | <LOD | <LOD |
| Ligstroside | 63.9 ± 9.4 | <LOD | <LOD | <LOD |
| Oleuropein aglycone | 12.8 ± 0.9 | −4.3 ± 0.2 | 0.9 ± 0.1 | 0.25 ± 0.01 |
| Oleacein | 149.7 ± 4.5 | −142.3 ± 0.3 | 0.4 ± 0.1 | <LOD |
| Oleocanthal | 10.3 ± 0.3 | <LOD | 1.5 ± 0.3 | <LOD |
| Hydroxytyrosol | 949 ± 6 | −113 ± 70 | 0.22 ± 0.02 | 0.8 ± 0.3 |
| Tyrosol | 27.7 ± 1.8 | −4.1 ± 0.2 | <LOD | <LOD |
| Elenolic acid | 472.2 ± 6.4 | −400 ± 4 | 1.9 ± 0.1 | <LOD |

### 5.3.2 Increasing the concentration of oleuropein in OLT and changing the source of enzyme to almond milk

It is possible that the concentration of the oleuropein (341 mg/kg) in OLT used in the initial experiment (Section 5.3.1) was not concentrated enough, resulting in a smaller amount of oleuropein aglycone. To end up with a higher concentration of oleuropein aglycone,

OLT15-2-23 with a higher concentration of oleuropein (3668 mg/kg) was investigated. It was also possible that the small amount of oleuropein aglycone formed from OLT3-2-23 was establishing a favourable interaction with the lipids present on the surface of the almond flour hindering the mass transfer into the oil. It is known that almonds are made up of lipids (around 50%), proteins (around 25%) and carbohydrates (around 20%),[289] hence a different source of enzyme was investigated; almond milk. The use of almond milk should reduce the amount of particulate matter present in the enrichment experiments. The experiments using almond milk were carried out following methods described in Chapter 2, Section 2.10.2.1; with incubation for 30 minutes and vortex mixing every 5 minutes for 30 seconds. Preliminary results are summarised in Table 5.6. Almond milk as the source of enzyme increased the concentration of oleuropein aglycone in the oil (6.5 mg/kg) using OLT15-2-23 compared to only 0.2 mg/kg using OLT3-2-23. This was likely influenced by the starting oleuropein concentration. To confirm that almond flour as a source of enzyme was influencing the transfer of the aglycone, it was substituted for the almond milk in a reaction using OLT15-2-23; this resulted in an aglycone concentration <LOD. Increasing the oleuropein content in the OLT and changing the source of enzyme did achieve a higher concentration of oleuropein aglycone in the oil, however the amount was still below desirable levels (250 mg/kg). Therefore, other experimental parameters (discussed below) were investigated with the use of OLT15-2-23 and almond milk as a source of β-glucosidase.

**Table 5.6. Change in oleuropein aglycone concentration in oil using different OLTs and almond milk and almond flour as the source of enzyme (*n* = 1)*.**

| Analyte | Control oil* | OLT15-2-23 | OLT3-2-23 | OLT15-2-23 |
|---|---|---|---|---|
| | | Almond milk | | Almond flour |
| | mg/kg | Δ mg/kg | | |
| Oleuropein aglycone | 0.8 ± 0.1 | 6.5 | 0.2 | <LOD |

**Control oil is n = 2.*

### 5.3.3 Increasing the volume of oil and changing the type of mixing

There were several possibilities as to why the concentration of oleuropein aglycone was low (6.5 mg/kg, Section 5.3.2); the oil was possibly saturated with aglycone and/or the level of emulsification to facilitate mass transfer was poor. Two experiments were set up to investigate these potential influences – doubling the amount of oil and generate a more stable emulsion through different types of mixing. Emulsions are complex systems comprising of two or more liquids with one being dispersed in the other.[290,291] They are considered a thermodynamically unstable system because of the positive free energy needed to increase the surface area between the oil and water phase.[292] As a result, emulsions tend to separate into a system that consists of a layer of oil (lower density) on top of a layer of water (higher density) over time.[292] This was observed in previous enrichment experiments using a vortex; initially an emulsion was formed but after a few minutes phase separation occurred between the OLT and oil. To form emulsions that are kinetically stable for a longer period of time, chemical substances known as emulsifiers can be added before thorough homogenisation.[292] Emulsifiers are surface-active compounds that adsorb to the surface of freshly formed droplets during homogenisation, forming a protective 'membrane' that prevents the droplets from coming close enough together to aggregate.[292] In this research, an Eppendorf ThermoMixer® C (dry block for heating, cooling, and mixing) and a laboratory homogeniser (LabServ) were investigated to generate a more thorough emulsion. The methods used are described in Chapter 2, Section 2.10.2.1, with vortex mixing every 5 minutes for 30 seconds and an incubation period of 30 minutes; the experiment using the homogeniser was scaled 20-fold as samples needed to be >20 mL.

The results of increasing the oil volume and changing the mixing type are summarised in Table 5.7. Altering these experimental parameters did not increase the oleuropein aglycone concentration in the oil which ranged between 6.3 and 6.5 mg/kg. The results suggest the original volume of oil (550 µL or 0.5 g based on the density of OO) was not saturated with aglycone and preventing mass transfer. This is not unexpected as the concentration of aglycone in EVOO can be high with one study reporting values ranging between 39.5 and

1785.9 mg/kg.[92] The homogeniser generated a more stable emulsion compared to the other mixing types but samples using this technique did not have any further increase in aglycone concentration. Since the homogeniser required a larger sample size, the mixing type in subsequent experiments were undertaken using an Eppendorf ThermoMixer® C. Interestingly, the use of almond milk with these new mixing techniques resulted in a more stable emulsion. Almonds contain 16-22% protein on a dry weight basis with many functional attributes of almonds linked to these proteins.[293,294] It has been reported by Sze-Tao *et al.* (2000)[295] that almond protein isolate (API) has good emulsifying properties.[295] Almond proteins contain one major and several minor proteins that are water soluble[295] so it is plausible they are present in the almond milk produced. Thus, generating a more stable emulsion with homogenisation.

**Table 5.7. Change in oleuropein aglycone concentration by increasing oil volume (shown in grams)* and changing the mixing type. Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | Type of mixing and amount of OO added (g)* | | | |
|---|---|---|---|---|
| | Control | Vortex (0.5) | ThermoMixer (1.0) | Homogeniser (1.0) |
| | mg/kg | Δ mg/kg | | |
| Oleuropein aglycone | 0.81 ± 0.04 | 6.49 ± 0.03 | 6.50 ± 0.05 | 6.26 ± 0.03 |

**Mass of oil added (g) is given in the parentheses where 0.5 g = 550 µL based on OO density.*

## 5.4 Analyte behaviour using almond milk as the source of enzyme

This section will further investigate almond milk as a source of β-glucosidase; the unusual phenomena occurring and the influence of temperature on hydrolysis to gain a better understanding of this new source of enzyme.

### 5.4.1 Presence of oleuropein aglycone in the aqueous portion

In previous experiments, almond milk as a source of β-glucosidase resulted in oleuropein aglycone being quantified in the aqueous portion, e.g., 98 ± 0.8 mg/kg (*n* = 2). This was an unexpected phenomenon as oleuropein aglycone is hydrophobic. Thus, this was

investigated following the methods described in Chapter 2, Section 2.10.2 using 40 °C for incubation. The amount of oleuropein hydrolysed and the aglycone quantified in the aqueous portion using the different sources of enzyme (almond milk and almond flour) are summarised in Table 5.8. Firstly, the amount of oleuropein hydrolysed in OLT using the two sources of enzyme were similar with a percentage difference of 8.5%. Corroborating earlier results, oleuropein aglycone was quantified in the aqueous portion of both samples. Almond milk as the source of enzyme achieved the highest concentration of aglycone (545 mg/kg) compared to almond flour (235 mg/kg). Considering oleuropein aglycone is hydrophobic, its presence in an aqueous matrix was unexpected. There are currently no studies in literature that corroborate the results achieved. Criado-Navarro *et al.* (2022)[296] investigated the partition of bioactive compounds in two olive cultivars during EVOO extraction. In both instances, a minimal amount of oleuropein aglycone was measured in the wastewater and olive pomace compared to EVOO. In Chapter 4, Section 4.3.2 and 4.4.2, oleuropein aglycone was also quantified in OLT but in lower amounts (<26 mg/kg). This was attributed to the additional matrix components extracted from olive leaf material modifying the chemical composition of OLT. Furthermore, the results of this section suggest that the addition of almond milk further modifies the aqueous matrix allowing oil soluble non-polar analytes such as oleuropein aglycone to favour this region in much higher concentrations.

**Table 5.8. Change in oleuropein and oleuropein aglycone concentration in OLT using almond flour/meal versus almond milk as the source of enzyme. Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | Control OLT | Utilising almond flour | Utilising almond milk |
|---|---|---|---|
| | mg/kg | Δ mg/kg | |
| Oleuropein | 3897 ± 84 | −1442 ± 92 | −1222 ± 157 |
| Oleuropein aglycone | 16 ± 1 | 219 ± 5 | 529 ± 13 |

## 5.4.2 β-glucosidase activity in almond milk at different temperatures

Given the possibility to quantify oleuropein aglycone in the aqueous portion with the use of almond milk as a source of β-glucosidase. It was possible to investigate the hydrolysis of

oleuropein and the formation of oleuropein aglycone at different temperatures (30, 40, 50 and 60 °C). It was important to understand the optimal hydrolysis and formation temperature of these analytes for commercial benefit when carrying out enrichment experiments using almond milk as the source of enzyme. The methods used are described in Chapter 2, Section 2.10.3.

***Oleuropein and ligstroside***

Figure 5.3 reveals the decrease in oleuropein, ligstroside and increase in glucose concentrations at different temperatures. The initial concentrations of oleuropein and ligstroside in the OLT were 3761 and 623 mg/kg, respectively. Hydrolysis at a temperature of 60 and 50 °C were more favourable with 60 °C resulting in the largest decrease for oleuropein and ligstroside (3175 and 561 mg/kg, respectively) after 120 minutes (2 hours). Conversely, 30 °C was the least favourable temperature for hydrolysis, resulting in the smallest decreases; 1499 and 331 mg/kg, respectively after 120 minutes. The BGM qualitatively confirmed the results obtained by LC-QqQ-MS/MS where 60 °C resulted in the highest glucose concentration (4 mmol/L) and 30 °C resulted in the lowest (1 mmol/L). Generally, glycosides such as oleuropein are more stable than their respective aglycones but readily degrade through enzymatic hydrolysis at elevated temperatures.[34] However, a favourable temperature of 60 °C was unexpected. A study by Hati *et al*. (2021)[277] reported an optimum temperature of 30 °C for purchased almond β-glucosidase (Merck) to bio-transform isoflavones in soy milk. Aside from β-glucosidase in almonds, studies using endogenous β-glucosidase from soy have revealed a variety of optimal temperatures. Matsuura *et al.* (1993)[297] reported maximum activity at 45 °C with inactivation at 60 °C where the optimal pH was between 5.0 and 7.0. Lopes Barbosa *et al.* (2006)[298] studied the influence of temperature and pH on the production of soy aglycone isoflavones. They reported an optimal temperature of 45 °C and deactivation at 60 °C with an optimal pH between 4.5 and 5.5. Conversely, Bau *et al.* (2015)[299] and Sanches de Lima *et al.* (2014)[300] reported higher optimal activities at temperatures of 50 and 55 °C for enzymatic hydrolysis of glycosylated isoflavones in soy. The latter study also reported that higher temperatures

produced greater quantities of the isoflavone aglycones, which is contradictory to results obtained in this research. It is interesting to note that the optimal pH in the studies discussed are within range of teas used in the research, an average of 5 ± 0.2 (Chapter 2 Section 2.7.1).

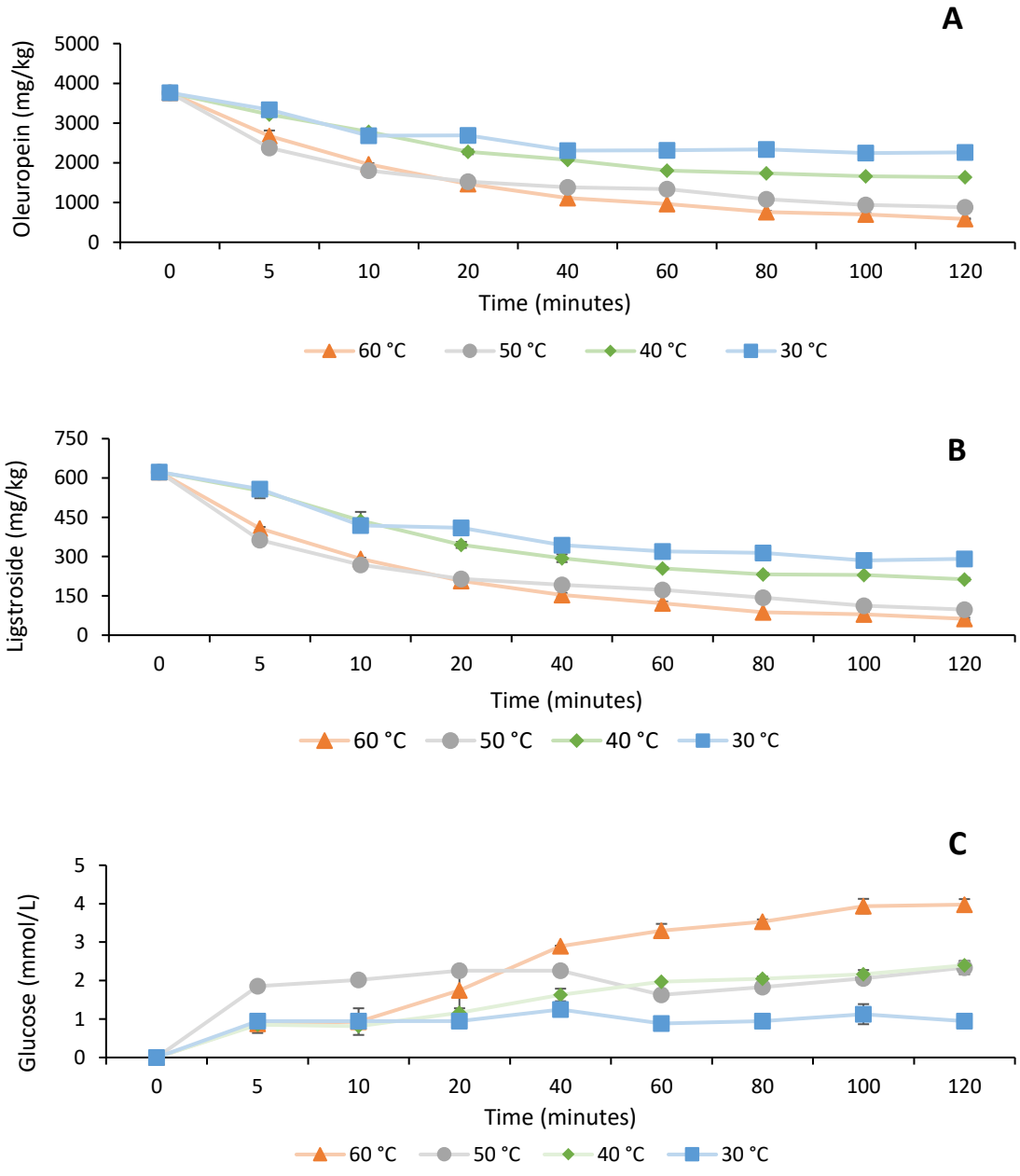



**Figure 5.3. The concentration of A) oleuropein, B) ligstroside and C) glucose versus time at 30 °C (blue square), 40 °C (green diamond), 50 °C (grey circle) and 60 °C (orange triangle) using almond milk as the enzymatic source. Results are presented as the average of duplicate samples ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

***Oleuropein aglycone***

As discussed, high amounts of oleuropein aglycone could be measured in the aqueous portion using almond milk as the enzyme source. Hence, its formation was monitored under different temperatures (Figure 5.4). Up until 10 minutes, a temperature of 40 °C resulted in the highest amount of oleuropein aglycone (613 mg/kg). However, this was not the most effective temperature for oleuropein hydrolysis. There was also an increase in aglycone concentration up until 10 minutes at 50 and 60 °C. At 30 °C, there was a maximum increase up until 40 minutes (361 mg/kg). At this temperature, a slower increase was not unexpected as hydrolysis of oleuropein at 30 °C was slow.

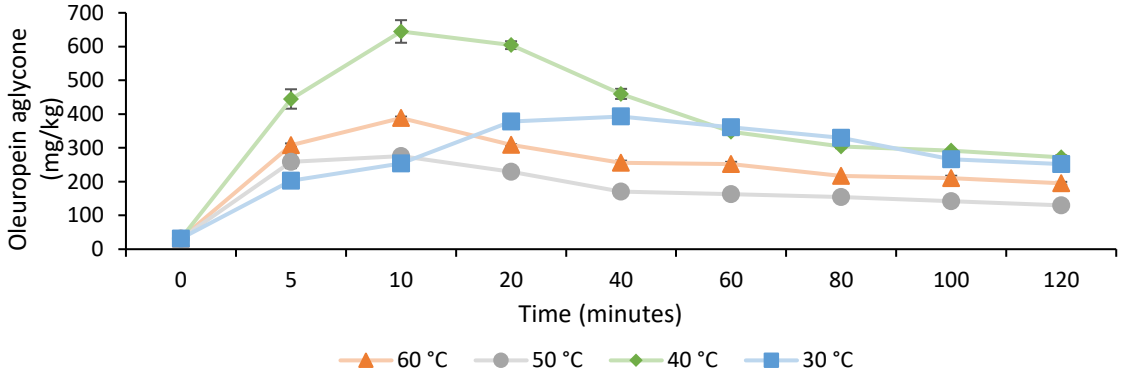



**Figure 5.4. Concentration of oleuropein aglycone versus time at 30 °C (blue square), 40 °C (green diamond), 50 °C (grey circle), and 60 °C (orange triangle) using almond milk as the source of enzyme. Results are presented as the average of duplicate samples ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

As discussed, aglycones are generally unstable compared to their glycoside derivatives.[34] It is likely the favourable oleuropein hydrolysis temperatures of 50 and 60 °C were too high and adversely degraded the oleuropein aglycone as it was formed. Whereas a lower temperature (40 °C) preserved more of the aglycone formed. Similar results were reported by Lozano-Castellón *et al.* (2020).[301] This study investigated the influence of domestic sauteing on OO at temperatures of 120 and 170 °C. During the cooking process, secoiridoids decreased by 45% at the low temperature and 70% at the high temperature where oleuropein aglycone was affected by the interaction of time and temperature. The reactivity

corresponded to compounds with an *o*-diphenol group such as oleuropein aglycone. *Ortho*-diphenols are more reactive and can be easily converted to *ortho*-quinones through a radical reaction (Figure 5.5).[302-304]

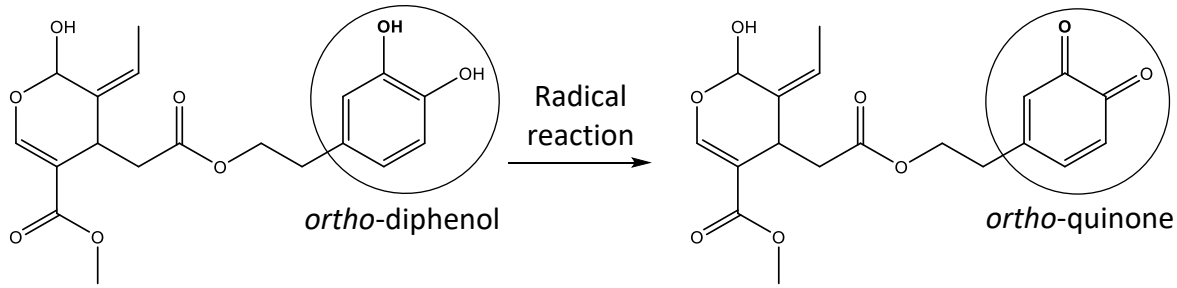



**Figure 5.5. The structure of oleuropein aglycone with the presence of the *ortho*-diphenol group which can be easily converted to quinones through a radical reaction.**

Commercially, the results obtained are impractical. If wanting to achieve rapid hydrolysis of oleuropein, 60 °C is required but this temperature will also degrade the desired target analyte (oleuropein aglycone) required for OO enrichment. As a result, future enrichment experiments should be carried out at 40 °C within 10 minutes to achieve higher amounts of oleuropein aglycone and avoid degradation before mass transfer has taken place.

***Other target analytes***

The concentration of hydroxytyrosol (Figure 5.6 A) and tyrosol (Figure 5.6 B) increased under all temperatures, while elenolic acid (Figure 5.6 C) increased then remained stable or decreased. Oleacein and oleocanthal were <LOD. For the quantified analytes, there was no trend observed between formation at different temperatures. The increase was expected for hydroxytyrosol and elenolic acid as its precursor, oleuropein aglycone, degraded at all temperatures. It is also likely the precursor for tyrosol (ligstroside aglycone, not quantified in this research) degraded at all temperatures hence the increase in tyrosol.

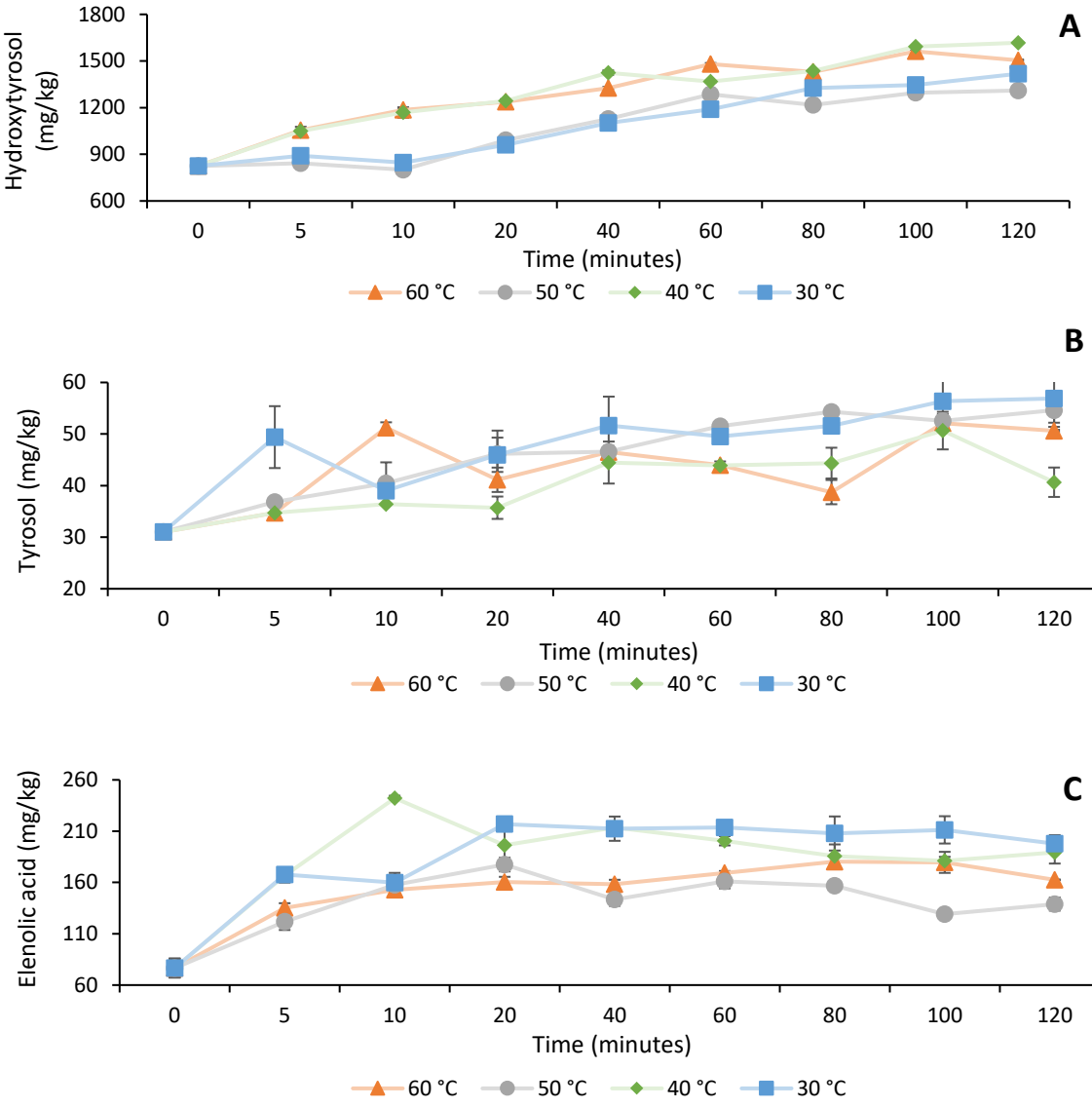



**Figure 5.6. Concentration of A) hydroxytyrosol, B) tyrosol and C) elenolic acid versus time at 30 °C (blue square), 40 °C (green diamond), 50 °C (grey circle) and 60 °C (orange triangle) using almond milk as the enzymatic source. Results are presented as the average of duplicate samples ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

### 5.4.3 Stability of oleuropein aglycone in 50% MeOH olive leaf tea

Results in Section 5.4.2 alluded to poor stability of oleuropein aglycone at elevated temperatures. Therefore, its stability in 50% MeOH OLT at different temperatures (30, 40 and 60 °C) was investigated using the method described in Chapter 2, Section 2.10.4. Since

the β-glucosidase in OLT samples was inactivated with MeOH in a 1:1 ratio, the stability of oleuropein aglycone would be in 50% MeOH OLT. At present there is no literature on the stability of oleuropein aglycone in MeOH OLT as it has never been quantified in this matrix.

***Half-lives of oleuropein aglycone***

An initial experiment (*n* = 2) was conducted and the OLT was periodically sub-sampled up until 40 minutes. Heating at 60 °C resulted in quick degradation of oleuropein aglycone (455 mg/kg) compared to 40 and 30 °C, decreasing 151 and 84 mg/kg, respectively (Figure 5.7).

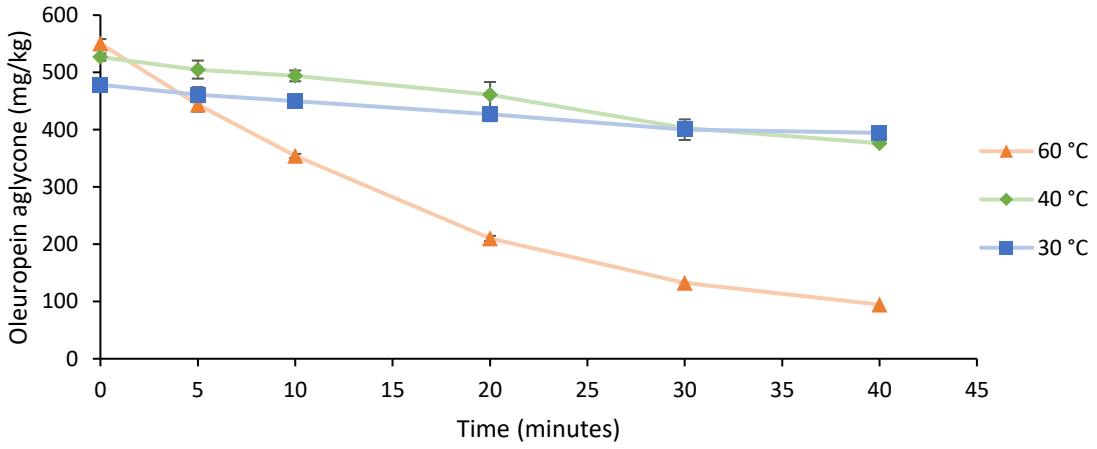



**Figure 5.7. Degradation of oleuropein aglycone in 50% MeOH OLT at 30 °C (blue square), 40 °C (green diamond) and 60 °C (orange triangle). Results are presented as the average of duplicate samples ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

To determine the order of the degradation for oleuropein aglycone at 30, 40 and 60 °C, zero-order ([oleuropein aglycone] vs. time), first-order (ln[oleuropein aglycone] vs. time) and second-order (1/[oleuropein aglycone]) plots were created. A zero-order reaction rate is constant over time and independent of reactant concentration; however, the half-life ($t_{1/2}$) depends on the initial concentration as it is the time required for the concentration to decrease by half. For a first-order reaction, the reaction rate linearly depends on the concentration of one reactant; however, the half-life is constant throughout the reaction.

In second-order reactions, the rate depends on the concentration of the reactants, with the half-life being inversely proportional to these concentrations. A plot that describes a particular order properly will be linear when the data is fitted. The initial experiment carried out for 40 minutes at 60 °C showed first-order reaction kinetics for oleuropein aglycone. However, degradation at 40 and 30 °C showed zero-order and second-order kinetics, respectively. It is unlikely the same analyte would have different reaction orders under different temperature conditions within the same experiment. Hence, the latter two temperature experiments were repeated, however time points were taken until 300 minutes (*n* = 2). These results highlight the importance of using data from samples that have achieved 80% or higher loss for identifying the reaction order to avoid misinterpretation.[305] After increasing the reaction time, degradation of oleuropein aglycone at 30, 40, and 60 °C showed linearity for the first-order plots, and deviation from linearity for the second-order and zero-order plots; an example of the three plots for oleuropein aglycone at 60 °C are shown in Figure 5.8. At 30 °C, 80% or higher loss of oleuropein aglycone (loss of 56%) was not achieved due to a slower reaction; however, the kinetic plot still showed linearity for first-order degradation, so a half-life was determined. The disappearance of oleuropein aglycone in 50% MeOH OLT at 30, 40 and 60 °C were treated as first-order reactions and calculated using Equation 5.3 and Equation 5.4:

$$\frac{d[oleuropein\ aglycone]}{dt} = -k[oleuropein\ aglycone] \qquad \textbf{Equation 5.3}$$

The equation was integrated to give:

$$\ln[oleuropein\ aglycone] = -kt + \ln[oleuropein\ aglycone]_0 \qquad \textbf{Equation 5.4}$$

A plot of ln[oleuropein aglycone] vs. time gave a slope equal to −k, where k is the first-order rate constant for the disappearance of oleuropein aglycone and the intercept of the equation is equal to [oleuropein aglycone]$_0$. Using Equation 5.5, the half-lives of oleuropein aglycone in 50% MeOH OLT at 30, 40 and 60 °C were calculated:

$$t_{\frac{1}{2}} = \frac{0.693}{k}$$ **Equation 5.5**

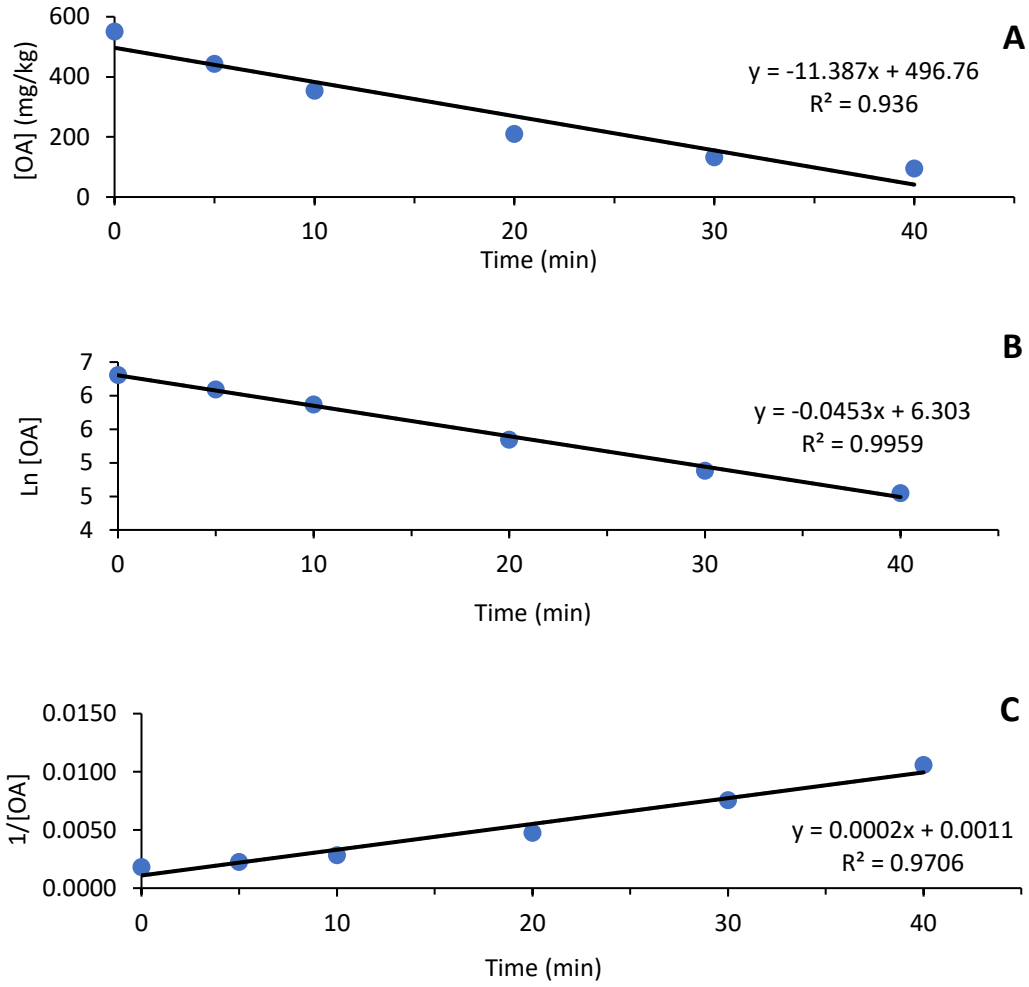



**Figure 5.8. Reaction order plots for oleuropein aglycone in 50% MeOH OLT stored at 60 °C. The Zero-order (A. [oleuropein aglycone (OA)] (mg/kg) vs. time), first-order (B. ln[oleuropein aglycone] vs. time) and second-order (C. 1/[oleuropein aglycone] vs. time) plots with zero and second-order plots deviating from linearity**

The initial and final concentration of oleuropein aglycone (mg/kg), rate constant and half-lives for oleuropein aglycone at 30, 40 and 60 °C are presented in Table 5.9. The half-lives of oleuropein aglycone at 30, 40 and 60 °C were 256, 125 and 16 minutes respectively. There

was a percentage difference of 176% between the half-lives calculated at 30 and 60 °C suggesting there is a large variation in aglycone stability. In the literature, there are no studies analysing oleuropein aglycone stability in an aqueous media with MeOH. However, studies have been conducted on its precursor; oleuropein. Malik *et al.* (2008)[262] investigated oleuropein stability in olive leaf extracts and reported a higher stability in MeOH compared to water. This suggests oleuropein aglycone may have a shorter half-live without the presence of MeOH. In an oil matrix, Lozano-Sánchez *et al.* (2013)[306] reported an oleuropein aglycone half-life of 405 days at 25 °C. This suggests the aglycone is far more stable when present in oil. The results achieved corroborate results in Section 5.4.2; oleuropein aglycone in an aqueous matrix is unstable and transfer into oil needs to occur quickly (within 10 minutes) to avoid degradation of the forming aglycone.

**Table 5.9. Initial and final concentration (mg/kg), rate constant (k) and calculated half-life period ($t_{1/2}$) of oleuropein aglycone content lost in 50% MeOH OLT samples at 30, 40 and 60 °C. Concentration results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Temperature (°C) | Oleuropein aglycone concentration (*n* = 2, mg/kg) | | Kinetic parameters for oleuropein aglycone | |
|---|---|---|---|---|
| | Initial (0 min) | Final* | k (minutes$^{-1}$) | $T_{1/2}$ (minutes) |
| 30 | 451± 7 | 200 ± 6 | 0.003 | 256 |
| 40 | 353 ± 5 | 67.1 ± 0.2 | 0.006 | 125 |
| 60 | 550 ± 7 | 95 ± 1 | 0.044 | 16 |

**Experiments at 40 and 30 °C were conducted for 300 minutes and the experiment at 60 °C was conducted for 40 minutes.*

## 5.5 Investigating the mass transfer of oleuropein aglycone in an emulsion

Changing various experimental parameters in Section 5.3 did not result in aglycone concentrations at or above the health claim (250 mg/kg). It was also determined (Section 5.4) that oleuropein aglycone was unstable in the aqueous portion, thus enrichment experiments would need to be conducted for a shorter time (10 minutes) compared to previous experiments (30 minutes). This section considers the above results and further

investigates why the mass transfer of oil soluble aglycone was insufficient through changing the phenolic content of OO and altering the composition of the emulsion through salting and sugaring out.

### 5.5.1 Influence of phenolic content in olive oils

As discussed in Section 5.3.3, emulsions were formed when mixing almond milk, OLT and OO. Whether two immiscible layers form a stable emulsion is related to surface active compounds (emulsifiers) as these determine the interfacial tension.[307] Interfacial tension is the force acting between two different liquids; a higher interfacial tension means both liquids tend to separate into two phases.[307,308] Thus, decreasing the interfacial tension is essential to create a stable emulsion and better mass transfer.[307] There are various ways to reduce interfacial tension; Gmach *et al.* (2019)[309] revealed that interfacial tension increased in the order of corn oil, peanut oil, sunflower oil and rapeseed oil. This observation was attributed to the fatty acid composition of the oils. However, fatty acid composition may not be the only factor determining the interfacial tension. Depending on the oil type (vegetable/seed) and production process, composition can vary (e.g., phenolic compounds). These compounds can be surface-active (surfactants) and have the capacity to adsorb to liquid interfaces, lowering the interfacial tension between the two liquids.[308,310-313] Some studies have focused on surface-active compounds affecting the interfacial tension between vegetable oils and an aqueous phase.[309,314,315] A study by Dopierala *et al*. (2011)[316] investigated OO. The interfacial tension of six EVOOs varied, this was attributed to the difference in composition originating from chemical reactions (hydrolysis, oxidation) and the presence of different minor constituents as a result of oil processing, cultivar type and storage.

These considerations were applied to an experiment in this research following methods described in Chapter 2, Section 2.10.2.1 with incubation at 40 °C, use of OLT15-2-23 and almond milk as the source of enzyme. Refined OO, such as Pams, had a lower phenolic content (<3 mg/kg) compared to EVOO. The higher phenolic content of EVOOs may decrease the interfacial tension, increasing mass transfer of oleuropein aglycone. Two

EVOOs were selected based on total phenolic content; The Olive Press – Picholine and Frantoio had a phenolic content of 23 and 118 mg/kg, respectively. The results are summarised in Table 5.10. Increasing the phenolic content of oil resulted in no further increase in the concentration of oleuropein aglycone compared to using Pams oil (6.6-6.3 mg/kg; Section 5.3.3). The lower phenolic content oil (The Olive Press – Frantoio) resulted in an aglycone increase of 5.8 mg/kg. Conversely, in the higher phenolic content oil (The Olive Press – Picholine), the concentrations of all target analytes decreased during the enrichment process. This was not unexpected for the target analytes (excluding oleuropein aglycone) as they are known to be soluble in oil and water (Figure 5.1 in Section 5.1). In future, measuring the fatty acid composition of these oils would be useful to compare interfacial tension and a possible correlation in mass transfer.

**Table 5.10. Change in target analyte concentration in high and low phenolic content oils and the aqueous portion after the enrichment process. Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | Control oils | | Enriched oils | |
|---|---|---|---|---|
| | High content[◊] | Low content[†] | High content[◊] | Low content[†] |
| | mg/kg | | Δ mg/kg | |
| Oleuropein | <LOD | <LOD | <LOD | <LOD |
| Ligstroside | <LOD | <LOD | <LOD | <LOD |
| Oleuropein aglycone | 73.8 ± 3.1 | 8.1 ± 0.2 | −36.9 ± 0.7 | 5.8 ± 1.8 |
| Oleacein | 14.9 ± 1.0 | 0.3 ± 0.1 | −10.2 ± 0.3 | −0.2 ± 0.1 |
| Oleocanthal | 18.8 ± 0.2 | 4.6 ± 0.1 | −10.4 ± 0.2 | −2.3 ± 0.1 |
| Hydroxytyrosol | 8.7 ± 0.3 | 7.9 ± 0.2 | −7.8 ± 0.2 | −6.9 ± 0.04 |
| Tyrosol | 1.6 ± 0.1 | 2.7 ± 1 | <LOD | <LOD |
| Elenolic acid | 61 ± 1 | 124 ± 1 | −46.3 ± 1.2 | −109 ± 1 |
| | Aqueous portion | | | |
| | High content | | Low content | |
| Oleuropein aglycone | 226 ± 10 | | 187 ± 7 | |

[◊]*The Olive Press – Picholine;* [†]*The Olive Press – Frantoio.*

In the same experiments, high amounts of the aglycone (187-226 mg/kg) were present in the aqueous portion even with the presence of oil (Table 5.10). As briefly mentioned in Section 5.4.1, the addition of almond milk likely modifies the aqueous portion resulting in

minimal transfer of aglycone to the oil present. There are several plausible explanations as to why this is occurring. Secoiridoids such as oleuropein aglycone have antioxidant properties[317] and it is known that antioxidant behaviour is more complex in emulsions than in bulk oils.[318,319] In an emulsion, the oil-water interface is where emulsifiers and other surface-active compounds such as phenolics are located. The compounds will then partition themselves among the three different layers of the emulsion (dispersed phase, interface and continuous phase) according to their solubility and surface activity which is determined by their chemical structures and polarity.[319,320] However, the "polar paradox theory" was proposed.[319,321] According to this theory, less polar/non-polar compounds are more effective in emulsions due to their greater affinity for the oil-water interface. On the contrary, more polar antioxidants are less effective because they are predominantly dissolved in the aqueous phase.[318,319,322] The order of polarity for olive phenolics based on the partition coefficient between *n*-octanol and water ($\log K_{ow}$) was determined by Gordon *et al.* (2001);[323] hydroxytyrosol > oleuropein > oleacein > oleuropein aglycone.[318,323] With regards to the polar paradox theory, the low polarity of oleuropein aglycone may explain why it is not present in the bulk oil layer, possibly concentrating at the oil-water interface after centrifuging.

However, in the last ten years, new evidence from more comprehensive assessments have emerged that contradict the polar paradox theory where more complex factors in addition to polarity must be taken into account to explain antioxidant efficacy.[319,324] One factor of interest is the effect of the quantity and type of emulsifier in a heterogeneous system. This can drastically change the behaviour of antioxidant compounds, altering the polarity-effectiveness relationship proposed in the theory.[319] The effect of the emulsifier on antioxidant activity is mostly through modification of antioxidant distribution in the emulsified medium.[319] The emulsifier saturates the interfacial membrane, thus leaving less interfacial area available for antioxidants. In other words, emulsifiers compete with antioxidants for localisation at the interface. Moreover, emulsifiers in high quantities (above the critical micelle concentration) form thermodynamically stable structures known as micelles.[319] These self-assembled structures form because they minimise the contact

area between polar and non-polar regions of an emulsion.[292] The surfactant (emulsifier) compounds arrange themselves so that the nonpolar tails are located in the interior (away from the water) and the hydrophilic head groups are located at the surface (in contact with the water) (Figure 5.9).[292] It is possible the emulsion generated with almond milk as the source of enzyme resulted in micelle formation. The micelles are capable of solubilising nonpolar molecules such as antioxidants into their hydrophobic cores and can transfer them into the water phase.[292,319] As a result, the concentration of normally insoluble or slightly soluble nonpolar molecules tends to increase in the aqueous phase.[292,325] This mechanism could explain why hydrophobic oleuropein aglycone was quantified in the aqueous portion of experiments as the thermodynamically stable micelles would prevent mass transfer into the oil.

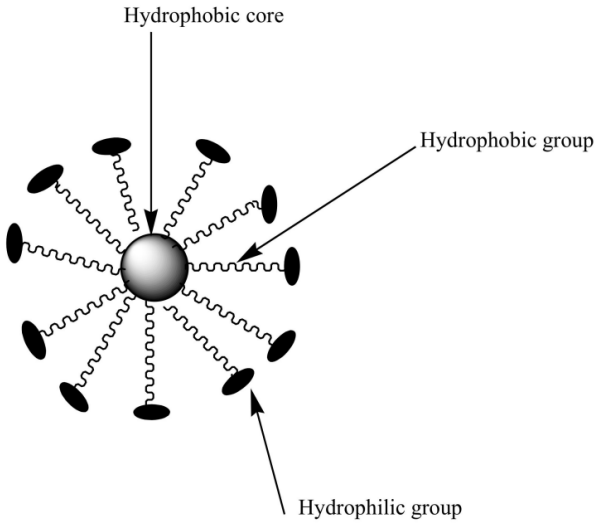



**Figure 5.9. Schematic diagram of a micelle. Adapted from Mhlwatika *et al.* (2018).[326]**

## 5.5.2 Filtering the aqueous portion

Filtering the aqueous portion of samples was carried out to determine if this would reduce the concentration of oleuropein aglycone present. It is possible the aglycone was absorbing to the particulate matter present in the aqueous portion of enrichment experiments resulting in poor mass transfer. Two filter pore sizes were used (0.45 and 0.20 µm) and

samples were filtered following methods described in Chapter 2, Section 2.10.2.2 using 40 °C incubation, OLT15-2-34 and almond milk as the source of enzyme. The results are summarised in Table 5.11. When considering the experimental error, there is no difference in oleuropein aglycone concentration between the samples. The typical size of a micelle is between 10 and 100 nm.[327] As a result, they would be capable of moving through the filter pore sizes used. With filtration having removed particulate matter, it is plausible there is some kind of stable structure such as a micelle existing in the aqueous portion of samples.

**Table 5.11. The concentration of oleuropein aglycone measured in the aqueous portion after no filtration and filtration with a 0.45 and 0.20 µm filter. Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | Not filtered | Filtered | |
|---|---|---|---|
| | | 0.45 µm | 0.20 µm |
| | | mg/kg | |
| Oleuropein aglycone | 454 ± 7 | 474 ± 26 | 459 ± 14 |

## 5.5.3 Salting and sugaring out of the aqueous portion

With the possibility of micelle formation, salting (NaCl) and sugaring out (glucose) of the aqueous portion was investigated in this research. These extractions are designed around a similar mechanism and will be discussed briefly. Salting out is where salt is added to an amphipathic solvent. The solvent is affected by the changes of salt-induced surface properties, electric charge and various forces such as hydrophobic, hydrogen and ionic bonds.[328] Salting out is often used to enhance extractions into nonpolar immiscible organic solvents, where the solubility of a compound in water decreases with an increase in the concentration of salt.[329] Sugaring out is the addition of a monomeric sugar or a disaccharide rather than salt.[328,330] The sugaring out agents compete with the solute to attract water molecules and form a new phase desirable for lower polarity compounds, the sugars reside in the water phase allowing separation of analytes.[328] The difference between salting and sugaring out is salt dissociates in water to form ions (sodium, $Na^+$ and chloride, $Cl^-$) whereas sugars are polar and do not dissociate. Investigating these extraction methods could

transfer more target analytes into the oil, particularly oleuropein aglycone. However, considerations need to be made when utilising salt and sugar. The addition of high amounts may critically affect the solubility, binding, stability and the biological activities of enzymes, possibly leading to deactivation.[331,332] This occurs due to changes in water activity; salt ions and glucose molecules are known to disrupt the local water structure, diminishing the number of intermolecular hydrogen bonds which ultimately prevents the hydration of the enzyme.[333,334] Enzymes such as the β-glucosidase require a certain level of water in their structures to maintain their natural conformation, delivering their full functionality.[335] To confirm the addition of salt and glucose was not going to drastically reduce the water activity (1.0 being pure water and 0.0 being pure anhydrous) it was measured in this research (Chapter 2, Section 2.10.2.3). The water activity ($a_w$) for saturated NaCl and glucose OLT solutions at 22 °C were 0.75305 ± 0.0002 and 0.9127 ± 0.004, respectively. Some enzymes are more resistant to lower water activities; however, enzyme activity is virtually non-existent between $a_w$ of 0.0 and 0.25. Enzyme activity increases gradually between $a_w$ of 0.3 and 0.75, then increases rapidly in the loosely bound water ($a_w$ 0.75 to 1.0).[336] Thus, the water activity was still high enough to delivery full enzymatic functionality.

In the experiments carried out, low and high phenolic content oils were used (Pams and The Olive Press – Picholine, respectively). Samples in the first experiment had “no treatment” of salt or glucose and differed by addition of oil pre- and post-incubation. Samples in the second experiment were “treated” with the addition of NaCl and glucose. Details on experimental methods are described in Chapter 2, Section 2.10.2.3, using 40 °C incubation, OLT15-2-23 and almond milk as the source of β-glucosidase. It should be noted that the aqueous portion of salting and sugaring out experiments could not be analysed due to the precipitate present in the saturated solutions. For example, precipitated salts can accumulate or deposit within the interior parts of the mass spectrometer such as the skimmer cones.[337] This can interfere with the ionisation of analytes and reduce the amount of ions reaching the mass spectrometers subsequently reducing the intensity measured.[337]

The results of these experiments are summarised in Table 5.12. “No treatment” samples with low content oil added pre-incubation and post-incubation had a similar concentration of oleuropein aglycone, 14 mg/kg and 12 mg/kg, respectively. The amount of aglycone obtained in no treatment samples was the highest achieved compared to earlier results (6.3-6.6 mg/kg, Section 5.3.3). This likely relates to the period left in incubation; previous experiments were left for >30 minutes whereas these experiments were reduced to 10 minutes based on results in Section 5.4.

**Table 5.12. Change in target analyte concentration in high and low phenolic OOs with no treatment (addition of oil pre- and post-incubation) and treatment (addition of NaCl and glucose). Results are presented as the average of duplicate samples ± SD (*n* = 2)*.**

| Analyte | Control oils* | No treatment | | Treatment | |
|---|---|---|---|---|---|
| | Low content◊ | Pre-incubation | Post-intubation | NaCl | glucose |
| | mg/kg | Δ mg/kg | | | |
| Oleuropein | <LOD | <LOD | <LOD | <LOQ | <LOD |
| Ligstroside | <LOD | <LOD | <LOD | <LOD | <LOD |
| Oleuropein aglycone | **0.8** | **13.532 ± 0.004** | **11.8 ± 0.3** | **30 ± 1** | **11.2 ± 0.1** |
| Oleacein | 0.3 | <LOD | <LOD | <LOQ | <LOD |
| Oleocanthal | 1.2 | <LOD | <LOQ | −0.721 ± 0.001 | <LOQ |
| Hydroxytyrosol | <LOD | 0.90 ± 0.01 | 1.3 ± 0.2 | 2.8 ± 0.1 | 0.674 ± 0.001 |
| Tyrosol | <LOD | <LOD | <LOD | 1.6 ± 0.2 | <LOD |
| Elenolic acid | 2.0 | 1.78 ± 0.01 | 1.5 ± 0.1 | 5.1 ± 0.2 | 1.1 ± 0.1 |
| | High content† | | | | |
| Oleuropein | <LOD | <LOD | <LOD | <LOD | <LOD |
| Ligstroside | <LOD | <LOD | <LOD | <LOD | <LOD |
| Oleuropein aglycone | **67.6** | **−33.4 ± 0.1** | **−29.3 ± 0.4** | **0.5 ± 2.0** | **−32 ± 0.7** |
| Oleacein | 10.8 | −5.531 ± 0.003 | −3.49 ± 0.04 | −3.0 ± 0.1 | −5.3 ± 0.3 |
| Oleocanthal | 12.1 | −4.5 ± 0.1 | −2.2 ± 0.1 | −1.0 ± 0.5 | −5.1 ± 0.3 |
| Hydroxytyrosol | 8.9 | −7.918 ± 0.002 | −7.93 ± 0.02 | −6.4 ± 0.1 | −8.15 ± 0.03 |
| Tyrosol | 2.2 | <LOD | <LOD | −0.6 ± 0.2 | <LOD |
| Elenolic acid | 55.3 | −39.7 ± 0.2 | −38.2 ± 0.4 | −3.7 ± 0.1 | −41.6 ± 0.5 |

**Control oils n = 1; ◊Pams OO; †The Olive Press – Picholine EVOO.*

"No treatment" samples with high phenolic content oils had a decrease in the oleuropein aglycone concentration; similar decreases were experienced for the oil added pre-incubation (33 mg/kg) and post-incubation (29 mg/kg). The decreases observed could be linked to similar mechanisms seen during OO production. Ideally malaxing temperatures and times should range between 25-30 °C and 30-45 minutes, respectively.[338-341] These temperature conditions are much lower compared to enrichment experiments in this research where oils are exposed to temperatures of 40 or 60 °C. Olmo-Cunillera *et al.* (2021)[342] reported a decreasing concentration of oleuropein aglycone with increasing malaxing temperature (20-30 °C) and time (30 versus 45 minutes). These results were corroborated by Lukić *et al.* (2018)[343] and Gómez-Rico *et al.* (2009).[344] Conversely, Taticchi *et al.* (2013)[345] reported oleuropein aglycone concentration was highest at a temperature of 35 °C compared to 20 °C for a time of 40 minutes. The discrepancies between studies could relate to variety, ripening index of fruit, activity of oxidoreductases (polyphenol oxidase and peroxidase) and variation in hydrolytic enzymes (β-glucosidase) reacting differently to malaxation conditions.[342,343,346]

Conversely, low phenolic content oil treated with salt had the highest concentration of oleuropein aglycone achieved thus far (30 mg/kg); equivalating to a 3650% increase compared to the control oil. With regards to high phenolic content oil, no decrease in aglycone was observed as experienced previously (Section 5.5.1). The increase in concentration observed with the addition of salt again supports the idea of micelle formation within the system. Several studies have investigated the effect of salting out on the formation of emulsions, interfacial tension and micellisation (formation of micelles). In an emulsion, water segregation is increased as salinity is increased. As a result, the ability of the surfactants (emulsifiers) to create and stabilise the emulsion are decreased due to the mobilisation of surfactant compounds to the oil phase (salting out effect).[347-349] Since NaCl is considered a water-structure maker, its presence means ions in the aqueous phase (such as OLT) become more uniform. As a result, they compete for the water molecules associated with micelle formation which promotes micelle dehydration by interfering with the hydration of the surfactant head groups. Micelles can then approach each other more

easily, leading to aggregation, ensuing a phase separation as NaCl has no effect in the oil.[347,350] This phenomenon may explain why the mass transfer of aglycone was higher in the presence of salt. Contradicting salting out results, sugaring out did not result in the same increases in oleuropein aglycone concentration. Low phenolic content oil had an increase of 11 mg/kg whereas the high phenolic content oil experienced a decrease of 32 mg/kg. Additional glucose to the system may have had minimal effect on the matrix composition compared to salt.

With regards to other target analytes, no treatment low phenolic content oil added pre- and post-incubation had an increase in the concentration of hydroxytyrosol (0.9 and 1.3 mg/kg) and elenolic acid (1.8 and 1.5 mg/kg) but no increase in tyrosol, oleacein and oleocanthal. This may relate to the analyte concentrations quantified in the aqueous portion (Table 5.13) and their water/oil solubility. Prior to enzymatic hydrolysis, the aqueous portion had a higher concentration of hydroxytyrosol (775 mg/kg) compared to tyrosol (32 mg/kg) where elenolic acid had a concentration of 67 mg/kg. The concentration for oleocanthal and oleacein were <LOD.

**Table 5.13. Change in target analyte concentration in the aqueous portion of samples with no treatment (oil added pre- and post-incubation). Results are presented as the average of duplicate samples ± SD (*n* = 2).**

| Analyte | Control OLT | Aqueous portion, no treatment | | | |
|---|---|---|---|---|---|
| | | Low content oil[◊] | | High content oil[†] | |
| | | Pre | Post | Pre | Post |
| | mg/kg | Δ mg/kg | | | |
| Oleuropein | 3897 ± 84 | −2547 ± 58 | −2433 ± 12 | −2350 ± 77 | −2379 ± 29 |
| Ligstroside | 589 ± 22 | −405 ± 3 | −372 ± 1 | −383 ± 26 | −392 ± 23 |
| Oleuropein aglycone | 16 ± 1 | 272 ± 3 | 268 ± 6 | 385 ±17 | 441 ± 18 |
| Oleacein | <LOD | 2 ± 0.1 | 1.7 ± 0.1 | 22 ± 1 | 33 ± 3 |
| Oleocanthal | <LOD | <LOD | <LOD | <LOD | <LOD |
| Hydroxytyrosol | 775 ± 1 | 248 ± 6 | 325 ± 31 | 382 ± 87 | 373 ± 32 |
| Tyrosol | 32 ± 2 | 4 ± 2 | 5 ± 6 | 19 ± 2 | 14 ± 3 |
| Elenolic acid | 67 ± 0.3 | 43 ± 2 | 76 ± 3 | 279 ± 28 | 301 ± 27 |

*[◊]Pams OO; [†]The Olive Press – Picholine EVOO*

Salting out resulted in increased amounts of hydroxytyrosol, tyrosol and elenolic acid (2.8, 1.6 and 5.1 mg/kg) except for oleacein (−0.5 mg/kg). Whereas sugaring out only resulted in an increase of hydroxytyrosol and elenolic acid (0.7 and 1.1 mg/kg). High phenolic content oil regardless of treatment type experienced a decrease in concentration even though increases were experienced in the aqueous portion.

While the addition of salt increased the oleuropein aglycone concentration, the levels were low compared to some of the commercial EVOOs analysed in Chapter 4, Section 4.2.2 and were below the target of 250 mg/kg. However, similar results have been reported in previous enrichment studies. Sánchez de Medina *et al.* (2011)[144] investigated the enrichment of edible oil with olive leaf extract and olive pomace. Enrichment of OO with leaf extracts did not increase the oleuropein aglycone content. However, their experiments did not add β-glucosidase to convert the oleuropein present. In the same study, hydroxytyrosol increased (4 mg/kg) but tyrosol did not, similar to the results achieved in this research. They also reported a higher concentration of oleacein with the addition of leaf extract (43 mg/kg) and a small increase in oleocanthal (5 mg/kg). Conversely, enrichment with olive pomace was more successful. Oleuropein aglycone had the highest transferred amount (36 mg/kg). However, this amount was still low compared to the concentration measured in the pomace prior to enrichment (372 mg/kg). Different oils such as refined soy oil and refined high-oleic sunflower oil enriched with olive pomace resulted in higher concentrations of oleuropein aglycone (56 and 41 mg/kg, respectively) and hydroxytyrosol (40 and 25 mg/kg, respectively). They suggested that the oleic acid content of oil was a factor in the amounts transferred; polar phenolic compounds such as hydroxytyrosol experienced a higher transfer to oils with a lower content of oleic acid and higher concentration of polyunsaturated acids. However, they observed no trend for oleuropein aglycone albeit transferred lowest in oils with a composition least similar to EVOO. Girón *et al.* (2009)[351] investigated the dependence of fatty acid composition of edible oils on enrichment using an olive phenolic solution obtained from olive pomace. In the study, the distribution factor between the phenolic concentration in different oils (coconut, sunflower, high-oleic sunflower, linseed and orujo) and that in the other phase was

determined. The order established was deemed a function of polarity and molecular weight. High polarity, low molecular weight olive phenolics had a lower distribution factor where the presence of mono- and polyunsaturated fatty acids increased the distribution factor. However, the study claimed oleuropein aglycone (higher molecular weight) to be an exception, having a lower partition coefficient than expected regardless of oil type. In both studies, the interfacial tension was not considered. As discussed in Section 5.5.1, interfacial tension seems to be an important factor in the level of mass transfer that takes place. In future experiments, different edible oils should be investigated to determine their influence on the amount of oleuropein aglycone transferred. Overall, this research corroborates the above studies where oleuropein aglycone behaviour in an enrichment experiment is complex. As a result, the aims of transferring oleuropein aglycone into OOs to levels at or above the health claim (250 mg/kg) have been unsuccessful and the development of an enriched oil for commercial purposes needs to be investigated further.

## 5.6 Summary of enrichment experiments

The aims of this research were to investigate the use of almond flour/meal to enzymatically convert oleuropein to oil soluble oleuropein aglycone which could be later transferred into an OO. While the conversion of oleuropein with the β-glucosidase from almond flour/meal was successful, mass transfer of the resulting aglycone was unsuccessful. Based on the results of this chapter, enrichment experiments need to be conducted in a minimal amount of time as oleuropein aglycone is very unstable in the aqueous portion prior to transfer into the oil. The addition of salt achieved an aglycone concentration of 30 mg/kg which was still below the target concentration in this research (250 mg/kg). Thermodynamically stable micelle formation which can solubilise non-polar analytes such as oleuropein aglycone was a plausible explanation to the low amounts transferred into the oil. Therefore, the preparation of a tailor-made enriched OO with a high content of olive phenolics such as oleuropein aglycone is complex and would need to be investigated further.

# CHAPTER 6

# Conclusion and Future Work

## 6.1 Conclusion

Olive oil is considered an excellent source of minor bioactive compounds such as oleuropein aglycone which is gaining increasing attention due to its biological properties. In the OO market, the concentration of bioactive compounds such as the aglycone can vary between batches of OO and some batches may contain bioactive compounds that are below the threshold which provides health benefits. This thesis presented the opportunity to explore the utilisation of oleuropein present in olive leaves to enhance the bioactive component of OO to produce a product with a high, consistent concentration of oleuropein aglycone. Olive leaves can be used to produce an OLT that contains high amounts of oleuropein glycoside which can be hydrolysed to oleuropein aglycone via an exogenous source of β-glucosidase; almond flour/meal (unexplored in literature). To better understand the OO market, analysis of commercial EVOOs was undertaken to later compare to enriched levels. The effect that drying olive leaves had on the phenolic composition of OLT and the stability of target analytes in the OLT were also briefly explored. However, most of the research focused on investigating the possibility to enrich OO with higher amounts of oleuropein aglycone from hydrolysis of oleuropein in OLT. A concentration above 250 mg/kg was the target as this would give certainty of health benefits to consumers.

Optimisation and validation of the methods used in this research (LLE extraction and a LC-QqQ-MS/MS analysis method) was carried out to ensure that reliable results were produced (Chapter 3). All validation parameters of the methods were comparable to those in the literature and suitable for the quantification of target analytes in this research.

In Chapter 4, the concentration of target analytes in EVOOs ($n$ = 9) on the New Zealand market had large variation across all analytes, with none meeting the health claim level (250 mg/kg). It was also observed that oils with a lower cost had higher amounts of target

analytes compared to more expensive oils, highlighting the uncertainty of quality in the market. Chapter 4 also explored the composition of OLT. The content of oleuropein in OLT from two different batches of leaves increased under different drying conditions in the order fresh<RT<70 °C while other target analytes tended to increase with drying at RT. However, the results also seemed to be dependent on the time leaves spent drying which can result in oxidation and endogenous enzyme activities. A stability experiment of OLT (−20, RT, 40 °C, 189 days) determined that the majority of target analytes were stable at −20 °C and RT, with precursors such as oleuropein and ligstroside degrading at 40 °C. Consequently, this resulted in an increase of their respective derivatives, where unstable derivatives such as oleocanthal and oleacein further degraded.

In Chapter 5, almond flour/meal was an effective source of exogenous β-glucosidase. Almond meal hydrolysed oleuropein the quickest, with differences in rate likely attributed to prior processing of the almond. This research highlighted that the mass transfer of aglycone into the oil was complex. Changing the source of enzyme to almond milk, increasing the concentration of oleuropein in the OLT and changing the mixing type resulted in an aglycone concentration of 6 mg/kg in the oil. Unusually, almond milk as a source of β-glucosidase resulted in a considerable amount of hydrophobic oleuropein aglycone to be quantified in the aqueous portion of samples (the highest being 613 mg/kg). As a result, the use of this enzyme was explored further in an aqueous media; the optimal oleuropein hydrolysis temperature was 60 °C, while the optimal oleuropein aglycone formation was at 40 °C. The half-life of aglycone in 50% MeOH/OLT was 16 minutes at 60 °C. The presence of aglycone in the aqueous portion and its short half-life was a plausible explanation to the poor mass transfer in initial experiments. Therefore, other experimental parameters were investigated. Decreasing the interfacial tension by changing the phenolic content of oil did not increase the aglycone measured in the oil with much larger amounts quantified in the aqueous portion. It was thought that the emulsion generated when using almond milk as the source of enzyme resulted in thermodynamically stable micelle formation which were capable of solubilising oleuropein aglycone into their hydrophobic cores. As a result, the concentration of aglycone increased in the aqueous phase, reducing the mass transfer into

the oil. Salting out the reaction resulted in the largest increase of the aglycone (30 mg/kg). It was likely the salt dehydrated the micelle surfactant head groups causing anything contained in them to congregate to the oil layer. The highest aglycone quantity transferred into the oil was still below the target concentration of 250 mg/kg and lower than some of the concentrations measured in commercial EVOOs. These results corroborated other studies where oleuropein aglycone behaviour in an enrichment experiment was complex. As a result, the aims of transferring oleuropein aglycone into a low content oil to levels above the health claim (250 mg/kg) were unsuccessful and the possibility of developing an enriched OO for commercial purposes would need to be investigated further.

## 6.2 Recommendations for future research

Throughout the research, improvements or ideas for future work were identified. Improvements included alterations to the experimental methods as well as experimental design. The recommendations based on the chapters are discussed below.

For experimental methods:

- For several of the analytes (elenolic acid, oleacein, ligstroside, and oleocanthal) the standard preparation could not be undertaken by mass difference as the vial containing the compound was too heavy for the balance. In future, purchasing "accurate weight" from the manufacturer should be done to obtain a known amount of standard which can be accurately prepared.
- It would be advantageous to purchase a ligstroside aglycone standard to see if it behaves in a similar way to oleuropein aglycone during enrichment experiments.
- During the research elenolic acid had a poor peak shape. In future, the analysis method could be further optimised to improve the shape allowing for more accurate quantification of this analyte.
- Oleuropein aglycone had multiple peaks in the chromatography which correlated to the presence of different isomers. NMR could be used to distinguish isomers present making the analysis more accurate.

- After standards had been prepared, it was determined that MeOH can cause the formation of isomers in oleocanthal and oleacein. Purchasing more standards and preparing them with ACN would be beneficial to understanding this potential effect.
- An internal standard could have been used to improve the accuracy and precision of quantification as well as the robustness of the method.
- With the use of the quadratic regression, linearity could be improved with the use of a stable-isotope-labelled internal standard.

For the experimental design:

- During the research, several dilutions of OLT had to be carried out to obtain reliable results. It would be useful to screen all OLT prior to their analysis to reduce the number of dilutions required.
- During validation, different volumes of the mixed standard were spiked into the oil via MeOH. In future, the MeOH should be evaporated off to reduce variation in the matrix.

In Chapter 4, analysis of commercial EVOOs, effect of leaf drying on oleuropein content of OLTs and stability of target analytes in OLT at different storage temperatures were investigated. There are several avenues that could be changed or explored such as:

- Purchase a newer Olive Black EVOO sample to confirm the low content measured is age related. The older sample should also be subjected to rancidity testing which can be an indicator of age.
- Undertake leaf drying experiments at a temperature greater than 100 °C. Some studies suggested higher temperatures were more favourable in achieving a higher oleuropein concentration. This would provide more information on optimal drying conditions.
- One OLT was put into different storage temperatures for stability. In future, it would be beneficial to include a different batch of OLT. Different initial concentrations of target analytes may alter their respective stabilities.

- The half-life of oleuropein and ligstroside in OLT fitted a zero-order kinetic plot, this was unexpected because a zero-order reaction rate is constant over time and independent of reactant concentration. Extending the storage period (>189 days) may result in a first-order plot as reported in the literature.
- During storage at 40 °C, oleocanthal and oleacein degraded, likely due to oxidation. Testing for their oxidation products (oleocanthalic and oleaceinic acid) would determine if this type of degradation occurred.

In Chapter 5, almond flour/meal and later almond milk were successful exogenous sources of β-glucosidase. The mass transfer of oleuropein aglycone into OO was not successful and amounts transferred were well below the target level of 250 mg/kg. More avenues to investigate this area of research as well as better under the system are:

- Optimise the amount of almond flour/meal required to hydrolyse oleuropein by conducting an enzyme assay. This would determine the enzymatic activity of β-glucosidase present.
- Obtain almonds from known origin to better understand how their prior processing parameters affect the activity of β-glucosidase.
- Test the OLT matrix for the interferences listed in the BGM manual such as fructose, galactose, maltose, mannose and xylose. This would determine if they were responsible for the lowering of actual glucose readings.
- Develop a non-toxic extraction and purification method for β-glucosidase from almonds. This would reduce the amount of matrix components during enrichment experiments and possibly result in a better mass transfer of the aglycone.
- Increase the hydrolysis temperature during enzymatic reactions to determine if 60 °C was optimal. This would also determine when the enzyme is inactivated.
- Centrifuge the almond milk at a low temperature to remove particulate matter and precipitate out the lipids. The removal of additional matrix components could result in a better mass transfer of the aglycone.

- Measure the fatty acid content of the oils. This could provide more clarity on the trends observed during the transfer of some analytes. Some studies reported that the amounts transferred and the fatty acid composition were correlated.
- Look at the emulsion mixture (OLT, oil, and almond milk) under a suitable microscope (scanning and/or transition electron microscope) to confirm micelle formation.
- Investigate different salting and sugaring out reagents. Changing the reagents could result in higher amounts of aglycone transferred into the oil.
- Spike a Type 1 water/oil mixture with a known concentration of oleuropein, add almond milk as the enzyme source and measure the partition coefficient between the aqueous and oil phases. With a 1:1 hydrolysis ratio and a known concentration spiked; it would be possible to track the movement of the resulting aglycone.
- Change the source of enzyme to a purchased β-glucosidase from almonds and monitor whether the aglycone is still quantified in the aqueous portion. This would confirm if the addition of almond milk results in minimal mass transfer of the aglycone.
- Future work could include investigating the use of different edible oils in enrichment experiments to observe any variation in the mass transfer of oleuropein aglycone. It was evident based on other studies that amounts transferred varied among oil types.

# References


1. Xu, F.; Li, Y.; Zheng, M.; Xi, X.; Zhang, X.; Han, C. Structure properties, acquisition protocols, and biological activities of oleuropein aglycone. *Frontiers in Chemistry* **2018**, *6*, 239.
2. Therios, I. Oleuropein, olive leaf extract, olive oil and the benefits of the Mediterranean diet to human health. In *Olives*: Wallingford, United Kingdom, 2008; pp 303-318.
3. Kaniewski, D.; Van Campo, E.; Boiy, T.; Terral, J. F.; Khadari, B.; Besnard, G. Primary domestication and early uses of the emblematic olive tree: palaeobotanical, historical and molecular evidence from the Middle East. *Biological Reviews* **2012**, *87*, 885-899.
4. Hava F. Rapoport; Andrea, F.; Luca, S. Olive Biology. In *The Olive Tree Genome*; E. Rugini; L. Baldoni; R. Muleo and L. Sebastiani, Eds.; Springer International Publishing, 2016; pp 13-22.
5. Ghanbari, R.; Anwar, F.; Alkharfy, K. M.; Gilani, A. H.; Saari, N. Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.) - A review. *international Journal of Molecular Sciences* **2012**, *13*, 3291-3340.
6. Souilem, S.; Fki, I.; Kobayashi, I.; Khalid, N.; Neves, M. A.; Isoda, H.; Sayadi, S.; Nakajima, M. Emerging technologies for recovery of value-added components from olive leaves and their applications in food/feed industries. *Food and Bioprocess Technology* **2017**, *10*, 229-248.
7. Unsplash. Esencia de Plantas - Olive tree. https://unsplash.com/photos/yUR0JP2DSPo (accessed 13th of September 2023).
8. Pixabay. Olive Fruit. https://pixabay.com/photos/olives-fruit-olive-tree-oil-fruit-15016/ (accessed 13th of September 2023).
9. Adobe Stock Images. Olive green leaves isolated on white background. https://stock.adobe.com/nz/search?k=%22olive+leaves%22&asset_id=315348332 (accessed 13th of September 2023).
10. Besnard, G.; Terral, J.-F.; Cornille, A. On the origins and domestication of the olive: a review and perspectives. *Annals of botany* **2018**, *121*, 385-403.
11. Torres, M.; Pierantozzi, P.; Searles, P.; Rousseaux, M. C.; García-Inza, G.; Miserere, A.; Bodoira, R.; Contreras, C.; Maestri, D. Olive cultivation in the Southern Hemisphere: flowering, water requirements and oil quality responses to new crop environments. *Frontiers in Plant Science* **2017**, *8*, 1830.
12. Stempfle, S.; Carlucci, D.; de Gennaro, B. C.; Roselli, L.; Giannoccaro, G. Available pathways for operationalizing circular economy into the olive oil supply chain: Mapping evidence from a scoping literature review. *Sustainability* **2021**, *13*, 9789.
13. Behl, T.; Kumar, K.; Brisc, C.; Rus, M.; Nistor-Cseppento, D. C.; Bustea, C.; Aron, R. A. C.; Pantis, C.; Zengin, G.; Sehgal, A.; Kaur, R.; Kumar, A.; Arora, S.; Setia, D.; Chandel, D.; Bungau, S. Exploring the multifocal role of phytochemicals as immunomodulators. *Biomedicine & Pharmacotherapy* **2021**, *133*, 110959.

14. Abbas, M.; Saeed, F.; Anjum, F. M.; Afzaal, M.; Tufail, T.; Bashir, M. S.; Ishtiaq, A.; Hussain, S.; Suleria, H. A. R. Natural polyphenols: An overview. *International Journal of Food Properties* **2016**, *20*, 1689-1699.
15. Heleno, S. A.; Martins, A.; Queiroz, M. J. R.; Ferreira, I. C. Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food chemistry* **2015**, *173*, 501-513.
16. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*, 1231-1246.
17. Shahidi, F.; Ambigaipalan, P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of Functional Foods* **2015**, *18*, 820-897.
18. Martinez, K. B.; Mackert, J. D.; McIntosh, M. K. Polyphenols and intestinal health. In *Nutrition and Functional Foods for Healthy Aging*; R. R. Watson, Ed.; Academic Press, 2017; pp 191-210.
19. Talhaoui, N.; Taamalli, A.; Gómez-Caravaca, A. M.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits. *Food Research International* **2015**, *77*, 92-108.
20. Lamuela-Raventós, R. M.; Vallverdú-Queralt, A.; Jáuregui, O.; Martínez-Huélamo, M.; Quifer-Rada, P. Improved characterization of polyphenols using liquid chromatography. In *Polyphenols in Plants*; Elsevier, 2014; pp 261-292.
21. Bucciantini, M.; Leri, M.; Nardiello, P.; Casamenti, F.; Stefani, M. Olive polyphenols: Antioxidant and anti-inflammatory properties. *Antioxidants (Basel)* **2021**, *10*, 1044.
22. Hano, C.; Tungmunnithum, D. Plant polyphenols, more than just simple natural antioxidants: Oxidative stress, aging and age-related diseases. *Medicines (Basel, Switzerland)* **2020**, *7*, 26.
23. Chaiprasongsuk, A.; Panich, U. Role of phytochemicals in skin photoprotection via regulation of Nrf2. *Frontiers in Pharmacology* **2022**, *13*, 1-22.
24. Annunziata, F.; Pinna, C.; Dallavalle, S.; Tamborini, L.; Pinto, A. An overview of coumarin as a versatile and readily accessible scaffold with broad-ranging biological activities. *International Journal of Molecular Sciences* **2020**, *21*, 4618.
25. Boskou, D. Olive fruit, table olives, and olive oil bioactive constituents. In *Olive and Olive Oil Bioactive Constituents*; D. Boskou, Ed.; AOCS Press, 2015; pp 1-30.
26. Jimenez-Lopez, C.; Carpena, M.; Lourenço-Lopes, C.; Gallardo-Gomez, M.; Lorenzo, J. M.; Barba, F. J.; Prieto, M. A.; Simal-Gandara, J. Bioactive compounds and quality of extra virgin olive oil. *Foods* **2020**, *9*, 1014.
27. Celano, M.; Maggisano, V.; Lepore, S. M.; Russo, D.; Bulotta, S. Secoiridoids of olive and derivatives as potential coadjuvant drugs in cancer: A critical analysis of experimental studies. *Pharmacological Research* **2019**, *142*, 77-86.
28. Wang, C.; Gong, X.; Bo, A.; Zhang, L.; Zhang, M.; Zang, E.; Zhang, C.; Li, M. Iridoids: Research advances in their phytochemistry, biological activities, and pharmacokinetics. *Molecules* **2020**, *25*, 287.

29. Sidda, J. D.; Song, L.; Parker, J. L.; Studholme, D. J.; Sambles, C.; Grant, M. Diversity of secoiridoid glycosides in leaves of UK and Danish ash provide new insight for ash dieback management. *Scientific Reports* **2020**, *10*, 19566.
30. Covas, M.-I.; Fitó, M.; de la Torre, R. Minor bioactive olive oil components and health: Key data for their role in providing health benefits in humans. In *Olive and Olive Oil Bioactive Constituents*; D. Boskou, Ed.; AOCS Press, 2015; pp 31-52.
31. Charoenprasert, S.; Mitchell, A. Factors influencing phenolic compounds in table olives (*Olea europaea*). *Journal of Agricultural and Food Chemistry* **2012**, *60*, 7081-7095.
32. Hassen, I.; Casabianca, H.; Hosni, K. Biological activities of the natural antioxidant oleuropein: Exceeding the expectation – A mini-review. *Journal of Functional Foods* **2015**, *18*, 926-940.
33. Křen, V. Glycoside vs. Aglycon: The role of glycosidic residue in biological activity. In *Glycoscience: Chemistry and Chemical Biology*; B. O. Fraser-Reid; K. Tatsuta and J. Thiem, Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2008; pp 2589-2644.
34. Johnson, R.; Melliou, E.; Zweigenbaum, J.; Mitchell, A. E. Quantitation of oleuropein and related phenolics in cured Spanish-style green, California-style black ripe, and Greek-style natural fermentation olives. *Journal of Agricultural and Food Chemistry* **2018**, *66*, 2121-2128.
35. Velázquez-Palmero, D.; Romero-Segura, C.; García-Rodríguez, R.; Hernández, M. L.; Vaistij, F. E.; Graham, I. A.; Pérez, A. G.; Martínez-Rivas, J. M. An oleuropein β-glucosidase from olive fruit is involved in determining the phenolic composition of virgin olive oil. *Frontiers in Plant Science* **2017**, *8*, 1902.
36. Yuan, J. J.; Wang, C. Z.; Ye, J. Z.; Tao, R.; Zhang, Y. S. Enzymatic hydrolysis of oleuropein from *Olea europea* (olive) leaf extract and antioxidant activities. *Molecules* **2015**, *20*, 2903-2921.
37. Ramírez, E.; Brenes, M.; García, P.; Medina, E.; Romero, C. Oleuropein hydrolysis in natural green olives: Importance of the endogenous enzymes. *Food Chemistry* **2016**, *206*, 204-209.
38. Rodis, P. S.; Karathanos, V. T.; Mantzavinou, A. Partitioning of olive oil antioxidants between oil and water phases. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 596-601.
39. Galiano, V.; Villalaín, J. Oleuropein aglycone in lipid bilayer membranes. A molecular dynamics study. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2015**, *1848*, 2849-2858.
40. Guggenheim, K. G.; Crawford, L. M.; Paradisi, F.; Wang, S. C.; Siegel, J. B. β-glucosidase discovery and design for the degradation of oleuropein. *ACS Omega* **2018**, *3*, 15754-15762.
41. Briante, R.; La Cara, F.; Febbraio, F.; Barone, R.; Piccialli, G.; Carolla, R.; Mainolfi, P.; De Napoli, L.; Patumi, M.; Fontanazza, G.; Nucci, R. Hydrolysis of oleuropein by recombinant β-glycosidase from hyperthermophilic archaeon *Sulfolobus solfataricus* immobilised on chitosan matrix. *Journal of Biotechnology* **2000**, *77*, 275-286.

42. Francioso, A.; Federico, R.; Maggiore, A.; Fontana, M.; Boffi, A.; D'Erme, M.; Mosca, L. Green route for the isolation and purification of hyrdoxytyrosol, tyrosol, oleacein and oleocanthal from extra virgin olive oil. *Molecules* **2020**, *25*, 3654.
43. Starec, M.; Calabretti, A.; Berti, F.; Forzato, C. Oleocanthal quantification using $^{1}$H NMR spectroscopy and polyphenols HPLC analysis of olive oil from the Bianchera/Belica cultivar. *Molecules* **2021**, *26*, 242.
44. Diamantakos, P.; Giannara, T.; Skarkou, M.; Melliou, E.; Magiatis, P. Influence of harvest time and malaxation conditions on the concentration of individual phenols in extra virgin olive oil related to its healthy properties. *Molecules* **2020**, *25*, 2449.
45. Nikou, T.; Sakavitsi, M. E.; Kalampokis, E.; Halabalaki, M. Metabolism and bioavailability of olive bioactive constituents based on in vitro, in vivo and human studies. *Nutrients* **2022**, *14*, 3773.
46. Emma, M. R.; Augello, G.; Di Stefano, V.; Azzolina, A.; Giannitrapani, L.; Montalto, G.; Cervello, M.; Cusimano, A. Potential uses of olive oil secoiridoids for the prevention and treatment of cancer: A narrative review of preclinical studies. *International Journal of Molecular Sciences* **2021**, *22*, 1234.
47. Perez-Trujillo, M.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernandez-Gutierrez, A.; Parella, T. Separation and identification of phenolic compounds of extra virgin olive oil from *Olea Europaea* L. by HPLC-DAD-SPE-NMR/MS. Identification of a new diastereoisomer of the aldehydic form of oleuropein aglycone. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 9129-9136.
48. Ye, J.; Wijesundera, C.; Shi, M. Effects of agronomic and oil processing conditions on natural antioxidative phenolics in olive (*Olea europaea* L.). *Austin Journal of Nutrition and Food Sciences* **2014**, *2*, 1-8.
49. Quirantes-Piné, R.; Lozano-Sánchez, J.; Herrero, M.; Ibáñez, E.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC–ESI–QTOF–MS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts. *Phytochemical Analysis* **2013**, *24*, 213-223.
50. Omar, S. H. Oleuropein in olive and its pharmacological effects. *Scientia Pharmaceutica* **2010**, *78*, 133-154.
51. Taamalli, A.; Arráez Román, D.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Classification of 'Chemlali' accessions according to the geographical area using chemometric methods of phenolic profiles analysed by HPLC–ESI-TOF–MS. *Food Chemistry* **2012**, *132*, 561-566.
52. Altıok, E.; Bayçın, D.; Bayraktar, O.; Ülkü, S. Isolation of polyphenols from the extracts of olive leaves (*Olea europaea* L.) by adsorption on silk fibroin. *Separation and Purification Technology* **2008**, *62*, 342-348.
53. Bouallagui, Z.; Han, J.; Isoda, H.; Sayadi, S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food and Chemical Toxicology* **2011**, *49*, 179-184.
54. Fu, S.; Arráez-Roman, D.; Segura-Carretero, A.; Menéndez, J. A.; Menéndez-Gutiérrez, M. P.; Micol, V.; Fernández-Gutiérrez, A. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and

preliminary evaluation of cytotoxic activity against human breast cancer cells. *Analytical and Bioanalytical Chemistry* **2010**, *397*, 643-654.

55. Hachicha Hbaieb, R.; Kotti, F.; García-Rodríguez, R.; Gargouri, M.; Sanz, C.; Pérez, A. G. Monitoring endogenous enzymes during olive fruit ripening and storage: Correlation with virgin olive oil phenolic profiles. *Food Chemistry* **2015**, *174*, 240-247.
56. Konno, K.; Hirayama, C.; Yasui, H.; Nakamura, M. Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree. *Proceedings of the National Academy of Sciences of the United States of America* **1999**, *96*, 9159-9164.
57. De Leonardis, A.; Macciola, V.; Cuomo, F.; Lopez, F. Evidence of oleuropein degradation by olive leaf protein extract. *Food Chemistry* **2015**, *175*, 568-574.
58. Mazzuca, S.; Spadafora, A.; Innocenti, A. M. Cell and tissue localization of β-glucosidase during the ripening of olive fruit (*Olea europaea*) by in situ activity assay. *Plant Science* **2006**, *171*, 726-733.
59. Wang, W.; Li, C. Q.; Hu, X. L. Developmental expression of β-glucosidase in olive leaves. *Biologia Plantarum* **2009**, *53*, 138-140.
60. Vassão, D. G.; Wielsch, N.; Gomes, A. M. d. M. M.; Gebauer-Jung, S.; Hupfer, Y.; Svatoš, A.; Gershenzon, J. Plant defensive β-glucosidases resist digestion and sustain activity in the gut of a lepidopteran herbivore. *Frontiers in Plant Science* **2018**, *9*, 1389-1389.
61. Esen, A. Beta-glucosidase. In *Handbook of Food Enzymology*; J. R. Whitaker; A. G. J. Voragen and D. W. S. Wong, Eds.; Marcel Dekker: New York, 2003; pp 791-804.
62. Romero-Segura, C.; García-Rodríguez, R.; Sánchez-Ortiz, A.; Sanz, C.; Pérez, A. G. The role of olive β-glucosidase in shaping the phenolic profile of virgin olive oil. *Food Research International* **2012**, *45*, 191-196.
63. Romero-Segura, C.; Sanz, C.; Perez, A. G. Purification and characterization of an olive fruit β-glucosidase involved in the biosynthesis of virgin olive oil phenolics. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 7983-7988.
64. Abaza, L.; Taamalli, A.; Arráez-Román, D.; Segura-Carretero, A.; Fernández-Gutierrérez, A.; Zarrouk, M.; Youssef, N. B. Changes in phenolic composition in olive tree parts according to development stage. *Food Research International* **2017**, *100*, 454-461.
65. Marx Í, M. G. Co-extraction technique improves functional capacity and health-related benefits of olive oils: A mini review. *Foods* **2023**, *12*, 1667.
66. Johnson, R. L.; Mitchell, A. E. Reducing phenolics related to bitterness in table olives. *Journal of Food Quality* **2018**, *2018*, 3193185.
67. Nikolaivits, E.; Termentzi, A.; Skaltsounis, A.-L.; Fokialakis, N.; Topakas, E. Enzymatic tailoring of oleuropein from *Olea europaea* leaves and product identification by HRMS/MS spectrometry. *Journal of Biotechnology* **2017**, *253*, 48-54.
68. Ciafardini, G.; Marsilio, V.; Lanza, B.; Pozzi, N. Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation. *Applied and Environmental Microbiology* **1994**, *60*, 4142-4147.
69. Rigacci, S.; Guidotti, V.; Bucciantini, M.; Parri, M.; Nediani, C.; Cerbai, E.; Stefani, M.; Berti, A. Oleuropein aglycon prevents cytotoxic amyloid aggregation of human amylin. *The Journal of Nutritional Biochemistry* **2010**, *21*, 726-735.

70. Grossi, C.; Rigacci, S.; Ambrosini, S.; Ed Dami, T.; Luccarini, I.; Traini, C.; Failli, P.; Berti, A.; Casamenti, F.; Stefani, M. The polyphenol oleuropein aglycone protects TgCRND8 mice against Aß plaque pathology. *PLoS One* **2013**, *8*, e71702.
71. Luccarini, I.; Grossi, C.; Rigacci, S.; Coppi, E.; Pugliese, A. M.; Pantano, D.; la Marca, G.; Ed Dami, T.; Berti, A.; Stefani, M.; Casamenti, F. Oleuropein aglycone protects against pyroglutamylated-3 amyloid-ß toxicity: biochemical, epigenetic and functional correlates. *Neurobiology of Aging* **2015**, *36*, 648-663.
72. Luccarini, I.; Pantano, D.; Nardiello, P.; Cavone, L.; Lapucci, A.; Miceli, C.; Nediani, C.; Berti, A.; Stefani, M.; Casamenti, F. The polyphenol oleuropein aglycone modulates the PARP1-SIRT1 Interplay: An  In Vitro and In Vivo Study. *Journal of Alzheimer's Disease* **2016**, *54*, 737-750.
73. Walter, W. M., Jr.; Fleming, H. P.; Etchells, J. L. Preparation of antimicrobial compounds by hydrolysis of oleuropein from green olives. *Applied Microbiology* **1973**, *26*, 773-776.
74. Guiso, M.; Marra, C. Highlights in oleuropein aglycone structure. *Natural Product Research* **2005**, *19*, 105-109.
75. Jemai, H.; Bouaziz, M.; Fki, I.; El Feki, A.; Sayadi, S. Hypolipidimic and antioxidant activities of oleuropein and its hydrolysis derivative-rich extracts from Chemlali olive leaves. *Chemico-Biological Interactions* **2008**, *176*, 88-98.
76. Delgado-Povedano, M. D. M.; Priego-Capote, F.; Luque de Castro, M. D. Selective ultrasound-enhanced enzymatic hydrolysis of oleuropein to its aglycon in olive (*Olea europaea* L.) leaf extracts. *Food Chemistry* **2017**, *220*, 282-288.
77. Thodberg, S.; Del Cueto, J.; Mazzeo, R.; Pavan, S.; Lotti, C.; Dicenta, F.; Jakobsen Neilson, E. H.; Møller, B. L.; Sánchez-Pérez, R. Elucidation of the amygdalin pathway reveals the metabolic basis of bitter and sweet almonds (*Prunus dulcis*). *Plant Physiology* **2018**, *178*, 1096-1111.
78. Del Cueto, J.; Møller, B. L.; Dicenta, F.; Sánchez-Pérez, R. β-Glucosidase activity in almond seeds. *Plant Physiology and Biochemistry* **2018**, *126*, 163-172.
79. Bak, S.; Paquette, S. M.; Morant, M.; Morant, A. V.; Saito, S.; Bjarnholt, N.; Zagrobelny, M.; Jørgensen, K.; Osmani, S.; Simonsen, H. T.; Pérez, R. S.; van Heeswijck, T. B.; Jørgensen, B.; Møller, B. L. Cyanogenic glycosides: A case study for evolution and application of cytochromes P450. *Phytochemistry Reviews* **2006**, *5*, 309-329.
80. He, S.; Withers, S. G. Assignment of sweet almond β-glucosidase as a family 1 glycosidase and identification of its active site nucleophile. *Journal of Biological Chemistry* **1997**, *272*, 24864-24867.
81. Jin, M. Y.; Zhang, T.; Yang, Y. S.; Ding, Y.; Li, J. S.; Zhong, G. R. A simplified and miniaturized glucometer-based assay for the detection of β-glucosidase activity. *Journal of Zhejiang University Science* **2019**, *20*, 264-272.
82. Mazzuca, S.; Giorno, L.; Spadafora, A.; Mazzei, R.; Drioli, E. Immunolocalization of β-glucosidase immobilized within polysulphone capillary membrane and evaluation of its activity in situ. *Journal of Membrane Science* **2006**, *285*, 152-158.
83. Chiari, M.; Gelain, A.; Riva, S.; Tura, D. Analysis of mandelonitrile lyase and β-glucosidase from sweet almonds by combined electrophoretic techniques. *Electrophoresis* **1997**, *18*, 2050-2054.

84. Joubert, F. J.; Van der Walt, T. N. The purification and physicochemical properties of β-glucosidase from sweet almonds. *South African Journal of Chemistry* **1964**, *17*, 79-94.
85. Boskou, D. Phenolic compounds in olives and olive oil. In *Olive Oil : Minor Constitutents and Health*; CRC Press: Boca Raton, 2009; pp 11-44.
86. Peri, C. Part II The process. In *The Extra-Virgin Olive Oil Handbook*; John Wiley & Sons, 2014; pp 6-100.
87. Viola, P.; Nobili, F.; Viola, M. Olive oil as a skin protector. In *Handbook of Diet, Nutrition and the Skin*; V. R. Preedy, Ed.; Wageningen Academic Publishers: Wageningen, 2012; pp 282-299.
88. Lozano-Castellón, J.; Rinaldi de Alvarenga, J. F.; Vallverdú-Queralt, A.; Lamuela-Raventós, R. M. Cooking with extra-virgin olive oil: A mixture of food components to prevent oxidation and degradation. *Trends in Food Science & Technology* **2022**, *123*, 28-36.
89. Peres, F.; Martins, L. L.; Ferreira-Dias, S. Influence of enzymes and technology on virgin olive oil composition. *Critical Reviews in Food Science and Nutrition* **2017**, *57*, 3104-3126.
90. Lozano-Castellón, J.; López-Yerena, A.; Domínguez-López, I.; Siscart-Serra, A.; Fraga, N.; Sámano, S.; López-Sabater, C.; Lamuela-Raventós, R. M.; Vallverdú-Queralt, A.; Pérez, M. Extra virgin olive oil: A comprehensive review of efforts to ensure its authenticity, traceability, and safety. *Comprehensive Reviews in Food Science and Food Safety* **2022**, *21*, 2639-2664.
91. Lechhab, T.; Lechhab, W.; Cacciola, F.; Salmoun, F. Sets of internal and external factors influencing olive oil *(Olea europaea* L.) composition: a review. *European Food Research and Technology* **2022**, *248*, 1069-1088.
92. Różańska, A.; Russo, M.; Cacciola, F.; Salafia, F.; Polkowska, Ż.; Dugo, P.; Mondello, L. Concentration of potentially bioactive compounds in Italian extra virgin olive oils from various sources by using LC-MS and multivariate data analysis. *Foods* **2020**, *9*, 1120.
93. López-Huertas, E.; Lozano-Sánchez, J.; Segura-Carretero, A. Olive oil varieties and ripening stages containing the antioxidants hydroxytyrosol and derivatives in compliance with EFSA health claim. *Food Chemistry* **2021**, *342*, 128291.
94. Criado-Navarro, I.; Lopez-Bascon, M. A.; Priego-Capote, F. Evaluating the variability in the phenolic concentration of extra virgin olive oil according to the commission regulation (EU) 432/2012 health claim. *Journal of Agricultural and Food Chemistry* **2020**, *68*, 9070-9080.
95. Klisović, D.; Novoselić, A.; Lukić, I.; Brkić Bubola, K. Extra virgin olive oil under simulated consumption conditions: Evaluation of quality, health, and flavour properties. *Journal of Food Composition and Analysis* **2022**, *110*, 104570.
96. Dugo, L.; Russo, M.; Cacciola, F.; Mandolfino, F.; Salafia, F.; Vilmercati, A.; Fanali, C.; Casale, M.; De Gara, L.; Dugo, P.; Mondello, L.; Rigano, F. Determination of the phenol and tocopherol content in Italian high-quality extra-virgin olive oils by using LC-MS and multivariate data analysis. *Food Analytical Methods* **2020**, *13*, 1027-1041.

97. Kalaboki, I.; Koulougliotis, D.; Kleisiari, D.; Melliou, E.; Magiatis, P.; Kampioti, A.; Eriotou, E.; Destouni, A. A study on the clustering of extra virgin olive oils extracted from cultivars growing in four Ionian Islands (Greece) by multivariate analysis of their phenolic profile, antioxidant activity and genetic markers. *Foods* **2021**, *10*, 3009.
98. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J. P.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & Redox Signaling* **2013**, *18*, 1818-1892.
99. Finicelli, M.; Squillaro, T.; Galderisi, U.; Peluso, G. Polyphenols, the healthy brand of olive oil: Insights and perspectives. *Nutrients* **2021**, *13*, 3831.
100. Gorzynik-Debicka, M.; Przychodzen, P.; Cappello, F.; Kuban-Jankowska, A.; Marino Gammazza, A.; Knap, N.; Wozniak, M.; Gorska-Ponikowska, M. Potential health benefits of olive oil and plant polyphenols. *International Journal of Molecular Sciences* **2018**, *19*, 686.
101. Ellis, L. Z.; Liu, W.; Luo, Y.; Okamoto, M.; Qu, D.; Dunn, J. H.; Fujita, M. Green tea polyphenol epigallocatechin-3-gallate suppresses melanoma growth by inhibiting inflammasome and IL-1β secretion. *Biochemical and Biophysical Research Communications* **2011**, *414*, 551-556.
102. Sahin, S.; Bilgin, M. Olive tree (*Olea europaea* L.) leaf as a waste by-product of table olive and olive oil industry: a review. *Journal of the Science of Food and Agriculture* **2018**, *98*, 1271-1279.
103. Carluccio, M. A.; Siculella, L.; Ancora, M. A.; Massaro, M.; Scoditti, E.; Storelli, C.; Visioli, F.; Distante, A.; Caterina, R. D. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2003**, *23*, 622-629.
104. Edgecombe, S. C.; Stretch, G. L.; Hayball, P. J. Oleuropein, an antioxidant polyphenol from olive oil, is poorly absorbed from isolated perfused rat intestine. *Journal of Nutrition* **2000**, *130*, 2996-3002.
105. Maalej, A.; Mahmoudi, A.; Bouallagui, Z.; Fki, I.; Marrekchi, R.; Sayadi, S. Olive phenolic compounds attenuate deltamethrin-induced liver and kidney toxicity through regulating oxidative stress, inflammation and apoptosis. *Food and Chemical Toxicology* **2017**, *106*, 455-465.
106. Takashima, T.; Sakata, Y.; Iwakiri, R.; Shiraishi, R.; Oda, Y.; Inoue, N.; Nakayama, A.; Toda, S.; Fujimoto, K. Feeding with olive oil attenuates inflammation in dextran sulfate sodium-induced colitis in rat. *The Journal of Nutritional Biochemistry* **2014**, *25*, 186-192.
107. Camargo, A.; Rangel-Zuñiga, O. A.; Haro, C.; Meza-Miranda, E. R.; Peña-Orihuela, P.; Meneses, M. E.; Marin, C.; Yubero-Serrano, E. M.; Perez-Martinez, P.; Delgado-Lista, J. Olive oil phenolic compounds decrease the postprandial inflammatory response by reducing postprandial plasma lipopolysaccharide levels. *Food chemistry* **2014**, *162*, 161-171.

108. Malik, N. S. A.; Bradford, J. M. Changes in oleuropein levels during differentiation and development of floral buds in 'Arbequina' olives. *Scientia Horticulturae* **2006**, *110*, 274-278.
109. Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer* **2000**, *36*, 1235-1247.
110. Tripoli, E.; Giammanco, M.; Tabacchi, G.; Di Majo, D.; Giammanco, S.; La Guardia, M. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutrition Research Reviews* **2005**, *18*, 98-112.
111. Zheng, A.; Li, H.; Xu, J.; Cao, K.; Li, H.; Pu, W.; Yang, Z.; Peng, Y.; Long, J.; Liu, J. Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of db/db mice: Role of AMP-activated protein kinase activation. *British Journal of Nutrition* **2015**, *113*, 1667-1676.
112. Manna, C.; D'Angelo, S.; Migliardi, V.; Loffredi, E.; Mazzoni, O.; Morrica, P.; Galletti, P.; Zappia, V. Protective effect of the phenolic fraction from virgin olive oils against oxidative stress in human cells. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 6521-6526.
113. Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life Sciences* **1998**, *62*, 541-546.
114. Leri, M.; Oropesa-Nuñez, R.; Canale, C.; Raimondi, S.; Giorgetti, S.; Bruzzone, E.; Bellotti, V.; Stefani, M.; Bucciantini, M. Oleuropein aglycone: A polyphenol with different targets against amyloid toxicity. *Biochimica et Biophysica Acta (BBA)-General Subjects* **2018**, *1862*, 1432-1442.
115. Luccarini, I.; Dami, T. E.; Grossi, C.; Rigacci, S.; Stefani, M.; Casamenti, F. Oleuropein aglycone counteracts Aβ42 toxicity in the rat brain. *Neuroscience Letters* **2014**, *558*, 67-72.
116. Bisignano, G.; Tomaino, A.; Cascio, R. L.; Crisafi, G.; Uccella, N.; Saija, A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *Journal of Pharmacy and Pharmacology* **2010**, *51*, 971-974.
117. Federici, F.; Bongi, G. Improved method for isolation of bacterial inhibitors from oleuropein hydrolysis. *Applied and Environmental Microbiology* **1983**, *46*, 509-510.
118. Clodoveo, M. L.; Crupi, P.; Annunziato, A.; Corbo, F. Innovative extraction technologies for development of functional ingredients based on polyphenols from olive leaves. *Foods* **2022**, *11*, 103.
119. Papastavropoulou, K.; Pasias, I. N.; Dotsika, E.; Oz, E.; Oz, F.; Proestos, C. Separation and determination of biophenols in olive oil samples based on the official method of the international olive council and commission regulation (EU) No. 432/2012. *Separations* **2022**, *9*, 101.
120. European Commission Regulation EC No. 432/2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children's development and health. *Official Journal of the European Union* **2012**, *136*, 1-40.

121. EFSA Panel on Dietetic Products. Scientific opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage. *EFSA Journal* **2011**, *9*, 2033.
122. Celano, R.; Piccinelli, A. L.; Pugliese, A.; Carabetta, S.; di Sanzo, R.; Rastrelli, L.; Russo, M. Insights into the analysis of phenolic secoiridoids in extra virgin olive oil. *Journal of Agricultural and Food Chemistry* **2018**, *66*, 6053-6063.
123. Bongiorno, D.; Di Stefano, V.; Indelicato, S.; Avellone, G.; Ceraulo, L. Bio-phenols determination in olive oils: Recent mass spectrometry approaches. *Mass Spectrometry Reviews* **2023**, *42*, 1462-1502.
124. Casadei, E.; Valli, E.; Panni, F.; Donarski, J.; Farrús Gubern, J.; Lucci, P.; Conte, L.; Lacoste, F.; Maquet, A.; Brereton, P.; Bendini, A.; Gallina Toschi, T. Emerging trends in olive oil fraud and possible countermeasures. *Food Control* **2021**, *124*, 107902.
125. Taiti, C.; Marone, E.; Fiorino, P.; Mancuso, S. The olive oil dilemma: To be or not to be EVOO? chemometric analysis to grade virgin olive oils using 792 fingerprints from PTR-ToF-MS. *Food Control* **2022**, *135*, 108817.
126. Eyres, L.; Wong, M.; Cho, S.; Cundy, M. Exploding the myth - NZ extra virgin olive oil is an ideal frying oil. *Food New Zealand* **2019**, *19*, 12-16.
127. Erbay, Z.; Icier, F. The importance and potential uses of olive leaves. *Food Reviews International* **2010**, *26*, 319-334.
128. Topuz, S.; Bayram, M. Oleuropein extraction from leaves of three olive varieties (*Olea europaea* L.): Antioxidant and antimicrobial properties of purified oleuropein and oleuropein extracts. *Journal of Food Processing and Preservation* **2022**, *46*, 7-12.
129. Selim, S.; Albqmi, M.; Al-Sanea, M. M.; Alnusaire, T. S.; Almuhayawi, M. S.; AbdElgawad, H.; Al Jaouni, S. K.; Elkelish, A.; Hussein, S.; Warrad, M.; El-Saadony, M. T. Valorizing the usage of olive leaves, bioactive compounds, biological activities, and food applications: A comprehensive review. *Frontiers in Nutrition* **2022**, *9*, 1008349.
130. Salta, F.; Mylona, A.; Chiou, A.; Boskou, G.; Andrikopoulos, N. Oxidative stability of edible vegetable oils enriched in polyphenols with olive leaf extract. *Food Science and Technology International* **2007**, *13*, 413-421.
131. Bouaziz, M.; Fki, I.; Jemai, H.; Ayadi, M.; Sayadi, S. Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves. *Food Chemistry* **2008**, *108*, 253-262.
132. Abaza, L.; Taamalli, A.; Nsir, H.; Zarrouk, M. Olive tree (*Olea europeae* L.) leaves: Importance and advances in the analysis of phenolic compounds. *Antioxidants* **2015**, *4*, 682-698.
133. Briante, R.; Patumi, M.; Terenziani, S.; Bismuto, E.; Febbraio, F.; Nucci, R. *Olea europaea* L. leaf extract and derivatives: antioxidant properties. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 4934-4940.
134. Tsimidou, M. Z.; Papoti, V. T. Bioactive ingredients in olive leaves. In *Olives and Olive Oil in Health and Disease Prevention*; Elsevier, 2010; pp 349-356.
135. Şahin, S.; Elhussein, E.; Bilgin, M.; Lorenzo, J. M.; Barba, F. J.; Roohinejad, S. Effect of drying method on oleuropein, total phenolic content, flavonoid content, and

antioxidant activity of olive (*Olea europaea*) leaf. *Journal of Food Processing and Preservation* **2018**, *42*, e13604.

136. Ahmad-Qasem, M. H.; Ahmad-Qasem, B. H.; Barrajón-Catalán, E.; Micol, V.; Cárcel, J. A.; García-Pérez, J. V. Drying and storage of olive leaf extracts. Influence on polyphenols stability. *Industrial Crops and Products* **2016**, *79*, 232-239.
137. Ramírez, E. M.; Brenes, M.; Romero, C.; Medina, E. Olive leaf processing for infusion purposes. *Foods* **2023**, *12*, 591.
138. Boudhrioua, N.; Bahloul, N.; Ben Slimen, I.; Kechaou, N. Comparison on the total phenol contents and the color of fresh and infrared dried olive leaves. *Industrial Crops and Products* **2009**, *29*, 412-419.
139. Cör Andrejč, D.; Butinar, B.; Knez, Ž.; Tomažič, K.; Knez Marevci, M. The effect of drying methods and extraction techniques on oleuropein content in olive leaves. *Plants (Basel)* **2022**, *11*, 865.
140. Al-Rimawi, F.; Afaneh, I.; Jaraiseh, H. Effect of olive leaves drying on the content of oleuropein. *American Journal of Analytical Chemistry* **2015**, *6*, 246-252.
141. Babu, A. K.; Kumaresan, G.; Raj, V. A. A.; Velraj, R. Review of leaf drying: Mechanism and influencing parameters, drying methods, nutrient preservation, and mathematical models. *Renewable and Sustainable Energy Reviews* **2018**, *90*, 536-556.
142. Safarzadeh Markhali, F. Roles of drying, size reduction, and blanching in sustainable extraction of phenolics from olive leaves. *Processes* **2021**, *9*, 1662.
143. Crupi, P.; Dipalmo, T.; Clodoveo, M. L.; Toci, A. T.; Coletta, A. Seedless table grape residues as a source of polyphenols: Comparison and optimization of non-conventional extraction techniques. *European Food Research and Technology* **2018**, *244*, 1091-1100.
144. Sánchez de Medina, V.; Priego-Capote, F.; Jiménez-Ot, C.; Luque de Castro, M. D. Quality and stability of edible oils enriched with hydrophilic antioxidants from the olive tree: The role of enrichment extracts and lipid composition. *Journal of Agricultural and Food Chemistry* **2011**, *59*, 11432-11441.
145. Abaza, L.; Ben Youssef, N.; Manai Djebali, H.; Faouzia, H.; Methenni, K.; Zarrouk, M. Chétoui olive leaf extracts: Influence of the solvent type on phenolics and antioxidant activities. *Grasas y Aceites* **2011**, *62*, 96-104.
146. da Rosa, G. S.; Vanga, S. K.; Gariepy, Y.; Raghavan, V. Comparison of microwave, ultrasonic and conventional techniques for extraction of bioactive compounds from olive leaves (*Olea europaea* L.). *Innovative Food Science & Emerging Technologies* **2019**, *58*, 102234.
147. Şahin, S.; Bilgin, M.; Dramur, M. U. Investigation of oleuropein content in olive leaf extract obtained by supercritical fluid extraction and soxhlet methods. *Separation Science and Technology* **2011**, *46*, 1829-1837.
148. Martín-García, B.; Pimentel-Moral, S.; Gómez-Caravaca, A. M.; Arráez-Román, D.; Segura-Carretero, A. Box-Behnken experimental design for a green extraction method of phenolic compounds from olive leaves. *Industrial Crops and Products* **2020**, *154*, 112741.

149. Kiritsakis, K.; Kontominas, M. G.; Kontogiorgis, C.; Hadjipavlou-Litina, D.; Moustakas, A.; Kiritsakis, A. Composition and antioxidant activity of olive leaf extracts from Greek olive cultivars. *Journal of the American Oil Chemists' Society* **2010**, *87*, 369-376.
150. Malik, N. S. A.; Bradford, J. Recovery and stability of oleuropein and other phenolic compounds during extraction and processing of olive (*Olea europaea* L.) leaves. *Journal of Food, Agriculture and Environment* **2008**, *6*, 8-13.
151. Stamatopoulos, K.; Chatzilazarou, A.; Katsoyannos, E. Optimization of multistage extraction of olive leaves for recovery of phenolic compounds at moderated temperatures and short extraction times. *Foods* **2014**, *3*, 66-81.
152. Frankel, E.; Bakhouche, A.; Lozano-Sánchez, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Literature review on production process to obtain extra virgin olive oil enriched in bioactive compounds. Potential use of byproducts as alternative sources of polyphenols. *Journal of Agricultural and Food Chemistry* **2013**, *61*, 5179-5188.
153. Medina, E.; Romero, C.; García, P.; Brenes, M. Characterization of bioactive compounds in commercial olive leaf extracts, and olive leaves and their infusions. *Food & Function* **2019**, *10*, 4716-4724.
154. Farhoosh, R. The effect of operational parameters of the Rancimat method on the determination of the oxidative stability measures and shelf-life prediction of soybean oil. *Journal of the American Oil Chemists' Society* **2007**, *84*, 205-209.
155. International Organization for Standardization (ISO). Animal and vegetable fats and oils. Determination of peroxide value: Iodometric (visual) endpoint determination. https://www.iso.org/standard/39158.html (accessed 25th of July 2023).
156. Rubió, L.; Motilva, M.-J.; Macià, A.; Ramo, T.; Romero, M.-P. Development of a phenol-enriched olive oil with both its own phenolic compounds and complementary phenols from thyme. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 3105-3112.
157. Perestrelo, R.; Silva, C.; Silva, P.; Câmara, J. S. Global volatile profile of virgin olive oils flavoured by aromatic/medicinal plants. *Food Chemistry* **2017**, *227*, 111-121.
158. Caponio, F.; Durante, V.; Varva, G.; Silletti, R.; Previtali, M. A.; Viggiani, I.; Squeo, G.; Summo, C.; Pasqualone, A.; Gomes, T.; Baiano, A. Effect of infusion of spices into the oil vs. combined malaxation of olive paste and spices on quality of naturally flavoured virgin olive oils. *Food Chemistry* **2016**, *202*, 221-228.
159. Japón-Luján, R.; Luque de Castro, M. Liquid–liquid extraction for the enrichment of edible oils with phenols from olive leaf extracts. *Journal of Agricultural and Food Chemistry* **2008**, *56*, 2505-2511.
160. Japon-Lujan, R.; Janeiro, P.; Luque de Castro, M. D. Solid–liquid transfer of biophenols from olive leaves for the enrichment of edible oils by a dynamic ultrasound-assisted approach. *Journal of Agricultural and Food Chemistry* **2008**, *56*, 7231-7235.
161. Achat, S.; Tomao, V.; Madani, K.; Chibane, M.; Elmaataoui, M.; Dangles, O.; Chemat, F. Direct enrichment of olive oil in oleuropein by ultrasound-assisted maceration at laboratory and pilot plant scale. *Ultrasonics Sonochemistry* **2012**, *19*, 777-786.
162. Bayram, B.; Esatbeyoglu, T.; Schulze, N.; Ozcelik, B.; Frank, J.; Rimbach, G. Comprehensive analysis of polyphenols in 55 extra virgin olive oils by HPLC-ECD and

their correlation with antioxidant activities. *Plant Foods for Human Nutrition* **2012**, *67*, 326-336.

163. McDonald, S.; Prenzler, P. D.; Antolovich, M.; Robards, K. Phenolic content and antioxidant activity of olive extracts. *Food Chemistry* **2001**, *73*, 73-84.
164. Leporini, M.; Loizzo, M.; Tenuta, M.; Falco, T.; Vincenzo, S.; Pellicanò, T.; Tundis, R. Calabrian extra-virgin olive oil from Frantoio cultivar: Chemical composition and health properties. *Emirates Journal of Food and Agriculture* **2018**, *30*, 631-637.
165. Fanali, C.; Della Posta, S.; Vilmercati, A.; Dugo, L.; Russo, M.; Petitti, T.; Mondello, L.; De Gara, L. Extraction, analysis, and antioxidant activity evaluation of phenolic compounds in different Italian extra-virgin olive oils. *Molecules* **2018**, *23*, 3249.
166. Gómez-Caravaca, A. M.; Lozano-Sánchez, J.; Gámez Contreras, M. D. M.; Carretero, A. S.; Taamalli, A. Bioactive phenolic compounds from *olea europaea*: A challenge for analytical chemistry. In *Olive and Olive Oil Bioactive Constituents*; D. Boskou, Ed.; AOCS Press, 2015; pp 261-298.
167. Schneider, S. Agilent Technologies: Quality analysis of extra virgin olive oils - part 6 nutritive benefits - phenolic compounds in virgin oil. https://www.agilent.com/cs/library/applications/5991-3801EN.pdf (accessed 23rd of August 2022).
168. International Olive Council (IOC). Standards, methods and guides: Testing methods. https://www.internationaloliveoil.org/what-we-do/chemistry-standardisation-unit/standards-and-methods/ (accessed 26th of November 2022).
169. Owen, R.; Mier, W.; Giacosa, A.; Hull, W.; Spiegelhalder, B.; Bartsch, H. Phenolic compounds and squalene in olive oils: The concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food and Chemical Toxicology* **2000**, *38*, 647-659.
170. Montedoro, G.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A. Simple and hydrolyzable compounds in virgin olive oil: Spectroscopic characterizations of the secoiridoid derivatives. *Journal of Agricultural and Food Chemistry* **1993**, *41*, 2228-2234.
171. Pirisi, F. M.; Cabras, P.; Cao, C. F.; Migliorini, M.; Muggelli, M. Phenolic compounds in virgin olive oil: Reappraisal of the extraction, HPLC separation, and quantification procedures. *Journal of Agricultural and Food Chemistry* **2000**, *48*, 1191-1196.
172. Suárez, M.; Macià, A.; Romero, M.-P.; Motilva, M.-J. Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. *Journal of Chromatography A* **2008**, *1214*, 90-99.
173. Sánchez de Medina, V.; Miho, H.; Melliou, E.; Magiatis, P.; Priego-Capote, F.; Luque de Castro, M. D. Quantitative method for determination of oleocanthal and oleacein in virgin olive oils by liquid chromatography–tandem mass spectrometry. *Talanta* **2017**, *162*, 24-31.
174. Luque-Muñoz, A.; Tapia, R.; Haidour, A.; Justicia, J.; Cuerva, J. M. Direct determination of phenolic secoiridoids in olive oil by ultra-high performance liquid chromatography-triple quadruple mass spectrometry analysis. *Scientific Reports* **2019**, *9*, 1-9.

175. Kıvrak, Ş.; Kıvrak, İ. Ultrasonic-assisted extraction method of phenolic compounds in extra-virgin olive oils (EVOOs) by ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). *Separation Science and Technology* **2021**, *56*, 322-329.
176. Brenes, M.; García, A.; García, P.; Garrido, A. Rapid and complete extraction of phenols from olive oil and determination by means of a coulometric electrode array system. *Journal of Agricultural and Food Chemistry* **2000**, *48*, 5178-5183.
177. Pizarro, M. L.; Becerra, M.; Sayago, A.; Beltrán, M.; Beltrán, R. Comparison of different extraction methods to determine phenolic compounds in virgin olive oil. *Food Analytical Methods* **2013**, *6*, 123-132.
178. Murkovic, M.; Lechner, S.; Pietzka, A.; Bratacos, M.; Katzogiannos, E. Analysis of minor components in olive oil. *Journal of Biochemical and Biophysical Methods* **2004**, *61*, 155-160.
179. Mateos, R.; Espartero, J. L.; Trujillo, M.; Ríos, J. J.; León-Camacho, M.; Alcudia, F.; Cert, A. Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 2185-2192.
180. Liberatore, L.; Procida, G.; d'Alessandro, N.; Cichelli, A. Solid-phase extraction and gas chromatographic analysis of phenolic compounds in virgin olive oil. *Food Chemistry* **2001**, *73*, 119-124.
181. Hrncirik, K.; Fritsche, S. Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. *European Journal of Lipid Science and Technology* **2004**, *106*, 540-549.
182. Brahim, S. B.; Kelebek, H.; Ammar, S.; Abichou, M.; Bouaziz, M. LC–MS phenolic profiling combined with multivariate analysis as an approach for the characterization of extra virgin olive oils of four rare Tunisian cultivars during ripening. *Food chemistry* **2017**, *229*, 9-19.
183. Carrasco-Pancorbo, A.; Neusüß, C.; Pelzing, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. CE-and HPLC-TOF-MS for the characterization of phenolic compounds in olive oil. *Electrophoresis* **2007**, *28*, 806-821.
184. Saitta, M.; Curto, S. L.; Salvo, F.; Bella, G. D.; Dugo, G. Gas chromatographic–tandem mass spectrometric identification of phenolic compounds in Sicilian olive oils. *Analytica Chimica Acta* **2002**, *466*, 335-344.
185. Capriotti, A. L.; Cavaliere, C.; Crescenzi, C.; Foglia, P.; Nescatelli, R.; Samperi, R.; Laganà, A. Comparison of extraction methods for the identification and quantification of polyphenols in virgin olive oil by ultra-HPLC-QToF mass spectrometry. *Food Chemistry* **2014**, *158*, 392-400.
186. Impellizzeri, J.; Lin, J. A simple high-performance liquid chromatography method for the determination of throat-burning oleocanthal with probated antiinflammatory activity in extra virgin olive oils. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 3204-3208.
187. Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Gallina-Toschi, T.; Fernández-Gutiérrez, A. Analytical determination of polyphenols in olive oils. *Journal of Separation Science* **2005**, *28*, 837-858.

188. Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable phenolic compounds in virgin olive oil: Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *Journal of Agricultural and Food Chemistry* **1992**, *40*, 1571-1576.
189. Ben-Hassine, K.; Taamalli, A.; Ferchichi, S.; Mlaouah, A.; Benincasa, C.; Romano, E.; Flamini, G.; Lazzez, A.; Grati-kamoun, N.; Perri, E.; Malouche, D.; Hammami, M. Physicochemical and sensory characteristics of virgin olive oils in relation to cultivar, extraction system and storage conditions. *Food Research International* **2013**, *54*, 1915-1925.
190. Bakhouche, A.; Lozano-Sánchez, J.; Ballus, C. A.; Martínez-García, M.; Velasco, M. G.; Govantes, Á. O.; Gallina-Toschi, T.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Monitoring the moisture reduction and status of bioactive compounds in extra-virgin olive oil over the industrial filtration process. *Food Control* **2014**, *40*, 292-299.
191. Taamalli, A.; Gómez-Caravaca, A. M.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area. *European Food Research and Technology* **2010**, *231*, 965-975.
192. Paradiso, V. M.; Di Mattia, C.; Giarnetti, M.; Chiarini, M.; Andrich, L.; Caponio, F. Antioxidant behavior of olive phenolics in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry* **2016**, *64*, 5877-5886.
193. Litridou, M.; Linssen, J.; Schols, H.; Bergmans, M.; Posthumus, M.; Tsimidou, M.; Boskou, D. Phenolic compounds in virgin olive oils: Fractionation by solid phase extraction and antioxidant activity assessment. *Journal of the Science of Food and Agriculture* **1997**, *74*, 169-174.
194. Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Lercker, G.; Gallina Toschi, T. Liquid–liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. *Journal of Chromatography A* **2003**, *985*, 425-433.
195. Jobgen, W. S.; Jobgen, S. C.; Li, H.; Meininger, C. J.; Wu, G. Analysis of nitrite and nitrate in biological samples using high-performance liquid chromatography. *Journal of Chromatography B* **2007**, *851*, 71-82.
196. Cunha, S. C.; Amaral, J. S.; Fernandes, J. O.; Oliveira, M. B. P. Quantification of tocopherols and tocotrienols in Portuguese olive oils using HPLC with three different detection systems. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 3351-3356.
197. Ben Mohamed, M.; Guasmi, F.; Ben Ali, S.; Radhouani, F.; Faghim, J.; Triki, T.; Kammoun, N. G.; Baffi, C.; Lucini, L.; Benincasa, C. The LC-MS/MS characterization of phenolic compounds in leaves allows classifying olive cultivars grown in South Tunisia. *Biochemical Systematics and Ecology* **2018**, *78*, 84-90.
198. Lopez-Fernandez, O.; Dominguez, R.; Pateiro, M.; Munekata, P. E. S.; Rocchetti, G.; Lorenzo, J. M. Determination of polyphenols using liquid chromatography-tandem mass spectrometry technique (LC-MS/MS): A Review. *Antioxidants (Basel)* **2020**, *9*, 479.

199. González-Hedström, D.; Granado, M.; Inarejos-García, A. M. Protective effects of extra virgin olive oil against storage-induced omega 3 fatty acid oxidation of algae oil. *NFS Journal* **2020**, *21*, 9-15.
200. Bendini, A.; Cerretani, L.; Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Lercker, G. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules* **2007**, *12*, 1679-1719.
201. Sacchi, R.; Addeo, F.; Paolillo, L. H and $^{13}C$ NMR of virgin oil. An overview. *Magnetic Resonance in Chemistry* **1997**, *35*, 5133-5145.
202. Vlahov, G. Application of NMR to the study of olive oils. *Progress in Nuclear Magnetic Resonance Spectroscopy* **1999**, *35*, 341-357.
203. Monasterio, R. P.; Fernández, M. d. l. Á.; Silva, M. F. Olive oil by capillary electrophoresis: Characterization and genuineness. *Journal of Agricultural and Food Chemistry* **2013**, *61*, 4477-4496.
204. Nevado, J. J. B.; Peñalvo, G. C.; Robledo, V. R.; Martínez, G. V. New CE–ESI-MS analytical method for the separation, identification and quantification of seven phenolic acids including three isomer compounds in virgin olive oil. *Talanta* **2009**, *79*, 1238-1246.
205. Robards, K.; Ryan, D. Principles and practice of modern chromatographic methods. Elsevier Science & Technology: United Kingdom, 2021; pp 1-40.
206. Green, J. M. Peer reviewed: A practical guide to analytical method validation. *Analytical Chemistry* **1996**, *68*, 305A-309A.
207. McMillan, J. Principles of analytical validation. In *Proteomic Profiling and Analytical Chemistry (Second Edition)*; P. Ciborowski and J. Silberring, Eds.; Elsevier: Boston, 2016; pp 239-251.
208. Romani, A.; Mulas, S.; Heimler, D. Polyphenols and secoiridoids in raw material (*Olea europaea* L. leaves) and commercial food supplements. *European Food Research and Technology* **2017**, *243*, 429-435.
209. Romero, C.; Medina, E.; Mateo, M. A.; Brenes, M. Quantification of bioactive compounds in Picual and Arbequina olive leaves and fruit. *Journal of the Science of Food and Agriculture* **2017**, *97*, 1725-1732.
210. Martínez-Navarro, M. E.; Cebrián-Tarancón, C.; Oliva, J.; Salinas, M. R.; Alonso, G. L. Oleuropein degradation kinetics in olive leaf and its aqueous extracts. *Antioxidants* **2021**, *10*, 1963.
211. Oe, T.; Tian, Y.; O'Dwyer, P. J.; Roberts, D. W.; Malone, M. D.; Bailey, C. J.; Blair, I. A. A validated liquid chromatography/tandem mass spectrometry assay for *cis*-amminedichloro(2-methylpyridine)platinum(ii) in human plasma ultrafiltrate. *Analytical Chemistry* **2002**, *74*, 591-599.
212. Yuan, L.; Zhang, D.; Jemal, M.; Aubry, A.-F. Systematic evaluation of the root cause of non-linearity in liquid chromatography/tandem mass spectrometry bioanalytical assays and strategy to predict and extend the linear standard curve range. *Rapid Communications in Mass Spectrometry* **2012**, *26*, 1465-1474.
213. Liu, Q.; Jiang, F.; Zhu, J.; Zhong, G.; Huang, M. Development, validation, and application of a new method to correct the nonlinearity problem in LC-MS/MS

quantification using stable isotope-labeled internal standards. *Analytical Chemistry* **2019**, *91*, 9616-9622.

214. Shrivastava, A.; Gupta, V. B. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists* **2011**, *2*, 21-25.
215. Gustavo González, A.; Ángeles Herrador, M. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *TrAC Trends in Analytical Chemistry* **2007**, *26*, 227-238.
216. Borman, P.; Elder, D. Q2(R1) Validation of analytical procedures. In *ICH Quality Guidelines*, 2017; pp 127-166.
217. Araujo, P. Key aspects of analytical method validation and linearity evaluation. *Journal of Chromatography B* **2009**, *877*, 2224-2234.
218. Stöckl, D.; D'Hondt, H.; Thienpont, L. M. Method validation across the disciplines—Critical investigation of major validation criteria and associated experimental protocols. *Journal of Chromatography B* **2009**, *877*, 2180-2190.
219. Australian Pesticides & Veterinary Medicines Authority. Validation of analytical methods for active constituents and agricultural products. https://apvma.gov.au/node/1048 (accessed 9th of February 2023).
220. Monteiro, M.; Silva, A. F. R.; Resende, D.; Braga, S. S.; Coimbra, M. A.; Silva, A. M. S.; Cardoso, S. M. Strategies to broaden the applications of olive biophenols oleuropein and hydroxytyrosol in food products. *Antioxidants* **2021**, *10*, 444.
221. Cavaca, L. A. S.; Afonso, C. A. M. Oleuropein: A valuable bio-renewable synthetic building block. *European Journal of Organic Chemistry* **2018**, *2018*, 581-589.
222. Zhang, X.; Zhao, J.; Wang, C.; Zhu, L.; Pan, X.; Liu, Y.; Li, J.; Guo, X.; Chen, D. Measurement of sucrose in beverages using a blood glucose meter with cascade-catalysis enzyme particle. *Food Chemistry* **2023**, *398*, 133951.
223. Heinzerling, P.; Schrader, F.; Schanze, S. Measurement of enzyme kinetics by use of a blood glucometer: Hydrolysis of sucrose and lactose. *Journal of Chemical Education* **2012**, *89*, 1582-1586.
224. Hardee, J. R.; Delgado, B.; Jones, W. Kinetic parameters for the noncatalyzed and enzyme-catalyzed mutarotation of glucose using a blood glucometer. *Journal of Chemical Education* **2011**, *88*, 798-800.
225. Galant, A. L.; Kaufman, R. C.; Wilson, J. D. Glucose: Detection and analysis. *Food Chemistry* **2015**, *188*, 149-160.
226. LGC. Preparation of calibration curves: A guides to best practice. https://biosearch-cdn.azureedge.net/assetsv6/Calibration-curve-guide.pdf (accessed 12th of July 2023).
227. Lockyer, S.; Rowland, I. Authorised EU health claims for polyphenols in olive oil. In *Foods, Nutrients and Food Ingredients with Authorised EU Health Claims*; M. J. Sadler, Ed.; Woodhead Publishing, 2014; pp 212-228.
228. García-Rodríguez, R.; Belaj, A.; Romero-Segura, C.; Sanz, C.; Pérez, A. G. Exploration of genetic resources to improve the functional quality of virgin olive oil. *Journal of Functional Foods* **2017**, *38*, 1-8.

229. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Quantitative measurement of major secoiridoid derivatives in olive oil using qNMR. Proof of the artificial formation of aldehydic oleuropein and ligstroside aglycon isomers. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 600-607.
230. Miho, H.; Diez, C. M.; Mena-Bravo, A.; Sanchez de Medina, V.; Moral, J.; Melliou, E.; Magiatis, P.; Rallo, L.; Barranco, D.; Priego-Capote, F. Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. *Food Chemistry* **2018**, *266*, 192-199.
231. Miho, H.; Moral, J.; Barranco, D.; Ledesma-Escobar, C. A.; Priego-Capote, F.; Díez, C. M. Influence of genetic and interannual factors on the phenolic profiles of virgin olive oils. *Food Chemistry* **2021**, *342*, 128357.
232. Caporaso, N.; Savarese, M.; Paduano, A.; Guidone, G.; De Marco, E.; Sacchi, R. Nutritional quality assessment of extra virgin olive oil from the Italian retail market: Do natural antioxidants satisfy EFSA health claims? *Journal of Food Composition and Analysis* **2015**, *40*, 154-162.
233. Fregapane, G.; Gómez-Rico, A.; Inarejos, A. M.; Salvador, M. D. Relevance of minor components stability in commercial olive oil quality during the market period. *European Journal of Lipid Science and Technology* **2013**, *115*, 541-548.
234. Diamantakos, P.; Ioannidis, K.; Papanikolaou, C.; Tsolakou, A.; Rigakou, A.; Melliou, E.; Magiatis, P. A new definition of the term "high-phenolic olive oil" based on large scale statistical data of Greek olive oils analyzed by qNMR. *Molecules* **2021**, *26*, 1115.
235. Kritikou, E.; Kalogiouri, N. P.; Kostakis, M.; Kanakis, D.-C.; Martakos, I.; Lazarou, C.; Pentogennis, M.; Thomaidis, N. S. Geographical characterization of olive oils from the North Aegean region based on the analysis of biophenols with UHPLC-QTOF-MS. *Foods* **2021**, *10*, 2102.
236. Bellumori, M.; Cecchi, L.; Innocenti, M.; Clodoveo, M. L.; Corbo, F.; Mulinacci, N. The EFSA health claim on olive oil polyphenols: Acid hydrolysis validation and total hydroxytyrosol and tyrosol determination in Italian virgin olive oils. *Molecules* **2019**, *24*, 2179.
237. Bimbo, F.; Roselli, L.; Carlucci, D.; de Gennaro, B. C. Consumer misuse of country-of-origin label: Insights from the Italian extra-virgin olive oil market. *Nutrients* **2020**, *12*, 2150.
238. Directorate-General for Agriculture and Rural Development. Producing 69% of the world's production, the EU is the largest producer of olive oil. https://agriculture.ec.europa.eu/news/producing-69-worlds-production-eu-largest-producer-olive-oil-2020-02-04_en (accessed 11th of July 2023).
239. The Village Press. About Us - From Humble Beginnings https://thevillagepress.co.nz/about-us/ (accessed 1st of August 2023).
240. Latino, M. E.; De Devitiis, B.; Corallo, A.; Viscecchia, R.; Bimbo, F. Consumer acceptance and preference for olive oil attributes - a review. *Foods* **2022**, *11*, 3805.
241. Groves, A. Authentic british food products: A review of consumer perceptions. *International Journal of Consumer Studies* **2008**, *25*, 246-254.
242. Spielmann, N.; Charters, S. The dimensions of authenticity for terroir products. *International Journal of Wine Business Research* **2014**, *25*, 310-324.

243. Sanaeifar, A.; Jafari, A. Determination of the oxidative stability of olive oil using an integrated system based on dielectric spectroscopy and computer vision. *Information Processing in Agriculture* **2019**, *6*, 20-25.
244. Cicerale, S.; Conlan, X. A.; Barnett, N. W.; Keast, R. S. J. Storage of extra virgin olive oil and its effect on the biological activity and concentration of oleocanthal. *Food Research International* **2013**, *50*, 597-602.
245. Gargouri, B.; Zribi, A.; Bouaziz, M. Effect of containers on the quality of Chemlali olive oil during storage. *Journal of Food Science and Technology* **2015**, *52*, 1948-1959.
246. Sanmartin, C.; Venturi, F.; Macaluso, M.; Nari, A.; Quartacci, M. F.; Sgherri, C.; Flamini, G.; Taglieri, I.; Ascrizzi, R.; Andrich, G.; Zinnai, A. Preliminary results about the use of argon and carbon dioxide in the extra virgin olive oil (EVOO) storage to extend oil shelf life: Chemical and sensorial point of view. *European Journal of Lipid Science and Technology* **2018**, *120*, 1800156.
247. Martín-Tornero, E.; Fernández, A.; Durán-Merás, I.; Martín-Vertedor, D. Fluorescence monitoring oxidation of extra virgin olive oil packed in different containers. *Molecules* **2022**, *27*, 7254.
248. Sanmartin, C.; Venturi, F.; Sgherri, C.; Nari, A.; Macaluso, M.; Flamini, G.; Quartacci, M. F.; Taglieri, I.; Andrich, G.; Zinnai, A. The effects of packaging and storage temperature on the shelf-life of extra virgin olive oil. *Heliyon* **2018**, *4*, 1-18.
249. Morelló, J. R.; Motilva, M. J.; Tovar, M. J.; Romero, M. P. Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chemistry* **2004**, *85*, 357-364.
250. Morales, M. T.; Przybylski, R. Olive oil oxidation. In *Handbook of Olive Oil: Analysis and Properties*; J. Harwood and R. Aparicio, Eds.; Springer US: Boston, MA, 2000; pp 459-490.
251. Tarapoulouzi, M.; Agriopoulou, S.; Koidis, A.; Proestos, C.; Enshasy, H. A. E.; Varzakas, T. Recent advances in analytical methods for the detection of olive oil oxidation status during storage along with chemometrics, authenticity and fraud studies. *Biomolecules* **2022**, *12*, 1180.
252. Rodrigues, N.; Dias, L. G.; Veloso, A. C. A.; Pereira, J. A.; Peres, A. M. Monitoring olive oils quality and oxidative resistance during storage using an electronic tongue. *LWT* **2016**, *73*, 683-692.
253. Krichene, D.; Salvador, M. D.; Fregapane, G. Stability of virgin olive oil phenolic compounds during long-term storage (18 months) at temperatures of 5–50 °C. *Journal of Agricultural and Food Chemistry* **2015**, *63*, 6779-6786.
254. Helvaci, H. U.; Menon, A.; Aydemir, L.; Korel, F.; Gökçen Akkurt, G. Drying of olive leaves in a geothermal dryer and determination of quality parameters of dried product. *Energy Procedia* **2019**, *161*, 108-114.
255. Kamran, M.; Hamlin, A. S.; Scott, C. J.; Obied, H. K. Drying at high temperature for a short time maximizes the recovery of olive leaf biophenols. *Industrial Crops and Products* **2015**, *78*, 29-38.
256. Cabrera-Gomez, A.; Garrido, A.; Guerrero, J.; Ortiz, V. Nutritive value of the olive leaf: effects of cultivar, season of harvesting and system of drying. *The Journal of Agricultural Science* **1992**, *119*, 205-210.

257. Ghomari, O.; Sounni, F.; Massaoudi, Y.; Ghanam, J.; Drissi Kaitouni, L. B.; Merzouki, M.; Benlemlih, M. Phenolic profile (HPLC-UV) of olive leaves according to extraction procedure and assessment of antibacterial activity. *Biotechnology Reports* **2019**, *23*, e00347.
258. Chang, C.-H.; Lin, H.-Y.; Chang, C.-Y.; Liu, Y.-C. Comparisons on the antioxidant properties of fresh, freeze-dried and hot-air-dried tomatoes. *Journal of Food Engineering* **2006**, *77*, 478-485.
259. Kozłowska, M.; Ścibisz, I.; Przybył, J.; Ziarno, M.; Żbikowska, A.; Majewska, E. Phenolic contents and antioxidant activity of extracts of selected fresh and dried herbal materials. *Polish Journal of Food and Nutrition Sciences* **2021**, *71*, 269-278.
260. Garcìa, L. M.; Ceccanti, C.; Negro, C.; De Bellis, L.; Incrocci, L.; Pardossi, A.; Guidi, L. Effect of drying methods on phenolic compounds and antioxidant activity of *Urtica dioica* L. Leaves. *Horticulturae* **2021**, *7*, 10.
261. Hata, S. Effect of drying temperature on the oleuropein content of olive (*Olea europea* L.) leaves. *Food Preservation Science* **2004**, *30*, 191-193.
262. Malik, N. S. A. Recovery and stability of oleuropein and other phenolic compounds during extraction and processing of olive (*Olea europaea* L.) leaves. *Journal of Food, Agriculture & Environment* **2008**, *6*, 8-13.
263. Ahmad-Qasem, M. H.; Barrajón-Catalán, E.; Micol, V.; Mulet, A.; García-Pérez, J. V. Influence of freezing and dehydration of olive leaves (var. Serrana) on extract composition and antioxidant potential. *Food Research International* **2013**, *50*, 189-196.
264. Hirun, S.; Utama-Ang, N.; Roach, P. D. Turmeric (*Curcuma longa* L.) drying: an optimization approach using microwave-vacuum drying. *Journal of Food Science Technology* **2014**, *51*, 2127-2133.
265. De Leonardis, A.; Aretini, A.; Alfano, G.; Macciola, V.; Giancarlo, R. Isolation of a hydroxytyrosol-rich extract from olive leaves (*Olea Europaea* L.) and evaluation of its antioxidant properties and bioactivity. *European Food Research and Technology* **2008**, *226*, 653-659.
266. Attya, M.; Benabdelkamel, H.; Perri, E.; Russo, A.; Sindona, G. Effects of conventional heating on the stability of major olive oil phenolic compounds by tandem mass spectrometry and isotope dilution assay. *Molecules* **2010**, *15*, 8734-8746.
267. Stamatopoulos, K.; Katsoyannos, E.; Chatzilazarou, A. Antioxidant activity and thermal stability of oleuropein and related phenolic compounds of olive leaf extract after separation and concentration by salting-out-assisted cloud point extraction. *Antioxidants (Basel)* **2014**, *3*, 229-244.
268. Gonzalez-Ortega, R.; Di Mattia, C. D.; Pittia, P.; Natasa, P. U. Effect of heat treatment on phenolic composition and radical scavenging activity of olive leaf extract at different pH conditions: a spectroscopic and kinetic study. *Journal of the Science of Food and Agriculture* **2023**, *103*, 2047-2056.
269. Mousavi, S.; Mariotti, R.; Stanzione, V.; Pandolfi, S.; Mastio, V.; Baldoni, L.; Cultrera, N. G. M. Evolution of extra virgin olive oil quality under different storage conditions. *Foods* **2021**, *10*, 1945.

270. Di Stefano, V.; Melilli, M. G. Effect of storage on quality parameters and phenolic content of Italian extra-virgin olive oils. *Natural Product Research* **2020**, *34*, 78-86.
271. Feng, S.; Zhang, C.; Liu, L.; Xu, Z.; Chen, T.; Zhou, L.; Yuan, M.; Li, T.; Ding, C. Comparison of Phenolic Compounds in Olive Leaves by Different Drying and Storage Methods. *Separations* **2021**, *8*, 156.
272. Karković Marković, A.; Torić, J.; Barbarić, M.; Jakobušić Brala, C. Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. *Molecules* **2019**, *24*, 2001.
273. Yalcin, S.; Schreiner, M. Stabilities of tocopherols and phenolic compounds in virgin olive oil during thermal oxidation. *Journal of Food Science Technology* **2018**, *55*, 244-251.
274. Gómez-Alonso, S.; Salvador, M. D.; Fregapane, G. Phenolic compounds profile of Cornicabra virgin olive oil. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 6812-6817.
275. Hrncirik, K.; Fritsche, S. Relation between the endogenous antioxidant system and the quality of extra virgin olive oil under accelerated storage conditions. *Journal of Agricultural and Food Chemistry* **2005**, *53*, 2103-2110.
276. Olajuyigbe, F. M.; Nlekerem, C. M.; Ogunyewo, O. A. Production and characterization of highly thermostable β-glucosidase during the biodegradation of methyl cellulose by *Fusarium oxysporum*. *Biochemistry Research International* **2016**, *2016*, 3978124.
277. Hati, S.; Ningtyas, D. W.; Khanuja, J. K.; Prakash, S. β-Glucosidase from almonds and yoghurt cultures in the biotransformation of isoflavones in soy milk. *Food Bioscience* **2020**, *34*, 100542.
278. Montero, M. A.; Romeu, A. Properties of almond beta-glucosidase immobilized on concanavalin A-sepharose. *Biochemistry and Molecular Biology Education* **1993**, *30*, 685-689.
279. Sigma. Sigma quality control test procedure - enzymatic assay of β-glucosidase. https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/205/287/g0395enz.pdf (accessed 27th of July 2023).
280. Sista, R. V.; Erickson, M. C.; Shewfelt, R. L. Quality deterioration in frozen foods associated with hydrolytic enzyme activities. In *Quality in Frozen Food*; M. C. Erickson and Y.-C. Hung, Eds.; Springer US: Boston, MA, 1997; pp 101-110.
281. Bennett, B. L. The new kid on the shelves. In *Almond flour: The high-protein, gluten-free choice for baking and cooking*; Book Publishing Company, 2016; pp 6-7.
282. Altan, A.; McCarthy, K. L.; Tikekar, R.; McCarthy, M. J.; Nitin, N. Image analysis of microstructural changes in almond cotyledon as a result of processing. *Journal of Food Science* **2011**, *76*, E212-E221.
283. Mandalari, G.; Tomaino, A.; Arcoraci, T.; Martorana, M.; Turco, V. L.; Cacciola, F.; Rich, G. T.; Bisignano, C.; Saija, A.; Dugo, P.; Cross, K. L.; Parker, M. L.; Waldron, K. W.; Wickham, M. S. J. Characterization of polyphenols, lipids and dietary fibre from almond skins (*Amygdalus communis* L.). *Journal of Food Composition and Analysis* **2010**, *23*, 166-174.
284. Deeth, H. C. Heat-induced inactivation of enzymes in milk and dairy products. A review. *International Dairy Journal* **2021**, *121*, 105104.

285. Ann Lila, M. The nature-versus-nurture debate on bioactive phytochemicals: the genome versus terroir. *Journal of the Science of Food and Agriculture* **2006**, *86*, 2510-2515.
286. Chen, C. O.; Milbury, P. E.; Blumberg, J. B. Polyphenols in almond skins after blanching modulate plasma biomarkers of oxidative stress in healthy humans. *Antioxidants (Basel)* **2019**, *8*, 95.
287. Ivancic, T.; Jakopic, J.; Veberic, R.; Vesel, V.; Hudina, M. Effect of ripening on the phenolic and sugar contents in the meso- and epicarp of olive fruits (*Olea europaea* L.) cultivar "Leccino" *Agriculture* **2022**, *12*, 1347.
288. Negro, M. J.; Alvarez, C.; Ballesteros, I.; Romero, I.; Ballesteros, M.; Castro, E.; Manzanares, P.; Moya, M.; Oliva, J. M. Ethanol production from glucose and xylose obtained from steam exploded water-extracted olive tree pruning using phosphoric acid as catalyst. *Bioresource Technology* **2014**, *153*, 101-107.
289. Barreca, D.; Nabavi, S. M.; Sureda, A.; Rasekhian, M.; Raciti, R.; Silva, A. S.; Annunziata, G.; Arnone, A.; Tenore, G. C.; Süntar, İ.; Mandalari, G. Almonds (*Prunus Dulcis* Mill. D. A. Webb): A source of nutrients and health-promoting compounds. *Nutrients* **2020**, *12*, 672.
290. Ghelichi, S.; Hajfathalian, M.; Yesiltas, B.; Sørensen, A.-D. M.; García-Moreno, P. J.; Jacobsen, C. Oxidation and oxidative stability in emulsions. *Comprehensive Reviews in Food Science and Food Safety* **2023**, *22*, 1864-1901.
291. Gutiérrez, J. M.; González, C.; Maestro, A.; Solè, I.; Pey, C. M.; Nolla, J. Nano-emulsions: New applications and optimization of their preparation. *Current Opinion in Colloid & Interface Science* **2008**, *13*, 245-251.
292. Coupland, J. N.; McClements, D. J. Lipid oxidation in food emulsions. *Trends in Food Science & Technology* **1996**, *7*, 83-91.
293. Sathe, S. Solubilization, electrophoretic characterization and in vitro digestibility of almond (*Prunus amygdalus*) proteins. *Journal of Food Biochemistry* **1992**, *16*, 249-264.
294. Young, C.; Cunningham, S. Exploring the partnership of almonds with cereal foods. *Cereal Foods World* **1991**, *36*, 412-418.
295. Sze-Tao, K. W. C.; Sathe, S. K. Functional properties and in vitro digestibility of almond (*Prunus dulcis* L.) protein isolate. *Food Chemistry* **2000**, *69*, 153-160.
296. Criado-Navarro, I.; Ledesma-Escobar, C. A.; Parrado-Martínez, M. J.; Marchal-López, R. M.; Olmo-Peinado, J. M.; Espejo-Calvo, J. A.; Priego-Capote, F. Monitoring the partition of bioactive compounds in the extraction of extra virgin olive oil. *LWT - Food Science and Technology* **2022**, *162*, 113433.
297. Matsuura, M.; Obata, A. β-glucosidases from soybeans hydrolyze daidzin and genistin. *Journal of Food Science* **1993**, *58*, 144-147.
298. Lopes Barbosa, A. C.; Lajolo, F. M.; Genovese, M. I. Influence of temperature, pH and ionic strength on the production of isoflavone-rich soy protein isolates. *Food Chemistry* **2006**, *98*, 757-766.
299. Bau, T. R.; Ida, E. I. Soymilk processing with higher isoflavone aglycone content. *Food Chemistry* **2015**, *183*, 161-168.

300. Sanches de Lima, F.; Ida, E. I. Optimisation of soybean hydrothermal treatment for the conversion of β-glucoside isoflavones to aglycones. *LWT - Food Science and Technology* **2014**, *56*, 232-239.
301. Lozano-Castellón, J.; Vallverdú-Queralt, A.; Rinaldi de Alvarenga, J. F.; Illán, M.; Torrado-Prat, X.; Lamuela-Raventós, R. M. Domestic sautéing with EVOO: Change in the phenolic profile. *Antioxidants (Basel)* **2020**, *9*, 77.
302. Vallverdú-Queralt, A.; Regueiro, J.; Rinaldi de Alvarenga, J. F.; Torrado, X.; Lamuela-Raventos, R. M. Home cooking and phenolics: Effect of thermal treatment and addition of extra virgin olive oil on the phenolic profile of tomato sauces. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 3314-3320.
303. Hvattum, E.; Ekeberg, D. Study of the collision-induced radical cleavage of flavonoid glycosides using negative electrospray ionization tandem quadrupole mass spectrometry. *Journal of Mass Spectrometry* **2003**, *38*, 43-49.
304. Singleton, V. L. Oxygen with phenols and related reactions in musts, wines, and model systems: Observations and practical implications. *American Journal of Enology and Viticulture* **1987**, *38*, 69-77.
305. Grainger, M. N. C. Kinetics of conversion of dihydroxyacetone to methylglyoxal in honey. Doctoral Thesis, University of Waikato, Hamilton, New Zealand, 2015.
306. Lozano-Sánchez, J.; Bendini, A.; Quirantes-Piné, R.; Cerretani, L.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Monitoring the bioactive compounds status of extra-virgin olive oil and storage by-products over the shelf life. *Food Control* **2013**, *30*, 606-615.
307. Misono, T. Interfacial tension between water and oil. In *Measurement Techniques and Practices of Colloid and Interface Phenomena*; M. Abe, Ed.; Springer Singapore: Singapore, 2019; pp 39-44.
308. Lee, T. K.; Hur, G.; Choi, J.; Ban, C.; Kim, J.-Y.; Yang, H.; Park, J. H. Y.; Lee, K. W.; Kim, J. H. Enhancing stability and bioavailability of sulforaphene in radish seed extracts using nanoemulsion made with high oleic sunflower oil. *Food Science and Biotechnology* **2023**, *32*, 1269-1279.
309. Gmach, O.; Bertsch, A.; Bilke-Krause, C.; Kulozik, U. Impact of oil type and pH value on oil-in-water emulsions stabilized by egg yolk granules. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2019**, *581*, 123788.
310. Anestopoulos, I.; Kiousi, D. E.; Klavaris, A.; Galanis, A.; Salek, K.; Euston, S. R.; Pappa, A.; Panayiotidis, M. I. Surface active agents and their health-promoting properties: Molecules of multifunctional significance. *Pharmaceutics* **2020**, *12*, 688.
311. Dugan, P. J.; Siegrist, R. L.; Crimi, M. L. Coupling surfactants/cosolvents with oxidants for enhanced DNAPL removal: A review. *Remediation Journal* **2010**, *20*, 27-49.
312. Mulligan, C. N.; Yong, R. N.; Gibbs, B. F. Surfactant-enhanced remediation of contaminated soil: a review. *Engineering Geology* **2001**, *60*, 371-380.
313. Besha, A. T.; Bekele, D. N.; Naidu, R.; Chadalavada, S. Recent advances in surfactant-enhanced In-Situ chemical oxidation for the remediation of non-aqueous phase liquid contaminated soils and aquifers. *Environmental Technology & Innovation* **2018**, *9*, 303-322.
314. Gaonkar, A. G. Interfacial tensions of vegetable oil/water systems: Effect of oil purification. *Journal of the American Oil Chemists' Society* **1989**, *66*, 1090-1092.

315. Fisher, L. R.; Mitchell, E. E.; Parker, N. S. Interfacial tensions of commercial vegetable oils with water. *Journal of Food Science* **1985**, *50*, 1201-1202.
316. Dopierala, K.; Javadi, A.; Krägel, J.; Schano, K. H.; Kalogianni, E. P.; Leser, M. E.; Miller, R. Dynamic interfacial tensions of dietary oils. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2011**, *382*, 261-265.
317. Ranieri, G.; Mazzei, R.; Poerio, T.; Bazzarelli, F.; Wu, Z.; Li, K.; Giorno, L. Biorefinery of olive leaves to produce dry oleuropein aglycone: Use of homemade ceramic capillary biocatalytic membranes in a multiphase system. *Chemical Engineering Science* **2018**, *185*, 149-156.
318. Paiva-Martins, F.; Gordon, M. H. Effects of pH and ferric ions on the antioxidant activity of olive polyphenols in oil-in-water emulsions. *Journal of the American Oil Chemists' Society* **2002**, *79*, 571-576.
319. Shahidi, F.; Zhong, Y. Revisiting the polar paradox theory: A critical overview. *Journal of Agricultural and Food Chemistry* **2011**, *59*, 3499-3504.
320. Chaiyasit, W.; Elias, R. J.; McClements, D. J.; Decker, E. A. Role of physical structures in bulk oils on lipid oxidation. *Critical Reviews in Food Science and Nutrition* **2007**, *47*, 299-317.
321. Porter, W. L. Paradoxical behavior of antioxidants in food and biological systems. *Toxicology and Industrial Health* **1993**, *9*, 93-122.
322. Frankel, E. N.; Huang, S.-W.; Kanner, J.; German, J. B. Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. *Journal of Agricultural and Food Chemistry* **1994**, *42*, 1054-1059.
323. Gordon, M. H.; Paiva-Martins, F.; Almeida, M. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 2480-2485.
324. Zhong, Y.; Shahidi, F. Antioxidant behavior in bulk oil: Limitations of polar paradox theory. *Journal of Agricultural and Food Chemistry* **2012**, *60*, 4-6.
325. Tadros, T. F. Surfactants, Industrial Applications. In *Encyclopedia of Physical Science and Technology (Third Edition)*; R. A. Meyers, Ed.; Academic Press: New York, 2003; pp 423-438.
326. Mhlwatika, Z.; Aderibigbe, B. Polymeric nanocarriers for the delivery of antimalarials. *Molecules* **2018**, *23*, 2527.
327. Koopaie, M. Nanoparticulate systems for dental drug delivery. In *Nanoengineered Biomaterials for Advanced Drug Delivery*; M. Mozafari, Ed.; Elsevier, 2020; pp 525-559.
328. Fu, C.; Li, Z.; Sun, Z.; Xie, S. A review of salting-out effect and sugaring-out effect: driving forces for novel liquid-liquid extraction of biofuels and biochemicals. *Frontiers of Chemical Science and Engineering* **2021**, *15*, 854-871.
329. Poole, C. F. Milestones in the development of liquid-phase extraction techniques. In *Liquid-Phase Extraction*; C. F. Poole, Ed.; Elsevier, 2020; pp 1-44.
330. Wang, B.; Ezejias, T.; Feng, H.; Blaschek, H. Sugaring-out: A novel phase separation and extraction system. *Chemical Engineering Science* **2008**, *63*, 2595-2600.
331. Yaghoub, M.; Farideh, E. Membrane and bioseparation. In *Advances in Membrane Technologies*; A. Amira, Ed.; IntechOpen: Rijeka, 2020; pp 1-35.

332. Karan, R.; Capes, M. D.; DasSarma, S. Function and biotechnology of extremophilic enzymes in low water activity. *Aquatic Biosystems* **2012**, *8*, 4.
333. Mancinelli, R.; Botti, A.; Bruni, F.; Ricci, M. A.; Soper, A. K. Hydration of sodium, potassium, and chloride ions in solution and the concept of structure maker/breaker. *The Journal of Physical Chemistry B* **2007**, *111*, 13570-13577.
334. Spitzer, J. From water and ions to crowded biomacromolecules: in vivo structuring of a prokaryotic cell. *Microbiology and Molecular Biology Reviews* **2011**, *75*, 491-506.
335. Rezaei, K.; Jenab, E.; Temelli, F. Effects of water on enzyme performance with an emphasis on the reactions in supercritical fluids. *Critical Reviews in Biotechnology* **2007**, *27*, 183-195.
336. DeMan, J. M.; Finley, J. W.; Hurst, W. J.; Lee, C. Y. Water. In *Principles of Food Chemistry*; Springer, 1999; pp 29.
337. Panuwet, P.; Hunter, R. E., Jr.; D'Souza, P. E.; Chen, X.; Radford, S. A.; Cohen, J. R.; Marder, M. E.; Kartavenka, K.; Ryan, P. B.; Barr, D. B. Biological matrix effects in quantitative tandem mass spectrometry-based analytical methods: Advancing biomonitoring. *Critical Reviews in Analytical Chemistry* **2016**, *46*, 93-105.
338. Inarejos-García, A. M.; Gómez-Rico, A.; Salvador, M. D.; Fregapane, G. Influence of malaxation conditions on virgin olive oil yield, overall quality and composition. *European Food Research and Technology* **2009**, *228*, 671-677.
339. Clodoveo, M. L. Malaxation: Influence on virgin olive oil quality. Past, present and future–An overview. *Trends in Food Science & Technology* **2012**, *25*, 13-23.
340. Peri, C. Quality excellence in extra virgin olive oils. In *Olive Oil Sensory Science*; M. Erminio and L. Susan, Eds., 2014; pp 1-32.
341. Plasquy, E.; García Martos, J. M.; Florido Fernández, M. d. C.; Sola-Guirado, R. R.; García Martín, J. F. Adjustment of olive fruit temperature before grinding for olive oil extraction. Experimental study and pilot plant trials. *Processes* **2021**, *9*, 586.
342. Olmo-Cunillera, A.; Lozano-Castellón, J.; Pérez, M.; Miliarakis, E.; Tresserra-Rimbau, A.; Ninot, A.; Romero-Aroca, A.; Lamuela-Raventós, R. M.; Vallverdú-Queralt, A. Optimizing the malaxation conditions to produce an Arbequina EVOO with high content of bioactive compounds. *Antioxidants (Basel)* **2021**, *10*, 1819.
343. Lukić, I.; Krapac, M.; Horvat, I.; Godena, S.; Kosić, U.; Brkić Bubola, K. Three-factor approach for balancing the concentrations of phenols and volatiles in virgin olive oil from a late-ripening olive cultivar. *LWT - Food Science and Technology* **2018**, *87*, 194-202.
344. Gómez-Rico, A.; Inarejos-García, A. M.; Salvador, M. D.; Fregapane, G. Effect of malaxation conditions on phenol and volatile profiles in olive paste and the corresponding virgin olive oils (*Olea europaea* L. Cv. Cornicabra). *Journal of Agricultural and Food Chemistry* **2009**, *57*, 3587-3595.
345. Taticchi, A.; Esposto, S.; Veneziani, G.; Urbani, S.; Selvaggini, R.; Servili, M. The influence of the malaxation temperature on the activity of polyphenoloxidase and peroxidase and on the phenolic composition of virgin olive oil. *Food Chemistry* **2013**, *136*, 975-983.
346. Ortega-García, F.; Peragón, J. Phenylalanine ammonia-lyase, polyphenol oxidase, and phenol concentration in fruits of *Olea europaea* L. cv. Picual, Verdial, Arbequina, and

Frantoio during ripening. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 10331-10340.

347. Bansal, V.; Shah, D. The effect of ethoxylated sulfonates on salt tolerance and optimal salinity of surfactant formulations for tertiary oil recovery. *Society of Petroleum Engineers Journal* **1978**, *18*, 167-172.
348. Tatiana Lezama, L.; Rafati, R.; Sharifi Haddad, A. The effect of formate brines on the emulsification of ionic, non-ionic, and enzymatic surfactants. *Chemical Engineering Science* **2022**, *263*, 118016.
349. Kanicky, J. R.; Lopez-Montilla, J.-C.; Pandey, S.; Shah, D. O. Surface chemistry in the petroleum industry. *Handbook of Applied Surface and Colloid Chemistry* **2001**, *1*, 251-267.
350. Hierrezuelo, J. M.; Molina-Bolívar, J. A.; Ruiz, C. C. An energetic analysis of the phase separation in non-ionic surfactant mixtures: The role of the headgroup structure. *Entropy* **2014**, *16*, 4375-4391.
351. Girón, M. V.; Ruiz-Jiménez, J.; Luque de Castro, M. D. Dependence of fatty-acid composition of edible oils on their enrichment in olive phenols. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 2797-2802.

# Appendix A. OLT Stability Plots with Error Bars

This section contains the stability trial plots of target analytes discussed in Chapter 4, Section 4.4. The respective error bars have been included and are set to the SD of duplicate samples (*n* = 2).

***Trial 1 plots***

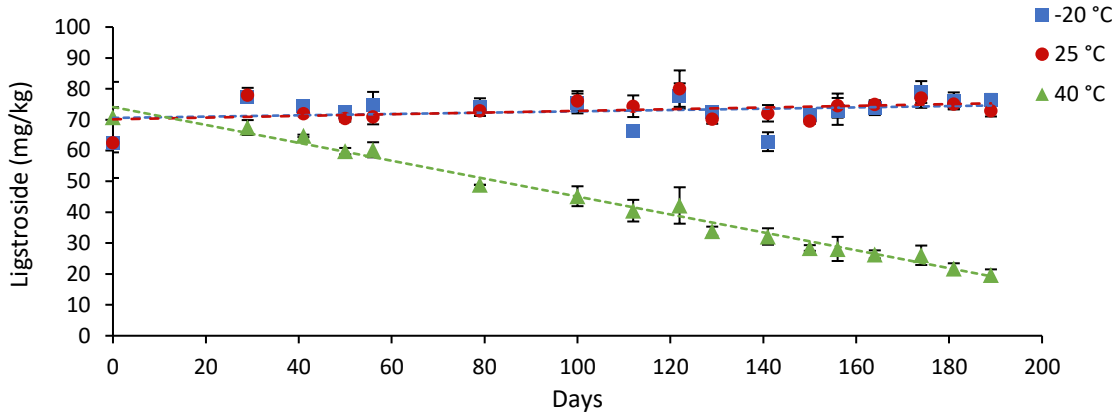



**Figure A.1. The concentration of ligstroside versus time stores at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

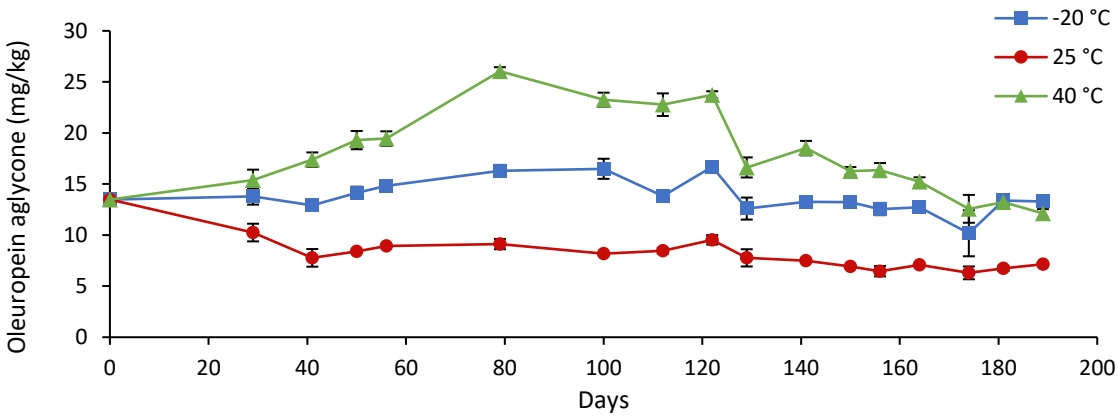



**Figure A.2. The concentration of oleuropein aglycone versus time for stability samples stored at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

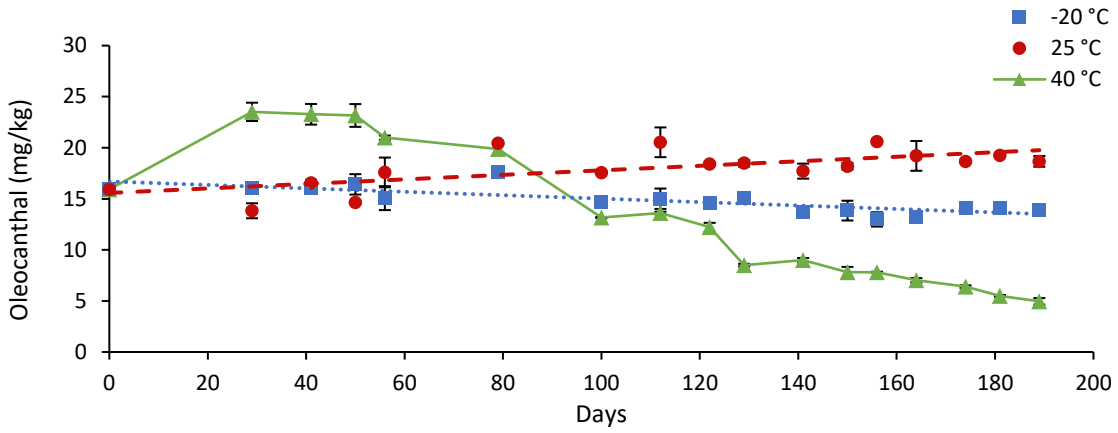



**Figure A.3. The concentration of oleocanthal versus time for stability samples stored at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* =2). Some error bars (± SD) are too small to be visible.**

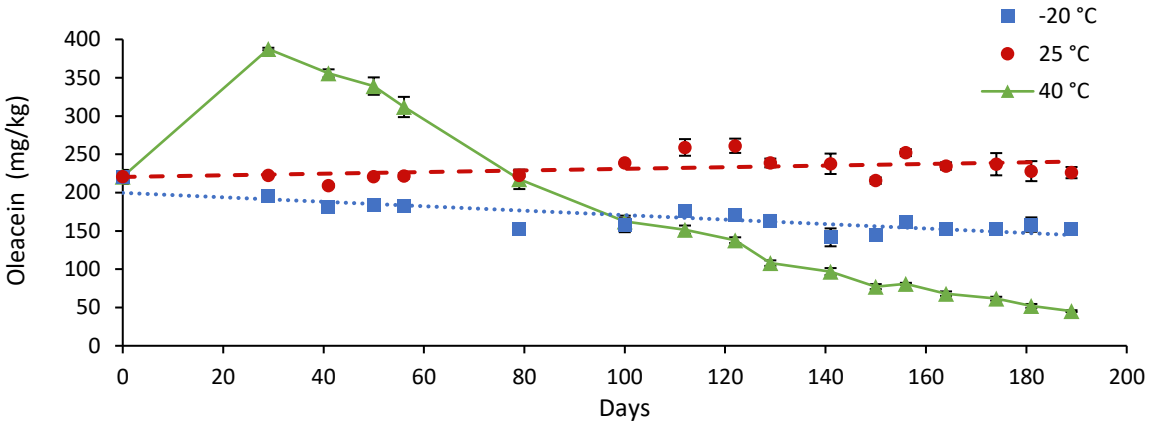



**Figure A.4. The concentration of oleacein versus time for stability samples stored at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* =2). Some error bars (± SD) are too small to be visible.**

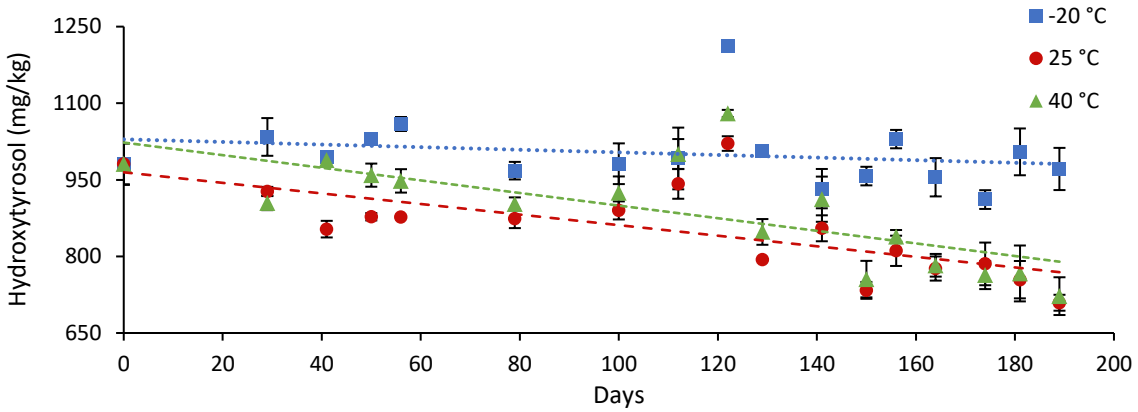



**Figure A.5. The concentration of hydroxytyrosol versus time for stability samples stored at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* =2). Some error bars (± SD) are too small to be visible.**

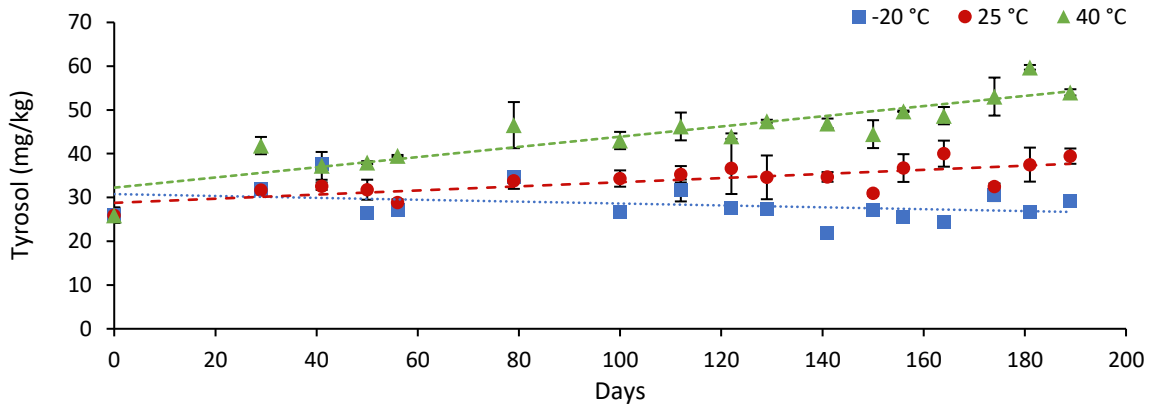



**Figure A.6. The concentration of tyrosol versus time for stability samples stored at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* =2). Some error bars (± SD) are too small to be visible.**

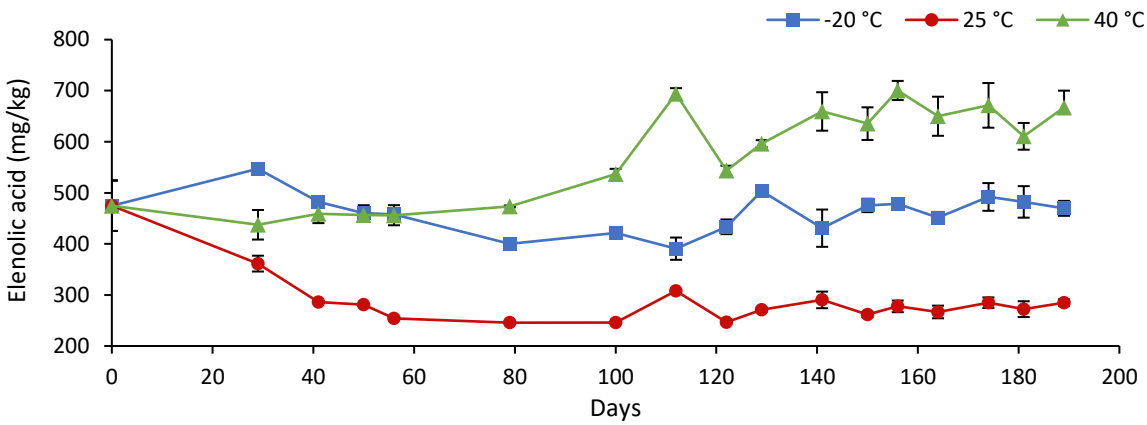



**Figure A.7. The concentration of oleuropein aglycone versus time for stability samples stored at −20 °C (blue square), 25 °C (red circle) and 40 °C (green triangle). Results are presented as the average ± SD (*n* = 2). Some error bars (± SD) are too small to be visible.**

***Trial 2 plots***

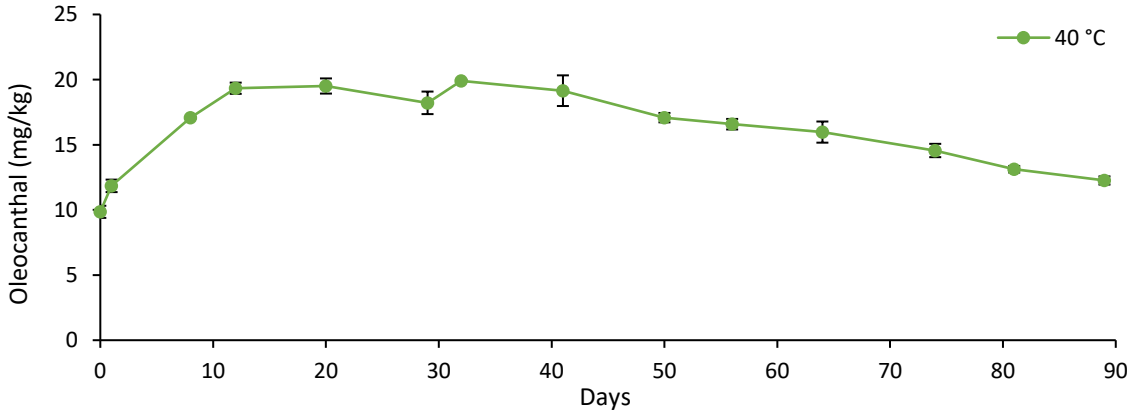



**Figure A.8. The concentration of oleocanthal versus time for stability samples stored at 40 °C (green triangle). Results are presented as the average ± SD (*n* =2). Some error bars (± SD) are too small to be visible.**

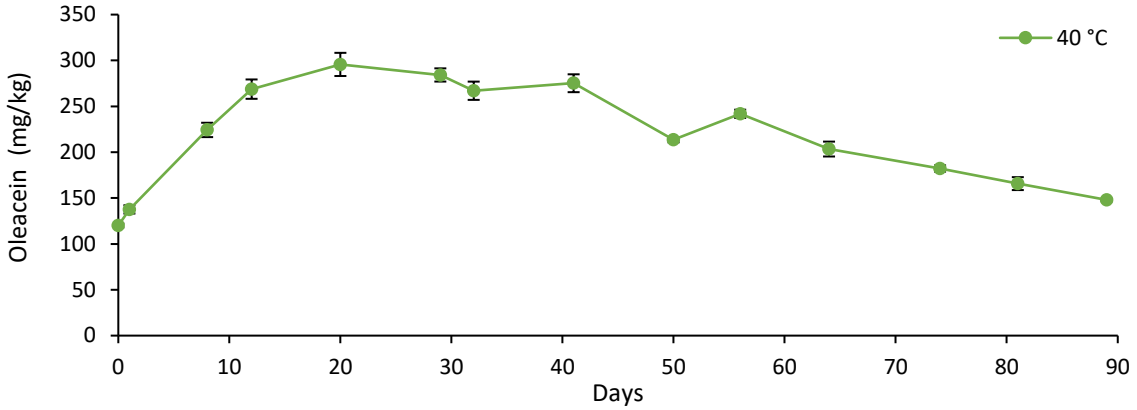



**Figure A.9. The concentration of oleacein versus time for stability samples stored at 40 °C (green triangle). Results are presented as the average ± SD (*n* =2). Some error bars (± SD) are too small to be visible.**