The Extraction, Quantification and Application of High-value Biological Compounds from Olive Oil Processing Waste

Marthie Postma-Botha

NELSON MANDELA UNIVERSITY

2018

The Extraction, Quantification and Application of High-value Biological Compounds from Olive Oil Processing Waste

Marthie Postma-Botha

Submitted in the fulfilment of the requirements for the degree of

PHILOSOPHIAE DOCTOR

In the Faculty of Science at the
Nelson Mandela University

Dr. Nicole Vorster: Promotor
Prof. Saartjie Roux: Co-promotor

December 2017

NELSON MANDELA
UNIVERSITY

DEPARTMENT OF ACADEMIC ADMINISTRATION
EXAMINATION SECTION
SUMMERSTARND NORTH CAMPUS
PO Box 77000
Nelson Mandela Metropolitan University
Port Elizabeth
6013

Enquiries: Postgraduate Examination Officer

DECLARATION BY CANDIDATE

NAME: **MS MARTHIE POSTMA-BOTHA**

STUDENT NUMBER: **196191500**

QUALIFICATION: **PH D (CHEMISTRY)**

TITLE OF PROJECT:

THE EXTRACTION, QUANTIFICATION AND APPLICATION OF HIGH-VALUE BIOLOGICAL COMPOUNDS FR0M OLIVE OIL PROCESSING WASTE

DECLARATION:

In accordance with Rule G4.6.3, I hereby declare that the above-mentioned treatise/ dissertation/ thesis is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

SIGNATURE: M Postma-Botha

DATE: **08/04/2018**

Acknowledgements

This thesis would not have been possible without the guidance and the help of several individuals who in one way or another contributed their valuable assistance in the completion of this study.

I would like to express my sincere gratitude and appreciation, with a deep sense of respect, to the following people:

- My God and Creator who gave me this talent and carried me on His wings.
- My parents, Willem and Leonie Postma who motivated and believed in me and kept me in their prayers.
- My husband, Piet Botha, and two lovely daughters, Rouxlé and Leona-Kumi, who supported me, loved me, understood me and provided the opportunity and space for working on this challenging project.
- Dr Nicole Vorster, and her husband, Dr Freddie Vorster, for their instrumental guidance and support throughout my study. I really appreciated your patience and making yourselves available.
- Prof Saartjie Roux for her supportive role and guidance, and allowing me the use of the biochemistry laboratory.
- Coos Bosma from NMU who guided and assisted with the statistical analysis.
- Michael Carolus and Elna Kruger at Aspen Pharmacare, Port Elizabeth, for their assistance and guidance with regard to high performance liquid chromatography analysis and interpretation.
- Henriette and Hannes Brümmer for providing the pomace and accommodation at their lovely olive farm in Stilbaai. It was a memorable experience.
- Abraham Londt at De Rustica for providing pomace for final analysis and comparison of the two olive cultivars, *Coratina* and *Frantoio*.
- Mit for his continuous effort of freeze-drying the pomace samples.
- Lynne Cairncross for assisting with the spectrophotometric analysis during total phenol content and antioxidant activity determination.
- My good friends who kept me close to their hearts and carried me in their prayers.

Abstract

Olive oil processing waste (pomace) as a by-product of the olive oil industry is regarded as a rich source of high-value biological compounds exhibiting antioxidant potential. The objective of the present work was to obtain a concentrated extract of high-value biological antioxidants from the pomace. The effect of extraction conditions on the concentration of the bioactive compounds in the extracts was investigated. The simultaneous recovery of both hydrophilic and lipophilic high-value biological compounds exhibiting antioxidant potential was achieved through a one-step extraction method under reduced pressure using a non-toxic solvent blend. A multilevel experimental design was implemented with the aim of optimising the recovery of selected compounds, namely, hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene from olive pomace by using solvent blends of *n*-heptane, *d*-limonene, ethanol and water. The factors considered were: (i) extraction time, (ii) percentage composition of solvent blends and (iii) extraction temperature. The results suggested that a good recovery of the hydrophilic polyphenolic compounds, namely, hydroxytyrosol, tyrosol and oleuropein, as well as the lipophilic compounds, α-tocopherol and squalene may be achieved at a solvent temperature of 60°C at 400 mbar with a solvent blend of 30% *n*-heptane, 50% ethanol and 20% water and an extraction time of two hours. It was found that freeze-drying the pomace before extraction minimised production of artefacts, avoided degradation of biophenols, ensured long term stability of a reproducible sample and achieved better recovery of important hydrophilic and lipophilic bioactive compounds. Since the bioactive compounds are temperature sensitive, the extraction was performed under reduced pressure in order to reduce solvent reflux temperature and to improve extraction efficiency. The quantitative and qualitative determinations of the aforementioned high-value compounds were performed by high-performance liquid chromatography (HPLC), which revealed that the hydrophilic polyphenolic as well as the lipophilic α-tocopherol and squalene were present. In this study hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene were extracted from the pomace of two olive cultivars (*Frantoio* and *Coratina*). A comparison among the two cultivars showed quantitative differences between the two cultivars in all five high-value biological compounds and in the antioxidant capacity of the extracts evaluated by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. *Coratina* cultivar was found to have a significantly higher antioxidant capacity than *Frantoio* due to the much greater oleuropein content in

the *Coratina* compared to the *Frantoio* although *Frantoio* had a significantly greater amount of hydroxytyrosol. The stability of olive waste extracts stored at four temperatures was also investigated and the results show that increased temperatures caused greater extent of degradation of both the hydrophilic polyphenolic and lipophilic compounds. The proposed optimum storage condition for the olive pomace extracts was found to be at 5°C in the absence of light. The extracts were incorporated into two cosmetic formulations and were found, from a stability study, to be stable at room temperature and optimally stable at 5°C in the absence of light.

Table of Contents

Table of Contents

List of Tables

List of Figures

Abbreviations

OMW - Olive mill waste

OMWW – Olive mill waste waters

TPOMW – Two-phase olive mill waste

Cod – Chemical oxygen demand

BOD – Biological oxygen demand

HPLC – High performance liquid chromatography

DD – Diode array detector

MeOH – Methanol

EtOH – Ethanol

GC – Gas chromatography

IPA – Isopropyl alcohol

RP – Reverse-phase

SP – Solubility parameter

RS – reference standard

DPPH – 2,2-diphenyl-1-picryl-hydrazyl

UV – Ultra-violet

F-C – Folin-Ciocalteu

%AO – Percentage antioxidant activity

TP – Total phenols

GA – Gallic acid

DM – Dry matter

GAE – Gallic acid equivalents

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Introduction

Olive oil extraction is an ancient process and the olive oil or “liquid gold” from *Olea europaea* L. was historically used by Egyptians as fuel for their lamps, as a cosmetic and preservative for mummification, as well as food (1). Today, both olives and olive oil have a prominent place in the diet of the population around the Mediterranean Basin promoting longevity.

During the olive oil extraction process, waste material or pomace is generated which includes solid waste and/or liquid waste depending on the technique implemented. In the modern two-phase olive oil extraction system, a solid-liquid OMW, also known as *alperuj*o or pomace, 72.5 – 80% of the olive fruit which consists of both the solid waste (approximately 40% skin, seed, pulp and stone pieces) and vegetation liquid, is generated (2). The olive pomace pose a potential threat to environmental conservation because of the phytotoxicity which is mainly attributed to the high phenolic content. On the other hand, owing to the potential health-benefits of these biological compounds, two-phase system pomace is now regarded as a potent source of natural antioxidants for use in the pharmaceutical (medicinal and therapeutic), nutraceutical, cosmetic and food industry (3).

The potential recovery of these naturally occurring compounds from olive pomace could not only provide economic benefits but it could also render the waste less toxic. By implementing appropriate methods, two applications are indicated during the processing of the olive mill waste: firstly, the recovery of these hydrophilic and lipophilic bioactive value-added compounds and secondly, bioconversion of the waste into utility products. A single, integrated extraction process of both hydrophilic and lipophilic bioactives should reduce aspects such as time and cost, while enhancing the biological activity in the relevant utility application.

Olive fruits are a rich source of valuable nutrients and compounds and contain a range of hydrophilic (phenolic alcohols, phenolic acids, secoiridoids, and flavonoids) and lipophilic compounds (tocopherols and tripertenes such as squalene) that are known to possess multiple biological activities. Moreover, these compounds may provide a

defence mechanism (antioxidant activity) that delays aging and prevents inflammation, atherosclerosis, carcinogenesis, dyslipidemia, cardiovascular incidences and microbial infection (4, 5). Consequently, the consumption of table olives and olive oil by health-conscious consumers continues to increase worldwide.

The European Union (EU) is the largest producer of olive oil in the world, accounting for almost three-quarters of global production. Most olive oil production takes place in Southern Europe, North Africa and the Near East as 95% of the olive trees in the world are cultivated in the Mediterranean region. Main European olive producers are Spain (65.6%), Italy (18.3%), Greece (8.6%) and Portugal (6.8%) (6). Lower incidences of chronic illnesses such as atherosclerosis, cardiovascular and neurodegenerative diseases, and certain cancers have been observed in these countries and this phenomenon is correlated with higher consumption of olives and/or olive oil in the Mediterranean population's diet. The rising popularity of olive oil encouraged other countries such as Australia, Argentina, United States of America and South Africa as emerging olive tree cultivators (7).

1.2 Olive and olive oil industry in South Africa

Olives were introduced to South Africa by Jan van Riebeeck, the first Governor of what was then known as a Dutch settlement. According to Karsten (1955) (8), the first reference to olives was on 6 August 1659 when Jan van Riebeeck recorded in his diary: 'The season is also approaching for planting and grafting the olive and all kinds of home and Indian fruit trees…'. The current olive industry was established by an innovative Italian nurseryman, Ferdinando Costa, who arrived in South Africa in 1903.

The greater part of the olive industry is based within the Paarl Valley in the Western Cape Province and has recently expanded to include the Riebeek Kasteel, McGregor and Hermanus farming districts. A number of orchards have successfully been established in other regions of the country, namely the. Western Cape Province (Prince Albert, Oudtshoorn, Beaufort West and Laingsburg), Northern Cape Province (Vaalharts, Prieska and Upington), Eastern Cape Province (Alicedale), North West Province (Brits) and Limpopo Province (Modimolle). (8)

The South African olive industry in 2012 comprised more than 300 olive producers, the majority of them based in the Western Cape because of its relative cool, frost free winters followed by hot, dry summers. At least 20 different imported olive cultivars are grown locally; however the commercially important varieties range from the popular oil-

producing varieties like *Leccino, Frantoio* and *Coratina,* to *Mission* and *Calamata* olives, which are preferred for table olive production. It is estimated that the cultivation of olives in South Africa covers an approximate area of 2600 hectares (ha) of olive groves. New trees are constantly being planted with olive farms growing at a rate of 20 percent annually and doubling in size every four to five years. In 2011, an annual production of 1200 tons of olive oil contributed to 20% of the local consumption or was processed into speciality style olive products such as pastes while 80% was imported. Although the South African olive industry has made great advances in the past 10 years in terms of cutting-edge farming technology and oil-extracting and blending methods, producers are still unable to meet local demand. At present, they produce approximately 2500 tons of table olives per year and the 2016 harvest delivered approximately 2.2 million litres of olive oil. Local households use around three times the quantity of olive oil produced per year (80 millilitres per South African per annum) to a value of R250 million and therefore South Africa has to import about 66% of its olive oil requirements. Consumption of olive oil has grown by roughly 20% a year over the last eight years according to South African Olive Association (SA Olive). On the other hand, the shortage in terms of table olives are supplemented with only 20% imported (9). Above information was provided verbally and confirmed via email on the 8th May 2017 by a representative from the SA Olive.

1.3 Regulations on olive oil in South Africa

The interests of the country's olive growers, olive oil producers, table olive producers and olive tree nurseries are represented by the SA Olive, a voluntary association representing the country's olive industry. They are committed to supporting a dynamic future for South Africans, representing the interests of the South African Olive Industry and helping to promote South African olive oil brands in international markets. SA Olive holds an annual olive oil competition among domestic producers and awards the winners with a seal or label of "SA Olive" on the bottle. Currently, the one hundred members follow SA Olive's published Codes of Practice which are based on international quality standards. The SA Olive Commitment to Compliance initiative allows producers to display a seal on local bottles of olive oil which indicates when the olives were pressed and that the producer complies with the standards set out by SA Olive. This seal guarantees the olive oil meets International Olive Council (IOC) standards for extra virgin olive oil, indicates the freshness of the oil by displaying the year of harvest, and guarantees that the olive oil is 100% South African. During the past five years, South African Extra Virgin Olive Oils have constantly received

international quality awards, which proves South African products are as competitive as European olive oil.

Table 1.1 shows the components of South African Olive Oil consumption in 2004, 2011 and 2016 (9). South Africa has a competitive advantage in its location regarding the olive oil production although the current market heavily depends on the imports from Europe. Local olive farmers press their oils in the European off-season, when oil is scarce in the northern hemisphere countries.

Table 1.1 Data for Olive Oil Consumption in South Africa (1000 tonnes).

	2004	**2011**	**2016**
Own production	0.5	1.2	1.046
Imports	2	4.8	5.84
Total consumption	2.5	6	7.3

The International Olive Council (IOC) is an intergovernmental organization based in Madrid, Spain, with 23 member states. It promotes olive oil around the world by tracking production, defining quality standards, and monitoring authenticity. More than 85% of the world's olives are grown in International Olive Council (IOC) member nations. Over 750 million olive trees are cultivated worldwide, 95% of global production areas from Southern Europe, North Africa and the Near East situated in the Mediterranean region (10).

1.4 Generation of olive waste/pomace

During the modern two-phase olive oil processing technique, where water addition is excluded, oil and a combination of liquid and solid phase by-product (*alperujo)* is produced. This organic waste or wet pomace is a semi-solid high-humidity paste with a thick sludge consistency that contains 80% of the olive fruit including skin, pulp and broken stone pieces. The pomace possesses high amounts of organic substances (14 – 15%) including sugars, nitrogenous compounds, volatile fatty acids, polyalcohols, pectins and fats and high concentrations of phenolic compounds (up to 10 g/L) (11). Because of the high content of organic matter which is not easily degradable, waste discharged during the olive oil extraction period into the environment poses a major ecological concern. This effluent is generally untreated and leads to land degradation, soil contamination as well as contamination of groundwater and of the water-table itself with eventual pollution of drinking water. Both the soil and water quality are affected as indicated by foul odour, change in colour and oily shine visible on their surfaces. Together with a high chemical oxygen demand (COD) to biological oxygen demand

(BOD) ratio, phytotoxicity and antimicrobial properties to natural occurring bacteria, olive oil producing countries face challenges to find an ecologically sound and economically viable solution to the handling and disposal of the large quantities of pomace generated (12, 13).

Several research groups have been working on alternative uses of this organic by-product which may also be regarded as an economical resource for recovery of valuable bioactive compounds in the form of extracts which can be used in the cosmetic and health food sector. Diverse extraction methods for obtaining high-value bioactives from pomace have been described in literature, however, no literature on obtaining both hydrophilic compounds such as hydroxytyrosol, tyrosol and oleuropein, and lipophilic α-tocopherol (Vitamin E) and squalene in a single extraction process were found. Solvents frequently used for the extraction of the hydrophilic compounds are ethanol, methanol, water, formic acid, and aqueous-alcoholic mixtures whereas *n*-hexane with or without ethyl acetate and isopropyl alcohol have been frequently used for defatting and extraction of lipophilic components (14-18). However, solvents such as methanol and *n*-hexane are not suited to industrial exploitation to obtain valuable bioactive compounds for human use due to their flammability and toxicity (19, 20).

1.5 Problem statement

Olive mill waste (OMW) or pomace generated from olive oil extraction presents a major environmental disposal problem, but is also rich in hydrophilic and lipophilic high-value bioactive compounds, also known as phytochemicals, that show remarkable health benefits. Phytochemicals are biologically active, naturally occurring chemical compounds that accumulate in different parts of plants (21). These chemicals contribute to the plant's flavour, colour and odour of plants and protect the plant cells against environmental hazards such as pollution, climatic stress, ultra-violet exposure and pathogenic attacks. Phytochemicals can be classified as primary metabolites, for example, sugars, amino acids, proteins, purines, pyrimidines and chlorophyll, and numerous secondary metabolites which exhibit biological properties such as antioxidant, antimicrobial, anticancer, anti-inflammatory and immune stimulation properties (22)

The OMW effluents produced from olive oil production exhibit highly phytotoxic and antimicrobial properties, mainly due to the high phenolic content. Beneficiation of this pomace by extracting the bioactive compounds will render it less toxic, safer to dispose of and will add value to the waste.

1.6 Research hypothesis

It is possible to extract and quantify compounds such as hydroxytyrosol, tyrosol, oleuropein, squalene and α-tocopherol from olive oil processing waste (pomace) in a manner which is environmentally-conscious and economical. Furthermore, it is possible to incorporate these high-value added bioactive compounds into a topical, stable and effective cosmetic formulation.

1.7 Aims and objectives

The aim of this study is to develop an integrated single extraction procedure to obtain extracts of high-value bioactive compounds specifically hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene from two-phase *alperujo* (or wet olive pomace) in the highest possible yield, and in a practical, economic and environmentally-conscious manner.

The research was divided into four interdependent activities, each with defined objectives as follows:

❖ **Extraction process development and HPLC analytical development**

- To determine the best extraction method for the hydrophilic compounds hydroxytyrosol, tyrosol and oleuropein, and lipophilic compounds α-tocopherol and squalene from two-phase pomace.

- To determine the optimum method for the HPLC analysis of the hydrophilic and lipophilic bioactive compounds.

❖ **Optimum extraction conditions and solvents**

- To determine the optimum solvent combination/blend and extraction conditions for good recovery of five high-value bioactive compounds in a single extraction process considering variables such as solvents, time, reflux temperature and relevant reduced pressure.

- To base the choice of extraction method and solvents on the six principles for “Green extraction of natural products” governed by the nature (polar or non-polar) of material to be extracted (23).

❖ **Comparison of the bioactive composition of two olive cultivars**

- To characterise and compare olive pomace obtained from two different cultivars, *Coratina* and *Frantoio* in terms of bioactive content, total phenolic content, antioxidant activity and antimicrobial properties.

❖ **Determination of the stability of olive pomace extracts alone and in cosmetic formulations**

- To investigate the effect of storage conditions on the stability of the hydrophilic and lipophilic bioactive compounds in olive pomace extracts obtained.

- To determine the physical stability of two cosmetic formulations containing pomace extracts.

CHAPTER 2

OLIVE POMACE BENEFICIATION

2.1 Olive cultivation and processing

2.1.1 Olive tree

Olea europaea L. belongs to the *Oleaceae* family and the genus of interest for economic or horticultural purposes is *Olea.* The cultivated olives belong to the *sativa* subspecies which is the only species that produces edible fruits. This is the oldest known tree; its origins date back 5000 – 6000 years in the Middle East (24). At present, although approximately 95% of the world's *Olea europaea* L. are cultivated in the Mediterranean region (Spain, Italy, Greece, etc.), they are farmed all around the world including America, Australia and South Africa (25).

Olive trees are cultivated for their edible fruits consumed either as table olives or as olive oil. It is well established that the beneficial activities of olive oil are attributed to its high oleic acid content and to the concerted action of several nutrient phytochemicals, namely polar phenolic compounds, lipophilic phenols i.e. α-tocopherols, and non-phenolic triterpenes (i.e. squalene) (26). The olive tree has a long history of medicinal and nutritional value. For instance, the leaves have been used to mummify Pharaohs, treat fevers and malaria (1). Different parts of the olive fruit are valued for their nutrients and functional food components and health-promoting bioactive compounds. The fresh pulp of the fruit contains a high concentration of phenolic compounds ranging between 1–3% of the weight of which the main classes present are: lignans, phenolic acids, phenolic alcohols, flavonoids and secoiridoids (27). Olive trees yield economic benefits as producers of nutritious edible oil and table olives.

Olive trees are evergreen that average heights of 4 – 8 m, depending on the variety and can remain productive for hundreds of years. The tree bears small, creamy white flowers and has the characteristic small elongated leaves of two-colours: pale green above and silvery below (28, 29). Cool to cold winters, limited frost (minimum of 2°C – 4°C with maximum June and July temperatures not exceeding 21°C), and warm, dry, long summers with an average annual rainfall of around 800 mm are preferred for the tree's main shooting and optimum flower development. The fruit development begins with sprouting of floral buds, followed by pollination (carried out by the wind),

fertilization, fruit development and ripening. A first time sizeable olive crop is expected only after 8 -10 years of cultivation but for some varieties this occurs within 4 – 6 years. An olive tree's yield is predominantly affected by a biennial cycle, or alternate bearing, with one year of growth bearing a lighter crop and the other year the delivery of a heavy crop, known as "off" and "on" seasons. The alternate bearing of the olive tree is as a result of the heavy crop which suppresses shoot growth and exhausts food reserves. This results in reduced flowering and fruit set in the following year (30). Prerequisites for a quality and quantitative olive crop are: enough water at the right time, fertiliser, insect control, pruning, and leaf and soil analyses to determine whether supplements are needed. Factors which influence the micro-climatological conditions are the type and variation of soil and water quantity (rainfall and irrigation). Soil requires a neutral to alkaline pH of 7 – 8. Olive trees grow and crop well with poor quality saline (salty) water with a conductivity of up to 2 400 micro S/cm (31).

2.1.2 Olive fruit

The olive fruit is an oval shaped drupe or stone fruit with a single hardened stone containing a kernel and derived from a single carpal. It consists of two main parts: the pericarp and the seed or stone. The *pericarp* is composed of three layers: the edible skin, or *epicarp* (1 – 3%), which is free of hair, the *mesocarp (70 – 80 %)* as the flesh or pulp underneath the skin, and the hardened *endocarp* (18 – 22%), or fruit pit, enclosing a single kernel as seen in Figure 2.1. Whereas the *pericarp* comprises approximately 96 – 98% of oil, 2 – 4% is found in the kernel (32). The *epicarp* protects the internal tissue and consists of cutin (insoluble polymer) and waxes (complex mixture of aliphatic and cyclic lipids). It changes colour due to a modification of pigment concentration as a result of accumulation of anthocyans together with degradation of chlorophylls (green colour) and carotenoids.

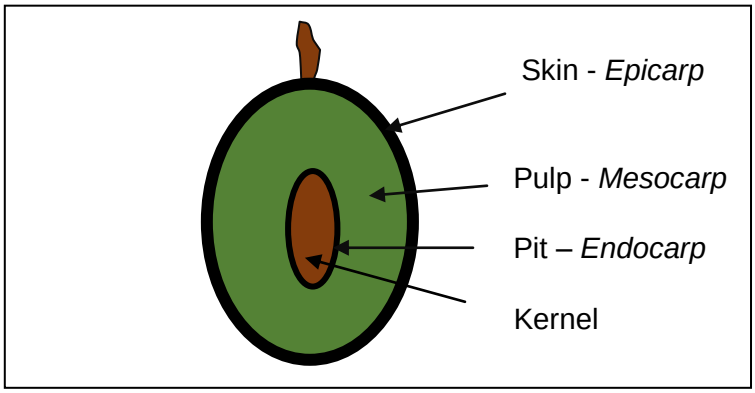


Figure 2.1 The olive fruit (drupe) (32)

According to Boskou *et al*. (33), the average chemical composition of the olive fruit is: 50% water or "vegetable" water although some varieties contain up to 70%; 1.6% protein; 22% oil; 19% carbohydrates; 5.8% cellulose; and 1.5% minerals/ash. Other important constituents include pectins, organic acids, pigments, and glycosides of phenols. The average composition of the olive fruit's main constituents is showed in Table 2.1.

Table 2.1 Main constituents of the olive fruit (adapted by Ryan et al.) (34)

Constituent	Flesh (%)	Stone (%)	Seed (%)
Water	50-60	9.3	30.0
Oil	15-30	0.7	27.3
Nitrogen	2-5	3.4	10.2
Sugars	3-7.5	41.0	26.6
Cellulose	3-6	38.0	1.9
Ash	1-2	4.1	1.5
Phenols	2-2.5	0.1	0.5-1.0
Others	-	3.4	24

2.1.3 Olive Cultivars

According to the Catalogue of Olive Varieties published by the International Olive Oil Council (IOOC), approximately 250 cultivated varieties are commercially exploited for either olive oil or table fruits (10). The olive fruits for oil extraction are small to medium in size, averaging 2.5 – 4 g and a high oil yield (18% and more). By contrast, table olives are medium to large sized (> 4 g) with a higher flesh/pit ratio and less oil content. Many cultivars have been imported to South Africa over the past century and those that have been successfully cultivated include: *Mission, Manzanilla, Kalamata, Barouni, Frantoio, Coratina, Leccino* and more recently *Favolosa* (FS-17).

Frantoio trees are highly productive with medium size oval shaped fruit (2.5 – 3 g) and start bearing fruit after 8 years. When mature, the fruit are purple-black in colour, but at the preferred harvest time for oil production are green and purple-green. The *Leccino* drupe is of medium size (2 to 2.5 g) and the mature fruit is purple-black although purple green at the optimal picking time. It has variable oil content of between 16% and 21%. *Coratina* is characterised by early production and high yields of slightly asymmetrical elongated oval shaped fruit of approximately 3 g. This variety adapts well to different soils and climates and is an excellent choice for hot climates without water logging. It is classified as one of the high phenolic olive cultivars together with *Rosciola* (35). *Frantoio* is self-fertile and an excellent pollinator for other cultivars whereas *Coratina* requires pollinators. The *Mission*, originally cultivated by Franciscan monks, is an American cultivar with lower oil content (12 – 16%) and is used for both oil and green

and black table olives. These trees are sensitive to wet soil, grow upright and bear fruit early after ample pollination by mainly the *Frantoio* olive variety. A more recent olive variety *Favolosa* for oil production offers numerous biological and agronomic characteristics: low vigour, self-fertile, high rooting attitude, early production, high productivity, high oil yield, early ripening, medium resistance to low temperatures and tolerance towards the most common plant diseases. The drupe is spherical in shape, reddish in colour when ripened and weighs approximately 2.5 – 4 g. Polyphenol content is medium to high with approximately 70% oleic acid (28).

2.1.4 Harvesting

Olives are harvested at different stages of ripeness. Initially, all olives are green and turn black upon ripening as seen in Figure 2.2. depending on the variety.

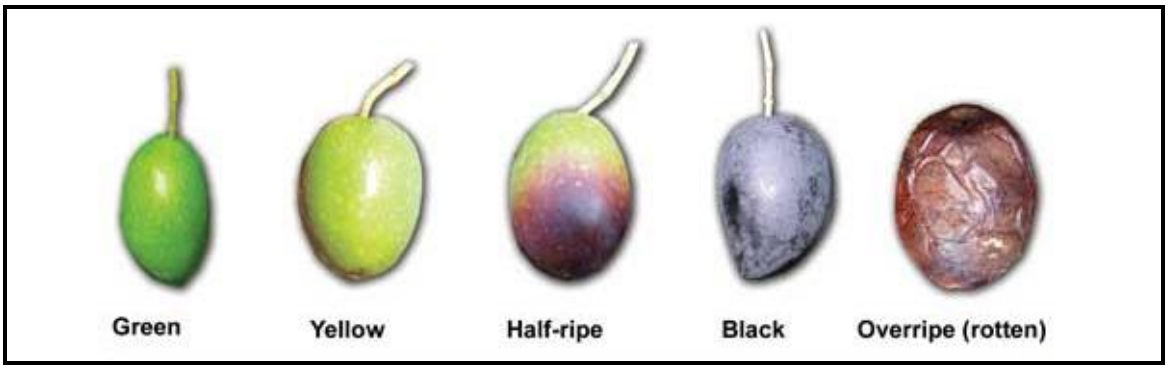


Figure 2.2 Stages of olive ripening (9)

Unripe, green olives produce very little oil. As the olives ripen, oil development peaks just before the olive falls from the tree. However, the best quality oil with excellent aromatic properties is made from half-ripe olives. Over-ripe olives have a higher percentage of oil, but it is of poorer quality with negative "sensory defects" that include: rancid, fusty, mouldy and winey–vinegary, which is associated with fermentative processes (36).

Harvest periods in South Africa depend on the cultivar and the purpose for which the fruit is intended and normally stretch from end of February to July. Fruit which is intended for green processing is picked at the stage when they have turned from bright green to yellow green and the first fruit show a light pink or purple blush. As the olives ripen further, their colour turns to red-brown. Purple or naturally black ripe olives are allowed to fully ripen on the tree and are harvested mid-April to May as seen in Figure 2.3 and 2.4 (9). Oil olives are harvested when most of the fruit on the tree are ripe enough and then the entire tree is picked. The oil content rises initially with colouring

and ripening, and then remains relatively constant, but delay in harvest will result in lowered oil quality.

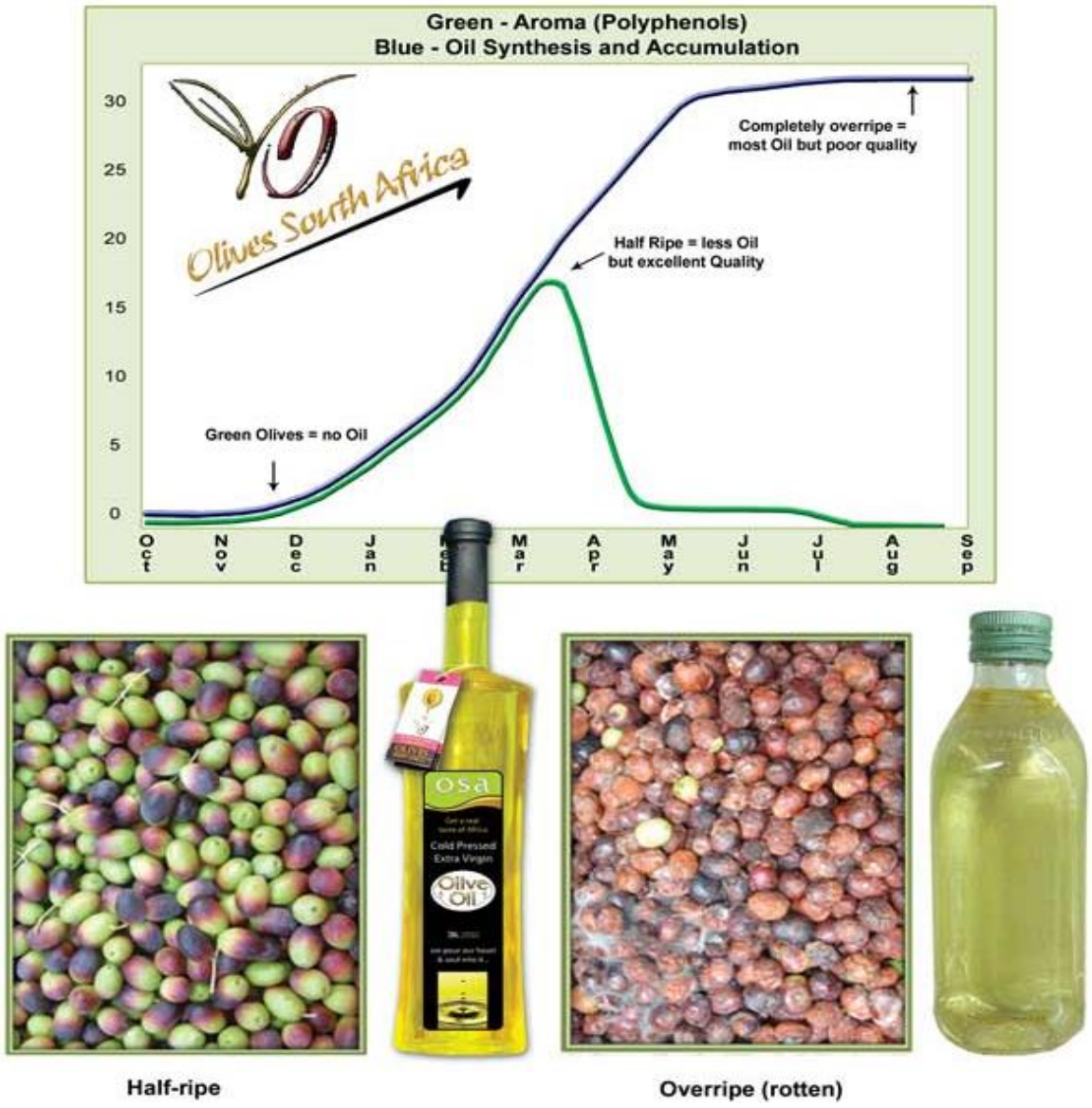


Figure 2.3 Ripening and olive harvesting times in South Africa (9)

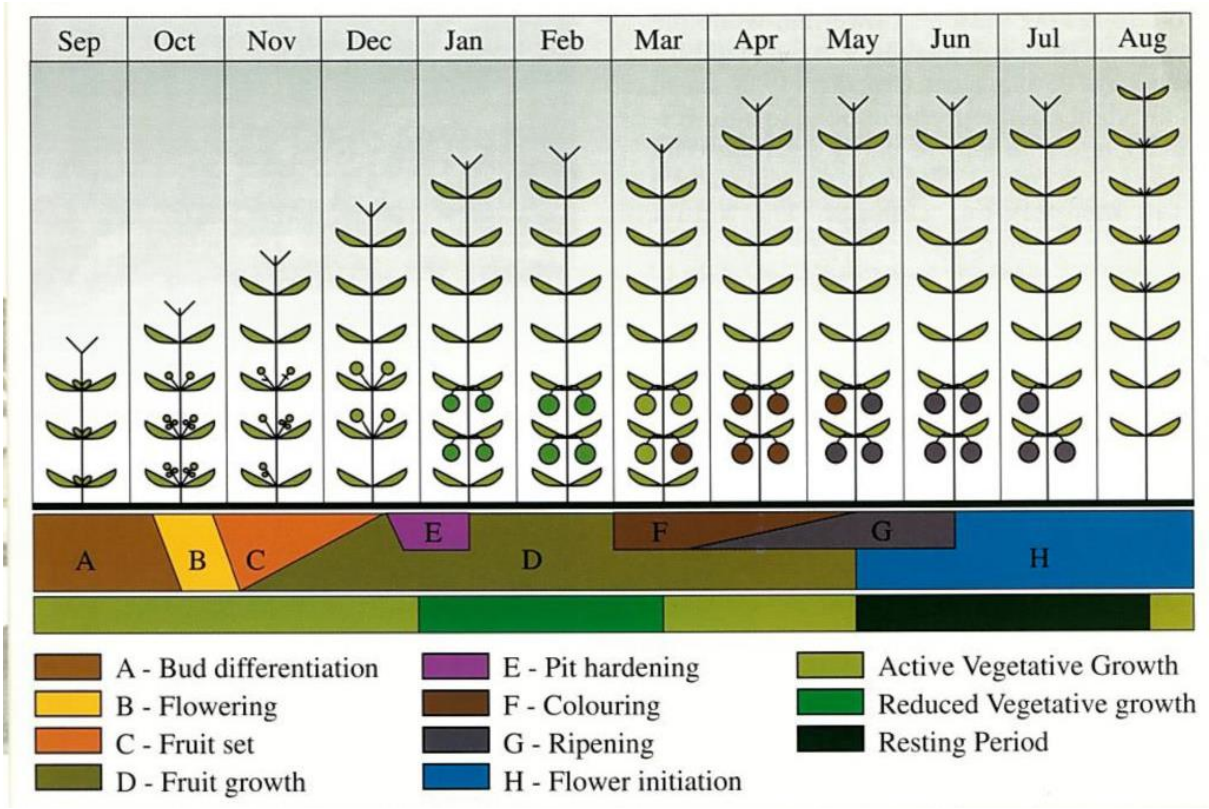


Figure 2.4 Annual growth cycle of the olive tree in South Africa (9).

2.1.5 Olive oil production

During olive oil production, different technological steps, mill types and various technological approaches towards the quality of virgin olive oil are implemented. Waste material or pomace is generated which includes solid waste and/or liquid waste depending on the technique used. A schematic representation of a two-phase system to extract olive oil from the olive fruit is illustrated in Figure 2.5.

Figure 2.5 Schematic representation of a continuous two-phase centrifugation system delivering olive oil and pomace at De Rustica Olive Farm in Oudtshoorn, Western Cape, South Africa

The production of olive oil consists of a number of processing steps:

a. Crushing: Olive crushing has an important influence on the organoleptic and nutritional qualities of virgin olive oil. The olives are washed and most of the olive grove residues removed during transit to the mill as seen in Fig. 2.5 (b)(c)(d). Mill or crusher types available for industrial application, namely, the stone mill, hammer crusher, disc crusher and de-pitter mill (partial or total de-pitting) can be selected from for grinding the olive into a paste (37-40). Factors such as fruit cultivar and level of maturity influence the type of mill used and paste fineness. When mill stones are used, the obtained oils have a lower intensity of bitterness and pungency because this crushing method helps to produce oil with a lower content of phenolic substances. The hammer mills, Fig. 2.5 (e)(f), are harsher than both the disc mill and stone mill which are better suited for varieties with very high polyphenol concentrations such as *Coratina*. Using the hammer mill, the fruit cells are cut without destroying the intracellular structure and this increases the oil yield during extraction. The pitter mill, utilised for its ability to remove the stones from the fruit, is fairly new but has no more benefit to the final product compared to the other mills.

b. Malaxation: The next step entails the extraction of the oil from the freshly milled paste and a process named malaxation, Fig. 2.5 (g)(h), or slow churning is implemented for its ability to reverse the homogenised state of the oil and fruit-water suspension by allowing the microscopic oil droplets to concentrate in larger drops and thus to increase the percentage of extracted oil. Olive paste malaxation influences the oil yields and also the antioxidant content of oil. Most malaxers are made of a horizontal trough with spiral mixing blades. During malaxation, the paste is stirred at approximately 28 rpm (20 – 30 rpm) in a stainless steel container consisting of double walls where hot water is circulated in order to provide the required heat for enhanced processing and at a temperature not exceeding 25 – 30°C for a time period of not more than 60 minutes (41). Both the temperature and time of malaxation influence the quality, bitterness and stability of the oil as well as the polyphenol concentration. Within a malaxation time period of 30 – 60 minutes, oil yield increases while the polyphenol content of the oil decreases, as does bitterness. At increased temperatures, more oil and polyphenols are extracted but bitterness is enhanced while volatile aromatics are decreased.

Furthermore, several studies have shown that enzyme activity is activated as a result of temperature increase during olive paste malaxation and at temperatures of 35°C,

polyphenyl oxidase, which is a oxidoreductase enzyme is rather high. Another enzyme namely, β-glucosidase, could be responsible for the hydrolysis of complex polyphenols such as oleuropein, dimethyl oleuropein and ligstroside while lipoxygenase, which catalyses the formation of hydroxiperoxides, could indirectly be responsible for the oxidation of these secoiridoids with a linear increase in hydroxytyrosol and tyrosol (42-45).

c. Phase separation: Phase separation or extraction of the oil from the solid and fruit-water liquid phases of olive paste is performed using either filtration (percolation), pressing or centrifuge systems (46). The selective filtration system uses no force on the paste while the Sinolea and Acapulco systems use gravity and the physical adhesion law between oil and stainless steel for phase separation. Both remove a maximum of 80% of the oil. Centrifugal decanters (two- and three-phase continuous centrifugation systems) spin on a horizontal axis at about 3 000 rpm and create enough force to separate the oil from the fruit-water and solids, Fig. 2.5 (i)(j). There is an auger inside a stainless steel cylinder that moves the paste through in a continuous flow for final separation of the oil and is a very efficient system. Vertical centrifugation operates at a speed of about 6 000 rpm on a vertical axis and attains a 4-fold separation compared to the horizontal centrifuge.

Three-phase centrifuge

The three phase decanter or horizontal centrifuge requires the addition of water to the system which dilutes out the hydrophilic components (polyphenols), separates the paste into three distinct phases: olive oil (20%) and two olive mill wastes (by-products) namely, the olive oil cake or *orujo* (30%) which consists of the olive pulp and stones, and olive mill waste water (OMWW) or *alpechin* (50%) which consists of vegetation water and olive fruit soft tissue. Principal disadvantages of this system include the necessity of water added as well as electricity for heating the additional water, lower polyphenols yields due to the washing away of the phenolic compounds of the product, with consequent decreases in this important antioxidant fraction, and excessive waste/vegetation water generated (47).

Two-phase centrifuge

In the modern “ecologically attractive” two phase decanter or horizontal centrifuge, two end-products namely, a solid-liquid OMW or *alperuj*o (72.5 – 80%) which consists of both the solid waste (seed, pulp and stone pieces) and vegetation liquid, is generated

(47, 48). This two phase system operates without adding water (or only a minimal amount of water if the moisture of the olive paste is < 50% to facilitate separation) (49). The main advantages are lower water and energy consumption (lower operating costs), the oils produced have higher polyphenol content (higher oxidation stability and organoleptic properties), and no fruit-water effluent is generated for disposal. Moreover, the construction of the two-phase system is less complicated and less expensive, and aspects such as remixing of the separated water and oil utilising the three-phase system, is excluded. The high moisture quantity as well as dissolved sugars represents constraints for storage (degradation), transportation (greater weight) and treatment (pre-treatment required) of the two-phase olive mill waste (2). Refer to Table 2.2 for a comparison of the quantity and composition of virgin olive mill pomace generated using the two-phase and three-phase extraction systems (50, 51).

Table 2.2 Characteristics of olive pomace obtained with the two-and three-phase centrifugation systems.

Parameters	**3-Phase system**	**2-Phase system**
Water added (%)	50	0-10
Pomace (kg/100 kg olive)	55-57	75-80
Pomace moisture (%)	45-55	60-75
Waste waters (kg/100 kg olive)	80-110	10
Oil (% on fresh pomace)	3.5-4.5	3-4
Pulp (%)	15-25	10-15
Stones (%)	20-28	12-18
Ash (%)	2-4	3-4
Nitrogen (mg/100 g)	200-300	250-350
Phosphorus (mg/100 g)	30-40	40-50
Potassium (mg/100 g)	100-150	150-250
Total phenolic compounds (mg/100 g)	200-300	400-600

2.1.6 Olive mill waste matrices and environmental concern

Solid waste (*orujo*), liquid waste (*alpechin*) and pomace sludge (*alperujo*) include mineral and organic fractions namely fats, proteins, sugars, organic acids, cellulose, hemi-cellulose, pectin, gums, tannins and polyphenols. Some of the organic materials are composed of complex substances which are not easily degradable. Gross characterisation of the waste has been done by several researchers and includes tests such as COD, BOD, pH, % moisture, % ash, odour, colour, % suspended solids**,** fats, sugars, nitrogen, volatile and total phenolic content (52, 53). Gross characterisation of OMMW provided the following characteristics: dark brown to black colour with strong acid smell; COD values of around 220 g/l and COD/BOD ratio of 2.5 – 5 which indicating low biodegradability; acidic pH of 3-5.9; and high phenolic content (52).

The presence of toxic organics, mainly originating from the broken pit, is detrimental to naturally occurring bacteria in the environment and contribute to pollution of both the soil and natural water supply due to their high organic content as well as the high BOD to COD ratio which indicates low biodegradability. COD does not differentiate between biologically available and inert organic matter and it is a measure of the total quantity of oxygen required to oxidize all organic material into carbon dioxide and water. COD values are always greater than BOD values. BOD is a measure of the amount of oxygen that bacteria will consume while decomposing the organic components present in water / waste water under aerobic conditions. The olive waste which contains significantly high levels of phenolic compounds, poses a major environmental threat as a result of its antimicrobial (including both bacteria and yeast) and phytotoxic properties, as well as its foul odour (50, 54).

As the demand for olive oil is rapidly increasing in South Africa and worldwide, environmental pollution posed by the olive oil processing waste will become a serious problem as it displaces beneficial bacterial flora in terrestrial and aquatic ecosystems and adversely affects the natural biodegradation process. According to the statistics of the International Olive Council, the worldwide consumption of olive oil increased by 85% between 1990 and 2015 (10). Since the olive oil comprises on average, less than 20% of the olive fruit, the increase of olive oil production has led to a proportional increase in olive mill processing waste.

However, the negative perception of “high phenolic content” in the olive pomace has moved towards embracing the positive characteristics of these high-value compounds exhibiting diverse biological activities on certain physiological parameters, for example:

plasma lipoproteins, inflammatory markers, in endothelial and epithelial cells, platelets, neurons, cells of the immune system, and neoplastic cells (54-56).

Several studies have indicated that both the solid and liquid waste may be regarded as an economic resource in the form of utility products such as soil conditioner, compost, biogas fuel and solid fuel (by drying of the stone) (7, 57). By-product utilisation of the olive oil mill waste could mean less environmental pollution of toxic substances and increased income from the extraction of selected added-value compounds. This support the European Directive 2008/98/EC on wastes which sets the basic concepts and definitions related to waste management, such as recycling and recovery (58). It explains when waste ceases to be waste and becomes a secondary raw material (so called end-of-waste criteria), and how to distinguish between waste and by-products.

2.2 Primary and secondary metabolites in olive fruits

In plants a wide variety of secondary metabolites are synthesized from primary metabolites (e.g., carbohydrates, lipids, proteins, chlorophyll) which have fundamental roles in plant protection against both biotic and abiotic stresses (Figure 2.6). Primary metabolites are essential to cell growth, and they are involved directly in metabolic reactions such as respiration and photosynthesis. Secondary metabolites are derived by pathways in which primary metabolites are involved and therefore are considered as the end products of primary metabolites (59, 60). The olive fruit *mesocarp* accumulates a wide range of secondary metabolites.

These secondary metabolites include: phenolics, terpenes and sterols. Phenolic compounds have, as a common characteristic, the presence of at least one aromatic ring hydroxyl-substituted and they are presented commonly bound to other molecules, frequently to sugars (glycosyl residue) and proteins. Interestingly, existing literature differ with regard to the number of olive phenol classes (61-63), however the polar phenolic classes include: phenolic acids namely hydroxybenzoic acid and hydroxycinnamic acids (i.e. vanillic acid and ferulic acid), flavonoids, simple phenol alcohols (i.e. hydroxytyrosol and tyrosol), secoiridoids, lignans and hydroxyl-isochromans. See Figure 2.7.

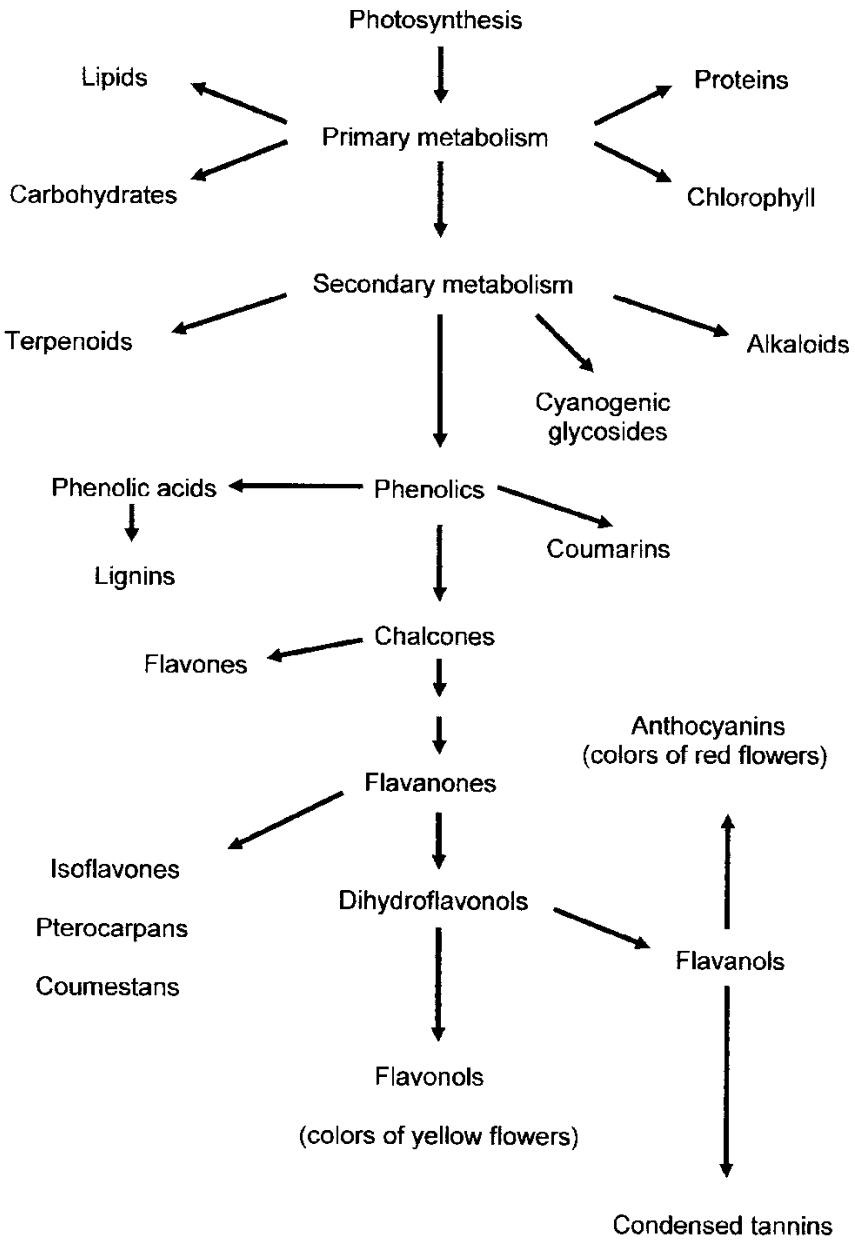


Figure 2.6 Primary and secondary metabolites in plants (60)

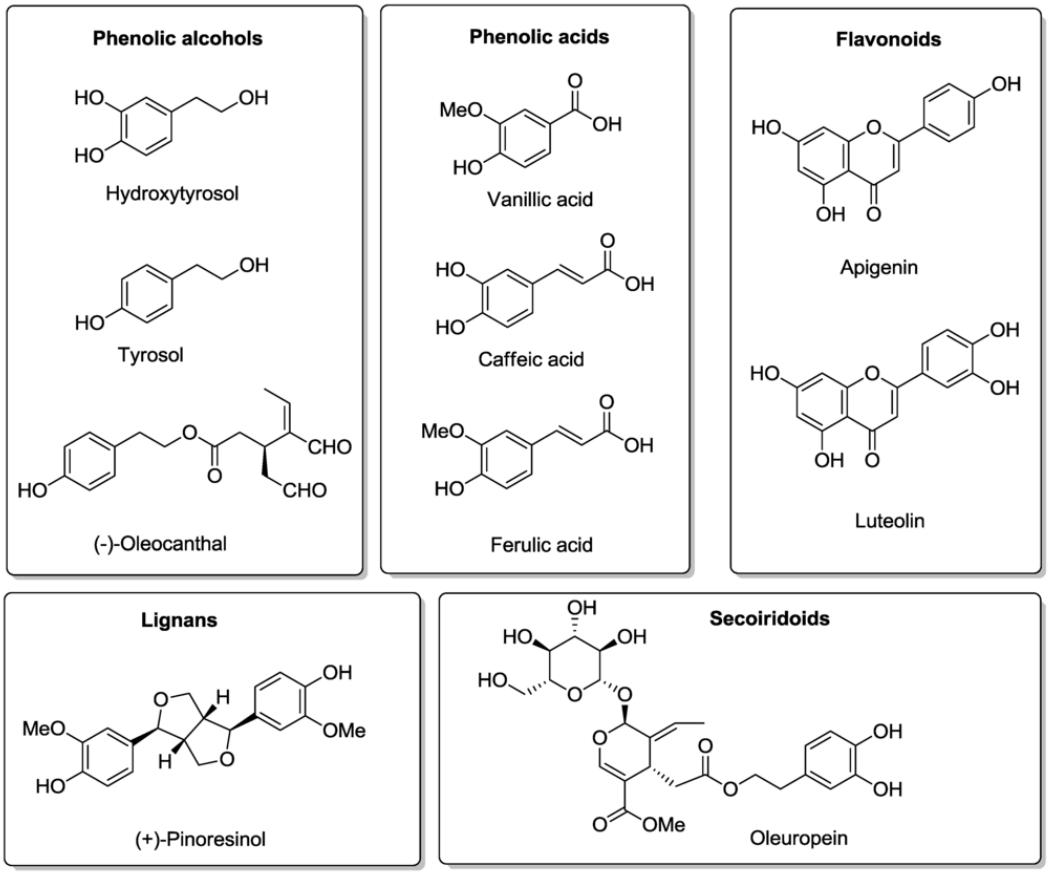


Figure 2.7 Chemical structures of five classes of olive fruit phenols: phenolic alcohols, phenolic acids, flavonoids, lignans and secoiridoids (62).

The main category of secondary metabolites is represented by secoiridoids which are abundant in olives as phenol-conjugated compounds that might contain a glycoside moiety. As seen in Figure 2.8, Servilli et al. (5, 64) proposes the biochemical transformation of secoiridoids, oleuropein and ligstroside, to their relative aglycones and respective decarboxymethylated derivatives as a result of enzymatic hydrolysis catalysed by endogenous β-glucosidases during the mechanical extraction process of the oil:These aglycones include: the dialdehydic form of decarboxymethyl elenolic acid linked to either hydroxytyrosol (3,4-DHPEA-EDA, also called oleocanthal found only in the oil) or tyrosol (p-HPEA-EDA), oleuropein aglycon (3,4-DHPEA-EA) and the ligstroside aglycon (p-HPEA-EA)

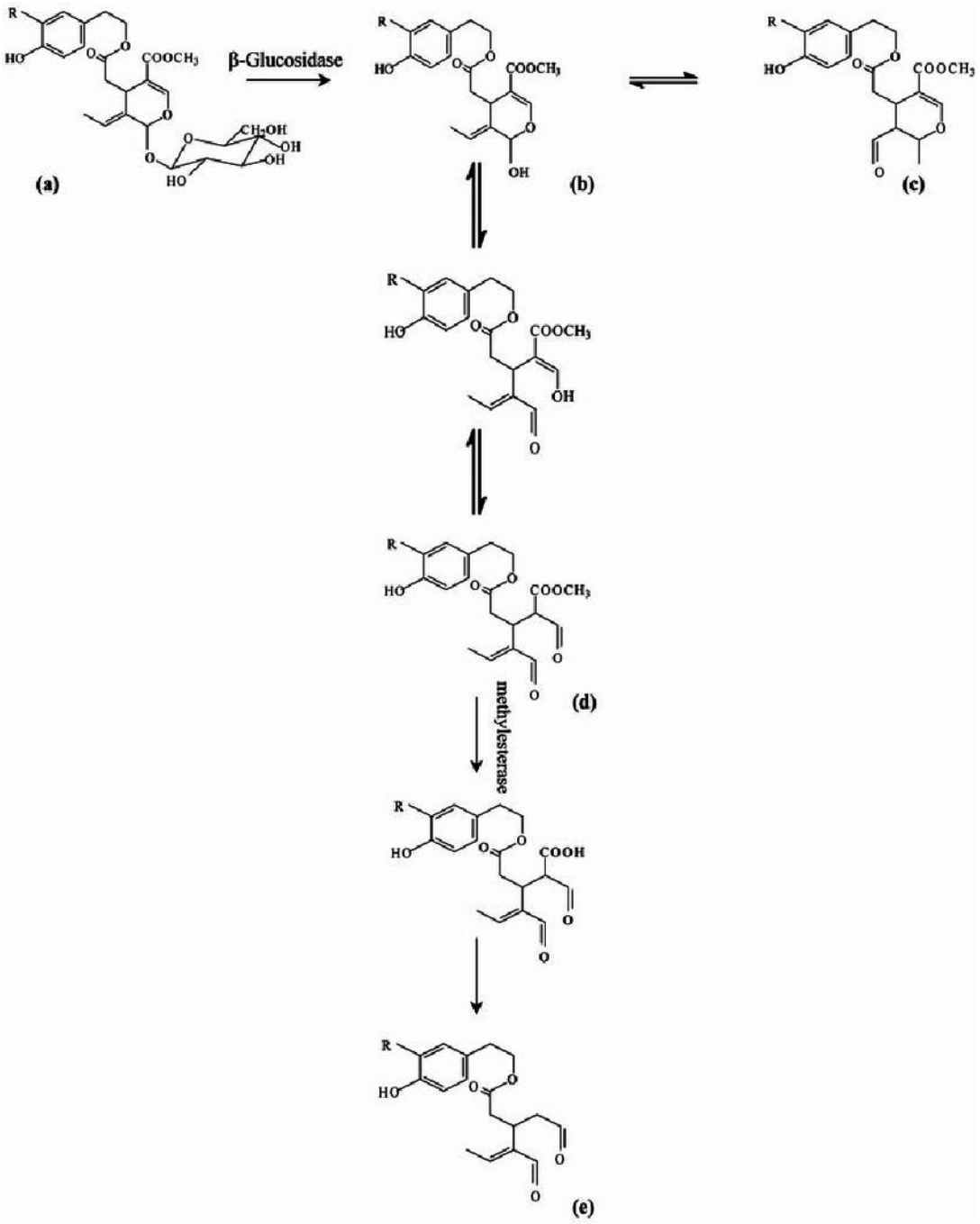


Figure 2.8 Proposed evolution of secoiridoid derivatives (5) (a) R=H: ligstroside; R=OH: oleuropein; (b) R=H: ligstroside aglycon; (c) R=OH: 3,4-DHPEA-EA; (d) R=H: dialdehydic form of oleuropein aglycon; (e) R=H: *p*-HPEA-EDA; R=OH: 3,4-DHPEA-EDA.

Squalene, an intermediate of the sterol pathway, is the precursor of α- and β-amyrins, as well as maslinic and oleanolic acid triterpenes which are concentrated in the skin of the olive fruit and exhibit anti-inflammatory and antioxidant properties *in-vitro*. As seen in Figure 2.9, it could be stated that these compounds could proceed from the acetate/mevalonate pathway and or plastidic MEP pathway (35, 65)

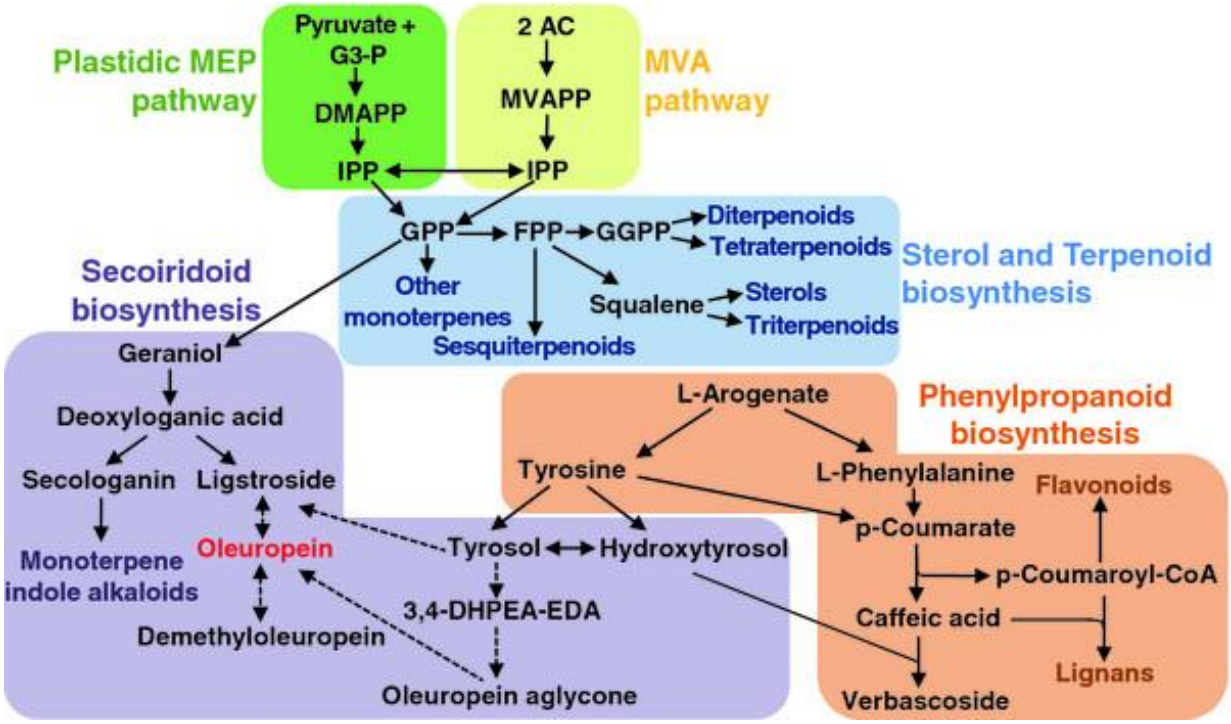


Figure 2.9 Schematic representations of the biosynthetic pathways of the main secondary compounds in the olive fruit (35).

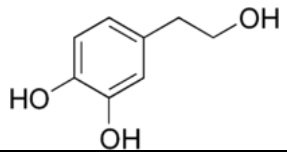
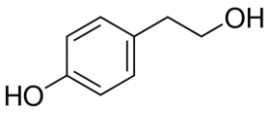
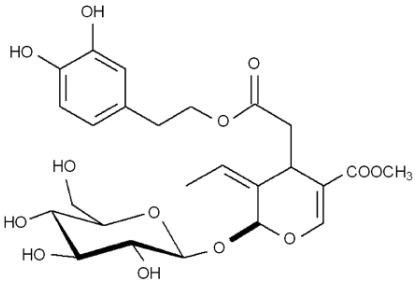
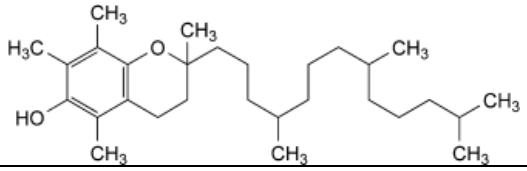
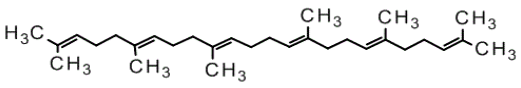
Cell wall derivate components include polysaccharides (pectins and hemicelluloses) and non-digestible oligosaccharides and are beneficial for their nutritional and physiological effects. Soluble sugars like glucose, sucrose and fructose, as well as sugar alcohol called mannitol can be extracted from the *alperujo*. Mannitol is used as excipient in the pharmaceutical industry, as a lubricant, and as a low calorie sweetener in the food industry. Polymerin, a dark multifaceted metal polymeric mixture composed of organic compounds polysaccharides (54%), melanin (26.1%), protein (10.4%) and minerals (11.06%), mainly potassium, can be used as a biofilter for toxic materials due to its resemblance with humic acid (66).

2.3 Selected high-value bioactive compounds in olive pomace and their potential health benefits.

Several useful compounds have been identified in olive mill pomace generated from olive oil processing can be classified as either insoluble, hydrophilic or lipophilic. The interest of this doctoral study is to focus on the potential pharmacological compounds for formulation purposes namely: the hydrophilic phenol alcohols hydroxytyrosol and tyrosol, and secoiridoid oleuropein, as well as the lipophilic squalene and α-tocopherol. The chemical structures of these high-value bioactive compounds are illustrated in

Table 2.4. Each of these selective bioactives will be discussed in terms of the chemistry and health benefits.

Table 2.4 Chemical structures of selected bioactive compounds in olive pomace.

Bioactive compound	Molecular formula	MW[a]	Chemical structure
Hydrophilic compounds			
Hydroxytyrosol	$C_8H_{10}O_3$	154.16	
Tyrosol	$C_8H_{10}O_2$	138.16	
Oleuropein	$C_{25}H_{32}O_{13}$	540.51	
Lipophilic compounds			
α-Tocopherols	$C_{29}H_{50}O_2$	430.71	
Squalene	$C_{30}H_{50}$	410.72	

[a] Molecular weight (Da)

2.3.1 Hydroxytyrosol / 2-(3,4-dihydroxyphenyl) ethanol

Chemistry

Hydroxytyrosol, the principle degradation product of oleuropein, in its pure form is a slightly yellow viscous liquid with a characteristic pungent odour and bitter taste. It has a boiling point of 174°C and is miscible in water at 25°C. From a structural viewpoint,

hydroxytyrosol has two adjacent OH groups on the benzene moiety and is therefore an ortho-diphenol or catechol.

Health benefits of hydroxytyrosol

Hydroxytyrosol shows a broad spectrum of biological properties due to its antioxidant and radical-scavenging properties. As an antioxidant and potent OH scavenger, hydroxytyrosol transform itself into a catechol quinone. Because of its amphiphilic characteristics (octanol-water partitioning coefficient = 1.1), hydroxytyrosol will readily cross cell membranes and provide protection in the cytosol and membranes, including the water-lipid interface (67, 68).

The anti-inflammatory activity of hydroxytyrosol is due to its inhibitory action on the production of inflammatory mediators: nitric oxide (NO) and prostaglandin E2 (PGE2). Moreover, the strong anti-inflammatory effect, antioxidant activity and detoxifying effect on cultured skin cells, proposes its application in products for skin care (69-71). Hydroxytyrosol is currently utilised in the preparation of functional foods such as spreads for its health-promoting properties and for increasing the storage periods of foods; pharmaceutical formulations for its pharmacological activity; and cosmetics for its antioxidative properties. Other biological properties include anticancer activity; maintenance of bone and prevention of osteoporosis development; antimicrobial activity against bacteria, viruses and protozoa; anti-inflammatory activity by decreasing the inflammatory markers such as leukotriene B4; antiviral activity as a class HIV-1 inhibitor of viral entry and integration; and use as an anti-nitrosating agent (72-74).

2.3.2 Tyrosol / 2-(4-hydroxyphenyl) ethanol)

Chemistry

Tyrosol, a phenol alcohol, is a colourless solid at room temperature, melting at 91–92°C, boiling at 158°C, and slightly soluble in water. The higher solubility of tyrosol in organic solvents with respect to hydroxytyrosol is shown by the partitioning coefficients between oil and water phases determined as 0.077 (71).

Health benefits of tyrosol

Tyrosol exerts anti-inflammatory properties; anti-diabetic effect; and protective effects on bone by reducing bone loss. It exhibits anti-inflammatory, neuro-protective, cardio-protective and anti-cancer properties (75-77). In addition, the anti-hyperglycemic effect

of tyrosol in streptozotocin (STZ)-induced diabetic rats reported by Chandramohan et al. (78) confirmed that tyrosol treatment reduced accumulation of glycoprotein components in rats in addition to its anti-diabetic effect and may be used in therapeutic approaches. It also demonstrates *in vitro* cytotoxicity properties (79).

2.3.3 Oleuropein

Chemistry

Oleuropein, the most abundant among these high-value components, is a white to off-white crystalline powder with a meling point of 88°C. Chemically, oleuropein is a complex phenol that can by hydrolysed to hydroxytyrosol, elenolic acid, oleuropein aglycone and glucose. The biosynthesis of oleuropein proceeds via a branching in the mevalonic acid pathway from the secondary metabolism, resulting in the formation of oleosides where secoiridoids are derived. This bitter secoiridoid is responsible for the pungent taste and browning of green olives during harvest and processing. During ripening of the fruit, the oleuropein content decreases, probably due to the β-glucosidase enzyme glycosidic activity, hydrolysis and esterase as seen in Figure 2.10 (80, 81).

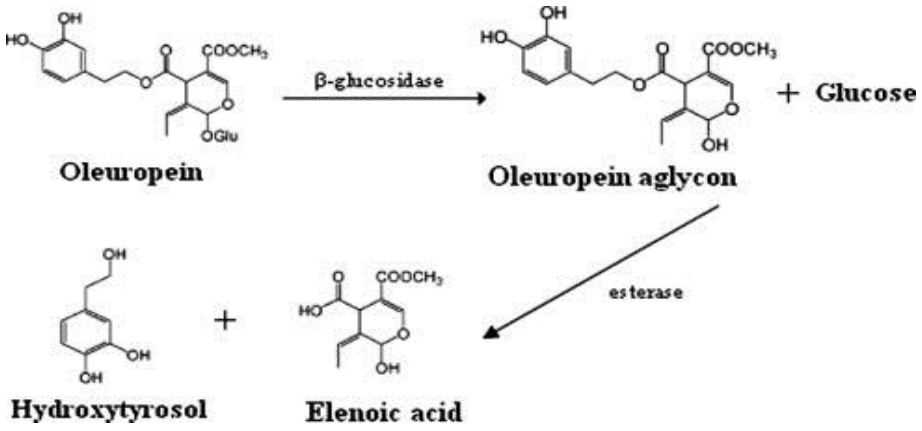


Figure 2.10 A simplified schematic description of the oleuropein degradation pathway (81).

Health benefits of oleuropein

There are a number of studies regarding oleuropein and its pharmacological activities including antioxidant and free radical-scavenger by scavenging chain-propagating lipid peroxyl radicals. Studies have shown the beneficial cardiovascular effects which include antiarrhythmic, spasmolytic, cardio-protective, hypotensive and anti-inflammatory properties (82). A variety of antimicrobial actions of oleuropein, a phenolic

glucoside, have been demonstrated to be effective against important human pathogenic bacteria (both gram positive *Staphyococcus aureus* and gram negative *Escherichia coli* bacteria), as well as yeasts, fungi and moulds. *In vitro* studies have established that this secoiridoid acts as an anti-cancer compound, inhibits platelet-activating factor activity, and shows hypolipidemic and hypoglycemic activity (83, 84).

2.3.4 α-Tocopherol (Vitamin E)

Chemistry

Naturally occurring α-tocopherol is primarily recognized as the most active source of vitamin E in the *d*-optical isomer form exhibiting antioxidant properties. It is a clear, colourless or pale yellow, viscous, oily liquid which is practically odourless and oxidises on exposure to air or light causing darkening. Its boiling point is 200 – 220°C. α-Tocopherol is practically insoluble in water, freely soluble in acetone, ethanol, ether and vegetable oils (85).

α-Tocopherol forms part of a family of related compounds grouped under the name of vitamin E which result from the chromanol ring linked to a C_{16} isoprenic chain (refer to structure in Table 2.4): tocopherols are characterized by a saturated isoprenic chain, while in tocotrienols, the chain is unsaturated. Tocopherols and tocotrienols occur in 4 different forms namely, α, β, γ and δ, depending the number and position of the methyl group. These Vitamin E analogues cannot be manufactured by the body and supplementation is necessary. All tocochromanols are amphipathic molecules i.e. the lipophilic isoprenoic side chain is associated to the membrane lipids and the polar chromanol ring is exposed to the membrane surface. The total tocopherols in olive oil are represented mainly by α-tocopherol (90%) and by minor amounts of β-, γ- and δ-tocopherol.

Health benefits of α-tocopherol

α-Tocopherol is a natural antioxidant, exerting the antioxidant effects both *in vivo* and *in vitro* and the free hydroxyl group on the aromatic ring reacts with free radicals which are the cause of oxidative damage to cell membranes by donating the hydrogen to the free radical, resulting in a relatively stable free radical form of vitamin E in the process. In addition, this fat soluble vitamin regulates and improves immune function, maintaining endothelial cell integrity and balancing normal coagulation. Moreover, α-tocopherol exhibits anti-coagulant, anti-atherogenic, anti-thrombotic, neuroprotective,

antiviral, immunomodulatory, cell membrane-stabilising and anti-proliferative actions (86). Retinal vascular dysfunction due to hyperglycaemia could be prevented by α-tocopherol via the diacylglycerol-protein kinase C pathway (87).

Anti-inflammatory effects of α-tocopherol are both *in vitro* and *in vivo* and therapeutic supplementation, especially at high doses, has shown to decrease release of pro-inflammatory cytokines (such as interleukin-1beta, interleukin-6 and tumour necrosis factor-alpha) and the chemokine interleukin-8, and to decrease adhesion of monocytes to endothelium and therefore prevent atherogenesis observed in cardiovascular disease (88)**.** Vitamin E is often used in skin creams and lotions because it is believed to play a role in encouraging skin healing and reducing scarring after injuries such as burns. As lipophilic compounds, tocopherols are easily incorporated into oil- and fat-based pharmaceutical and cosmetic formulations.

2.3.5 Squalene/2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene

Chemistry

Squalene, a highly unsaturated hydrocarbon ($C_{30}H_{50}$) widely distributed in both the plant and the animal kingdom, is an isoprenoid compound consisting of six isoprene units (C_5) with double bonds and appears as a colourless to light yellow liquid (oil). The boiling point is 258°C. It is freely soluble in diethyl ether, carbon tetrachloride and acetone as well as other fat solvents; sparingly soluble in alcohols and glacial acetic acid; but insoluble in cold water. It has a faint agreeable odour. Squalene is easily oxidised because of its many double bonds.

Olive squalene is a clear, non-greasy oil and has the same molecular structure as human squalene which is the principal hydrocarbon of human skin surface lipids and is manufactured in the liver of every human body and circulates in our bloodstream.

Health benefits of squalene

The double bond structure of squalene contributes the strong antioxidant properties this linear hydrocarbon isoprenoid exhibits (89). In addition, squalene enhances vitality when the unsaturated carbons binding hydrogen ions from water, release three unbound oxygen molecules which reach the cells and intensify cellular metabolism and functions of the liver and kidney. Squalene, usually located in the hydrophobic band between the lipid bilayer cellular zone, has an outstanding antioxidant capability recognized because of its highly stable structure instigating the protective action

against cancer (90). It is not very susceptible to peroxidation and appears to function in the skin as a quencher of singlet oxygen, protecting human skin surface from lipid peroxidation due to UV exposure and other sources of ionizing radiation (91). Supplementation of squalene to mice has resulted in marked increases in cellular and non-specific immune functions in a dose-dependent manner. Squalene may also act as a "sink" for highly lipophilic xenobiotics (92). Since it is a nonpolar substance, it has a higher affinity for un-ionised drugs.

In animals, supplementation of the diet with squalene can reduce cholesterol and triglyceride levels. Dietary squalene promotes changes by increasing the levels of high density lipoprotein (HDL)-cholesterol and paraoxonase 1 and decreases reactive oxygen species in lipoproteins and plasma malondialdehyde levels (93). The primary therapeutic use of squalene currently is as an adjunctive therapy in a variety of cancers. Although epidemiological, experimental and animal evidence suggests anti-cancer properties, to date no human trials have been conducted to verify the role this nutrient might have in cancer therapy regimens. Moreover, squalene is present in human sebaceous secretions (13%) as a precursor of cholesterol. Its terpene nature gives it particular physico-chemical properties which make it an exceptional emollient and moisturiser (94). In pharmaceutics and cosmetics, it is used in many formulations because of its properties which are emulsifying, anti-inflammatory and anti-ageing, strong antioxidant and natural antibiotic (95). Squalene is added to lipid emulsions as drug carrier in vaccine applications against hepatitis B and C, herpes simplex virus, and influenza virus, stimulating the immune response and increasing the patient’s response to vaccine (96).

2.4 Herbal supplement market and related regulations

Products containing designated active ingredients such as plant or herbal material, amino acids, vitamins, minerals, essential oils, certain nutritional substances derived materials, homoeopathic preparations and essential oils are referred to as 'complementary or alternative medicines' and are regulated by the Medicine Control Council in South Africa as medicines under the Regulations for Medicines and Related Substance Act, 1965 (act 101 of 1965) (97). There is an emerging interest in natural plant-based remedies as a source for commercial products**.** Only a few South African medicinal plants have been exploited to their full potential in terms of commercialisation, for example, rooibos and buchu, whereas a high percentage of the South African population use traditional medicines to meet their primary health care needs. Hence, this mine of unexploited high-value compounds which can be extracted

from plant material of bio-waste for their bioactive pharmaceutical properties provide a marketing opportunity in both local and international domains. The European Medicine Agency compiled a draft assessment report on *Olea europaea* L., folium which acts as a guideline for the pharmaceutical forms available, extraction processes and relevant pharmacological and clinical outcomes of olive bioactive compounds for human consumption (82).

2.5 Conclusion

The olive fruit (*Olea europaea*) is one of the oldest and most important fruits consumed either as table olives or used for extraction of olive oil. Numerous studies published have associated prevention of diseases and inhibitions against pathogenic microorganisms with the nutritional and health benefits of olives. The composition of olive fruits include a diverse class of secondary hydrophilic and lipophilic bioactive compounds which attribute disease preventative and healing properties, for example, antioxidant activity. However, the bioactive compound compositions were found to be very complex and depended on numerous factors such as cultivar, maturation stage and agro-climatic conditions. In addition, secoiridoid aglyconic forms arise from hydrolysis and endogenous β-glucosidases during crushing and malaxation. These newly formed substances, having hydrophilic, lipophilic and/or amphiphilic characteristics, partition between the oily layer and two-phase extraction system olive pomace which is a by-product during olive oil extraction.

Important hydrophilic and lipophilic bioactives present in the olive pomace are hydroxytyrosl, tyrosol, oleuropein, α-tocopherol and squalene known for their antioxidant, antimicrobial, anti-inflammatory properties. These high-value compounds were the primary focus of this research study in which their optimum extraction from the two-phase olive pomace was developed using a novel integrated extraction methodology.

CHAPTER 3

ANALYTICAL METHOD DEVELOPMENT

According to literature (16, 17, 98), the preferred analytical method for qualitative and quantitative analysis of hydrophilic bioactive polyphenolic compounds such as hydroxytyrosol, tyrosol, and oleuropein in olive oil processing waste, is by high performance liquid chromatography (HPLC). Similarly, α-tocopherol (or vitamin E) and squalene present in the unsaponifiable fraction of the olive fruit are also analysed using the same method (99-101).

This chapter describes the development an optimum HPLC method to obtain clean, clearly separated peaks in the HPLC chromatograms for optimal determination of the five above-mentioned bioactive compounds.

3.1 Principles of liquid chromatography

Liquid chromatography is a separation technique which involves the injection of a small volume of liquid sample into a column packed with porous particles (stationary phase). The individual components of the liquid sample are transported along the packed column with the aid of a liquid (mobile phase). The components of the sample are separated from one another by their varying chemical and/or physical interactions with the packed material in the column and their solubility in the mobile phase. The separated components are collected at the column exit and detected by an external measurement device such as a spectrophotometer which measures their ultraviolet-visible (UV-Vis) absorption intensity in the ultraviolet-visible spectral region (102). Sample compounds that display stronger interactions with the stationary phase (column) will move more slowly through the column than components with weaker interactions.

In HPLC, very small volumes of solvent (mobile phase) and the injected sample are continuously pumped through the column under high pressure. The sample mixture is resolved into its components within the column and these are sensed by the detector as they leave the column. The resulting detector signal (usually absorbance) is plotted against retention time (time that the component spends in the column). The resolution of signal peaks is dependent upon the interaction between the solutes and the liquid

mobile phase. The columns are typically between 150 and 250 mm in length with 4.6 mm internal diameter and particle size of 5 µm. The sample is injected in microliter quantities and the flow rate of mobile phase is usually between 0.5 and 1 ml/min.

HPLC columns can be broadly divided into two types: normal and reversed phase columns. Normal phase columns have a polar stationary phase and 100% organic mobile phase is used to retain polar analytes; reversed phase columns have a non-polar (hydrophobic) stationary phase and use a polar mobile phase (such as water) where separations are based upon intermolecular forces between non-polar analytes and non-polar compounds bonded to the silica beads in the column (103).

The HPLC system can be set up either for isocratic elution in which the mobile phase composition remains constant during the full analytical run, or gradient elution, used more frequently, in which the mobile phase composition is steadily changed during the chromatographic analysis and generally provides better resolution of the sample and/or decreases analysis time compared to isocratic separation. Sample retention can be controlled by varying the solvent strength of the mobile phase. A multistep linear solvent gradient delivers variable mobile phase composition during a specified analysis run/period and is the method of choice for analysing complex samples. During gradient conditions, the mobile phase solvent or eluent (solvent A) changes from a more polar (usually a mixture of water or aqueous buffer) to a less polar or organic solvent (solvent B) such as methanol or acetonitrile.

Three key chromatographic principles that govern the effectiveness of component separation include retention, resolution and separation factor (104). **Retention factor (k)**, also known as a column capacity factor, is defined as the time an analyte is retained in the column by the stationary phase relative to the time it resides in the mobile phase (Equation 3.1), whereas the **retention time (t_R)** is the time elapsed between the injection of the sample and the appearance of the maximum peak response as a parameter for identification of that compound. Retention time is related to the retention factor by Equation 3.2. This relationship is also shown in Figure 3.1.

$$k = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}} \qquad \text{Equation 3.1}$$

And for a compound,

$$k = (t_R - t_0)/t_0 \qquad \text{Equation 3.2}$$

where,

t_0 reflects the dead time or time required for the mobile phase to pass through the column.

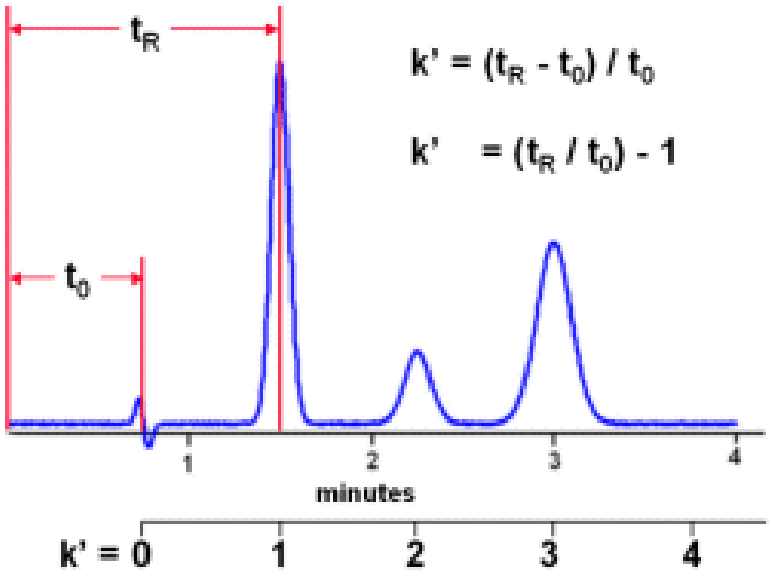


Figure 3.1 An HPLC chromatogram showing the relationship between retention factor and retention time (105).

Resolution (R_s) is the ability of the column to separate two compounds in a sample mixture showing two peaks on the chromatograph and is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line (Equation 3.3).

$$R_s = \frac{(t_{R1} - t_{r2})^2}{(w_1 + w_2)}$$ Equation 3.3

where,

t_{R1} = retention time of compound 1

t_{R2} = retention time of compound 2

w_1 = the peak width of compound 1

w_2 = the peak width of compound 2

The higher the resolution the less the overlap between two peaks. Separation only considers the distance or time between two peak maxima while resolution takes both the distance between two peaks and the width of the peaks into account. Figure 3.2 illustrates 3 resolution values. Figure 3.2 (a) shows that a resolution of < 1.50, the peaks overlap (co-elution). Figure 3.2 (b) shows the separated peaks at R = 1.50,

however, there is no baseline between the peaks. Resolution greater than 1.50 as seen in Figure 3.2 (c), indicates there is baseline between the peaks and the peaks are clearly separated. Sometimes percent resolution values are used. They are calculated by dividing the height of the valley between the peaks by the total peak height. It is an easier value to visualize than resolution numbers; however, it is not possible to distinguish between different amounts of full baseline resolution (106).

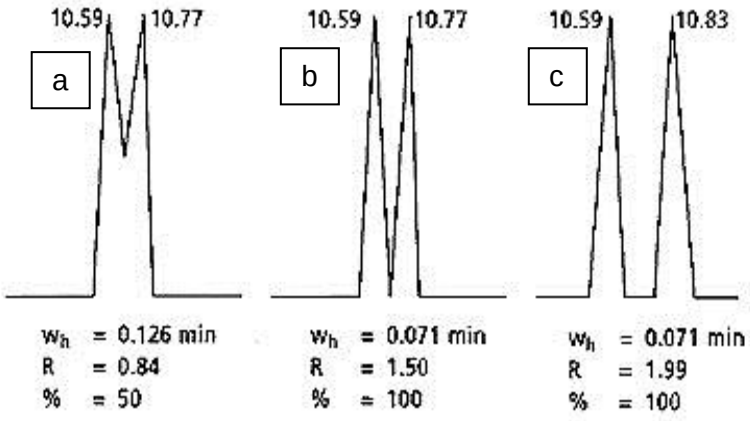


Figure 3.2 Examples of three resolution (R) values: (a) co-elution of two compounds (R < 1.50); (b) separation of two peaks (R = 1.50); and (c) ideal separation with baseline between peaks (R > 1.50).

It is essential that the detector selected will sense all the sample components of interest. Variable wavelength UV-Vis detectors are usually first choice because of convenience and applicability to most sample types.

Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector.

For quantitative analysis of component mixtures, three different calibration methods can be used, namely: external standard, internal standard and the standard addition method (105). The external standard method is the most prevalent of the three methods and was used in this research project for determination of the concentration of bioactive compounds in the olive extracts. In this study, linear regressions were performed based on a calibration curve constructed with six different dilutions of the reference standards for determination of the concentration of the analytes.

3.2 HPLC analysis of hydrophilic bioactives

3.2.1 Summary of literature methods

The first step for selecting an adequate analytical method is to define the objective of the analysis and to know the sample matrix and related complications with the analyte. Table 3.1 summarises literature methods to analyse hydroxytyrosol, tyrosol and oleuropein obtained from various plant sources. These methods were evaluated considering the above and the most suitable ones were chosen and slightly adapted for investigation. The remainder of this section describes the experimental development towards the optimum method to analyse hydroxytyrosol, tyrosol and oleuropein from the olive pomace extracts.

Table 3.1 Analytical methods according to a literature review for the quantification of hydroxytyrosol, tyrosol and oleuropein using HPLC.

Source of sample	Sample preparation	Instrument	Column used/ Detection method/ wavelength	Analytical conditions and elution method	Analytical results	Ref
1L Olive mill waste water	Extraction fluid: ethyl acetate (ratio: 2:1); Reconstitution for analysis: 25 ml methanol	HPLC (Alliance 2690, Waters)	ACE C18-R reverse phase column, 250 x 4.6 mm id 5 µm Wavelength: 280 nm	Solvent A: ultra pure water adjusted to pH 2.5 with acetic acid; Solvent B: acetonitrile. The elution program was as follows: 0–10 min 90% A; 10–15 min 70% A; 15–17 min 66% A; 17–22 min 5% A; 22–26 min 90% A. Run time: 26 min Flow rate: 0.7 ml/min	Gallic acid: 5.81 Hydroxytyrosol: 7.62 Tyrosol: 9.23 Oleuropein: 14.62	(107)
1L OMWW from triphasic mill – different cultivars & harvest years – lyophilised	Extraction fluid: n-hexane; ethyl acetate; acid MeOH (pH3.2) (200-300: 15 ml) Reconstitution: 1-2 ml with MeOH/CH_3CN/H_2O at pH 3.2 (60/20/20)	HP 1090L liquid chromatograph	DAD, UV-vis Wavelength: 240 nm 254 nm 280 nm 330 nm 350 nm	7 -step linear solvent gradient starting with 100% H_2O (adjust to pH 3.2 by H_3PO4) up to 100% CH_3CN. Run time: 106 min Flow rate: 1.0 ml/min Temp: 26 °C	4-Point regression curve ($R^2 \approx 0.9998$) obtained using the available standards, tyrosol, hydroxytyrosol and oleuropein at 280 nm.	(108)
Fresh Coratina SOR - 3 phase. (various years)	Soxhlet 15 hrs-(ratio:0.2g/ml); Extraction fluid: EtOH/H_20 at pH 2 (7:3 v/v) Reconstitution for analysis: 25 ml methanol Filtered: 0.45 µm	HPLC -DAD: HP 1100L liq chromatograph. Interface: HP 9000 workstation, Agilent HPLC-MS for identification	HP 1100L MSDMS with API/ electrospray interface. Varian Polaris C18-E, 250 x 4.6 mm, id 5 µm	Multistep linear solvent gradient starting from: 87% to 85% H_2O in 10 min; 10 min to 75% H_2O; then plateau of 3 min to 5% H_2O in 2 min, and final plateau for 3 min **Eluent:** H_2O (pH 3.2 by HCOOH - formic acid) and CH_3CN all HPLC grade. Run time: 28 min, equilibrium time: 20 min Flow rate: 0.8 ml/min Temp: 26 °C	Quantitative analysis: 4-Point regression curve ($R^2 \approx 0.9998$) obtained using the available standards, tyrosol, hydroxytyrosol and oleuropein at 280 nm.	(16)

Source of sample	Sample preparation	Instrument	Column used/ Detection method/wavelength	Analytical conditions and elution method	Analytical results	Ref
Two-phase olive mill waste or pomace		HPLC on binary LC pump. Varian Star chromatographic workstation (version 6.2)	Luna C-18(2) column attached to a Security Guard cartridge: 150 mm x 4.6 mm x 5 µm Perkin-Elmer LC-235 array detector. An LC 290 UV/vis detector (Perkin-Elmer) connected in series. Wavelength: 280 nm and 335 nm	Solvent A: mixture of 100:1 water/acetic acid (v/v); Solvent B: 90:10:1 methanol/acetonitrile/acetic acid. 6-step linear gradient analysis: 90% Sol A & 10% Sol B, increase to 30% Sol B over 10 min and then isocratic for 5 min, increase to 40% solvent B over 10 min, to 50% over 15 min, to 100% over 10 min, and finally isocratic for 10 min. Run time: 60 min Flow rate: 1.0 ml/min Temp: 21 °C	Standards: tyrosol, hydroxytyrosol, oleuropein, gallic. Standards were dissolved in 80% methanol to prepare stock solutions of 1mg/ml.	(17)
Methanolic extracts of olive stems or roots plus the corresponding standard compounds were used		HP 1100L liq chromatograph.	A reverse-phase Spherisorb ODS-2 column, 25 cm x 4.6 mm, i.d. 5 µm UV-visible Wavelength: 280 nm	2 x solvents: (A) 2ml/L acetic acid (pH 3.1) and (B) methanol. Elution conditions: initial A-B (90:10); in 10 min A-B (70:30); for 20 min A-B (70:30); in 10 min A-B 60:40); for 5 min A-B (60:40); in 5 min A-B (50:50), for 5 min A-B (50:50); in 5 min A-B (40:60); in 5 min A-B (30:70); in 5 min A-B (0:100); 15 min A-B (90:10). Run time: 100 min Flow rate: 1.0 ml/min Temp: 35 °C	Total injection volume was 20 µl: 10 µl methanolic extract + 10 µl standard. C onc. of standard compounds were 0.1 mg/ml for tyrosol, 0.1 mg/ml for hydroxytyrosol and 0.05 mg/ml for oleuropein.	(98)
Two-phase olive mill waste or pomace		HP 1100L liq chromatograph.	A reverse-phase Spherisorb ODS-2 column, 25 cm x 4.6 mm, i.d. 5 µm UV-VIS 280 nm	Mobile phase of trifluoracetic acid in water, pH 2.5 and acetonitrile with a gradient from 5 to 25 % of acetonitrile. Run time: 30 min Flow rate: 1.0 ml/min Temp: 25 °C		(109)

3.2.2 Materials and methods

Solvents and chemicals were obtained from various companies: methanol from Alfa Aesar (HPLC-grade); deionised water (DI) purified on a Millipore Milli Q Plus Ultra-pure water system, glacial acetic acid from Merck (technical grade), and HPLC-grade acetonitrile from Sigma-Aldrich Co. Phenolic reference standards namely, gallic acid (guaranteed reagent grade) was from Merck, tyrosol and oleuropein (both analytical standard) were from Sigma-Aldrich.

The reference stock solutions were prepared by dissolving reference standards in 50% aqueous methanol as a 10 mg/100ml) concentration. Initially, the three samples used for determination of extraction efficiency by HPLC analysis were extracts obtained using water, ethanol and 50% aqueous ethanol as solvents. These extracts were not evaporated and the required quantity for chromatographic analysis from each extract was filtered through a non-sterile 0.45 µm syringe filter from Pall into a 2 ml HPLC vial. Concentration of extracts by rotary evaporation was initiated from the third extraction trial onwards and the residues were reconstituted to 25 ml with 50 % aqueous methanol in a volumetric flask.

Phenolic compounds under investigation were initially identified based on the retention times of gallic acid, tyrosol and oleuropein reference standards using an Agilent high performance liquid chromatograph with diode array detector (DAD). Chromatic separation was achieved by gradient elution on a Zorbax Extend-C18 (Agilent, USA) reverse-phase column (250 mm x 4.6 mm, id 5 µm). A reference standard for hydroxytyrosol was costly and therefore not obtained for the initial development phase of HPLC analysis and literature was referred to for possible elution retention times.

3.2.3 Qualitative comparison of four literature HPLC methods for hydrophilic compounds

Four methods identified during the literature review were implemented for method development and are summarised in Table 3.2.

Table 3.2 Comparison of HPLC analytical gradient methods from literature review.

	Method 1			**Method 2**			**Method 3**			**Method 4**		
Flow rate *ml/min*	0.7			0.8			1.0			1.0		
Wavelength *nm*	280, 335			280, 335			280, 335			280, 335		
Temperature *°C*	25			25			25			35		
Injection volume *µl*	20			20			20			20		
Total analysis time *min*	26			28 + 20 equil			60 + 15 equil			100		
Gradient solutions	A: H_2O, pH 2.5 B: acetonitrile			A: H_2O, pH 3.2 B: acetonitrile (0.8% acetic acid)			A: H_2O/acetic acid (100:1) B: MeOH/ acetonitrile /acetic acid (90:10:1)			A: H_2O, pH 3.15 B: MeOH		
Gradient with 2 mobile phases A and B	Min	%A	%B	Min	%A	%B	Min	%A	%B	Min	%A	%B
	0	90	10	0	87	13	0	90	10	0	90	10
	10	70	30	10	85	15	10	70	30	10	70	30
	15	66	34	20	75	25	15	70	30	30	70	30
	17	5	95	23	75	25	25	60	40	40	60	40
	22	90	10	25	5	95	40	50	50	45	60	40
				28	5	95	50	0	100	50	50	50
				48	87	23	60	0	100	55	50	50
							75	90	10	60	40	60
										65	30	70
										70	0	100
										85	90	10

Table 3.3 reflects the retention times and peak areas obtained for the reference standards during chromatic graphic elution detected at a wavelength of 280 nm for each of the four methods.

Table 3.3 HPLC results for the reference standard solutions using adapted literature methods described in Table 3.2.

Peak	**Method 1 (107)**	**Method 2 (108)**	**Method 3 (17)**	**Method 4 (98)**
Gallic acid (10 mg/100 ml) **at 280 nm**				
Retention time (min)	5.721	4.425	5.213	-
Width (min)	0.357	0.151	0.134	-
Area (mAU*s)	6339	6392	5754	-
Height (mAU)	267	649	669	-
Area (%)	50.7	26.6	80.9	-
Tyrosol (10 mg/100 ml) **at 280 nm**				
Retention time (min)	9.436	9.546	10.815	12.057
Width (min)	0.104	0.769	0.137	0.1387
Area (mAU*s)	1799	546	1259	1259
Height (mAU)	263	132	143	141
Area (%)	75.7	27.7	51.5	50.3
Oleuropein (10 mg/100 ml) **at 280 nm**				
Retention time (min)	-	-	31.885	49.539
Width (min)	-	-	0.245	0.295
Area (mAU*s)	-	-	508	558
Height (mAU)	-	-	32	28.6
Area (%)	-	-	29.9	29.9

From the results obtained in Table 3.3, the polarity of the compounds decreases as follows: gallic acid, tyrosol and oleuropein, as indicated by the increase in retention times. During the gradient conditions where the mobile phase changed from more polar (acidic aqueous buffer) to less polar (organic solvent/s), the more polar substances eluted first.

Method 1:

The gallic acid standard separated into 2 peaks i.e. at 5.721 min. This is not ideal as one single peak would provide improved quantification. Oleuropein was detected with this method.

Method 2:

The gallic acid standard was detected at 4.425 min and tyrosol at 9.546 min. Oleuropein was not detected with this separation.

Method 3:

The gallic acid, tyrosol and oleuropein peaks were all detected and well separated. It appears that only the 50% aqueous ethanol extract revealed all three compounds.

Method 4:

This method had an extremely noisy baseline and thus would make quantification very difficult. Method 4 did not detect gallic acid, and the runtime was very long since oleuropein was consistently detected at 49 min.

Table 3.4 summarises the outcomes of these four different HPLC gradient methods for three sample extracts. These results are not quantitative but only for identification purposes

Table 3.4 Comparison of the retention times and areas of the relevant bioactive compounds implementing four different gradient HPLC methods.

	Gallic acid				**Tyrosol**				**Oleuropein**			
Method	**M1**	**M2**	**M3**	**M4**	**M1**	**M2**	**M3**	**M4**	**M1**	**M2**	**M3**	**M4**
Reference standards retention time (min)	5.721	4.425	5.213	ND	9.436	9.546	10.815	12.057	-	-	31.885	49.539
Ethanol extract												
Retention time (min)	-	-	-	-	-	-	-	-	-	-	-	-
Width (min)	-	-	-	-	-	-	-	-	-	-	-	-
Area (mAU*s)	-	-	-	-	-	-	-	-	-	-	-	-
Height (mAU)	-	-	-	-	-	-	-	-	-	-	-	-
Area (%)	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol/water extract												
Retention time (min)	5.757	-	5.420	-	-	9.551	10.813	12.059	-	-	32.095	-
Width (min)	0.0938	-	0.1345	-	-	0.1411	0.1468	0.1477	-	-	0.2048	-
Area (mAU*s)	146	-	55.2	-	-	330	357	386	-	-	20.2	-
Height (mAU)	24	-	6.2	-	-	36	37	39.6	-	-	1.6	-
Area (%)	0.7	-	0.4	-	-	1.3	2.9	2.4	-	-	0.16	-
Water extract												
Retention time (min)	5.772	-	5.446	5.222	-	9.558	10.816	12.073	-	-	-	-
Width (min)	0.0879	-	0.1103	0.1086	-	0.1561	0.1273	0.1438	-	-	-	-
Area (mAU*s)	281	-	396	92	-	412	353	506	-	-	-	-
Height (mAU)	47	-	52	14	-	40.7	43	54	-	-	-	-
Area (%)	1.5	-	3.8	0.8	-	1.6	3.4	4.5	-	-	-	-

ND- Not detected

Retention times between the reference standard peaks and sample peaks should vary only by approximately 0.01 minutes. In this method comparison, the retention times varied by about 0.1-0.2 min in some cases. This could be as a result of the difference in the diluents of the reference standards (50% aqueous methanol) and extraction samples (ethanol, water and 50% aqueous ethanol) since the extracts were not evaporated to dryness but were used as is. In the absence aforementioned sample preparation, gallic acid, tyrosol and oleuropein eluted from the 50% aqueous ethanol using Method 3. The most prominent parameters that can play a role in the selectivity of compounds by HPLC elution, are indicated in Table 3.5 (4).

Table 3.5 Factors affecting HPLC selectivity.

Parameter	Usage
Organic solvent	Changing to a different solvent phase will change the selectivity.
Mobile phase pH	Can alter the ionization of some analytes – affecting their hydrophobicity.
Solvent strength and additives	Can be adjusted to influence both retention time/factor and selectivity.
Stationary phase	Most popular way to alter selectivity.
Temperature	Can affect certain analytes.
Elution type	Gradient or isocratic.
Solvent polarity	The more polar the mobile phase, the less interaction will occur between the polar compounds and the column resulting in earlier elution (retention time).

In order to obtain the desired peak retention and peak separation, mobile phase composition (ratio A:B), solvent type, pH, temperature, and more can be changed to refine a separation. Another way of optimising the selectivity is to change the column type from C18 to C8. However, this is a very expensive route to follow and therefore, the approach in this study was to use the above mentioned variables to obtain the desired separation.

Solvent strength (%B)

Various gradients were evaluated. The solvent strength for Method 3 and 4 was less than that of Methods 1 and 2 (mobile phases B for Methods 3 and 4 were more polar than for Methods 1 and 2), hence the peaks eluted later. Therefore, Methods 3 and 4 provided better separation of peaks than Methods 1 and 2.

pH

The pH of the mobile phases used for method development ranged from ± 2.5 – 3.2. HPLC separations tend to be more robust when the pH of the mobile phase is controlled. During gradient elution the mobile phase composition changes and

therefore, if one of the mobile phases is pH adjusted, the pH will change during the separation. This will result in a “pH gradient” during the separation and could have an influence on the separation. Method 3 contains 1% acetic acid in both mobile phase A and mobile phase B. Therefore, this method is the most robust method, from a pH point of view of all the methods evaluated. This approach of ensuring that equal volumes of acetic acid are added to both mobile phases, should reduce the baseline noise.

Method 3 gradient elution was selected as the preferred method as it displayed the best responses and separation of peaks for identification of compounds gallic acid, tyrosol and oleuropein.

3.2.4 Comparison of isocratic vs gradient elution

For isocratic elution, the mobile phase composition remains constant during the analysis time whereas in the gradient elution, the ratio of polar to non-polar solvents changes in the mobile phase during the sample run.

Due to different polarities of the different compounds in the olive extracts, developing an isocratic method could be difficult. Gradient elution is usually considered more appropriate as it will allow changing the solvent strength of the mobile phase and ensure that all compounds are separated. However, isocratic elution was considered to assess the rising baseline observed in the gradient method, and if it could be reduced or eliminated.

Isocratic methods with various mobile phase combinations were set up and compared to gradient Method 3 selected. Method 3 was however shortened to a 55 min runtime since the oleuropein eluted before 35 minutes and the original 75 minutes was considered unnecessary long. For isocratic Method 1, mobile phase A [H_2O /acetic acid (100:1)] and mobile phase B [MeOH /acetonitrile /acetic acid (90:10:1)] from gradient Method 3 were selected and the percentage solvent compositions of A:B were as follows: 90:10, 60:40, 50:50, 40:60, and 10:90 in a series of 40 min runs. Isocratic method 2 comprised mobile phase A (H_2O with pH 2.5) and mobile phase B (acetonitrile) from gradient Method 1 to evaluate the solvent strength and polarity on phenolic compound separation. Acetonitrile is less polar than methanol and it was reasoned that this solvent would reduce the retention time of oleuropein. The percentage solvent compositions of A:B were as follow: 90:10, 70:30, 50:50, 30:70, and 10:90. The conditions of isocratic and gradient elution for these solvent systems are shown in Table 3.6.

Table 3.6 HPLC conditions of isocratic elution and gradient elution at different solvent compositions for analytical method development.

	Isocratic M1	**Isocratic M2**	**Gradient 3 (1st adaption) AGM3**		
Flow rate *ml/min*	0.7	0.8	1.0		
Wavelength *nm*	280, 335	280, 335	280, 335		
Temperature *°C*	25	25	25		
Injection volume *µl*	20	20	20		
Total analysis time *min*	40	40	55		
Gradient solutions	A: H_2O/acetic (100:1) B: MeOH/ acetonitrile/ acetic acid (90:10:1)	A: H_2O, pH 2.5 B: acetonitrile	A: H_2O/acetic acid (100:1) B: MeOH/ acetonitrile / acetic acid (90:10:1)		
Gradient phases or isocratic mobile phase composition	A90:B10 A60:B40 A50:B50 A40:B60 A10:B90	A90:B10 A70:B30 A50:B50 A30:B70 A10:B90	Min 0 10 15 25 35 40 45 55	%A 90 70 60 50 40 5 90 90	%B 10 30 40 50 60 95 10 10

During this set of experiments, samples from both the individual reference standards as well as a 1:1:1 concentration ratio mixture of the three reference standards were injected and compared.

The results from isocratic Methods 1 and 2 versus adapted gradient Method 3 using the mixed reference standards are shown in Table 3.7. It is clearly seen that isocratic Method 1 with mobile phases A50:B50 gave the best separation of the three compounds in the mixed reference standard (RS) solution. The peak separation was similar compared to the gradient elution using the same mobile phase system. The isocratic methods with mobile phase ratios A90:B10, A60:B40 and A30:B70 applying both mobile phase systems were not found suitable as oleuropein was not detected. Mobile phase ratios A70:B30, A40:B60 and A10:90 did not show good separation of the three peaks. During this evaluation, elution of the polar compounds gallic acid and tyrosol and the less polar oleuropein confirmed that gradient elution is the preferred method for analysis of complex samples.

Figure 3.3 shows a comparison of the chromatograms obtained for isocratic Methods 1 and 2 with mobile phase ratios of A50:B50 and the adapted gradient method. The chromatogram for AGM3 shows sharper, narrower peaks and a flat baseline, thus the gradient method was chosen for further analysis.

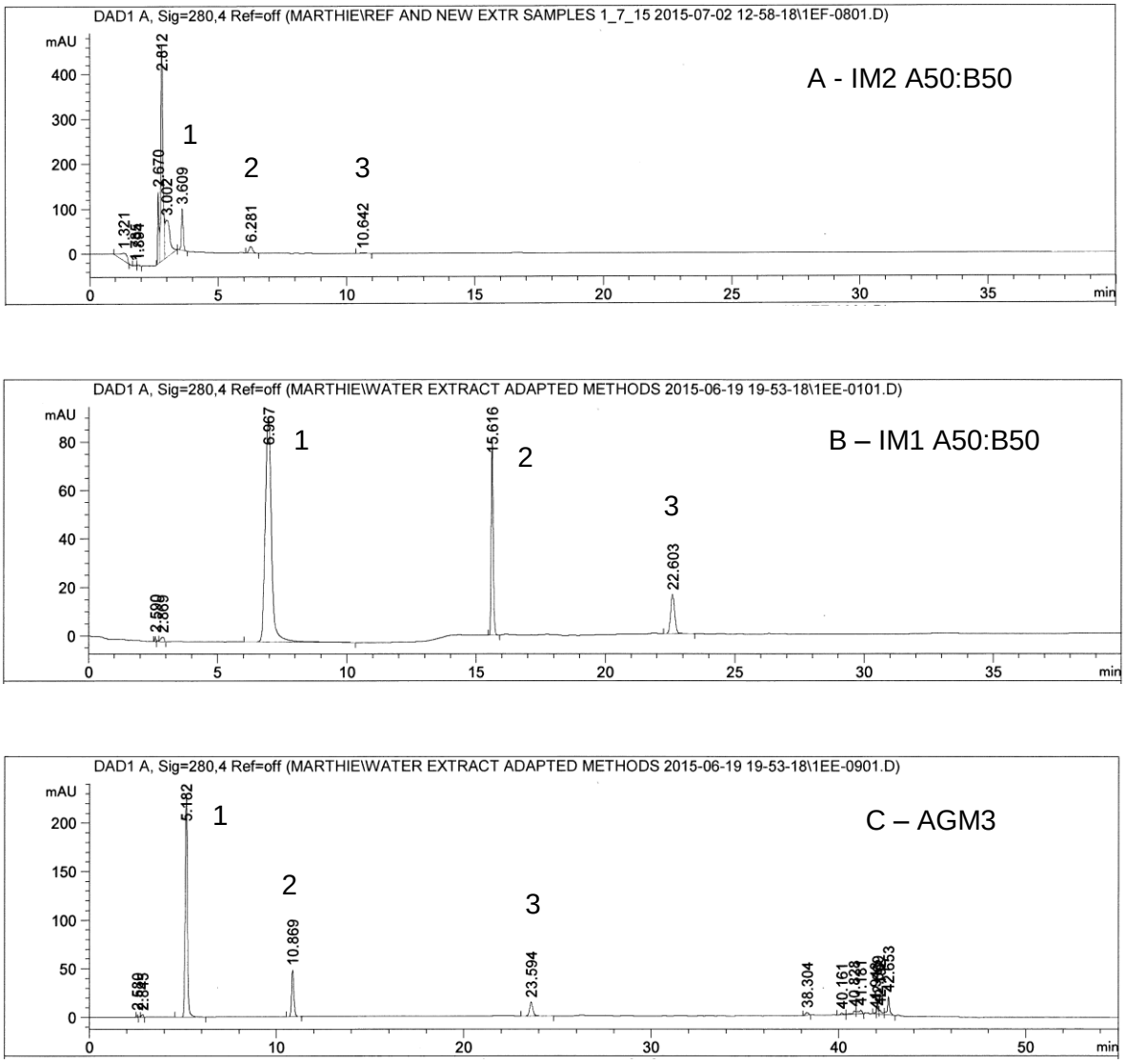


Figure 3.3 Chromatograms of mixed reference standards elution: A – IM2 A50:B50; B – IM1 A50:B50; and C - adapted gradient Method 3 (AGM3) - gallic acid (1), tyrosol (2) and oleuropein (3)

Table 3.7 HPLC results of the mixed reference standards during isocratic Methods 1 and 2 and AGM3 at 280 nm wavelength.

	A90 B10 IM1	**A90 B10 IM2**	**A70 B30 IM2**	**A60 B40 IM1**	**A50 B50 IM1**	**A50 B50 IM2**	**A40 B60 IM1**	**A30 B70 IM2**	**A10 B90 IM1**	**A10 B90 IM2**	**AGM3**
Gallic acid											
Retention time (min)	5.174	4.144	3.177	4.399	6.967	3.609	2.691	2.465	2.573	5.755	5.182
Width (min)	0.3012	0.1070	0.1632	0.0915	0.2622	0.0785	0.802	0.33	0.1023	0.4349	0.134
Area (mAU*s)	1927	1582	4993	426	1558	463	2237	2149	674	274	1966
Height (mAU)	101	233	533	71.5	92.6	92	420	93	83.4	9.6	229
Area (%)	81	75	82	15	71.5	8.7	75.7	68	23.7	6.6	66.4
Tyrosol											
Retention time (min)	17.919	10.960	3.579	14.726	15.616	6.281	3.153	2.683	2.656	8.073	10.869
Width (min)	1.07	0.2538	0.0691	0.3635	0.0804	0.1568	0.0724	0.1617	0.1806	0.4123	0.1324
Area (mAU*s)	393	405	419	167	419	151	493	1010	1238	247	413
Height (mAU)	4.8	25	95	7	81	15	106	82	85.7	9.2	48
Area (%)	16.5	19	6.9	6	19	2.84	16.7	32	43.5	5.9	14
Oleuropein											
Retention time (min)	-	-	4.84	-	22.603	10.642	3.8	-	2.959	10.381	23.594
Width (min)	-	-	0.1032	-	0.1663	0.208	0.0923	-	0.187	0.3540	0.1869
Area (mAU*s)	-	-	169	-	175	14.520	169	-	867	544	173
Height (mAU)	-	-	244	-	16	1.087	28	-	57.6	24	14.4
Area (%)	-	-	2.8	-	8	0.273	5.7	-	30.5	13	5.9

IM1 – Isocratic method 1: A = Mobile phase acetic water (1:100); B = Mobile phase methanol/acetonitrile/acetic acid (90:10:1).

IM2 – Isocratic method 2: A = Mobile phase acetic water (pH 2.5); B = Mobile phase acetonitrile.

AGM3 – A = Mobile phase acetic water (1:100); B = Mobile phase methanol/acetonitrile/acetic acid (90:10:1).

3.2.5 Further modification (adaption) of the selected gradient method

The preferred gradient method i.e. Method 3, was further modified to reduce the run time. See Table 3.8. This improvement still provided adequate separation as shown in Table 3.9.

Table 3.8 Modification of the preferred gradient method for elution of the hydrophilic bioactive compounds.

Parameters	**Method 3 (original)**			**Method 3 (1st adaptation)**			**Method 3 (2nd adaption)**		
Flow rate *ml/min*	1.0			1.0			1.0		
Wavelength *nm*	280, 335			280			280		
Temperature *°C*	25 / 21			25			25		
Injection volume *µl*	20			20			10		
Total analysis time *min*	60 + 15 equil			55			50		
Gradient solutions or mobile phases	A: H_2O/AcOH (100:1) B: MeOH/acetonitrile /acetic acid (90:10:1)								
Gradient	Min	%A	%B	Min	%A	%B	Min	%A	%B
	0	90	10	0	90	10	0	90	10
	10	70	30	10	70	30	20	50	50
	15	70	30	15	60	40	35	50	50
	25	60	40	25	50	50	40	90	10
	40	50	50	35	40	60	50	90	10
	50	0	100	40	5	95			
	60	0	100	45	90	10			
	75	90	10	55	90	10			

Table 3 .9 Comparison of retention times between original Method 3 and adapted method

	Method 3 (initial method) Rt	**At % B**	**Method 3 (2nd adaption) Rt**	**At % B**
Tyrosol	10.816 min	± 30 %	10.089 min	± 30 %
Oleuropein	32.095 min	± 55 %	21.615 min	± 50 %

Rt - Retention time

From the above summary, it can be seen that the tyrosol and oleuropein are eluting at approximately the same mobile phase strength for both Method 3 and Method 3 (2nd adaption). The advantage of second adaption of Method 3 is as follows:

- Reduced run time i.e. 50 min compared with 75 min.
- Increase in sample throughput.
- Reduction in gradient steps. This will provide a much smoother baseline.

3.2.6 Conclusion

The second adaption of Method 3 as shown in Table 3.8 and Table 3.9 was found to be the most suitable for quantification of the hydrophilic bioactives.

3.3 HPLC analysis lipophilic bioactives

3.3.1 Summary of literature methods

Alpha-tocopherol and squalene as non-polar compounds are part of a group of minor components present in the unsaponifiable fraction of olive pomace. Their importance in pharmaceutical and nutritional products makes determination of these lipophilic compounds of major interest.

If tocopherols have been extracted with hexane or heptane, and chromatography is to be performed by reversed-phase HPLC, the solvent must be evaporated and replaced by another solvent more similar to the mobile phase, or by the mobile phase itself. Reversed-phase systems show separation based on the saturation of the phytyl side chain of the tocopherols where the more saturated isomers are retained longer. C18 reversed-phase (RP) systems are preferred when mixtures of fat-soluble vitamins and free and esterified tocopherols are to be separated. Tocopherols are stable under HPLC conditions, easy to dissolve in appropriate solvents, and there are several detectors that can be combined with HPLC such as fluorescence detection (FLD) and ultraviolet detection which are the most commonly used. The polarity of tocopherols is mainly influenced by the number of methyl groups in the chromanol ring, and to a lesser extent by steric effects of the methyl groups.

Modern analytical methods for identification and quantitative determination of squalene are gas chromatography (GC) alone or coupled with mass spectrometry (GC/MS) or high performance liquid chromatography (HPLC) with or without mass spectrometry (HPLC/MS), using either ultraviolet (HPLC/UV) or diode array detector (HPLC/DAD).

A number of methods for analysis of α-tocopherol and squalene were found in the literature and these are summarised in Table 3.10. These methods were evaluated and four of them were selected for investigation. The remainder of this section describes the experimental development towards the optimum method analysis of α-tocopherol and squalene from olive extracts.

Table 3.10 Summary of HPLC methods from literature for identification and quantification of α-tocopherol and squalene.

Method	Analyte	Std or sample prep	Wavelength	Column	Mobile phase	Elution method	Flow rate	Col temp	Run time	Ref
1	α-tocopherol	10mg/100ml in IPA	292 nm	Lichrosorb/Spherisorb 250 x 4 mm, 5 µm (microparticulate silica)	Hexane/IPA (99.5/0.9 v/v)	isocratic	1 ml/min			(110)
2	α-tocopherol		292 nm free tocopherol	Lichrosorb RP18 120 x 4.6 mm, 5 µm	Methanol/water (98/2 v/v)	isocratic	1.5 ml/min			(99)
3	α-tocopherol		292 nm	Tracer Extrosil ODS-2 150 x 4.4 mm, 5 µm	Methanol/water (96/4 v/v)	isocratic	2 ml/min	45^{o}C	6 min	(111)
4	α-tocopherol	Sample dissolved in mobile phase	Fluorometric detection Excitation 290 nm Emission 330 nm	Supelcosil LC-Si, normal phase 250 x 4.6 mm, 5 µm	Ethyl acetate/acetic acid/hexane (or heptane) (1/1/198 v/v/v)	isocratic	1.5 ml/min		30 min	(112)
5	α-tocopherol	0.1g oil in 10 ml hexane	Fluorescent detector	LiChrospher Si60 250 x 4 mm, 5 mm	0.7% IPA in hexane	isocratic				(113)
6	squalene		280 nm	Agilent Zorbax Eclipes Plus C18 250 x 4.6 mm, 5 µm	Acetonitrile/acetone (60/40 v/v)	isocratic	1 ml/min	30^{o}C	20 min	(114)
5	squalene	10mg oil in 10 ml hexane	Photodiode 218 nm	LiCrospher RP-18 250 x 4.6 mm, 5 µm	A: acetonitrile B: IPA C: hexane	Gradient 0-12 min 20-22% B and 10-12% C in A 12-15 min 22-25% B and 12-25% C in A, 15-20 min 25% B and 25% C in A, 20-25 min 25-20% B and 25 -10% C in A	1 ml/min	30^{o}C	25 min	(113)
7	Squalene and α-tocopherol	Stds and samples in IPA	214 nm squalene 280 nm tocopherol	Whatman C18 280 x 4.6 mm, 5 µm	Methanol/IPA/acetic acid (91.95/8/0.05 v/v/v)	Isocratic	1.2 ml/min	Room temp		(100)
8	Squalene and α-tocopherol	Stds and samples dissolved in IPA	210 nm squalene 252 nm tocopherol	Chromolith RP-18C 100 x 3 mm, 2 µm	A: acetonitrile/methanol (7/3 v/v) B: IPA	Gradient 0-6 min A90 B10 6-8 min A70 B30 8-17 min A70 B30 17-20 min A90 B10 20-30 min A90 B10	5 µl/min		30 min	(101)

3.3.2 Materials and Methods

Solvents and chemicals were obtained from various companies: HPLC grade methanol from Merck; reagent grade isopropyl alcohol (IPA) from Alfa Aesar; deionised water (DI) purified on a Millipore Milli Q Plus Ultra-pure water system; reagent grade acetic acid from Radchem Lab; HPLC grade acetonitrile from Rankem; ethyl acetate from Alfa Aesar (99.5%), *d*-limonene (≥ 97%) from Fluka Chemika, chemically pure n-heptane and n-hexane from Merck, orthophosphoric acid (85%) from Loba Chemie. Both α-tocopherol (≥ 96%) and squalene (≥ 98%) reference standards (RS) were purchased from Sigma-Aldrich.

Reference standard stock solutions with a concentration of 0.1% were prepared by dissolving standards in isopropyl alcohol. The four samples used for determination of extraction efficiency by HPLC analysis were Soxhlet extracts obtained using four different solvent or solvent blends: *n*-heptane, IPA, 50% *n*-heptane:ethyl acetate and 50% *n*-hexane:ethyl acetate. These extracts were concentrated by rotary evaporation and reconstituted with IPA in a 25 ml volumetric flask. All extract samples containing lipophilic compounds were filtered through a non-sterile 0.45 µm syringe filter (with a GHP membrane) from Pall into 2 ml HPLC vials.

3.3.3 Qualitative comparison of four literature HPLC methods for lipophilic compounds

A chromatographic comparison for elution of squalene and α-tocopherol by reversed-phase chromatography was established. Four of the literature methods in Table 3.10 were selected to identify which method or combination of method parameters would provide the best peak separation and peak quality for simultaneous determination of both compounds. These methods and their parameters are summarised in Table 3.11. Chromatographic separation was achieved using an Agilent Eclipse XBD-C18 (Agilent, USA) reverse-phase (250 mm x 4.0 mm, 5 µm) column. The isolation of the lipophilic compounds was performed by using an Agilent 1290 Infinity system equipped with a binary gradient pump and a DAD detector. The wavelengths were set as indicated by the different analytical methods being evaluated for α-tocopherol and squalene. The column temperature was controlled at 30°C with a flow rate of 1.5 ml per minute during a maximum 30 minute run period. Sample volume was 20 µl. During the investigation to obtain the most effective chromatographic elution method, separation was achieved by both gradient and isocratic elution.

Table 3.11 Comparison of the selected HPLC analytical methods for qualitative determination of lipophilic compounds.

	Method 1 (99)	**Method 2 (114)**	**Method 3 (100)**	**Method 4 (101)**		
Flow rate (ml/min)	1.5	1	1.5	1.5		
Wavelength (nm)	292	280	214, 280	280, 335		
Temperature (°C)	30	30	30	30		
Injection volume (µl)	20	20	20	20		
Total analysis time (min)	30	30	30	30		
Elution method	Isocratic	Isocratic	Isocratic	Gradient		
Mobile phase(s)	MeOHl/H_2O (98/2 v/v)	Acetonitrile/ acetone (60/40 v/v)	MeOHl/IPA /acetic acid (91.95/8.0/0.05 v/v)	A: acetonitrile/ MeOH (7:3 v/v) B: IPA		
Gradient with 2 mobile phases A and B	Not applicable	Not applicable	Not applicable	Min 0 6 8 17 20 30	%A 90 70 70 30 90 90	%B 10 30 30 70 10 10

Figures 3.4 and 3.5 show the HPLC chromatograms of the 0.1% α-tocopherol RS and 0.1% squalene RS concentrations respectively implementing the four methods at the indicated wavelengths ranging between 210 - 292 nm.

Isopropyl alcohol diluent presented several peaks in the chromatograms which were observed in the diluent, RS and all the extract samples. This could be attributed to the poor quality of the solvent used at the time which was not HPLC grade. Broad peaks for both the squalene and α-tocopherol RS can be an indication of the samples being too concentrated and require further dilution or that the injection volume is too large and should be reduced.

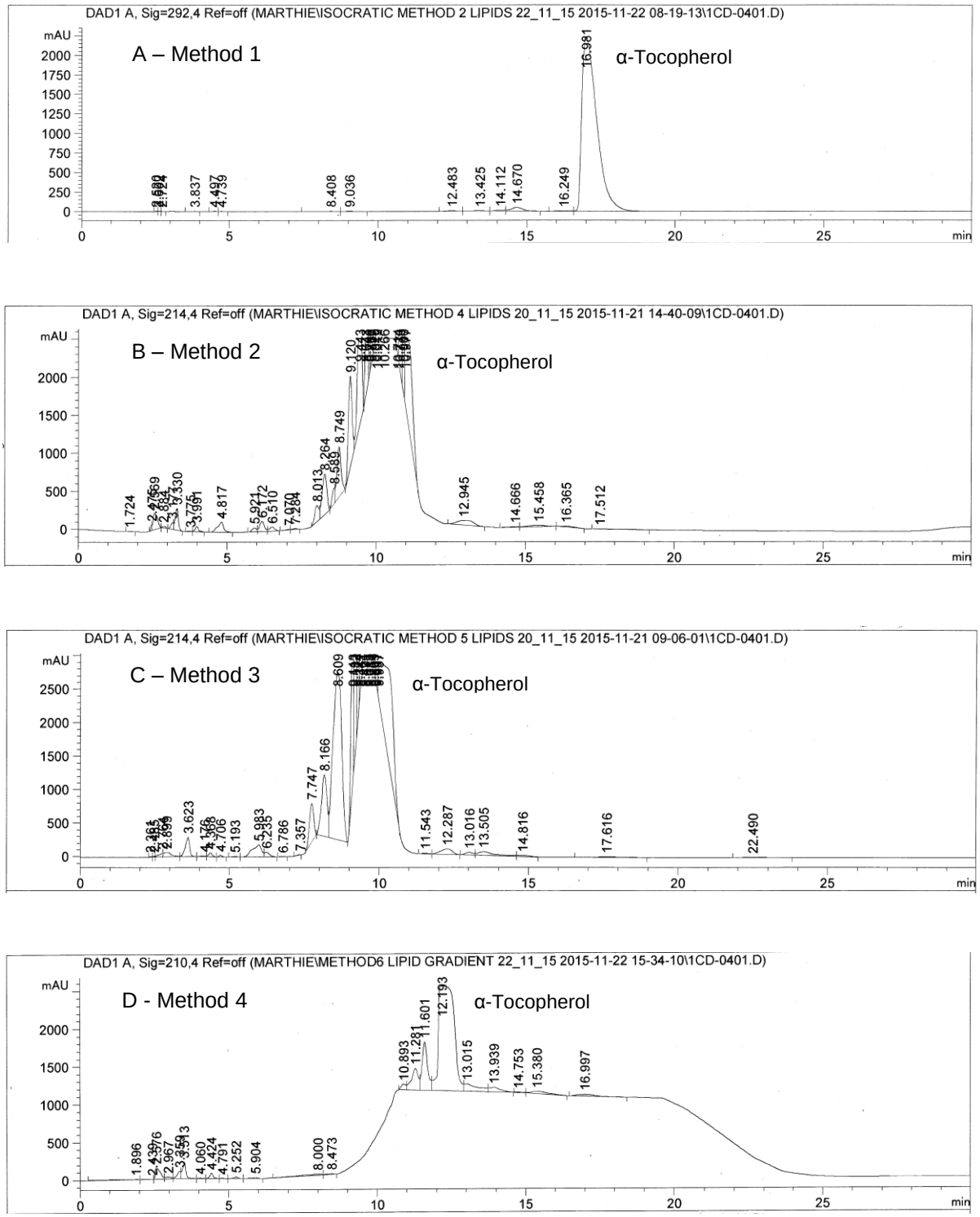


Figure 3.4 HPLC chromatograms of a 0.1% α-tocopherol RS solutions analysed using three isocratic methods (A-Method 1, B- Method 2, C- Method 3) and one gradient method (D – Method 4) as shown in Table 3.11.

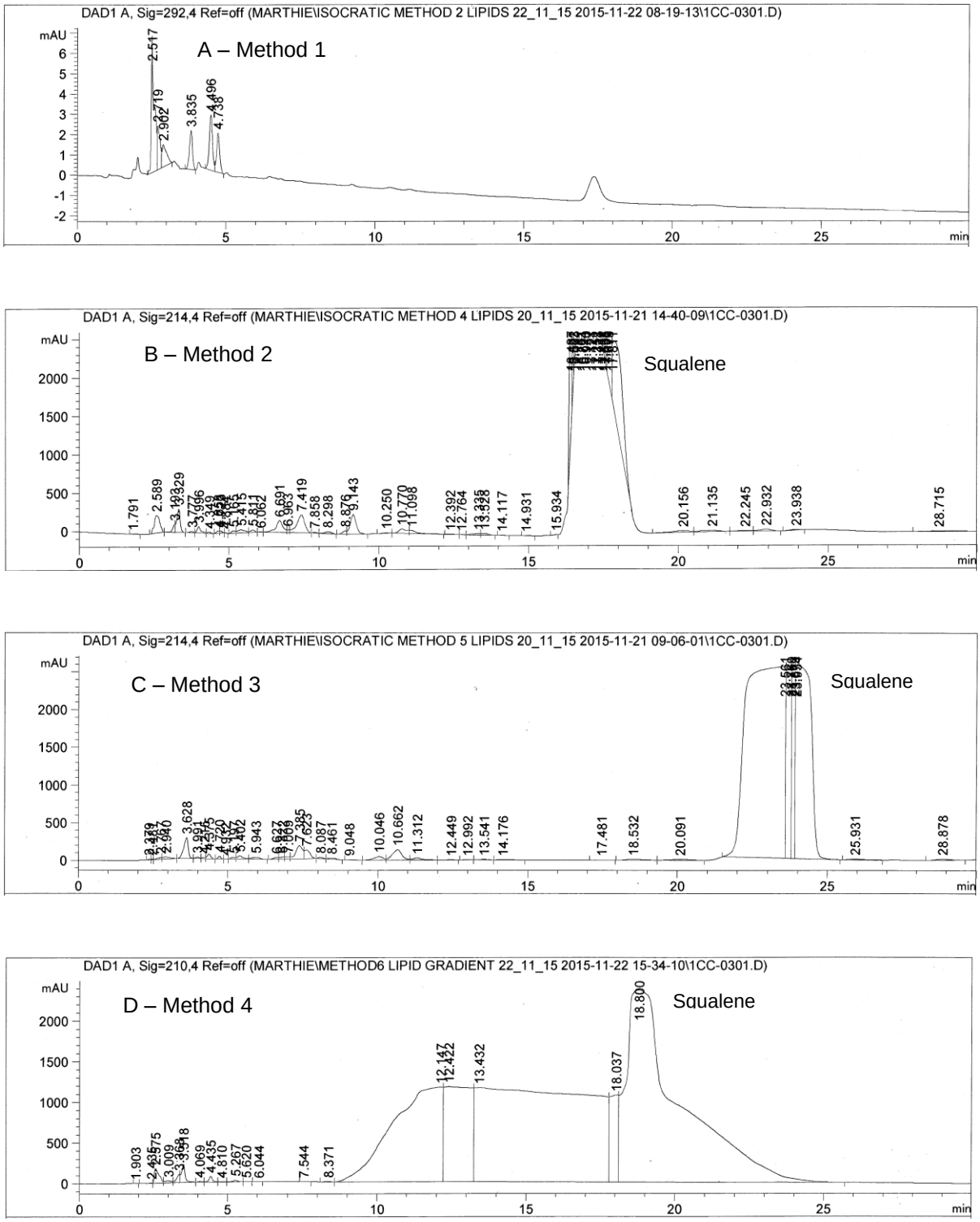


Figure 3.5 HPLC chromatograms of a 0.1% squalene RS solutions analysed using three isocratic methods ((A- Method 1, B- Method 2, C- Method 3) and one gradient method (D – Method 4) as shown in Table 3.11.

Table 3.12 shows the elution times and peak areas following execution of the above-mentioned methods for detection of lipophilic compounds α-tocopherol and squalene.

Table 3.12 HPLC retention times and peaks of reference standards and IPA-reconstituted extract samples following the different HPLC analytical methods.

α-Tocopherol RS								
Method	**M1**			**M2**	**M3**		**M4**	
Wavelenght (nm)	284	292	214	214	214	280	210	252
RS RT (min)	16.981	16.981	15.662 16.978	9.120 9.443	8.609 9.113	9.154	12.193	12.168
Area (mAU*s)	59919	7870	262851 187424	10277 13611	50787 12657	50787	51207	14100
Squalene RS								
Method	**M1**			**M2**	**M3**		**M4**	
Wavelenght (nm)	ND			214	214		210	
RS RT (min)	ND			16.427	23.561, 23.769, 23.879, 23.954		18.800	
Area (mAU*s)	ND			8466	221833, 29365, 78209, 92447		299541	

RS – Reference standard RT – Retention time ND – Not detected
Peak area: mAU/s

Method 1: Only α-tocopherol was detected in the RS solution at 284 and 292 nm at about 16.981 minutes and at 214 nm, two peaks for α-tocopherol were observed at 15.662 and 16.978 min. Squalene was not detected.

Method 2: At a wavelength of 214 nm, α-tocopherol in the RS solution separated into 2 peaks i.e. at 9.120 min and 9.443 min. This is not ideal as one single peak would provide improved quantification. Squalene RS eluted after 16.427 min. Both compounds showed very broad peaks. This could either be associated with the sample being too concentrated or the sample volume being too high.

Method 3: At a wavelength of 214 nm, α-tocopherol in the RS solution also separated into 2 peaks i.e. at 8.609 min and 9.113 min while a single peak was observed at 9.154 min at 280 nm. Squalene separated into 4 peaks i.e. at 23.561 min, 23.769 min, 23.879 min and 23.954 min with the largest peak area observed at 23. 561 min. Again, both compounds showed very broad peaks.

Method 4: Method 4 appears to be the best for the separation of α-tocopherol and squalene. This method uses gradient elution but is susceptible to baseline drift. The major cause of this baseline drift is due to the quality of the mobile phase solvents. Analytical grade solvents may be suitable for isocratic HPLC separation, however, they may not be suitable for gradient elution as these solvents may contain small amount of impurities which will result in baseline shifts. Therefore, it is best to use HPLC solvents

when performing HPLC gradient analysis. These HPLC gradient solvents are of a higher purity e.g. >99.5 % and will reduce the baseline drift.

3.3.4 Conclusion

In this study, the HPLC gradient Method 4 was selected in order to identify and qualify the lipophilic bioactive compounds α-tocopherol and squalene in the olive pomace extracts obtained from Soxhlet extraction. A six-step gradient as shown in Table 3.10 will be applied for a total run time of 30 minutes at a flow-rate 1.0 ml/min and ddetection recorded at 210 nm. The sample quantity injected will be 20 µl and only HPLC grade solvents were used. Calibration concentration ranges for both α-tocopherol and squalene reference standards. Stock solutions of a 0.1 mg/ml concentration were prepared in IPA for each compound and the concentration range for linear regression was 0.005 – 0.10 mg/ml.

3.3.5 Validation

Linearity and range. The linearity of the analytical method was evaluated by serial dilution of standard stock solutions over the broad concentration ranges using six-point calibration curves.

System suitability. Prior to quantification, the suitability of the HPLC system was checked in terms of the injector's reproducibility and inearity using commercially available standards.

Sensitivity. The LODs and LOQs were calculated from the *y*-intercept standard deviations (*Sb*) and slopes (*a*) of the calibration curves using signal-to-noise ratio criteria of 3.3 (LOD = 3.3 x *Sb/a*) and 10 (LOQ = 10 x *Sb/a*) in the concentration ranges expected for each bioactive compound in the extracts (mg/g pomace, dry weight): 0.011 / 0.333 (hydroxytyrosol), 0.012 / 0.036 (tyrosol), 0.010 / 0.031 (oleuropein), 0.0203 / 0.0615 (α-tocopherol) and 0.0213 / 0.0646 (squalene).

CHAPTER 4

EXTRACTION METHOD DEVELOPMENT

4.1 Introduction / rationale

The objective of an extraction method is to recover valuable compounds from a raw material by selectively dissolving them in liquid solvents so that these compounds can be separated from the other compounds in the inert material and recovered at a later stage from the liquid solvent. Plants contain a broad range of bioactive compounds such as lipids, nutraceuticals, phytochemicals, antimicrobial agents, flavours, fragrances and pigments, and extracts thereof are widely used in the food, pharmaceutical and cosmetics industries (21, 115).

Several factors must be considered during extraction of valuable compounds. These include the hydrophilic or lipophilic properties of the extractive compound, the polarity and stability of both the extractives and solvents, the toxicity, volatility, viscosity and grade of the extraction solvent, possible secondary by-product formation, and the quantity of bulk material required from the extraction (116).

Although extraction of natural products has been executed for thousands of years, recent trends have shifted focus on finding solutions that minimize duplications while also enabling process intensification delivering a high quality product within a cost-effective production industry. The European directive REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) concerning quality control of all chemical substances together with IPPC (Integrated Pollution Prevention Control) as a directive for instigating BAT (Best Available Technology) for product and processes promote “tomorrow’s products within a sustainable environmental-friendly development” using green technologies (23). As per definition for green extraction: “Green extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allow use of alternative solvents and renewable natural products, and ensure safe and high quality extract/products”, listing of the six principles of Green Extraction should endorse innovative and green label, charter and standard, within the process and all aspects of solid-liquid extraction, for research purposes and eventual worthwhile implementation by industry. These principles are:

Principle 1: Innovation by selection of plant varieties and use of renewable plant resources;
Principle 2: Use of alternative solvents and principally water or agro-solvents;
Principle 3: Reduction of energy consumption by energy recovery and using innovative technologies;
Principle 4: Production of co-products instead of waste to include the bio- and agro-refining industry;
Principle 5: Reduction of unit operations and favouring of safe, robust and controlled processes;
Principle 6: The production of a non-denatured and biodegradable extract without contaminants.

Solid-liquid extraction is applied when the soluble component which is incorporated in a solid matrix, is dissolved in a suitable solvent and recovered from the liquid phase. The efficiency of the extraction depends on the selectivity of the solvent whereas the extract contains the valuable compounds and the residue becomes the waste. The solvent used for extraction is known as the “menstruum’ and the residue left after extraction of the chosen compounds is known as the “marc” (117).

The choice of extraction procedure depends on the nature of the raw material and the components to be isolated. Some bioactive components such as free fatty acids and tocopherols as well as polyphenolic compounds are very sensitive to oxygen and heat. In this case, more care should be taken to prevent the oxidation and thermal degradation of those components. Therefore, the yield and quality of bioactive components should also be considered when an extraction method is selected. Conventional solid-liquid extraction methods (or leaching) are based on repeated extraction with fresh solvent and include Soxhlet extraction, maceration, percolation, extraction under reflux and steam distillation, turbo-extraction (high speed mixing) and sonication (118). Although these techniques are widely used, several shortcomings include the fact that they are often time-consuming, require relatively large quantities of toxic solvents, and the application of heat which can lead to the degradation of thermolabile metabolites and therefore low concentrations of the valuable compounds. Unconventional extraction methods more recently developed for extracting analytes from solid matrixes which are fast and efficient include: supercritical fluid extraction, microwave-assisted extraction and pressurised solvent extraction (119). These

extracted compounds are recovered from the solvents by way of crystallisation, evaporation, distillation or steam stripping (120).

Progression of leaching may involve simple physical solution or dissolution. The extraction procedures are affected by various factors, namely: the rate of transport of solvent into the mass; the rate of solubilisation of the soluble constituents by the solvent; and the rate of transport of solution out of the insoluble material. The extraction of crude plant bioactive compounds is mostly favoured by increasing the surface area of the material to be extracted and decreasing the radial distances traversed between the solid particles. Mass transfer theory states that the maximum surface area is obtained by particle size reductions which entail reduction of material into individual cells. However, this is not possible or desirable in many cases of vegetable material. It has been demonstrated that even 200 mesh particles contain hundreds of unbroken cells with intact cell walls. Therefore, it is pertinent to carry out extraction with unbroken cells to obtain an extract with a high degree of purity and to allow enough time for the diffusion of solvent through the cell wall for dissolution of the desired solute (groups of constituents) and for diffusion of the solution (extract) to the surface of the cell wall (121). Each of the extraction methods mentioned above will be discussed in more detail in the following section.

4.2 Methods of extraction

4.2.1 Conventional methods of extraction

4.2.1.1 Maceration

During ordinary maceration, the pulverised plant material is left to soak in a suitable solvent (also called menstruum) after mixing in a stoppered container at room temperature for several days with frequent agitation until the soluble matter has dissolved. The extract is collected from the plant-solvent mixture by straining. The process can be repeated with fresh solvent depending on the type of plant material. Finally, the last residue of extract is pressed out of the marc (damp solid material) using a mechanical press or a centrifuge and the combined liquids are clarified by filtration or decantation after standing. Because this process is static, it works by molecular diffusion. This method is suitable for both initial and bulk extraction, and for thermolabile compounds but the main disadvantage of maceration is that the process can be quite time-consuming, taking from a few hours up to several weeks and large volumes of solvent are used (122).

4.2.1.2 Turbo (vortical)-extraction

Simple maceration is a slow extraction process whereas kinetic maceration involves shaking and stirring. Turbo (vortical) extraction procedure uses a high speed stirrer or homogeniser which stirs the plant material for extraction in the menstruum. It induces hydrodynamic cavitation, thereby enhancing the extraction yield, the contact between the vegetal material and solvent is improved and therefore the diffusion process through the cell walls is increased (123). During shredding and shearing, the particle sizes are reduced leaving highly disintegrated cells allowing the high-value compounds to be washed out from this destroyed cellular tissue. Cavitation bubbles are produced and collapse in a similar way to the effect of ultrasonic devices. In addition, the energy supplied during the homogenation raises the temperature and increases the risk of decomposition of thermo-labile compounds which can be seen as a disadvantage when employing this method (124).

4.2.1.3 Percolation

This process involves packing the organic matter to be extracted into a tall cylinder or cone (known as a percolator) which allows constantly controlled removal of the extract via a valve at the bottom and adding fresh solvent from the top. The solid plant material is moistened with an appropriate amount of selected menstruum and allowed to stand for a specified time period in a closed container prior to packing and stoppering the top of the percolator. Hot solvent which may be either alcohol or ether, is then filled into the top of the percolator and allowed to slowly percolate or seep through the organic matter and to drain out of an opening at the bottom where it is collected in a beaker or flask. The process is repeated several times adding fresh quantities of solvent before the combined extracts are evaporated to retrieve the extracted matter. Percolation is adequate for both initial and large scale extraction. However, disadvantages include clogging of the percolator by resins or some plant materials containing mucilage which expand during addition of menstruum. Furthermore, complete extraction depends on the homogenous packing of the plant material which is essential for uniform distribution of the menstruum to reach all areas (122).

4.2.1.4 Soxhlet extraction

Soxhlet extraction is the automation of the percolation process for continuous extraction of nutraceuticals from plant matrices and is a well-established technique. German agricultural chemist Franz Ritter von Soxhlet was the inventor of the optimised

Soxhlet apparatus which included a constant level siphon returning the extract to the solvent flask after completion of a given extraction cycle (125). In a conventional Soxhlet system as shown in Figure 4.1, plant material, preferably a pulverised sample for extraction, is placed in a cellulose thimble in the extraction chamber which is placed on top of a collecting flask beneath a reflux condenser. Suitable solvent is added to the flask and heated under reflux. Vapour from the boiling solvent is condensed and passes through the sample. The extracted liquid with solutes remains in the chamber and siphons back into the solvent flask only after it has reached the critical volume determined by the height of the siphon, completing an extraction cycle. In the solvent flask, solute remains there while fresh solvent is evaporated, condensed and passes back into the solid plant matrix (126). At the end of the extraction, the solute is separated from the solvent by distillation of the solvent, leaving the solute as a residue in the flask.

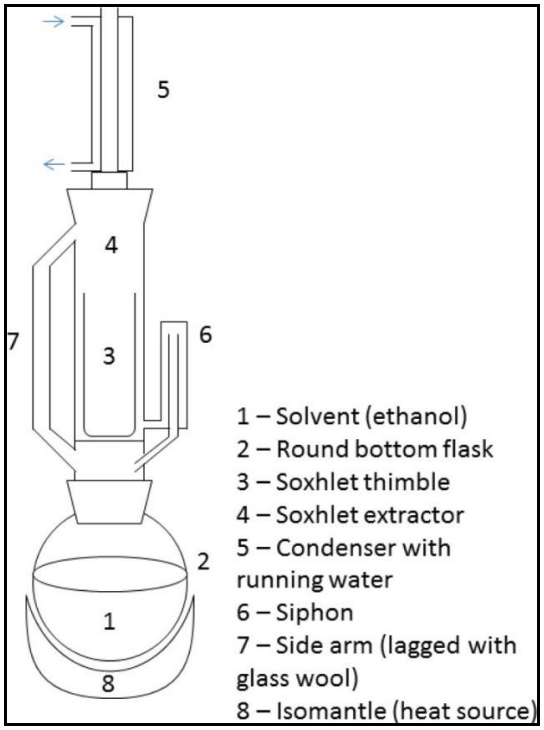


Figure 4.1 Conventional Soxhlet extraction apparatus.

The advantages of the Soxhlet extraction are the following:

- it is simple and inexpensive;
- it is less time and solvent consuming than maceration and percolation;
- sample in the thimble is completely surrounded with condensed warm solvent for the full extraction cycle which prolongs the period of extraction;

- just one batch of solvent is recycled;
- it is a continuous extraction process whereby fresh solvent is concentrated with plant material during each extraction cycle.

Large amounts of value-added compounds can be extracted with a much smaller quantity of solvent, hence Soxhlet extraction is used for both initial and bulk extractions. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale. Wide industrial applications, better reproducibility and efficiency, and less extract manipulation are the advantages over other unconventional or novel extraction methods such as ultrasound-assisted, microwave-assisted, supercritical fluid or accelerated solvent extractions. However, Soxhlet extraction method can be adapted by applying reduced pressure to reduce the boiling point of the solvent thus reducing risk of thermal degradation. The main disadvantages comprise: long extraction time and the possibility of decomposition of target thermo-labile compounds as extraction usually occurs at the boiling point of the solvent(s) for an extended time period.

4.2.1.5 Extraction under reflux and steam distillation

In extraction under reflux, plant material is immersed in a solvent in a round-bottomed flask which is connected to a condenser. The solvent is heated and when it reaches boiling point, it is recycled to the flask as a condensed vapour. Steam distillation is a comparable process where the flask containing the plant material covered in water, is also connected to a condenser. Upon heating, the vapours of both the volatile essential oils as well as the water condense and the distillate is collected in a graduated tube connected to the condenser. The distillate which consists of two immiscible layers is separated and the aqueous phase is recirculated back to the flask while the volatile oil is collected in a separate container. The main disadvantage of both of these methods is the degradation effect of heat on thermo-labile compounds (18).

4.2.1.6 Ultrasound (sonication-assisted) extraction

Ultrasound waves, as mechanical vibrations in a solid and liquid at frequencies higher than 20 kHz, are employed to extract high-value bioactive compounds from plant material by way of expansion and compression cycles during travel through the medium. The mechanical effect of ultrasound (kinetic energy) increases cell wall permeability, produces cavitation and facilitates the release of cellular contents into the

extraction solvent. Also, energy creates heat which indirectly increases the temperature of the plant material in contact with the surrounding extraction liquid and further enhances release of the compounds (127). Many factors govern the outcome of extraction and include: the moisture and particle size of the plant material; type of extraction solvent; and the frequency, pressure, temperature and sonication time to agitate the particles in the sample.

The main benefits of sonication in solid-liquid extraction include the cellular breakdown which increases the solubilisation of compounds in the solvent delivering higher extraction yields, quality extract and faster kinetics. This method is rarely applied in the case of large-scale extraction due to higher costs and is mostly used for the initial extraction of small amounts of material (126).

4.2.2 Unconventional or novel extraction methods

4.2.2.1 Supercritical fluid extraction (SFE)

There has been a rise in the demand for functional compounds obtained from plant material using “natural” clean processes for inclusion in food products and cosmo-pharmaceutical formulations. Supercritical fluid extraction is based on the supercritical state of a fluid which exhibits the characteristics of both gas and liquid when its temperature and pressure are raised above its critical value. During SFE raw plant material is loaded into an extraction vessel which is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The extraction is pressurised with the supercritical fluid by a pump. High-value compounds dissolved in the fluid are transported to separators where the recovery influence of the fluid is decreased by decreasing the pressure or increasing the temperature of the fluid (128).

Carbon dioxide (CO_2) is most commonly used as supercritical fluid because of its moderate critical temperature (31.3 ºC) and pressure (72.9 atm). There are several advantages to the use of CO_2 as extracting fluid because it is inexpensive, its diffusivity is one or two orders of magnitude higher than that of other liquids, it is generally recognized as safe (GRAS) and is abundant. However, CO_2 exhibits a low polarity and can be less effective to extract the most polar compounds in natural matrices. The addition of small amounts of modifiers (also called co-solvents such as methanol or ethanol), which are highly polar compounds, can produce substantial changes to the solvent properties of supercritical CO_2.

Other SFE process advantages are: (i) the extraction of constituents at low temperature; (ii) the elimination of a concentration step in the absence of solvent residue which is usually time-consuming; (iii) the higher diffusivity which permits rapid mass transfer resulting in larger extraction rate; (iv) and an eco-friendly extraction. However, the major deterrent in the commercial application of the extraction process are: the lipophilic nature of CO_2 as discussed above and the high-priced capital investment and operating conditions (126).

4.2.2.2 Microwave-assisted extraction (MAE)

Microwaves are electromagnetic radiations with a frequency from 300 MHz to 300 GHz and are transmitted as waves which can penetrate biomaterials and interact with polar molecules such as water in the biomaterials to create heat. Microwaves are made up of two oscillating perpendicular fields, namely, electric field and heat-creating magnetic field. Temperature increases penetration of the solvent into the matrix and constituents are released into the surrounding hot solvent. Microwave-assisted extraction is based on the principle of rapid delivery of energy to a total volume of solvent and solid matrix homogeneously and efficiently. Because moisture within the plant matrix absorbs microwave energy and internal superheating results, cell disruption is promoted as tremendous pressure on the cell wall is generated due to swelling of the plant cell. The expansion and rupturing of the cell walls is then followed by the liberation of chemicals into the surrounding solvent improving the recovery of nutraceuticals.

MAE has been considered to extract thermo-sensitive compounds as the chosen power can be set correctly to avoid excess temperatures and possible degradation. Other advantages include: reduced extraction time; improved extraction yield as agitation during extraction improves mass transfer; reduced solvent quantities; and industrial scale-up applications. However, the efficiency of microwaves can be poor when either the target compounds or the solvents i.e. hexane are non-polar, or are volatile (129).

4.2.2.3 Pressurised (accelerated) solvent extraction

Pressurized solvent extraction works according to the principle of static extraction with superheated liquids. Enhanced diffusivity of the solvent leads to an increase in extraction speed and efficiency. This solid-liquid extraction method uses organic solvents at elevated pressure and temperature in order to increase the efficiency of the extraction process while remaining in a liquid state. A solid or semi-solid sample is

placed into a stainless steel extraction cell which is filled with solvent and heated (50–200°C) in an oven. The heating process generates solvent expansion and thus pressure in the extraction cell, typically in the region of 500–3000 psi. A static valve pulses open and closed automatically when the cell pressure exceeds the set point in order to prevent over-pressurisation of the cell. A static extraction stage of about 5–10 min is followed by pumping fresh solvent through the system to rinse the sample and the tubing. All the solvent present in the system is then purged with a compressed gas, generally and the total solvent volume (extractant and rinsing) is collected in the vial (130). Accelerated solvent extraction is an automated extraction to achieve extraction from solid and semi-solid matrices in very short periods (12 – 20 minutes per sample) with a reduced amount of solvent. Disadvantages are: expensive equipment, a process which is not very selective and which will not be suitable for thermo-labile compounds as it requires high temperatures (131).

4.2.3 Factors influencing the choice and outcome of an extraction process

Various factors need to be considered when choosing the most suitable extraction process and sample preparation for a particular solid matrix and the quantity of the desired bioactive compounds in the plant material (132). These include:

1. Water content of sample:

Excessive moisture content in the pomace sample can adversely impact the physical properties of both the plant material and the extracts obtained. Weight, thermal expansion, microbial growth and content of the bioactive compounds are examples of the properties that can be altered by the presence of an unnecessary amount of moisture. Safety and stability of the active constituents in the sample material should be considered when drying processes are selected (133). For thermolabile compounds, freeze-drying is the method of choice since no heat is applied during the drying process.

2. Particle size and shape:

Grinding delivers a homogenous sample, often improving the kinetics of analytic extraction and also increasing the surface area of sample in contact with the solvent system.

3. Phytochemical nature of targeted biologic actives:

Thermolability of the constituents will determine which of the previously described extraction methods are suitable. In the case of thermolabile compounds, extraction

methods which does not use heat should be applied for example, maceration, turbo-extraction, ultrasound and SFE.

4. Extraction temperature, time and number of extractions:

In terms of extraction time, a compromise should be made between speedy extraction to minimise possible reactions such as oxidation and degradation of bioactive compounds and adequate time for complete wetting of the sample for dissolution of bioactives in the selected solvent.

Extraction temperature should be optimised for a particular sample type – sufficient heat should be applied to increase the solubility of bioactives in the solvent without resulting in degradation.

Extraction efficiency is increased by increasing the number of extraction cycles with fresh solvent in contact with the sample matrix in each cycle. This can be achieved by using Soxhlet extraction.

5. Amount and type of solvent:

The ratio of sample to solvent as well as the polarity of the solvents will influence the extraction yield of different classes of bioactive compounds. Therefore, a solvent of higher polarity, for example, water is effective in quantitative recovery of more polar simple phenolic alcohols, such as hydroxytyrosol and tyrosol. Oleuropein on the other hand is less polar and requires the addition of an alcohol such as methanol or ethanol. It has been confirmed that hydroalcoholic extractions improve recovery due to an increase of molecular affinity between solvent and solute as well as efficient cell wall disruptions to extract the intracellular ingredients from the plant material. Since the end product will contain traces of residual solvent, the solvent should be non-toxic (16, 17).

Commonly used non-toxic solvents for solid-liquid extraction of plant ingredients include water (at different temperatures) and supercritical carbon dioxide (CO_2) which have both environmental and economic benefits. Hexane, isopropanol, ethyl acetate, acetone, methanol and ethanol are organic solvents commonly used to extract bioactives of various polarities from plant materials since the molecular affinity between solvent and solute is critical to increase the yield and kinetics of extraction (134)**.** A co-solvent is sometimes added in order to increase the polarity of the liquid phase. The polarity of the organic solvent also determines the solubility of water-soluble target compounds in polar, moderately polar or non-polar organic solvents (135). Use of organic solvents, however, exhibit cost and environmental implications and are often

hazardous, therefore a carefully designed extraction process should address these issues. In the past and until recently, most extractions were performed using organic solvents but the potential safety of the residual solvent content for human consumption raised concern, especially if consumed in large doses. Residual solvents are classified as Class 1, Class 2 and Class 3 solvents as per United States Pharmacopoeia guideline depending on the type of solvent. Class 3 residual solvents, for example ethanol, may be regarded as less toxic and of lower risk to human health than Class 1 and Class 2 residual solvents and should be used where practical (136).

Considering the advantages and disadvantages of the various extraction methods discussed in the previous sections and the factors which influence the choice and outcome of an extraction process, the most suitable extraction method for the extraction of the hydrophilic and lipophilic bioactive from olive pomace had to be selected for further development. Soxhlet extraction was considered the most advantageous in terms of cost, ease of use, efficiency, adaptability, safety scalability.

4.3 Extraction trials of hydrophilic high-value bioactive components

For the purpose of selecting a starting point of the development of a suitable extraction method, the literature was reviewed for methods that had been applied to olive matrices in particular.

4.3.1 Review of literature methods

Polyphenolic compounds, the utmost important bioactive compounds from plant sources, are the most potent and therapeutically useful bioactive substances and their extraction is well described in literature. The extraction of polyphenols is reliant on two actions, namely the dissolution of each polyphenolic compound at cellular level in the plant material matrix, and their diffusion in the external solvent medium. According to the extraction process, the partitioning behaviour of polyphenols and their distribution between the oil and water fractions are affected by the solvent polarity, processing temperature and the quantity of solvent used for extraction. Partitioning of polyphenols into the extraction solvent is increased at higher temperatures and the more amphiphilic polyphenols are dissolved when water is added. Ethanol, regarded as a safe biological solvent, improves the solubility of the bioactive compounds when mixed with water in comparison to pure water. Nawaz et al. proposed an optimal solution which consisted of a mixture of 50 % ethanol and 50 % water (135).

Olive mill waste generated from *Olea europaea* L olives during triphasic milling, namely olive mill waste water (OMWW) or *alpechin* and the olive cake, also known as solid olive residue or *oruju,* as well as *alperujo* from biphasic olive processing waste are characterised by high contents of polyphenols (52) and are rich in the free (aglycone) forms of hydroxytyrosol, tyrosol and oleuropein (66). Extracting these bioactive compounds of interest need several issues to be taken into account. These include the polarity and stability of the extractives, and the toxicity, volatility, viscosity and purity of the extraction solvent, sample type and the amount of bulk material to be extracted (116).

Diverse extraction methods, including solvent extraction, for obtaining these high-value compounds quantitatively, qualitatively and with increased yields have been described in literature, and are summarised in Table 4.1.

Table 4.1 Methods and yield for extraction of hydrophilic bioactive polyphenols from olive wastes.

Sample type	Method	Solvent(s)	Conditions evaluated	Time & Temp (opt)	Results	Ref
Pomace (P) Solid fraction (SF) of OMWW	Solid-liquid with stirring	Ethanol	Sample type: 3g pomace or 7.5g solid fraction SF of OMWW; Extraction times: 60 / 70 / 80/ 90 / 100 / 110 / 120 min in dark; Agitation times: 10 / 30 / 50 min Solvent-to-solid ratio: 12.5 / 15 / 18.75 / 25 / 37.5 / 56.25 / 75 ml solvent; Temperature: 22 ± 2°C	1. Solvent: solid = 15 (solvent vol 112.5 ml) 2. 70 min extraction 3. 10 min agitation time	Total phenols: P: 2.19 ± 0.09 g/Lpolyphenols SF: 2.62 ± 0.01 g/Lpolyphenols	(137)
Olive pulp from 30 olives	Solid-liquid	Ethanol/water at pH 2.2 with HCOOH (80:20).	Olive pulp from 30 olives was frozen with liquid N_2, ground, extracted with 4 x 400ml solvent mixture.		TP content: *Frantoio* >8g/kg and between 1.68-2.86 g/kg for other cultivars Oleuropein: 57-65% Hydroxytyrosol:14-15%	(108)
Olive mill waste OMW – biphasic mill	Hydrothermal/ steam treatment	Water / steam	Hydrothermal/ steam treatment & aqua filtered through 0.45 µm filter paper and purification thereof; Lignocellulosic material & water / steam at 160 - 240°C ->auto-hydrolysis -> depolarization of polysaccharides (mainly of hemicelluloses) & breaking of lignin-carbohydrate bonds => solubilization of lignin fragments of low molecular weight. Thus, solid olive by-product was solubilized.	160 - 240°C 200-220 °C for 5 min and 1-1.5% of H2SO4	1-1.2% of hydroxytyrosol (grams per 100 g of dry matter) should provide 3-3.6 kg of soluble hydroxytyrosol per 1000 kg of alperujo (300 kg of dry matter). Yield of 65%; Hydroxytyrosol purity: 90-95% purity.	(109)
Fresh sample of SOR (solid olive residue) – triphasic Cultivar: *Corotina*	Stirring Soxhlet	1.Hydroalcoholic extraction – EtOH/acid H_2O (7:3 v/v) extraction solution/pH to 2 with HCOOH. 2.Ethanol 3. Soxhlet extraction with ethanol.	**For 1 & 2:** 25 g SOR extracted with 2 x 50 ml hydroalcoholic or ethanol solution; magnetic stirring in the dark for 2 hr at room temp; defatting with 3 x 25 ml *n*-hexane. **Soxhlet extraction:** Drug/solvent ratio of 0.2 g/ml for 15 hours; concentrated to dryness under reduced pressure; dissolved in ethanol/H_2O 7:3 v/v (pH of 2 adjusted with HCOOH). All samples pre-filtered through 0.45 µm filter prior HPLC analysis.	**Method 1 & 2:** 2hrs in the dark at room temp; **Soxhlet**: 15 hours	% Yield of the dried extracts: mg/g 1. Hydroalcoholic extract: 6.6-8.8 % 2. Ethanol extract:6.5 % 3. Soxhlet extract: 8.2 % TP: 142 mg/ml	(16)

Dried (convection dryer at 40-90 °C) and finely crushed sample from olive waste cake (OWC) Cultivar: *Frantoio*	Falcon tubes/orbital shaker	Abs Methanol	40 ml Absolute methanol and 2-3 g dried and finely crushed sample in 50 ml Falcon tubes; homogenise for 24hr in orbital shaker at amb temp + darkness; filtered through Whatman filter nr 1; filtered cake washed 2x with 20 ml methanol; filtrate evaporated to dryness under reduced pressure at 40^{o}C on a rotary; extract dissolved in 50 ml methanol in volumetric flask and kept refrigerated until analysis.	24hr Homogenise Ambient temp Darkness Rotary evaporation at 40^{o}C	<u>Fresh OWC sample</u> TP: 3003. 34 GA 100/g DM Dried at 90^{o}C& crushed sample: TP: ± 2800 GA 100/g DM DPPH: ± 64 µmol TE/g DM	(54)
5 g "Freeze-dried powder"	Stirring	1.Phytochem screening: aqua methanol (80% v/v pH 2 with HCl); 50 ml 2. Extraction: methanol: H_2O (60:40 v/v, 5ml) containing metabisulfite (2% w/w)	<u>1. Phytochem screening & ID:</u> 5 g Freeze-dried powder extracted with 50ml solvents for 1h at 25°C. (Dried extracts reconstituted with 5ml of extr solvent; UV-Vis: 0.2ml reconst to 10ml with water with/out HCl (2%), or 2M NaOH. <u>2.Optimized Extr of phenols:</u> 1. 1g Freeze-dried OMW (1g) extracted for 30 min with 50ml methanol: H2O (60:40 v/v, 5ml) containing metabisulfite (2% w/w) under stirring; filtered through Whatman no 1 filter paper; residue re-extracted as described previously for 15 min; combined extracts defatted with *n*-hexane (10 ml x 3) and filtered. 6. Filtrate filtered using 0.45µm plastic non-sterile filters at temp 20°C. 7. Biophenol extract stored at -20°C until analysed.	1. For 1h at 25°C. 2. Optimised extraction: 30 min + 15 min Continuous stirring	<u>1. Phytochem screening & ID:</u> 1a. Extractable matter: 26.2 ±1.3 % 1b. TP: 1.47 ± 0.01% GA w/w <u>2.Optimized extraction of phenols:</u> 50%	(17)
3g Olive fruit mesocarp & exocarp were homogenised	Homogenised	MeOH:H_2O (80:20)	Initial: 100ml MeOH:H_2O (80:20) Then 2 x more homogenations with 50ml extraction solvent; Evaporation of MeOH under N_2 flow at 37°C; sample: 1ml of olive extract + 50ml methanol as eluting solvent. Solid-phase extraction: HPLC analysis (C18 column, 4.6 x 250mm x 5 µm)		Major phenolic compounds: Oleuropein, Demethyloleuropein Tyrosol, Hydroxytyrosol, Flavanoids, Verbascosides, Lignans, Triterpenic acids: maslinic & oleanolic acids, Tocopherols Squalene	(35)

Referring to Table 4.1, the most common extraction method was solid-liquid extraction with stirring. Other extraction methods were homogenisation, hydrothermal steam and Soxhlet. Solvents used were pure ethanol, aqueous ethanolic and methanolic solutions which in some cases were acidified to pH 2. Extraction times varied between 15 minutes and 24 hours, temperature was ambient while the hydrothermal steam treatment temperatures range between to 160 – 240°C.

Obied et al. (17) followed a simple extraction procedure which included blending with an Ultra Turrax homogeniser (20 sec at 11 000 rpm twice), and stirring with a magnetic stirrer. They found that hydroalcoholic extractions delivered the highest extractable matter and measured total phenols when compared to methanolic or aqueous extractions alone due to a higher selectivity and an improved balance between recovery of the polar and less polar compounds. In addition, by increasing the molecular weight of the alcohol solvent, the lipophilicity and the recovery of the less polar biophenols, such as oleuropein, is increased as seen in the late-eluting HPLC peaks. Different combinations of methanol and water indicated that 60 – 80% methanol provided the highest total recoveries, however a 60% concentration achieved a balance between retrieval of both the more polar early-eluting and less polar later-eluting biophenol actives.

Exploration of extraction time and temperature produced the optimum extraction conditions of 20 ± 2°C with consecutive extraction time of 30 min + 15 min during the simple extraction procedure. An increase or decrease of temperature should reduce enzymatic degradation or hydrolysis in the plant, however sub-zero temperatures negatively affected the solubility of the biophenols. An evaluation of higher temperatures indicated that a 60% boiling aqueous methanol solution delivered an improved response of the major phenols such as hydroxytyrosol, verbascoside and oleuropein derivatives compared to ambient conditions although the total phenol content decreased. In addition, the humps observed in the chromatograms which are due to polymeric substances and could influence the elution of compounds were minimized.

4.3.2 Objectives

The main objective of this experimental exercise was to determine the most appropriate extraction method amongst proposed conventional solvent extraction methods and to determine extraction variables such as sample type, solvent blend and

pH for the recovery of the hydrophilic compounds hydroxytyrosol, tyrosol and oleuropein from two-phase olive mill pomace. An additional objective was to characterise the extracts and compare the phenol content.

4.3.3 Experimental methods

4.3.3.1 Solvents and chemical reagents

All reagents with their grades and suppliers used in the experiments are listed in Table 4.2.

Table 4.2 Solvents, chemicals and reference standards used in the extraction development experiments

Name	Chemical formula	Grade	Supplier
Ethanol	CH_3CH_2OH	Absolute, 96%	Emplura
Methanol	CH_3OH	HPLC	Alfa Aesar
Acetic acid, glacial	CH_3COOH	GR	Merck
Glycerol	$C_3H_8O_3$	Analytical standard	Sigma-Aldrich
D.i. water	H_2O	Purified, Millipore Milli-Q	Innoventon
Sodium metabisulfite	$Na_2O_5S_2$	Analytical standard	Sigma-Aldrich
n-Hexane	C_6H_{14}	Chemically pure	Merck
Gallic acid	$C_7H_6O_5$	97.5-102.5%	Sigma-Aldrich
Tyrosol RS	$C_8H_{10}O_2$	Analytical grade ≥ 99.5%	Sigma-Aldrich
Hydroxytyrosol RS	$C_8H_{10}O_3$	HPLC, ≥ 98%	Sigma-Aldrich
Oleuropein RS	$C_{25}H_{32}O_{13}$	HPLC, ≥ 98%	Sigma-Aldrich
Folin-Ciocalteu reagent	C_6H_6O	Analytical	Sigma-Aldrich
Sodium carbonate	Na_2CO_3	Analysed reagent	Merck chemicals

4.3.3.2 Plant material preparation

Two-phase olive oil pomace from the *Frantoio* cultivar picked at a ratio of 50:50 green:ripe was used. Pomace was collected in April 2016 at the time of olive oil pressing from a farm near Stilbaai in the Western Cape, and frozen immediately. As the extraction method development progressed, freeze-drying was considered as a better method of preserving the samples from degradation and ensuring a long-term stability of a reproducible sample for improved repeatability of the extractions.

Therefore, pomace was thawed in the fridge before decanting 50 g portions into 50 ml polypropylene centrifuge tubes with screw-on lids. These were immediately frozen at -80°C prior to freeze-drying. The tubes were removed from the freezer, lids removed and the opening of each tube covered with parafilm and pierced before inserting them into a vacuum-resistant open-mouthed glass flask (5 tubes per flask) and loaded onto a VirTis SP Scientific Sentry 2.0 with condenser temperature to -85^{o}C (refer to Figure 4.2

a). A vacuum pump removed air and other non-condensable vapours from the chamber to facilitate vapour migration in a nearly pressure-free environment from the frozen product towards the cold condenser surface until freeze-dried plugs of samples were formed with no moisture or stickiness. During freeze-drying, approximately 60% moisture loss resulted in a quantity of 20 g dried sample per plastic tube. The lyophilised pomace plugs appeared brownish-olive green and the material was coarse and hygroscopic. Just before extraction, the lyophilised plugs were removed from the tubes and ground in a mortar and pestle to produce a partly fine powder in the presence of the broken stone pieces. The purpose of grinding was to improve the subsequent extraction by making the sample more homogenous and increasing the surface area to facilitate better penetration of solvent into the matrix.

4.3.3.3 General extraction method

The solvents and solvent blends investigated during the various extraction trials are listed in Table 4.3.

Table 4.3 List of solvents and solvent blends used during preliminary extraction trials

100% Ethanol
50% Aueous ethanol
100% water
Water acidified with acetic acid (pH 2.00)
Water with acetic acid and 2% (w/w) sodium metabisulfite (pH 2.53)
Water containing 2% (w/w) sodium metabisulfite only (pH 3.84)
60% Aqueous methanol with 2% sodium metabisulfite
50% Aqueous methanol
100% Methanol
50% Aqueous glycerol

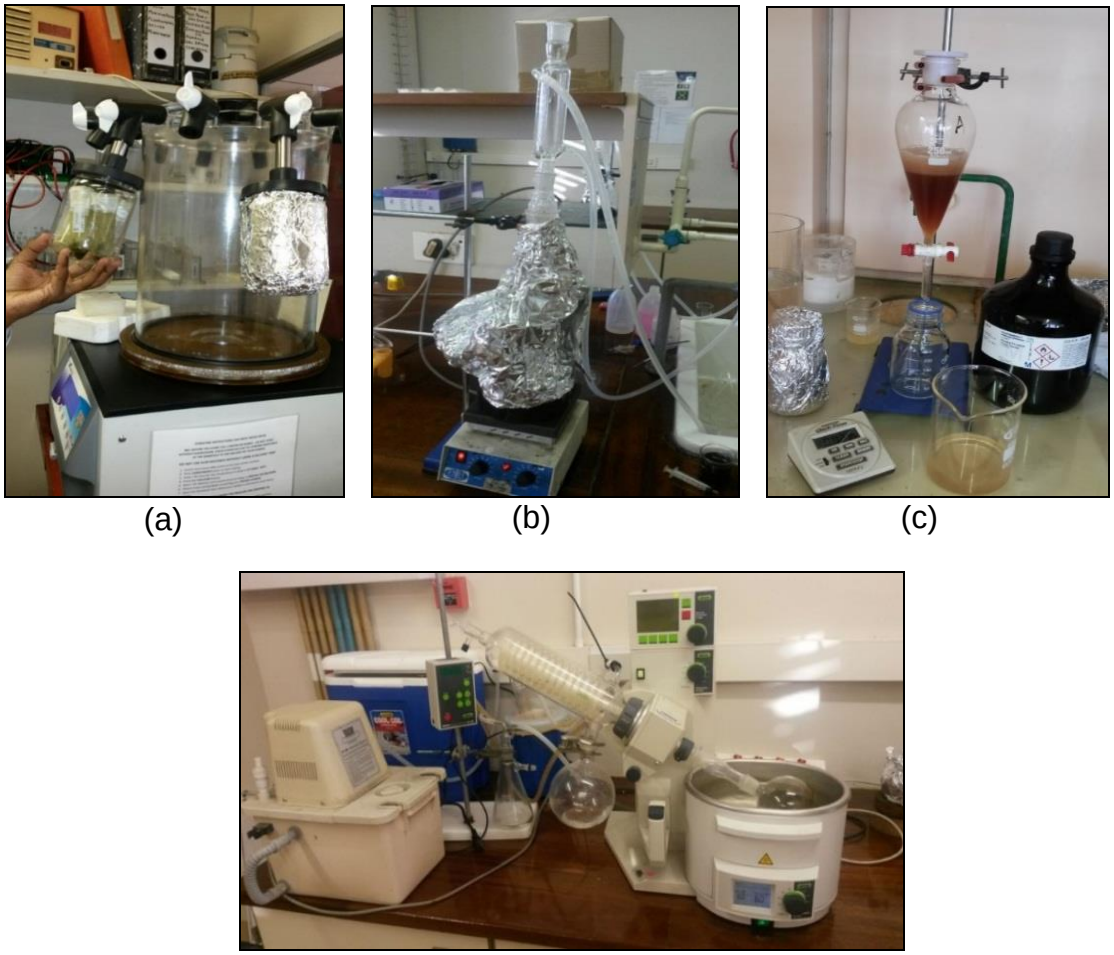
The Soxhlet method was selected as a continuous process for extraction of polyphenols from olive pomace. However, percolation and blend-stir methods were also carried out for comparison. For the Soxhlet method, a quantity of either 50 g freshly thawed pomace or 20 g freeze-dried pomace was weighed directly into a 80 x 30 mm cellulose extraction thimble before placing the thimble into the Soxhlet apparatus (Fig 4.2 b) and the round-bottomed flask containing 100 ml or 150 ml of extraction solvent and a magnetic stirrer bar was immersed into an oil bath on a hot plate/stirrer set at temperature setting of either 7 or 9 and stirrer speed setting of either

1 or 4. A condenser with circulating tap water was placed on top of the Soxhlet apparatus. The Soxhlet apparatus was covered with foil to protect the pomace from light and extraction was discontinued either when the overflow of solvent in the syphon stopped (when only 100ml solvent was used) or after a set time of either 1 or 2 hours.

For the percolation method, a weighed sample was loosely packed into a glass column stoppered with a cotton wool plug. Fresh solvent was poured into the top of the column and left to wet the pomace for 10 minutes. Solvent was drained through the tap at the bottom of the column and this process was repeated twice more with fresh solvent portions.

The blend/stir method was carried out as follows: weighed sample and fixed solvent quantity was placed in a glass beaker and was then homogenised twice for 20 seconds at 11 000 rpm. Magnetic stirrer was placed in the mixture and stirred initially for 30 minutes, stopped for a minute and stirred again for 15 minutes.

Each extract was filtered through a Whatman 541 (150 mm) filter. The 50% aqueous ethanol extract filtered more slowly than the water and ethanol extracts and required a second filtering through a Mockery Nagel 65 (90 mm) filter. Each filtered extract was then defatted with 3 x 10 ml or 3 x 50 ml hexane (Fig 4.2 c). In the first trial, the final defatted extracts were filtered again, kept in the refrigerator until HPLC analysis was performed on the extracts. In subsequent trials, the extracts were concentrated by rotary evaporation at 60°C (Fig 4.2 d) and reconstituted to 25 ml with a 50% aqueous methanol solution and kept in the refrigerator prior to analysis. solvent for a more accurate HPLC analysis.



(a) (b) (c)

(d)

Figure 4.2 (a) Pomace samples on the freeze-drier; (b) Soxhlet extraction system covered with foil for protection against light; (c) defatting with *n*-hexane; (d) rotary evaporator used for concentrating extracts.

4.3.3.4 HPLC analysis

The HPLC reference standard stock solutions were prepared by dissolving tyrosol and oleuropein standards in either pure methanol or 50% aqueous methanol (10 mg/100 ml concentration). Triplicate samples of the stock reference standards for both tyrosol and oleuropein were injected for quantification of the tyrosol and oleuropein content in the extracts. Tyrosol was detected at a retention time of approx. 10.8 min while oleuropein peaked after 22.3 min and the content was calculated based on the peak area ratio of sample to reference standard.

Chromatic separation of the hydrophilic bioactives was achieved by gradient elution on a Zorbax Extend-C18 (Agilent, USA) reverse-phase column (250 mm x 4.6 mm, id 5 µm) using the adapted gradient HPLC Method 3 with mobile phase A [H_2O /acetic acid

(100:1)] and mobile phase B [MeOH /acetonitrile /acetic acid (90:10:1)]. The column temperature was controlled at 25°C with a flow rate of 1.0 ml per minute during a maximum 50 minute run period. Sample volume was 10 µl and chromatographic elution was achieved at a wavelength of 280 nm.

4.3.3.5 Determination of total phenols

The method for total phenol determination by Costa et al. (138) was used with minor adjustments. Folin-Coicalteu (F-C) reagent was diluted to 1:10 in water; a gallic acid standard stock solution was prepared in methanol for a six-point regression curve (with concentrations from 25 – 250 ppm). A 7% sodium carbonate solution was prepared in deionised water. Volumes of 500 µl sample or reference standard solution, 2 ml sodium carbonate and 2.5 ml F-C reagent were transferred into glass pill vials, incubated at 45°C for 15 min and then at room temperature for 30 min. Total phenols (mgGAE/g) were determined by reading of the absorbance at 735 nm against a standard curve of gallic acid.

4.3.3.6 Determination of pH, conductivity, and % BRIX

The pH and conductivity were determined using a Crison Multimeter MM 41 with Crison conductivity cell and Crison combined glass pH electrode while the substance content or concentration of the samples were measured using an Atago PAL-3 Pocket Refractometer.

4.3.4 First extraction trial: Fresh pomace with ethanol, aqueous ethanol and water as solvents.

In the first extraction trial, ethanol, water and a 50:50 blend of the two solvents were selected as these two solvents are the most widely used for extraction of polyphenols. The results of the initial trial are summarised in Table 4.4.

Table 4.4 Initial extraction trial results using two-phase processing olive pomace from *Frantoio* cultivar.

A. Extraction Method:	**Soxhlet 1**	**Soxhlet 2**	**Soxhlet 3**
Solvent	Water	Ethanol/H_2O (50:50)	Ethanol
Solvent vol. (ml)	100	100	100
Pomace type	Fresh	Fresh	Fresh
Pomace mass (g)	50	50.01	50.01
Stir setting	1	1	1
Temp setting	7	7	7, reduced to 5
Reflux temp (^oC)	100	84	80
Extraction time (min)	180	210	150
Extract vol. (ml)	95	85	75
Extract colour	Dark brown	Red-brown	Amber-gold
Extract clarity	Clear, no filtering required	Murky and cloudy, filtered, less murky	Clear with particles, filtered
B. Defat extracts:	3 x 10 ml *n*-hexane, filtered		
C. Rota-evaporation:	Used extract as is and no rota-evaporation of sample		
D. Quantification: Tyrosol content: Oleuropein content:	 0.2 mg /100 ml (0.004 mg/g) Not detected	 2.54 mg /100 ml (0.051 mg/g) Not detected	 2.84 mg /100 ml (0.057 mg/g) Not detected

The murky appearance of the extracts could be due to the lipophilic components which do not dissolve in the ethanol / water blend when cooled. Browning of the aqueous extract (see Figure 4.3) during extraction is suggestive of oxidation and polymerisation. (Refer to Reaction scheme 4.1) Solvent loss was observed in the case where ethanol was employed. This could be partly due to evaporation and due to absorption of solvent into the cellulose thimbles. The quantities of tyrosol calculated were approximate values which were based on the peak areas of the reference standards. From these values, ethanol seemed to be the best solvent for extracting tyrosol but since the extraction times were not the same, and the extracts were evaporated and reconstituted, an accurate conclusion could not be made. These shortcomings were addressed in the next extraction trial and improvements were made.

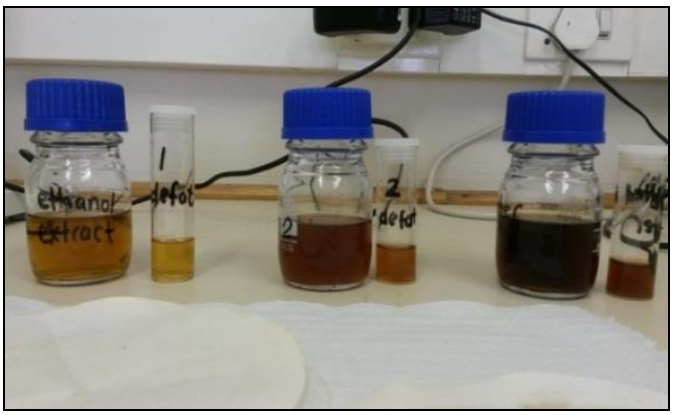


Figure 4.3 Appearance of first three extracts obtained before and after filtering: 1 – ethanol extract; 2 – 50% aqueous ethanol extract; 3 – water extract.

Some aspects for optimising a solid-liquid Soxhlet extraction process include the quantity of solvent as well as sample type. Usually before extraction, plant material is treated by milling, grinding and homogenisation, which may be preceded by air-drying or freeze-drying. Olive pomace represents a complex matrix in which oxidation, polymerisation, condensation and hydrolysis can take place concurrently. Generally, freeze-drying retains higher levels of phenolics content in plant samples than air-drying (139). Lyophilised or freeze-dried plant material has been shown to demonstrate good stability when stored in airtight screw-capped glass containers at –20°C for more than 12 months (17).

In subsequent experiments, it was thus decided to freeze-dry the freshly obtained olive pomace (*Frantoio*) samples before extraction. The amount of solvent was increased to 150 ml water to ensure continuous extraction cycles. The hot plate temperature was increased to setting 9 while the stirring speed was increased to setting 4 to increase the reflux rate. All extract t samples were vacuum filtered twice through 0.45 µm filters and defatted with 3 x 50 ml *n*-hexane.

4.3.5 Second extraction trial: Fresh versus freeze-dried pomace with water as solvent

Table 4.5 below summarises the extraction conditions and appearance of the extracts during the second Soxhlet extraction trial using both fresh and freeze-dried pomace with water as extraction solvent.

Table 4.5 Second extraction trials: fresh versus freeze-dried pomace with water as solvent.

A. Extraction Method:	**Soxhlet I**	**Soxhlet II**
Solvent	H_2O	H_2O
Solvent vol. (ml)	150	150
Pomace type	Fresh	Freeze-dried
Pomace mass (g)	50.26	20.77
Stir setting	4	4
Temp setting	9	9
Reflux temp (^oC)	100	100
Extraction time (min)	60	60
Extract vol. (ml)	±144	±120
Extract colour	Reddish-brown	Yellow-brown
Extract turbidity	Clear with particles, filtered	Clear with particles, filtered
B. Defat extracts:	3 x 50 ml *n*-hexane, filtered	
C. Rota-evaporation:	Samples concentrated on rota-evaporator at speed setting 5, bath temp 50 °C max.	
Mass RBF (g)	132.90	150.08
Mass residue + RBF (g)	136.43	152.35
Mass residue (g)	3.53	2.27
Extractable matter (mg/g pomace)	70	109
D. Quantification by HPLC	Reconstituted in 25 ml of 50% aqueous methanol	
Tyrosol content: **Oleuropein content:**	No chromatogram	2.14 mg /100 ml Not detected

The colour of the fresh pomace extraction sample was reddish brown while the freeze-dried sample appeared light yellow brown. The freeze-dried sample delivered an extractable yield of 109 mg/g pomace which is higher when compared to the 70 mg/g of the wet pomace sample. This might be due to the higher moisture content in the fresh sample which reduces the extractability of constituents as diffusion through the large amount of water must first take place. In addition, for the freeze-dried pomace, the concentration obtained for tyrosol (2.14 mg/100 ml) is 10 times higher than the 0.2 mg/100 ml obtained in the previous experiment using water as solvent and in the range of the aqueous ethanol concentration (2.54 mg/100 ml). Since the first two extraction trials gave a very low yield of tyrosol and were not able to yield oleuropein in the extracts, a few more solvent combinations that had been used by other researchers were investigated.

Stability of the polyphenols is compromised in the presence of oxygen. Oxygen is a co-substrate for polyphenol oxidation to quinone by the polyphenol oxidase (PPO) enzyme present in the plant material as shown by reaction scheme in Figure 4.4.
Polymerisation of quinones leads to melanins which produce the brown pigment observed in the previous extractions.

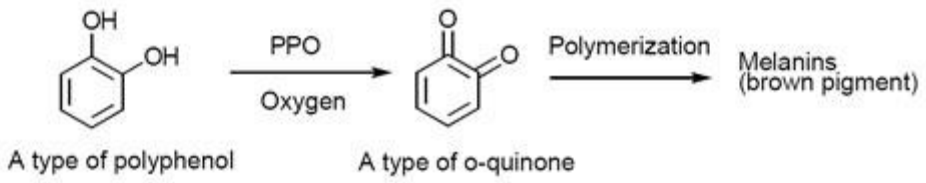


Figure 4.4 The oxidation reaction of a polyphenol molecule by the PPO enzyme

The addition of sodium metabisulphite ($Na_2O_5S_2$), a reducing agent, can aid in preserving the pomace from degradation caused by oxidation by inhibiting polyphenol oxidase activity (140). Obied et al. (24) showed that sodium metabisulphite has an optimum activity at a 2% (w/w) concentration as stabilising agent during olive mill waste extraction. They also showed that the presence of an acid yielded high amounts of hydroxytyrosol in a pH 2 extract, possibly as a result of acid hydrolysis of the hydroxytyrosol glucoside.

It was also found that pH can impact on the recovery of polyphenols according to different mechanisms and acidification increases solubility of the solutes because

polyphenolic compounds in plant material are often part of high molecular mass complexes, enhances cell wall disintegration, facilitating the solubilisation and diffusion of phenolic compounds; and increases stability of the phenolic compounds by inhibiting their oxidation. (17)

However, according to Obied *et al*., (24) the solvent of choice for extraction of the widest array of phenolic compounds and stabilisation of the resulting extract was best achieved using 60% (v/v) aqueous methanol with 2% (w/w) sodium metabisulfite.

Based on these findings it was decided to carry out a further extraction trial using various combinations of water, methanol, acetic acid and sodium metabisulfite in an attempt to increase extraction yield of both tyrosol and oleuropein.

4.3.6 Third extraction trial: Investigation of the effect of acetic acid, sodium metabisulfite and methanol on extraction yields and comparison of Soxhlet with two other conventional extraction methods

In the third trial, four water-based solvent mixtures were considered: A – water at pH 7.7; B - water at pH 2 (with acetic acid); C - water with acetic acid and 2% (w/w) sodium metabisulfite (pH 2.53); and D - water containing 2% (w/w) sodium metabisulfite only (pH 3.84) for bioactive compound extraction from 50 g fresh *Frantoio* pomace samples and 150 ml solvent blend using the Soxhlet method as described before.

The optimum solvent blend of 60% (v/v) aqueous methanol with 2% (w/w) sodium metabisulfite (SMB) claimed by Obied et al. (17) was also evaluated using both fresh and freeze-dried pomace. Fresh pomace was used in order to compare this extraction directly to extractions A, B, C and D which used fresh sample. In order to compare the Soxhlet extraction method with other well-known methods found in the literature, freeze-dried pomace samples were also extracted using a percolation method and a blend/stir method using the 60% (v/v) aqueous methanol with 2% (w/w) sodium metabisulfite solvent as well as pure water as a control.

Details of the extraction trials described above, observations and results are summarised in Appendix 4.1. Parameters of the extracts measured and evaluated included pH and conductivity of the extracts before and after extraction, colour, turbidity, and BRIX value (as determined by a handheld refractometer), of the extracts,

extractable matter, content of bioactives as determined by HPLC and total phenolic content of the extracts.

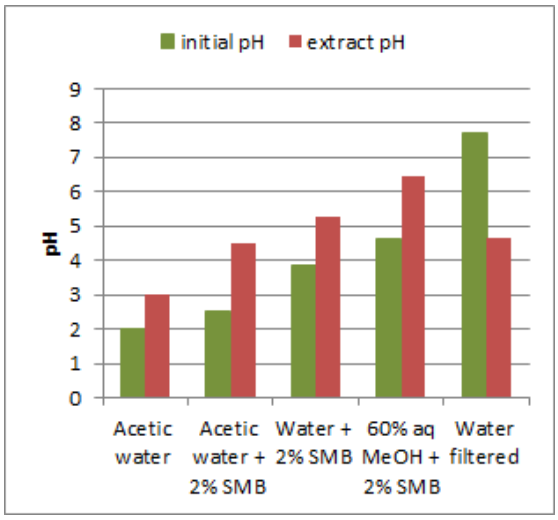
4.3.6.1 Comparison of solvents

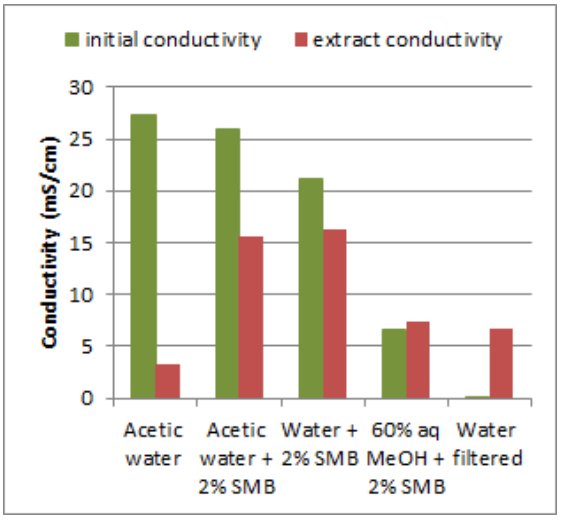
The results of all the measured parameters for the different solvents used in the Soxhlet extractions with fresh pomace samples are shown in Table 4.6 and plotted in Figure 4.5.

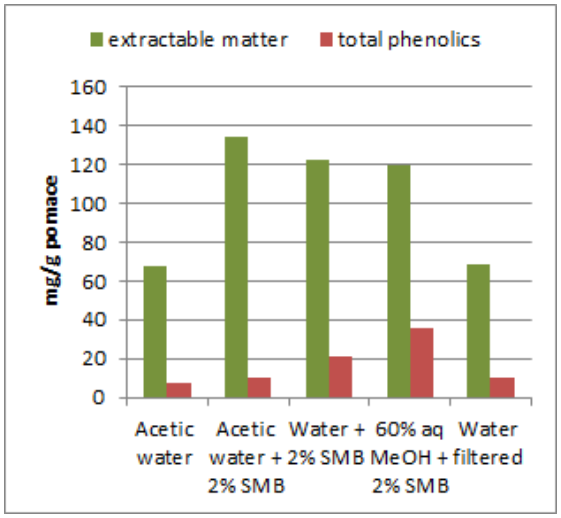
Table 4.6 Measured parameters for extractions performed with different solvents using the Soxhlet method and fresh *Frantoio* pomace

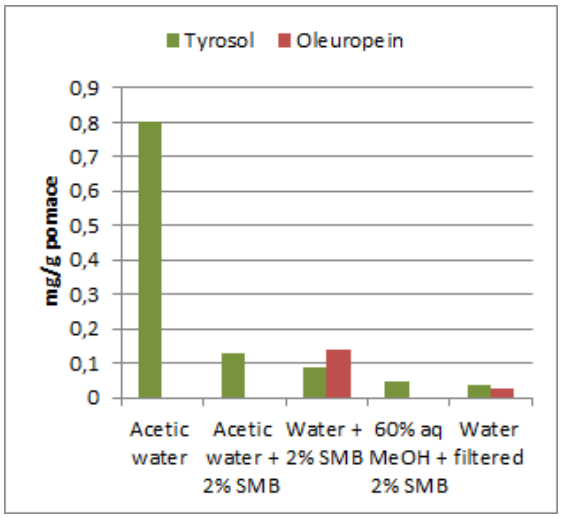
Solvent:	Initial pH	Extract pH	Initial cond *mS.cm*	Extract cond *mS.cm*	Colour	BRIX *%*	Extract matter *mg/g pomace*	Tyr *mg/g pomace*	Oleu *mg/g pomace*	TP *mg GAE/g pomace*
Acetic water	2	3	27.4	3.24	red-brown	3.1	67.8	0.8	ND	7.27
Acetic water + 2% SMB	2.53	4.51	25.9	15.67	amber	4.4	35	0.13	ND	10.45
Water + 2% SMB	3.84	5.28	21.2	16.27	gold	5.1	122.6	0.087	0.14	20.83
60% aq MeOH + 2% SMB	4.63	6.43	6.67	7.41	light yellow	9.8	119.6	0.049	ND	35.49
Water filtered	7.7	4.65	5.50 E-05	6.64	dark brown	2.3	68.4	0.034	0.024	10.45

MeOH – Methanol; SMB – Sodium metabisulfite ($Na_2S_2O_5$); SOX – Soxhlet F – Fresh; FD – Freeze-dried; Tyr – tyrosol; Oleu – oleuropein; TP – total phenolics









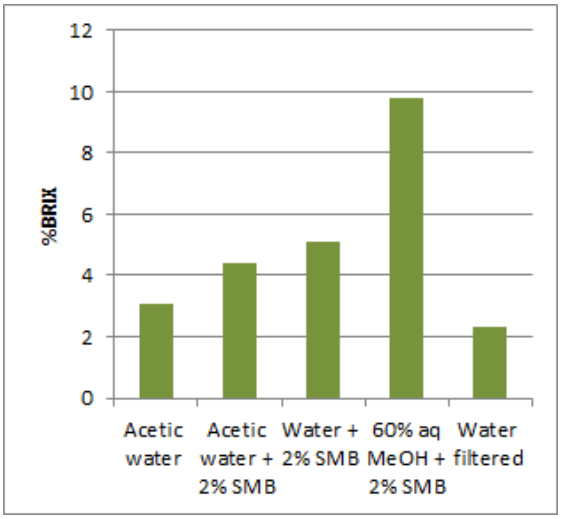


Figure 4.5 Graphical representation of measured parameters of Soxhlet extractions using various solvent blends and fresh pomace

pH

A comparison of the initial pH of the solvents versus the final pH of the extracts showed that except for pure water, pH increased after extraction. The extract pH values follow the same trend as the total phenolics for the different solvents. Thus the final pH value is a measure of the total phenolics present. The increased pH of the extracts can be explained by simultaneous acid-base equilibria which occur in the presence of weakly phenolic compounds in acidic medium, as shown by reaction scheme in Figure 4.6.

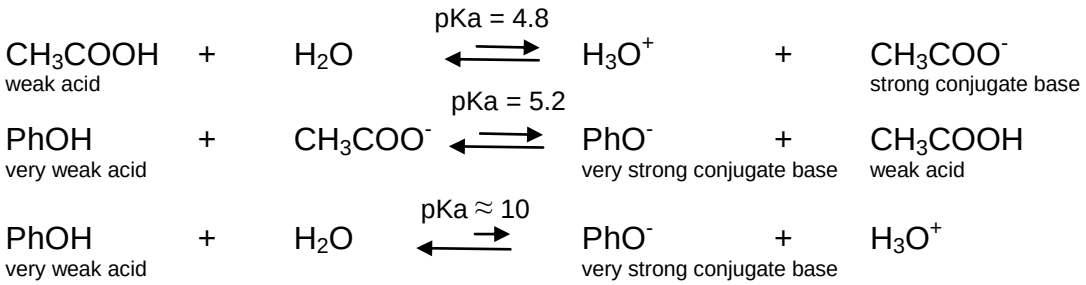


Figure 4.6 Acid/base equilibria in extracts

The phenolics are much weaker acids (pKa ≈ 10) than acetic acid (pKa = 4.8) thus the H_3O^+ ion concentration is reduced since the equilibrium of the PhOH ionisation reaction lies very far to the left.

The decrease in pH after extraction when water is used as extraction solvent can be explained by the fact that the phenolics, despite being very weak acids are still relatively stronger acids than water (pKw = 14).

Extractable matter

As shown in Figure 4.4, the extractable matter yield is highest when 2% SMB is present in the solvents. Acetic water and pure water yielded the same amount of extractable matter. This trend did not follow the pH and total phenols trend and this is probably due to the fact that the extractable matter consists of many varied compounds besides polyphenols such as polysaccharides, melanins, proteins, and other water-soluble polymers. It was noticed during the extractions that a white precipitate formed in the extracts containing 2% SMB and this could possibly account for the high yield of extractable matter. The SMB seemed to have limited solubility in aqueous methanol as a white precipitate formed during Soxhlet extraction where heat is applied.

Conductivity

Conductivity shows the presence of ionisable species. For acetic water, acetic water with 2% SMB and water with 2% SMB, the initial high conductivity decreased after extraction suggesting an increase in neutral species relative to ionic ones. Organic compounds, such as sugars and alcohols, do not readily form ions that conduct electricity. There was very little difference in conductivity for 60% methanol with 2% SMB after extraction and the conductivity was about half that of the water and acetic water with 2% SMB. This is due to the lower dielectric constant of aqueous methanol compared to water containing salts/ions. The conductivity of pure water after extraction was similar to that of 60% aqueous methanol. The conductivity of the extracts followed a similar trend as the pH and total phenolics, showing a direct correlation between conductivity and the amount of polyphenols

Extract colour and turbidity

The presence of SMB caused the extracts to be lighter in colour, (refer to Table 4.6) probably due to a bleaching effect as it is a strong reducing agent. Sulfites reduce quinones produced by catalysis of polyphenol oxidase to less reactive, and colourless compounds, (refer Figure 4.6) thereby preventing pigmentation (141). The quantity of sulfites necessary to prevent enzymatic browning varies according to the concentration and the nature of the substrate, the level of activity exhibited by the polyphenoloxidase (PPO), the desired period of control and the presence of other inhibitors.

The presence of methanol further lightened the colour of the extracts. In water alone, the extracts were dark brown. Most extracts were clear.

% BRIX values

The % BRIX values are an indication of the concentration and hence the density of the extract with regard to the soluble solids content and as can be seen in Figure 4.5, the % BRIX values follow the same trend as the total phenolic content namely, the % BRIX increased as the Soxhlet solvent was changed from water to acidified water and water with 2% sodium metabisulfite. This indicates that the concentration of the extracts intensified as the solvent selectivity and molar weight increased indicating that more soluble organic compounds, such as polyphenols were extracted. The % BRIX does

not follow the trend of extractable matter since much of this extractable matter is a residue which has a limited solubility in the various solvents. The highest %BRIX was measured for the aqueous methanolic solvent.

Content of bioactives

Acetic water yielded the highest tyrosol content while the water with 2% SMB yielded the highest oleuropein content. The lowest content of both bioactives was obtained with pure water. This seems to indicate that the presence of an acid and/or reducing agent both have the effect of inhibiting the PPO oxidation reaction to preserve the bioactives in their phenolic forms.

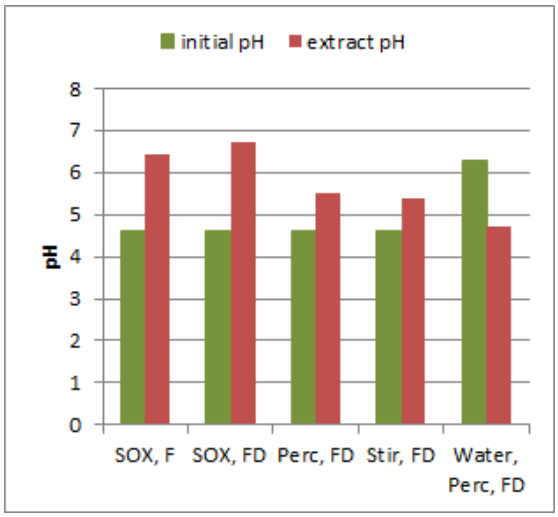
4.3.6.2 Comparison of extraction methods

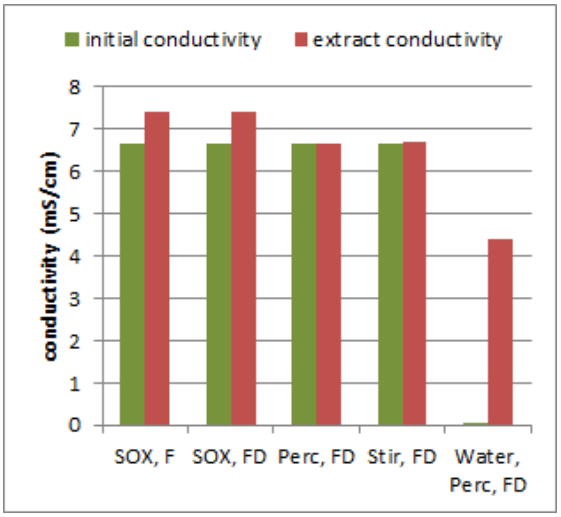
The results of all the measured parameters for the different extraction methods used with fresh and freeze-dried pomace samples are shown in Table 4.7 and plotted in Figure 4.7.

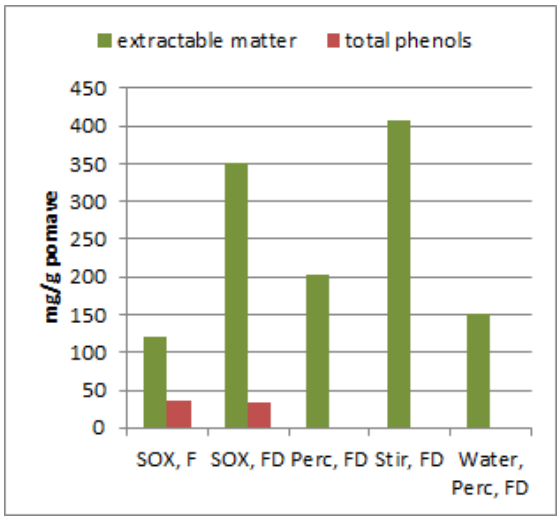
Table 4.7 Measured parameters for extractions performed with different extraction methods and both fresh and freeze-dried *Frantoio* pomace using 60% aqueous methanol with 2% sodium metabisulfite and water as control

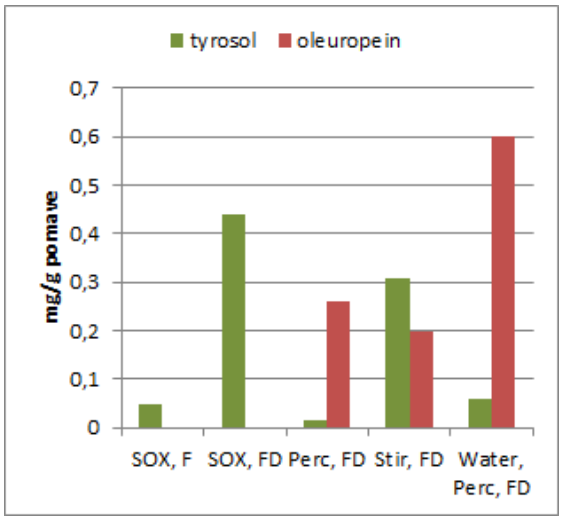
Method:	Initial pH	Extract pH	Initial cond	Extract cond	Colour	BRIX	Extract matter	Tyr	Oleu	TP
			mS.cm	*mS.cm*			*mg/g pomace*	*mg/g pomace*	*mg/g pomace*	*mg GAE/g pomace*
SOX, F	4.63	6.43	6.67	7.41	light yellow	9.8	119.6	0.049	ND	35.49
SOX, FD	4.63	6.71	6.67	7.41	yellow-brown	13.5	351	0.44	ND	34.06
Perc, FD	4.63	5.51	6.67	6.64	red-brown	13.1	204	0.016	0.26	ND
Stir, FD	4.63	5.38	6.67	6.72	amber	11.3	408.4	0.31	0.2	ND
Water, Perc, FD	6.27	4.73	5.50E-05	4.42	dark brown	4.7	152.2	0.06	0.6	ND

MeOH – Methanol; SMB – Sodium metabisulfite ($Na_2S_2O_5$); SOX – Soxhlet F – Fresh; FD – Freeze-dried; Tyr – tyrosol; Oleu – oleuropein; TP – total phenolics; ND – Not determined


initial pH
extract pH
pH
0
1
2
3
4
5
6
7
8
SOX, F
SOX, FD
Perc, FD
Stir, FD
Water, Perc, FD


initial conductivity
extract conductivity
conductivity (mS/cm)
0
1
2
3
4
5
6
7
8
SOX, F
SOX, FD
Perc, FD
Stir, FD
Water, Perc, FD


extractable matter
total phenols
mg/g pomawe
0
50
100
150
200
250
300
350
400
450
SOX, F
SOX, FD
Perc, FD
Stir, FD
Water, Perc, FD


tyrosol
oleuropein
mg/g pomawe
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
SOX, F
SOX, FD
Perc, FD
Stir, FD
Water, Perc, FD

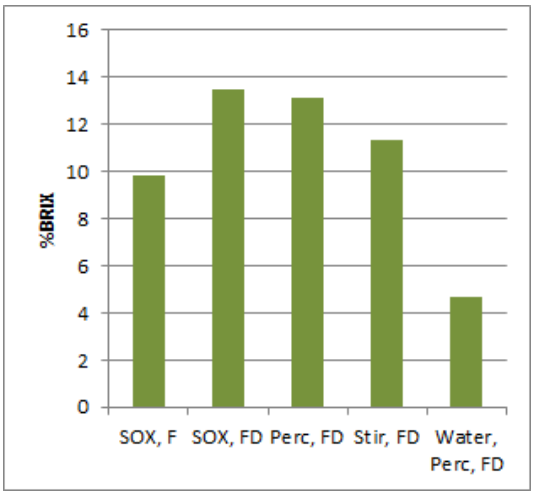


Figure 4.7 Graphical representation of measured parameters of different extraction methods using 60% aqueous methanol with 2% sodium metabisulfite with fresh and freeze-dried pomace (water as control)

Comparing methods:

The final pH of the extracts correlate with the % BRIX values as well as the conductivity indicating the extraction of very weakly acidic phenolic species.

Comparing different extraction methods based on these parameters it can be seen that the Soxhlet method is the most efficient followed by percolation and blend/stir. This is to be expected since Soxhlet is a method in which fresh solvent is continuously contacted with the plant matrix whereas in stirring all the plant material is exposed to all of the solvent in one step and the solvent becomes saturated. During percolation, the solvent moves through the plant material under the influence of gravity and thus the contact time is not as long as in stirring and the degree of saturation is thus not so high.

The addition of heat in the Soxhlet extraction method also improves the efficiency of the extraction since heat renders the plant cell walls more permeable, increases solubility and diffusion coefficients of the compounds to be extracted and decreases the viscosity of the solvent thus facilitating its passage through the solid substrate mass.

The blend-stir method, however, gave the highest amount of extractable matter, followed by the Soxhlet method and then the percolation method. This could possibly be due to the much longer contact time which also then allows unwanted polymeric

material to slowly dissolve in the water which was present in a much higher solvent: pomace ratio of 10:1.

Comparing fresh pomace vs freeze-dried pomace:

Very little difference in pH and conductivity and no difference in total phenolic content was found between freeze-dried and fresh pomace for the Soxhlet extraction. However, there was a significant difference in % BRIX. This difference correlated with the difference in extractable matter as well as tyrosol content. The freeze-dried pomace extraction yields a higher % BRIX, much higher total yield of extractable matter and nearly 4 times as much tyrosol than extraction with fresh pomace. Fresh pomace has a higher moisture content which could retard the diffusion of the solvent through the sample matrix during extraction thus reducing the extraction efficiency.

Comparing 60% aqueous methanol in 2% SMB with water:

The extraction of oleuropein was considerably higher for water as solvent than for 60% aqueous methanol with 2% SMB, while the opposite was true for tyrosol. Oleuropein and its derivative ligstroside (with one phenolic OH group instead of two) readily hydrolyse to form elenolic acid and hydroxytyrosol and tyrosol respectively. See reaction scheme in Figure 4.8. This hydrolysis reaction can possibly be increased by the lower pH of this solvent compared to water and the presence of sodium metabisulfite.

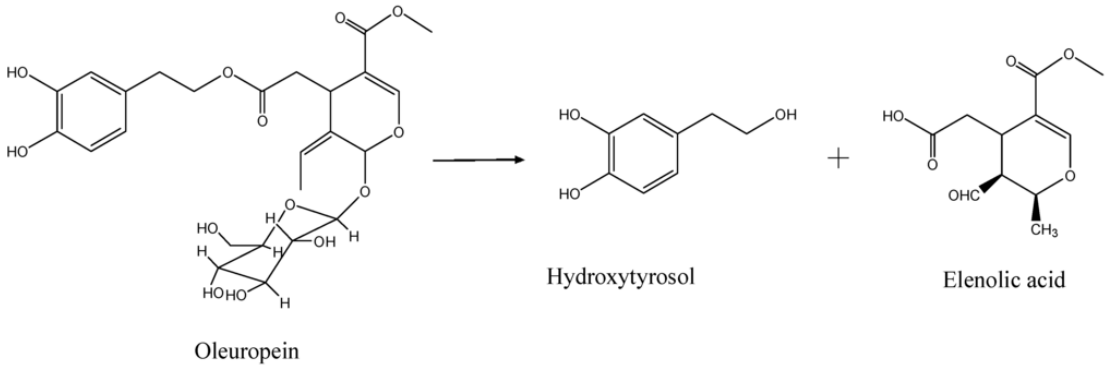


Figure 4.8 Hydrolysis of oleuropein

4.3.7 Fourth extraction trial: Effect of water, acidic water, glycerol, 60% methanol with 2% sodium metabisulfite, aqueous methanol, aqueous ethanol and 100% methanol

From the previous trial it was determined that only Soxhlet extraction and freeze-dried pomace would be used for all subsequent extractions. For confirmation of the results from the previous trial, it was decided to repeat the extractions with water, acetic water and 60% aqueous methanol with 2% sodium metabisulfite and add additional solvent combinations.

Glycerine (or glycerol $C_3H_8O_3$), a clear, colourless, viscous, sweet-tasting liquid was investigated as an alternative to ethanol or methanol as an eco-friendly “green” solvent for polyphenol extraction. Glycerol exhibits preservative properties (bacteriostatic in action) and has been shown to possess a high degree of extractive versatility for botanicals including the removal of numerous constituents and complex compounds that can be challenging using alcohol and aqueous alcohol solutions (142). It also fulfils the non-toxicity requirement. The chemical structure of glycerol shows that each carbon atom is bonded to a hydroxyl group which classifies glycerol as a polyol, an alcohol containing more than one hydroxyl group. Glycerol is soluble in water because of its three hydroxyl groups and exhibits a negligible volatility with a surface tension close to that of water. These hydroxyl groups are also responsible for the hygroscopic nature of glycerol, which means that it readily retains or takes up water (11, 143). The density of glycerol is 1.261 g/ml and its boiling point is 290°C, both of which are significantly higher than water which could pose a challenge to reflux in a Soxhlet extraction process (144). However, glycerol mixed with water could be suitable as an extraction solvent as both the boiling point and the viscosity can be reduced.

It was decided to evaluate the 50% aqueous glycerol concentration for extraction of the polar bioactive compounds since the boiling point for this mixture, determined in a separate experiment, was the same as water, and to compare this mixture with the 50% ethanol mixture in a previous extraction trial.

The solvent combinations used in this trial were 100% water, 50:50 glycerol:water, 50:50 ethanol:water, 50:50 methanol:water, 100% methanol, water at pH 2, and 60% methanol with 2% (w/w) sodium metabisulfite. All Soxhlet extraction parameters remained the same as in the previous trial; extracts were filtered prior and post defatting with the exception of the aqueous glycerol and acidic water extracts which were only filtered after defatting.

Appendix 4.2 summarises the extraction conditions and extraction results while the measured parameters are shown in Table 4.11 and depicted graphically in Figure 4.11.

Table 4.8 Measured parameters for extractions performed with Soxhlet extraction and freeze-dried *Frantoio* pomace

Solvent	Initial pH	Extract pH	Cond	Colour	%BRIX	Extractable matter	Tyr	Oleu	TP
			mS.cm			mg/g pomace	mg/g pomace	mg/g pomace	mg GAE/g pomace
Acetic water	2	2.97	2.26	light yellow	1.9	45.8	0.11	0.1	6.2
50% aq glycerol	3.96	4.95	0.0555	light yellow	53.8	ND	ND	ND	ND
60% aq MeOH + 2% SMB	4.02	6.24	10	amber	12.2	271.1	0.127	ND	20.17
Water (filtered)	5.52	4.7	2.13	red-brown	1.1	53.8	0.044	0.545	7.65
100% MeOH	ND	ND	ND	amber	not det	134.4	0.044	0.67	13.01
50% aq MeOH	6.67	5.34	2.44	light yellow-brown	6.6	111	0.045	0.52	10.93
50% aq EtOH	7.24	5.01	1.24E+00	red-brown	15.8	126.7	0.033	1.06	12.11

MeOH – Methanol; SMB – Sodium metabisulfite ($Na_2S_2O_5$); Cond – Conductivity; Tyr – tyrosol; Oleu – oleuropein; TP – total phenolics; ND – Not determined

In both the aqueous methanol and aqueous ethanol extracts, a yellowish ring formed around the round bottom flask as can be seen in Figure 4.9 while foam was formed during extraction. A slight white precipitate formed in both extracts and the extracts were thus filtered before being defatted. After the last defatting, the still murky extracts filtered very slowly through the Whatman no.1 filter paper. During defatting of the 100% methanol extract with the first 50 ml hexane portion, a blackish precipitate formed that blocked the separation funnel while the hexane portion changed colour to light green as seen in Figure 4.10. The black precipitate had to be removed for collection of the 1st defatted extract. A black ring formed in the separation funnel. The hexane portion remained light green during the remaining defatting process. This could be due to the presence of chlorophyll in the green unripe portion of the *Frantoio* olives.

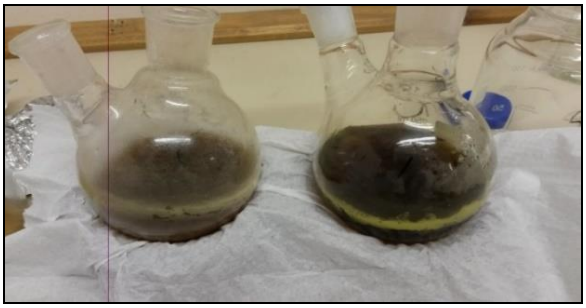


Figure 4.9 Aqueous methanol and ethanol extracts with yellowish rings in the round bottom flasks and a high concentration of precipitate or residue retained on the filters reducing filtration rate.

Figure 4.10 Blackish precipitate during defatting of methanol extract with *n-hexane* - a light green hexane layer at the top.

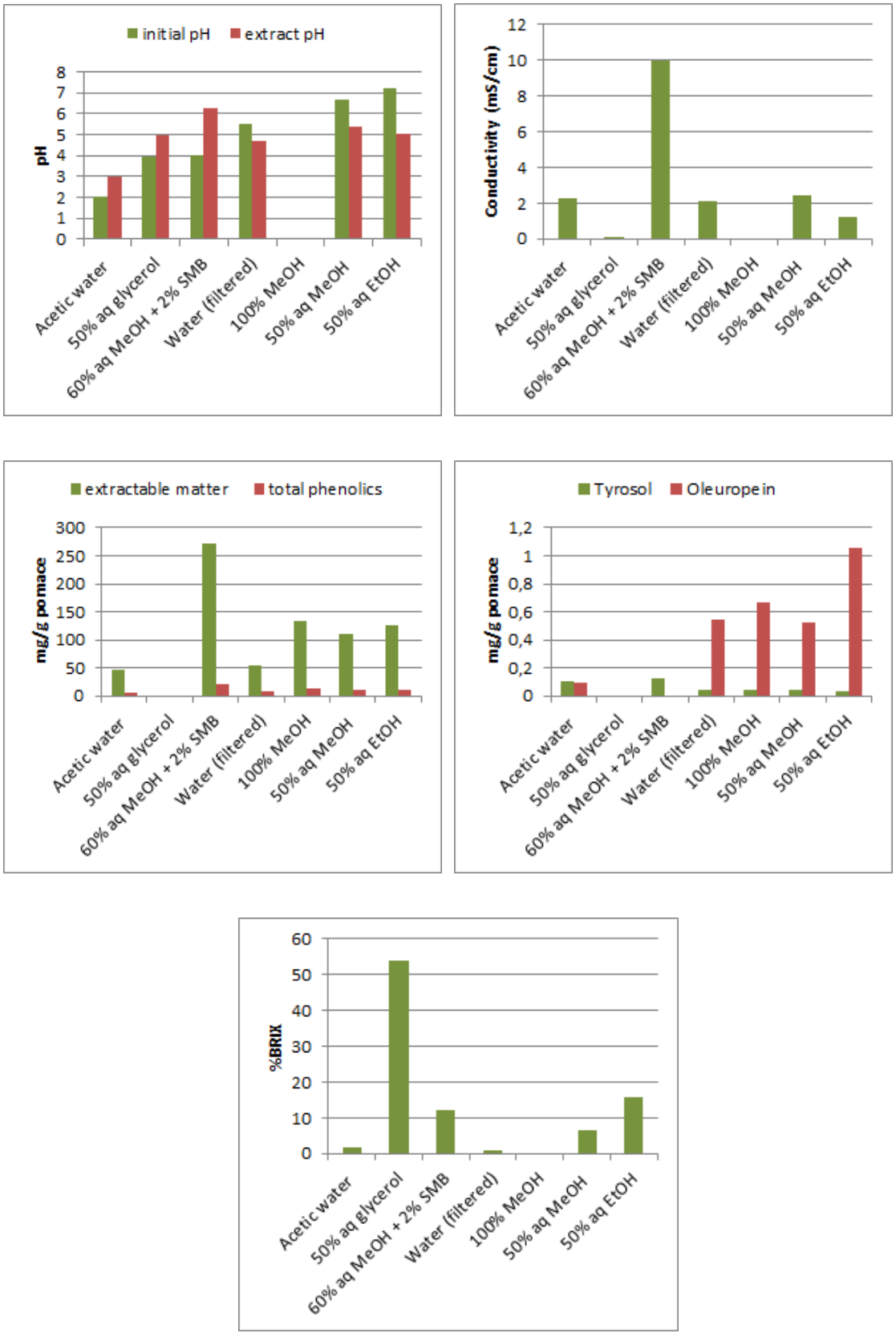


Figure 4.11 Graphical representation of measured parameters of different extraction solvents using freeze-dried pomace and Soxhlet extraction

pH of solvents and extracts

For the acidic solvents, the pH increased as expected, while for the more neutral water and aqueous methanol and ethanol, a decrease in pH was observed as expected.

Extract colour and turbidity

The colour of the extracts increased from light yellow (acidic water and aqueous glycerol) to red brown for the water and aqueous ethanol. The darker colours obtained as the solvents decrease in polarity, are probably due to extraction of some of the polymeric material in the pomace. See Figure 4.12.

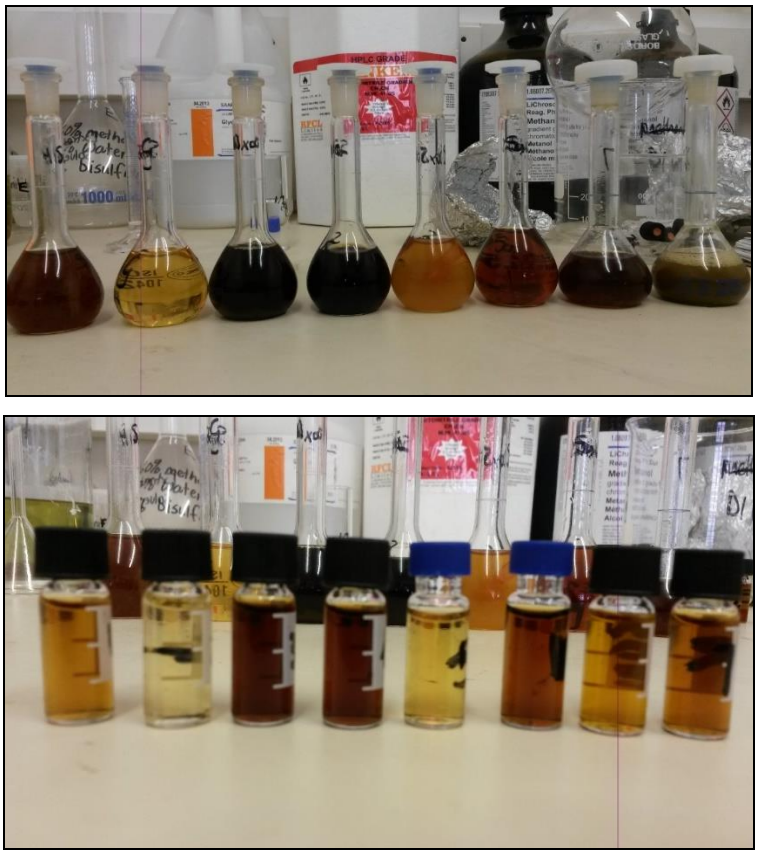


Figure 4.12 Reconstituted extracts with 50% methanol to 25 ml (top) and filtered samples in 2 ml vials for HPLC analysis (bottom).

Most extracts were turbid and filtered prior and post-defatting to provide a clear solution. The turbidity shows that there are components in the extract which have limited solubility in the solvent mixture. These components were extracted into the hot solvent during the Soxhlet extraction but precipitated upon cooling. Foaming was observed during both the aqueous methanol and ethanol Soxhlet extractions which left a light yellowish ring in the 2-necked flasks and a precipitate at the bottom. The same

was observed for the 60% methanol with 2% $Na_2S_2O_5$ extract. Foaming could be due to proteins extracted from pomace. Many of these are surface-active agents which create and stabilise foam.

Conductivity of extracts

The conductivity was highest for 60% aqueous methanol with 2% (w/w) sodium metabisulfite due to the presence of dissociated sodium and metabisulfite ions. The other extracts had low conductivity as a result of extracted weakly ionisable polyphenols.

% BRIX

The % BRIX values for all the solvents except the glycerol blend followed a similar trend to pH and conductivity as expected, and are in a similar range to what was found in the previous extraction trials. However, the glycerol blend has a very high % BRIX value which.in this case also reflects the high density and viscosity of glycerol.

Extractable matter

In aqueous alcoholic mixtures the total extractable matter was doubled compared to water and acidified water. This means that more organic material was extracted due to the lower polarity of the alcohols. However, a 60% aqueous methanol solvent mixture in combination with 2% (w/v) SMB delivered the highest residue mass and yielded twice the amount of extractable residue per gram compared with the aqueous alcoholic mixtures without SMB. This result could relate to the precipitation of SMB after solvent evaporation on the rota-vapor which contributes to the total mass. Aqueous glycerol extract was not able to be rota-evaporated as a result of its high viscosity and was thus not analysed.

Determination of Total phenols

Total phenols (mgGAE/g) were determined by reading of the absorbance at 735 nm against a standard curve of gallic acid. For the aqueous methanolic extract with 2% sodium metabisulfite, the highest total phenols measured was 20.17 mg GAE/g, followed by 100% methanol as 13.0 GAE/g, aqueous ethanol and aqueous methanol as 12.11 and 10.93 GAE/g respectively. Refer to Table 4.8.

Bioactive compounds content

Aqueous ethanol extract showed the highest oleuropein content and this could be explained by the lower polarity of the solvent mixture for extraction of this less polar bioactive compound. No oleuropein could be detected in the 60% aqueous methanol with 2% SMB extract. Tyrosol is a highly polar phenol alcohol and the content thereof in the water, pure methanol and aqueous methanol extracts were similar while 60% aqueous methanol with 2% SMB extracted the most, followed by acetic water, which had equal amounts of tyrosol and oleuropein.

4.3.8 Conclusion

During the initial extraction trial, fresh pomace as sample type was extracted with ethanol, aqueous ethanol (50:50) and water as solvents because these are widely used for extraction of plant biophenols, using the Soxhlet apparatus. Results from the extraction trials showed that a binary solvent system (ethanol/water) is more efficient than a mono-solvent system (water) in the extraction of phenolic compounds from olive pomace (tyrosol content: 2.54 mg/100 ml and 0.20 mg/100 ml respectively).

To investigate the effect of sample type, both fresh and freeze-dried pomace were extracted with water and the extracts evaluated. The extractable matter yield was highest for the freeze-dried as opposed to the fresh pomace sample (109 mg/g and 70 mg/g and) and the tyrosol content obtained reflected the same outcome namely 0.2 mg/100 ml and 2.14 mg/100 ml respectively.

In order to achieve the maximum recovery of the bioactive compounds investigated namely, tyrosol and oleuropein, acidification with 2% acetic acid, addition of sodium metabisulfite as preservative for extract stabilisation against polyphenol oxidase and 60% aqueous methanol together with different extraction methods were included in the third extraction trial. The recovery of the polar polyphenol alcohol tyrosol favoured the continuous Soxhlet extraction compared with two conventional extraction methods namely stir/blend and percolation which were also assessed. The highest tyrosol content namely, 0.44 mg/g dry weight was obtained using Soxhlet extraction and a freeze-dried sample in the presence of 60% methanol solution with 2% SMB. Comparable content for oleuropein was observed during Soxhlet extraction using a fresh sample and percolation using a freeze-dried sample with water (0.58 mg/g and

0.60 mg/g dry weight). Although polyphenol oxidase inhibitors such as sodium metabisulfite should prevent oxidation during extraction and preserve the extract, they may interfere with the assay determination of the individual bioactive compound being investigated and also deliver a higher extractable matter yield. The addition of an acid did not increase either the extractable matter or biophenol content.

During the final extraction trial, for confirmation of the results obtained during extraction development, it was decided to repeat some of the extractions with the Soxhlet apparatus using freeze-dried samples only and to add additional solvent combinations. These were water, acidic water, 50% aqueous glycerol, 60% methanol with 2% sodium metabisulfite, 50% aqueous methanol, 50% aqueous ethanol and 100% methanol. Aqueous glycerol delivered an amber-coloured treacle-like residue which could not be evaporated, and was thus considered an unsuitable extraction solvent.

The highest tyrosol quantity (0.127 mg/g) and total phenols (20.17 mg GAE/g) were obtained with 60% aqueous methanol and 2% SMB. Tyrosol content in the water, methanol and aqueous methanol extracts were in the same range (0.044 mg/g, 0.045 mg/g, and 0.044 mg/g dry weight) while oleuropein content was similar in water, 50% methanol and methanol (0.545 mg/g, 0.520 mg/g and 0.67 mg/g dry weight). However, 50% aqueous ethanol extracted the highest oleuropein quantity and could be explained by the reduced polarity of the solvent blend since this oleuropein is a less polar molecule. Total phenols results obtained for 50% aqueous methanol, aqueous ethanol and pure methanol namely: 10.93, 12.11 and 13.01 mg GAE/g, reflected an upwards trend with increase of solvent polarity.

4.4 Extraction of lipophilic bioactives from plant material

4.4.1 Review of literature methods

Some bioactive compounds such as free fatty acids and tocopherols are very sensitive to oxygen and heat. In this case, more care should be taken to prevent the oxidation and thermal degradation of those compounds during extraction. The most widely used solvent to extract lipophilic compounds from plant sources is non-polar *n*-hexane. Hexane, a petrochemical solvent, has a fairly narrow boiling point range of approximately 63 – 69°C and is preferred in terms of oil solubility and ease of recovery. However, *n*-hexane, the main component of commercial hexane, is classified as a Class 2 residual solvent and is “suspected of other significant but reversible toxicities” thus its use should be limited (136). In order to reduce emissions of volatile organic

compounds into the atmosphere, with hexane listed as the No. 1 on the list of 189 hazardous air pollutants by the US environmental Protection Agency, and in line with the new emphasis on environmental protection and the development of green chemistry, such solvent use is to be avoided as much as possible (145).

The use of alternative solvents such as isopropanol, *n*-heptane and ethanol have increased and these are classified as Class 3 solvents which have low toxic potential to man. Replacement of *n*-hexane with bio-solvents such as terpenes alleviates safety concerns towards both human health and promotes eco-conservation. Based on absorbance measurements, McConnell et al. (146) found that hexane and heptane extracted very similar material and that heptane extracts lipids faster. In a study by Buddrick et al (112), replacement of *n*-hexane with *n*-heptane provided comparable results during chromatographic isolation of the Vitamin E compounds**.** Few extraction methods for obtaining lipophilic squalene and α-tocopherol quantitatively have been descried in literature and Table 4.9 presents these extraction technologies for isolation of lipophilice squalene and α-tocopherol.

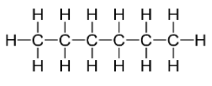
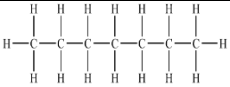
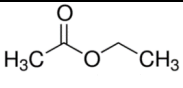
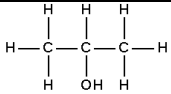
Table 4.9 Different extraction methods and solvents implemented for extraction of lipophilic squalene and α-tocopherol

Natural source	**Extraction procedure**	**Solvents**	**Recovery**	**Ref**
Olive biomass	Pressurized fluid extraction (Soxhlet)	Ethanol Isopropyl alcohol	0.2-0.5 mg/g squalene 0.01 mg/g α-tocopherol	(146)
Commercial table olives	Homogenised and centrifuged, then elution	*n*-Hexane *n*-Hexane/Ethyl acetate (9:1)	626 to 1,494 mg/kg squalene 25 to 90 mg/kg α-tocopherol	(112)
Soybean oil deodorizer distillate	Modified Soxhlet extraction	*n*-Hexane Ethyl acetate	6.29% squalene 2.39% tocopherols	(147)
Bark and leaves from *Trichilia catigua*	Soxhlet extraction Reflux Turbo extraction Maceration	Ethanol 50% Aqueous ethanol	*β*-sitosterol Bark: 252.33 – 396.31 µg/g Leaves: 581.65 – 777.14 µg/g	(101)
By-product from vegetable oil refining	Lab extraction vessel Column type extraction vessel	Supercritical CO_2 with modifiers: acetone, petroleum ether, ethanol	60-93 % squalene Tocopherols – not detected	(148)

The selection of method is governed by the nature of the compound and obtaining the maximum yield during extraction, as well as the nature and safety of the extraction

solvent selected. In Table 4.10, the different organic solvents and their chemical nature are summarised in support of selecting the most relevant solvent for extracting the non-polar compounds. Both *n*-hexane and *n*-heptane are non-polar solvents whereas ethyl acetate is classified as a borderline polar aprotic solvent. Polar aprotic solvents have moderately higher dielectric constants than the nonpolar solvents (between 5 and 20), have intermediate polarity for use in a wide range of reactions, and lack O-H or N-H bonds typical to polar solvents (149). On the other hand, isopropyl alcohol is a polar protic solvent (O-H bond) but falls within the range of intermediate polarity. It was decided to continue with Soxhlet extraction and evaluate single (*n*-heptane and isopropyl alcohol) and solvent combinations for obtaining the lipophilic bioactives squalene and α-tocopherol.

Table 4.10 Relevant chemical properties of solvents evaluated for use in extraction.

Solvent	***n*-Hexane**	***n*-Heptane**	**Ethyl acetate**	**Isopropyl alcohol**
Chemical structure				
Molecular formula	C_6H_{14}	C_7H_{16}	$C_4H_8O_2$	C_3H_8O
Molecular mass (g/mol)	86.18	100.2	88.1	60.1
Boiling point (°C)	69	98	77	88
Dielectric constant	1.9		6.0	18.0

4.4.2 Experimental methods

4.4.2.1 Solvents and chemical reagents

All materials used in the experiments are listed in Table 4.11.

Table 4.11 Solvents, chemicals and reference standards used in the extraction development experiments

Name	**Grade**	**Supplier**
n-Hexane	Chemically pure	Merck
n-Heptane	Chemically pure	Merck
Ethyl acetate	GR	Merck
Isopropyl alcohol	Technical grade	Spectrum
Squalene RS	Analytical grade ≥ 99.5%	Sigma-Aldrich
a-Tocopherol RS	HPLC, ≥ 98%	Sigma-Aldrich

4.4.2.2 Plant material preparation

Frantoio olive variety was evaluated. The olive pomace was produced from a two-phase centrifugation oil extraction process of 50% ripe olive maturation stage. Pomace

was collected in 50 ml plastic containers with a lid at the time of olive oil processing from a farm in the coastal regions of the Western Cape, in April 2016. It was immediately frozen prior to freeze-drying, as described previously.

4.4.2.3 Extraction method

Freeze-dried samples were ground and 10 g weighed into the extraction thimbles before placing these into the Soxhlet apparatus. A quantity of 100 ml solvent or solvent blend was placed in a 2-necked round flask (250 ml capacity) together with a magnetic stirrer bar (1:10 solid-solvent ratio) and connected to the Soxhlet extractor and a condenser. The solvent was heated in an oil bath on a hotplate to ensure reflux. The resulting extracts were filtered and concentrated by rotary evaporation at 60°C. The solvents and solvent blends investigated were: *n*-heptane; *n*-hexane/ethyl acetate (50:50); *n*-heptane/ethyl acetate (50:50); and isopropyl alcohol.

4.4.2.4 HPLC analysis

The identified suitable HPLC gradient method from Chapter 3 was selected in order to quantitatively identify the lipophilic bioactive compounds, squalene and α-tocopherol in the olive pomace extracts obtained from Soxhlet extraction. The total run time was 30 minutes at a flow-rate 1.0 ml/min and detection recorded at a wavelength of 210 nm. Mobile solvents used for elution were: mobile phase A [acetonitrile /methanol (7:3 v/v)] and mobile phase B [isopropyl alcohol]. Each extract was filtered through a non-sterile 0.45 µm syringe filter from Pall into a 2 ml HPLC vial. The standard stock solutions with a concentration of 0.1% were prepared by dissolving standards in isopropyl alcohol.

4.4.3 Results and discussion

Table 4.12 summarises the extraction trial results of each extraction of the lipophilic α-tocopherol and squalene.

Table 4.12 Extraction trial results of each extraction of the lipophilic α-tocopherol and squalene.

Extraction solvent(s)	***n*-Hexane + ethyl acetate 50:50**	**Isopropyl alcohol**	***n*-Heptane + ethyl acetate 50:50**	***n*-Heptane**
Extract colour	Light, olive green	Light green	Light green	Yellow
Extract turbidity	turbid	clear	clear	murky
Reflux temp (^{o}C)	64	84	96	95
Extract BRIX (%)	25.2	27.7	30.1	39.9
Extractable matter (mg/g)	169.8	197.8	123.7	165.7

The % BRIX increased as the solvent becomes more non-polar possibly indicating that more organic compounds were extracted by using only *n*-heptane as solvent. However, extractable matter obtained was highest for isopropyl alcohol (197.8 mg/g) whereas 50% *n*-hexane:ethyl acetate solvent blend and *n*-heptane delivered similar results (169.8 and 165.7 mg/g). The concentrated residues of three of the four extracts after rota-evaporation were yellow/green of colour and fairly clear or slightly turbid. These residues were dissolved in 10 ml isopropyl alcohol for HPLC-DAD analysis and stored in the refrigerator. However, a brown/yellow residue which included a brown precipitate formed in the isopropyl alcohol extract which did not dissolve entirely when reconstituted with the same solvent even after heating to improve dissolution. The isopropyl alcohol was not pure and also not HPLC grade.

Table 4.13 summarises the retention times and peak areas obtained by HPLC gradient analysis for 0.1% squalene and α-tocopherol reference standards as well as the percentage recovery of the lipophilic compounds from the four extractions.

Table 4.13 Identification of α-tocopherol and squalene in the different extracts during gradient HPLC elution at 210 nm.

Lipophilic compound	α-Tocopherol			Squalene		
	RT (min)	**Peak area** (mAu/s)	**Content** (mg/g)	**RT** (min)	**Peak area** (mAu/s)	**Content** (mg/g)
Reference standards	12.193	14101	0.1	18.800	299514	0.1
***n*-Hexane: ethyl acetate (50:50)**	11.978	7716	0.055	18.779	31664	0.011
***n*-Heptane: ethyl acetate (50:50)**	11.978	8017	0.057	18.752	12390	0.004
***n*-Heptane**	ND	ND	ND	18.753	96128	0.032
Isopropyl alcohol	12.082	10465	0.074	18.802	103005	0.034

RT – Retention time; ND – Not detected

The HPLC profile showed several peaks possibly corresponding to the two bioactives: squalene and α-tocopherol. α-Tocopherol has an OH group attached to the chromanol ring and also exhibits amphiphilic characteristics making it more polar than squalene. *n*-Heptane, the most non-polar of the solvents investigated was not able to extract α-tocopherol but it did extract squalene. Isopropyl alcohol, a solvent with intermediate polarity, extracted both non-polar compounds at retention times similar to that of the reference standard. The inclusion of ethyl acetate in the both *n*-heptane and *n*-hexane increased the polarity of the solvents and thus aided the extraction of α-tocopherol from the olive pomace. Similar quantities of squalene were extracted by both *n*-heptane and isopropyl alcohol (0.032 and 0.034 respectively).

4.4.4 Conclusion

The results showed that a pure non-polar solvent such as *n*-heptane is not suitable for extracting bioactives with different polarities. Organic solvents with some polarity, e.g. isopropanol would be more suitable. Filtration of the isopropyl alcohol extract was very slow which could be associated with the higher extractable matter content whereas the *n*-heptane extract filtered well. Since *n*-hexane is well known and widely used for its defatting properties, and that *n*-hexane and *n*-heptane showed interchangeability, it was decided to further investigate other “green” solvents in combination with *n*-heptane for extraction of both α-tocopherol and squalene bioactives.

CHAPTER 5

OPTIMISATION OF EXTRACTION CONDITIONS FOR THE COMBINED EXTRACTION OF HYDROPHILIC AND LIPOPHILIC BIOACTIVE COMPOUNDS

5.1 Introduction

As discussed in Chapter 2, the two-phase olive oil processing waste or pomace contains valuable bioactive compounds such as hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene that can be recovered for possible use in food, pharmaceutical and cosmetic industries. Natural products, either as pure compounds or as standardised extracts, provide unlimited opportunities for new applications because of their unmatched chemical diversity. Extraction is the most important first step for isolating these natural compounds. For it to be effective, the selected extraction procedure must consider the thermolability and polarity of each compound to be extracted, the sample form (for example, freeze-dried and ground to obtain a homogeneous sample), the choice of solvents, extraction time and extraction temperature. Literature was reviewed for methods of extracting both hydrophilic and lipophilic high-value bioactive compounds from plants in a single integrated process, but no documented procedures could be found. It became evident that various optimised methods only existed for extracting either hydrophilic or lipophilic compounds in separate processes with different solvent systems.

Soxhlet is a classic and well-established technique for the solvent extraction of active compounds from medicinal plant matrices and is based on the choice of solvent (alcohol-water mixture or other organic solvents) coupled with the use of heat and/or agitation. Soxhlet extraction relies on boiling solvents in order to ensure that fresh condensed solvent is continually in contact with the plant material to be extracted. This could be a problem for thermolabile compounds, as is the case with some of the bioactives found in the olive pomace. It was therefore decided to modify the conventional Soxhlet method to operate under reduced pressure in order to lower the boiling points of the solvents and reduce the heat supplied.

The objective of this part of the research was thus to:

a. determine an optimum blend of “green” or food-safe solvents to simultaneously extract the five desired bioactive compounds, hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene, and
b. to determine the optimum conditions for Soxhlet extraction such as time and solvent temperature at reduced pressure (19, 23, 150).

Selection of appropriate solvents was aided by the use of solubility parameters.

5.2 Determination of solubility parameters

Solubility parameter determination of the considered extraction solvents and desired bioactives is a tool which provides information on the extractability of each bioactive compound by a particular solvent either on its own or in combination with other solvents and can be used to guide solvent selection.

At the molecular level, solubility is controlled by the energy balance of intermolecular forces between solute-solute, solvent-solvent and solute-solvent molecules. The simple rule for solubility is “like dissolves like” and it is based on the polarity of the systems i.e. polar molecules dissolve in polar solvents (e.g. water, alcohols) and non-polar molecules in non-polar solvents (e.g. hydrocarbons). Liquids with similar intermolecular forces will be miscible, and compounds will dissolve in solvents whose intermolecular forces are not too different from their own. Thus a solute will dissolve best in a solvent that has a similar chemical structure to itself. The polarity of organic molecules is determined by the presence of polar bonds due to electronegative atoms (e.g. N, O) in polar functional groups such as amines ($-NH_2$) and alcohols (-OH).

For prediction of the energy when mixing solvents (polar and nonpolar), a method which involves the solubility parameter concept has been proposed by Hildebrand (151). His theory relates the energy of mixing (Equation 5.1) to the energies of vaporisation of the pure components (i.e. the energy necessary to convert a liquid into a gas) and defines the solubility parameter (δ) as the square root of the cohesive energy density (CED) of a substance (Equation 5.2). Thus, the solubility parameter is a numerical value that indicates the relative solvency behaviour of a specific solvent (152).

$$\frac{\Delta E_{mix}}{\Phi_1 \Phi_2} = V_m (\delta_1 - \delta_2)^2 \qquad \text{Equation 5.1}$$

And

$$\delta = (CED)^{½}$$

$= (\Delta E_{vap}/V_m)^{½}$ Equation 5.2

where;

ΔE_{mix} - energy of mixing

Φ_1, Φ_2 – volume fractions of solvents

V_m – average molar volume based on molar fraction

δ_1, δ_2 – solubility parameters of each solvent

CED - cohesive energy density

ΔE_{vap} - evaporation energy of solvent at a given temperature

The cohesive energy density of a liquid is a direct reflection of the degree of intermolecular forces holding the molecules of the liquid together.

The energy of vaporisation is a direct measure of the total (cohesive) energy holding the liquid's molecules together. Hildebrand's solubility parameter, however, does not take into account the various types of intermolecular bonds holding a liquid together, hence, the most established approach, Hansen's three-dimensional solubility parameter model, was considered. His theory lies within dividing total cohesive energy, E_t, into the three contributing energy components namely (atomic) dispersion forces, E_d, permanent dipole-dipole interactions, E_p, and hydrogen bonds, E_h. (Equation 5.3)

$$\Delta E_t = \Delta E_d + \Delta E_p + \Delta E_h \quad \text{Equation 5.3}$$

Dividing the energy of evaporation through molar volume (Equation 5.4), gives the square of the total solubility parameter or δ_t^2 expressed in terms of dispersion (δ_d) plus polarity (δ_p) plus hydrogen bonding (δ_h) (Equation 5.5) (153).

.

$$\frac{\Delta E_t}{V_m} = \frac{\Delta E_d}{V_m} + \frac{\Delta E_p}{V_m} + \frac{\Delta E_h}{V_m} \quad \text{Equation 5.4}$$

Or,

$$\delta_t^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \quad \text{Equation 5.5}$$

The application of the solubility parameters lies in correlating the solubility parameter of a solvent as closely as possible to that of the desired compound to be extracted. Solubility parameters for well-known compounds and common solvents can be found in literature, however, in cases where the solubility parameters of compounds are unavailable, values can be estimated by incremental methods. Contributions of the cohesion energy and the molar volume of the different group increments of each

compound or solvent can be used in the calculation of Hildebrand solubility parameters for qualitative predictions (Equation 5.6) (154).

$$\delta = (\sum \Delta E_{vi} / \sum V_i)^{½} \quad \text{Equation 5.6}$$

where,

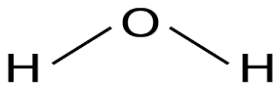
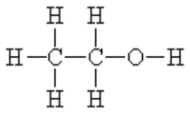
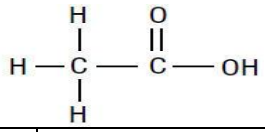
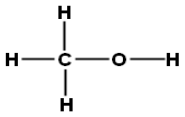
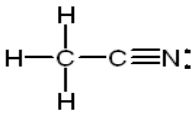
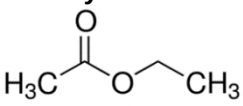
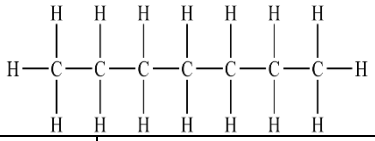
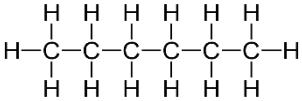
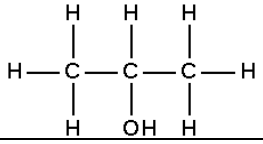
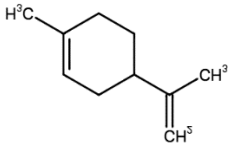
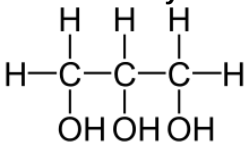
$\sum \Delta E_{vi}$ = Sum of the measurable cohesion energies of different groups in kJ/mol

$\sum V_i$ = Sum of the molar volumes of different groups in cm^3/mol

5.3 Determination of Hildebrand solubility parameters for desired bioactive compounds and preferred solvents

Due to the simplicity of the Hildebrand solubility parameters versus Hansen's parameters, the Hildebrand values were used in the determination. Based on the preliminary experiments in Chapter 4, the solvents investigated in this study were water, methanol, ethanol, glycerine, isopropyl alcohol, acetic acid, acetonitrile, ethyl acetate, *n*-hexane, *n*-heptane and *d*-limonene. *D*-limonene was included in the optimisation experiments as it was deemed a good alternative and environmentally acceptable solvent with the appropriate solubility parameter (155). Since solubility parameter values were not available for all of the preferred solvents and no values were available for the bioactive compounds, the solubility parameters for these were estimated by incremental methods using values obtained from a reference table (154). Equation 5.6 was used to calculate the Hildebrand solubility parameters from contributions of different group increments of the compounds. The solubility parameters for the solvents obtained from literature and those calculated from incremental values are shown in Table 5.1 while the calculated solubility parameters for the bioactive compounds are shown in Table 5.2.

Table 5.1 Solubility parameters of potential extraction solvents

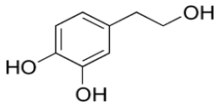
Water 		**Ethanol** 	
Solubility parameter	48 $MPa^{½}$ (152)	**Solubility parameter**	26.5 $MPa^{½}$ (152)
Acetic acid 		**Methanol** 	
Solubility parameter	21.4 $MPa^{½}$ (152)	**Solubility parameter**	29.6 $MPa^{½}$ (152)
Acetonitrile 		**Ethyl acetate** 	
Solubility parameter	24.4 $MPa^{½}$ (152)	**Solubility parameter**	18.1 $MPa^{½}$ (152)
***n*-Heptane** 		***n*-Hexane** 	
Solubility parameter	15.3 $MPa^{½}$ (152)	**Solubility parameter**	14.9 $MPa^{½}$ (152)
Isopropyl alcohol (IPA) 			
Solubility parameter	23.5 $MPa^{½}$ (152)		
Molecule: *d*-Limonene 		**Molecule: Glycerol** 	

Group	No of group	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i	Group	No of group	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i
-CH=	1	4,31	4,31	13,5	13,5	-OH	3	21,9	65,7	13	39
=CH2	1	4,31	4,31	28,5	28,5	-CH2-	2	4,94	9,88	16,1	32,2
-CH2-	3	4,94	14,82	16,1	48,3	>CH-	1	3,43	3,43	-1	-1
-CH3	2	4,71	9,42	33,5	67						
>CH-	1	3,43	3,43	-1	-1						
>C=	2	4,31	8,62	-5,5	-11						
conj in ring per =	1	1,67	1,67	-2,2	-2,2						
Total			47,63		159,1	**Total**			79,01		70,2
Solubility parameter:	17.33 $MPa^{½}$					**Solubility parameter:**	33,55 $MPa^{½}$				

ΔEvi: kJ/mol V_i: cm^3/mol

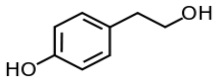
Table 5.2 Calculated solubility parameters of high-value bioactives (154)

Hydroxytyrosol



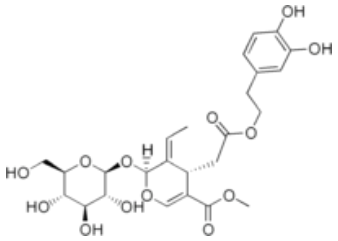
Group	No of groups	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i
-CH=	4	4,31	17,24	13,5	54
-CH2-	2	4,94	9,88	16,1	32,2
>C=	2	4,31	8,62	-5,5	-11
-OH	1	29,8	29,8	10	10
-OH adj C	2	21,9	43,8	13	26
conj in ring per =	3	1,67	5,01	-2,2	-6,6
6-membered ring	1	1,05	1,05	16	16
Total			115,4		120,6
Solubility parameter			30.93 $MPa^{½}$		

Tyrosol



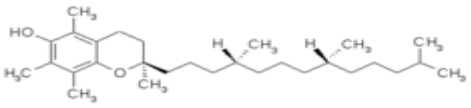
Group	No of groups	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i
-CH=	4	4,31	17,24	13,5	54
-CH2-	2	4,94	9,88	16,1	32,2
>C=	2	4,31	8,62	-5,5	-11
-OH, (disubst)	2	21,9	43,8	13	26
conj in ring per =	3	1,67	5,01	-2,2	-6,6
6-membered ring	1	1,05	1,05	16	16
Total			85,6		110,6
Solubility parameter			27.82 $MPa^{½}$		

Molecule: Oleuropein



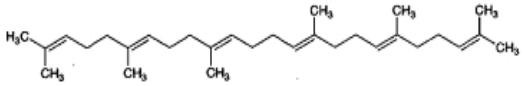
Group	No of groups	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i
-CH=	5	4,31	21,55	13,5	67,5
-CH2-	4	4,94	19,76	16,1	64,4
>C=	5	4,31	21,55	-5,5	-27,5
-OH	1	29,8	29,8	10	10
-OH adj C	5	21,9	109,5	13	65
-O-	3	3,35	10,05	3,8	11,4
conj in ring per =	4	1,67	6,68	-2,2	-8,8
6-membered ring	3	1,05	3,15	16	48
>CH-	7	3,43	24,01	-1	-7
-CH3	2	4,71	9,42	33,5	67
-COO- or (O-C=O)	2	18	36	10,8	21,6
Total			291,47		311,6
Solubility parameter			30.58 $MPa^{½}$		

α-Tocopherol



Group	No of groups	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i
>CH-	3	3,43	10,29	-1	-3
-CH2-	11	4,94	54,34	16,1	177,1
>C=	6	4,31	25,86	-5,5	-33
-OH	1	29,8	29,8	10	10
-CH3	8	4,71	37,68	33,5	268
-O-	1	3,35	3,35	3,8	3,8
conj in ring per =	3	1,67	5,01	-2,2	-6,6
6-membered ring	2	1,05	2,1	16	32
>C<	1	1,47	1,47	-19,2	-19,2
Total			169,9		429,1
Solubility parameter			19,90 $MPa^{½}$		

Squalene



Group	No of groups	ΔE_{vi}	$\Sigma\delta_{vi}$	V_i	ΣV_i
-CH=	6	4,31	25,86	13,5	81
-CH2-	10	4,94	49,4	16,1	161
>C=	6	4,31	25,86	-5,5	-33
-CH3	8	4,71	37,68	33,5	268
Total			138,8		477
Solubility parameter			17,06 $MPa^{½}$		

Table 5.3 shows the solvent solubility parameters of all the solvents evaluated during extraction method and analytical method development grouped into solubility ranges based on polarity. The solubility parameter value increases with polarity of the solvent / compound. The calculated solubility parameter ranges of the five bioactive compounds of interest are shown in Table 5.4.

Table 5.3 Solubility parameter (SP) ranges for solvents.

SP (14 - 15 MPa^{½})		**SP (17 - 18 MPa^{½})**		**SP (21 - 25 MPa^{½})**		**SP (26 - 34 MPa^{½})**	
n-Hexane	15.3	*d*-Limonene	17.3	Acetic acid	21.4	Ethanol	26.5
n-Heptane	14.9	Ethyl acetate	18.1	Isopropyl alcohol	23.5	Methanol	29.6
				Acetonitrile	24.4	Glycerol	33.35

Table 5.4 Solubility parameter (SP) ranges for bioactive compounds.

Lipophilic compound	**SP (17- 20 MPa^{½})**	**Hydrophilic compound**	**SP (27 – 31 MPa^{½})**
Squalene	17.06	Tyrosol	27.82
α-Tocopherol	19.9	Oleuropein	30.58
		Hydroxytyrosol	30.93
Average:	26,64		

Comparing Tables 5.3 and 5.4, it can be seen that the most closely matching solvents in terms of solubility parameters for the lipophilic compounds are *d*-limonene and ethyl acetate. However, preliminary extractions showed that ethyl acetate was not a suitable solvent because of its incompatibility with squalene. It was thus eliminated. *D*-limonene again has a high viscosity and boiling point and it was therefore decided to combine it with a lower boiling solvent from the hexane/heptane group. *n*-Hexane, although commonly used to extract lipophilic compounds from plant material, was excluded because it is considered a more toxic (Class 2 residual solvent) organic volatile chemical with a much smaller allowed residual limit when compared to *n*-heptane (Class 3 residual solvent). In pharmaceutical products, the prescribed daily exposure (PDE) for Class 3 solvents is 5000 ppm in contrast to the 290 ppm limit for *n*-hexane (136). Therefore, *n*-heptane was used in combination with *d*-limonene for extraction of lipophilic bioactive compounds.

Extraction solvents evaluated for the hydrophilic compounds included water, methanol, ethanol, glycerol and/or alcoholic/aqueous solutions with or without pH adjustment using acetic acid. Water has the highest solubility parameter of 48 MPa^{½} while glycerol, ethanol and methanol fall in the range of 26 – 33 MPa^{½}. The solubility parameters for hydroxytyrosol, tyrosol and oleuropein are in the range of 27 – 31 MPa^{½} which means

that ethanol, methanol and glycerol should be suitable as solvents for these compounds. Methanol, however, is a Class 2 solvent while ethanol is Class 3 and consequently methanol was excluded from this investigation. Water was included because previous extraction trials had shown that some water should be present to enhance solubility of the polyphenols.

In order to determine what the theoretical solubility parameter should be if all five bioactives are equally soluble in a mixture of the selected solvents, namely, *n*-heptane, *d*-limonene, ethanol, glycerol and water, the average solubility parameter of all the bioactives was calculated to be 26.64 $MPa^{½}$. This is very close to the solubility parameter of ethanol (26.3 $MPa^{½}$). Thus it was decided that ethanol should constitute the major proportion of the solvent blend and the other solvents would be added in smaller proportions to enhance ethanol's solubility characteristics as the contributions of the polar, dispersion and hydrogen-bonds must also be taken into account.

5.4 Determination of the composition of the solvent vapour phase

Since Soxhlet extraction involves solvent evaporation and subsequent condensation of the vapour during the reflux process, the condensed phase of a mixed solvent system falling on the solid matrix to be extracted will have a different composition to the original solvent blend as a result of the different boiling points and vapour pressures of the individual solvents in the blend. Thus, in order to define the real effect of the solvent blend under consideration on the dissolution of bioactive compounds, it is important to determine the composition of the condensed phase by considering the individual vapour pressures and mol fractions of the components of the solvent mixture.

A mentioned earlier, the energy of vaporisation is a direct measure of the total (cohesive) energy holding the liquid's molecules together. Raoult's law is a law of thermodynamics applied to ideal mixtures when determining the partial vapour pressure in mixtures. It states that the partial vapour pressure of a component in a mixture is equal to the vapour pressure of the pure component at that temperature multiplied by its mole fraction in the mixture (156). (Refer to Equation 5.7)

$$P_i = X_i P_i^o \qquad \text{Equation 5.7}$$

where,

P_i = partial vapour pressure of component, i, in a mixture

X_i = mole fraction of component, i, in a mixture

P_i^o = vapour pressure of pure component, i

Comparing two liquids, the liquid with a higher vapour pressure will have a lower boiling point and less heat is required for the molecules to escape from the surface. Therefore, a higher fraction of the more volatile component will be present in the vapour than the less volatile component. From an experimental perspective, by plotting the equilibrium boiling point as a function of the liquid and vapour phase compositions (usually mole fractions), a two-phase (binary) equilibrium system at constant pressure can be characterised. See Figure 5.1. When mixing two liquids in an ideal mixture, existing intermolecular attractions are broken (which requires energy) and new bonds made (which again releases energy) resulting in a zero enthalpy. For non-ideal mixtures, a positive deviation from zero indicates that the vapour pressure is higher than expected for an ideal mixture while a negative deviation indicates that the vapour pressures are less than expected. In cases of positive deviation, for example, mixing ethanol and water, the vapour pressure is higher than in an ideal mixture as the molecules break away more easily since the intermolecular forces are less than they would have been in the individual pure forms of the liquids and the boiling point of the mixture is below that of the highest boiling point of a pure component. See Figure 5.1. Because less heat is released when new bonds are made than heat required to break existing bonds within each liquid, the enthalpy change is endothermic (157).

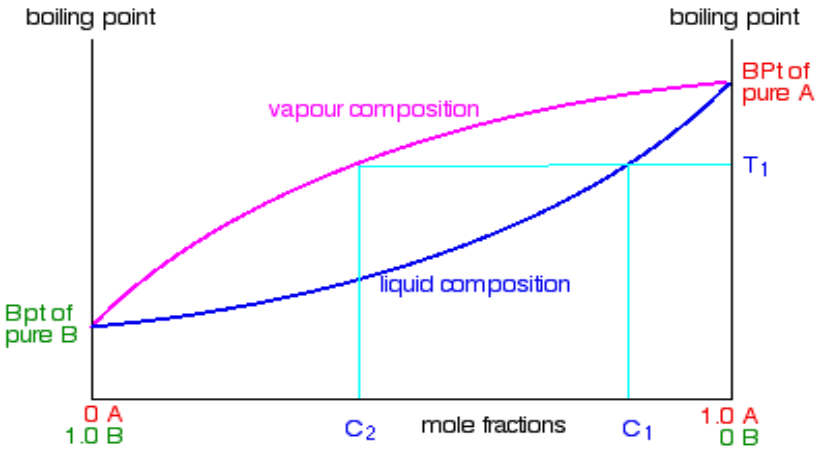


Figure 5.1 A vapour pressure diagram which shows that at the boiling temperature of composition C1 of a water and ethanol mixture, a vapour composition of C2 will be produced (156, 157)

Each of the five solvents (*n*-heptane, *d*-limonene, ethanol, glycerol and water) explored for extracting of both the hydrophilic and lipophilic bioactive compounds in a single process, has its own vapour pressure which contribute to the total vapour pressure of the gaseous phase. The condensed phase will consequently have a different composition to the original blend and therefor of interest to determine the condensed

phase vapour composition which comes in contact with the freeze-dried sample. Although these solvents were considered a non-ideal mixture of liquids, Equations 5.8 – 5.10 were applied to predict the volume percentage in the vapour for each blend. For the determination of the volume percentage solvent in the vapour, the following parameters must be available for each solvent, namely, its volume within the considered solvent blend, its mole mass, vapour pressure (Torr) at a specific temperature, and its density.

$m_i = V_i D_i$ Equation 5.8

where,

m_i = mass of solvent, i (g)

V_i = volume of solvent, i (ml)

D_i = density of solvent, i (g/ml)

and,

$n_i = m_i / M_i$ Equation 5.9

where,

n_i = moles of solvent, i

M_i = molar mass of solvent, i

$X_i = n_i / \Sigma n_i$ Equation 5.10

where,

X_i = mole fraction of solvent, i

In order to determine the percentage mole fraction of each solvent in the vapour, the partial pressure of each solvent as well as the total vapour pressure must be determined. The total vapour pressure equals the sum of the partial pressures of all the components in the vapour. Refer to Equations 5.11 and 5.12 below.

$P_T = \Sigma P_i$ Equation 5.11

where,

P_T = total vapour pressure (Torr)

The mole fraction of each component in the vapour is given by Equation 5.12. The volume percentage is thus determined by converting moles back to volume.

$X_{Vi} = P_i / P_T$ Equation 5.12

where,

X_{Vi} = mole fraction of solvent in vapour

Table 5.5 shows the theoretical calculation of the volume percentage of solvent vapour experimentally determined reflux temperature in a blend of the five solvents selected in section 5.3.

Table 5.5 Determination of volume percentage of vapour in a representative solvent blend of solvents considered for extraction of hydrophilic and lipophilic bioactive compounds from olive pomace.

Extraction temp at 77 °C Pressure: 1 atm	***n*-Heptane**	***d*-Limonene**	**Ethanol**	**Glycerol**	**Water**
Vol (ml) and percentage	10	10	50	10	20
Vapour press (Torr)	400	28	660	0,0315	314,1
Mass (g)	6.795	8.411	39.45	12.613	19.96
Total mass (g)	87.23				
Moles	0.0678	0.0617	0.8563	0.1370	1.1089
Mole fraction of liquid	0.0304	0.0277	0.3837	0.0614	0.4969
Partial press (Torr)	12.15	0.7746	253.2	0.0019	156.0793
Total vapour press (Torr)	426.095				
Mole fraction of vapour	0.0288	0.0018	0.599	4.58E-06	0.369
Mass (g) of 1 mol	2.88	0.25	27.63	0.00	6.65
Vol (ml) of 1 mol	2.24	0.30	35.02	0.00	6.67
Total vol of 1 mol (ml)	47.15				
Vol fraction of vapour	0.09	0.01	0.76	0.00	0.14
Vol % of vapour	9.18	0.64	75.75	0.00	14.42

As seen in Table 5.5, it is predicted that no glycerol fraction could possibly be detected in the vapour mixture. Glycerol as an odourless, viscous liquid with a high boiling point of 290°C was therefore excluded as extraction solvent for the hydrophilic compounds based on this outcome. Although a very low percentage of *d*-limonene might be present in the vapour, features such as being a “green, food-safe” solvent together with its pleasant odour contribution justified its inclusion for extraction of the oil-soluble compounds.

In the absence of glycerol, the percentage mole fraction in the vapour of two solvent blends with different solvent ratios, excluding glycerol and at different temperatures were calculated. Although the ethanol quantity in the two different blends remained 50% of the total volume, aspects such as the volume of each solvent, reflux temperature influencing the vapour pressure as well as the intermolecular forces to make a new mixture determined the change in the percentage mole fraction of each solvent in the vapour. These calculations are shown in Table 5.6.

Table 5.6 Determination of volume percentage of vapour in a solvent blends excluding glycerol.

Reflux temp at 82 °C Pressure: 1 atm	***n*-Heptane**	***d*-Limonene**	**Ethanol**	**Water**
Vol (ml) and percent:	15 (10%)	15 (10%)	75 (50%)	45 (30%)
Vapour press (Torr)	450	30	890	384.9
Mass (g)	10.19	12.62	59.18	44.91
Total mass (g)	126.9			
Moles	0.102	0.0926	1.285	2.495
Mole fraction of liquid	0.0256	0.0233	0.3232	0.6278
Partial press (Torr)	11.52	0.6992	287.7	241.7
Total vapour press (Torr)	541.6			
Mole fraction of vapour	0.0213	0.0013	0.5312	0.4462
Mass (g) of 1 mol	2.13	0.18	24.47	8.03
Vol (ml) of 1 mol	3.137	0.209	31.017	8.048
Total vol of 1 mol (ml)	42.41			
Vol fraction of vapour	0.07	0.00	0.73	0.19
Vol % of vapour	7.40	0.49	73.13	18.98

The calculated volume percentage of each condensed solvent that is in contact with the pomace, is different from the percentage of the original composition of the mixture as reflected in Table 5.6. The condensed phase composition will be 73% ethanol, 19% water, 7.4% *n*-heptane and 0.5% *d*-limonene. Ethanol will thus be primarily responsible for extraction of both the non-polar and polar bioactive compounds with the presence of *n*-heptane and *d*-limonene to enhance the extraction of the non-polar bioactive e.g. squalene and the water to enhance the extraction of the very polar bioactives such as hydroxytyrosol.

5.5 Optimisation of solvent blend and extraction time

A multilevel factorial D-Optimal mixture design was applied to obtain a predictive model which can be used to optimise the extraction solvent blend and extraction time by identifying the relationship between the responses, namely the extraction yield of each bioactive compound, and the extraction variables. The independent variables studied were the solvent volume ratios of the four solvents, namely, *n*-heptane, *d*-limonene, ethanol and water, with 100% ethanol as a control and extraction time of between 2 to 5 hours. The minimum and maximum values for each variable were predetermined as shown in Table 5.7.

Table 5.7 Variable ranges for the multilevel factorial D-Optimal mixture design.

	***n*-Heptane (%)**	***d*-Limonene (%)**	**Ethanol (%)**	**Water (%)**	**Time (hr)**
Min	0	0	50	0	2
Max	30	20	100	50	5

Parameters which were kept constant were, the mass of freeze-dried pomace, the pomace cultivar, the total volume of each solvent blend, the temperature of the solvent blend, and the pressure. The experimental design for this optimisation experiment is shown in Table 5.8.

Table 5.8 Multilevel factorial D-Optimal mixture for determination of optimal extraction solvent blend ratio and time.

Run	***n*-Heptane**	***d*-Limonene**	**Ethanol**	**Water**	**Time**
	Volume %				(hr)
1	15	0	85	0	2
2	0	0	100	0	4
3	0	0	75	25	3
4	30	10	60	0	2
5	0	0	100	0	4
6	0	20	80	0	3
7	15	20	65	0	2
8	0	20	50	30	2
9	30	20	50	0	5
10	30	0	70	0	5
11	30	0	50	20	4
12	0	0	50	50	3
13	30	0	50	20	5
14	15	0	68	18	4
15	30	20	50	0	5
16	15	10	50	25	3
17	0	20	50	30	2
18	0	0	50	50	3
19	6	4	57	32	4
20	0	20	65	15	5

5.5.1 Materials and General methodology

The solvents and reference standards used in this optimisation experiment are detailed in Table 5.9.

Table 5.9 Solvents and chemical reagents

Name	Chemical formula	Grade	Supplier
n-Heptane	C_7H_{16}	Chemically pure	Merck chemicals
d-Limonene	$C_{10}H_{16}$	97% purity	Sigma Chemicals
Ethanol	CH_3CH_2OH	Absolute, 96%	Emplura
D.i. water	H_2O	Purified, Millipore Milli-Q	Innoventon
Methanol	CH_3OH	HPLC	Alfa Aesar
Acetic acid, glacial	CH_3COOH	GR	Merck
Acetonitrile	C_2H_3N	HPLC	Rankem
Isopropyl alcohol	C_3H_8O	HPLC	Rankem
Tyrosol RS	$C_8H_{10}O_2$	Analytical grade ≥ 99.5%	Sigma-Aldrich
Hydroxytyrosol RS	$C_8H_{10}O_3$	HPLC, ≥ 98%	Sigma-Aldrich
Oleuropein RS	$C_{25}H_{32}O_{13}$	HPLC, ≥ 98%	Chromadex
α-Tocopherol RS	$C_{29}H_{50}O_2$	≥ 96%	Sigma-Aldrich
Squalene RS	$C_{30}H_{50}$	≥ 98%	Sigma-Aldrich
Folin-Ciocalteu 2M	C_6H_6O	Reagent	Sigma-Aldrich
DPPH	$C_{18}H_{12}N_5O_6$	95%	Alfa Aesar

RS: Reference D.i.: deionised water

5.5.2 Sample and preparation

Olive pomace was collected from olive fruits of *Frantoio* cultivar which were harvested from the olive grove of Oudewerf farm in the coastal region of the South Western Cape (Stilbaai, South Africa) during April 2016 for olive oil processing via a two-phase system. This cultivar was picked in the green to ripe ratio of 50:50. The pomace was collected at the end of centrifugation, filled as 50 g portions into 50 ml plastic tubes with screw-on lids and immediately frozen before being subjected to freeze-drying (VirTis SP Scientific Sentry 2.0).

5.5.3 Extraction methodology

Bioactive compounds were extracted according to the previously executed solid-liquid continuous Soxhlet methods used in both the single aqueous and lipid extractions described in Chapter 4, however, in consideration, it was decided to reduce the pressure in order to reduce the reflux temperatures of the solvent blends. A specially built extraction manifold of 4 Soxhlet units was used so that extractions could be performed simultaneously. See Figure 5.2.

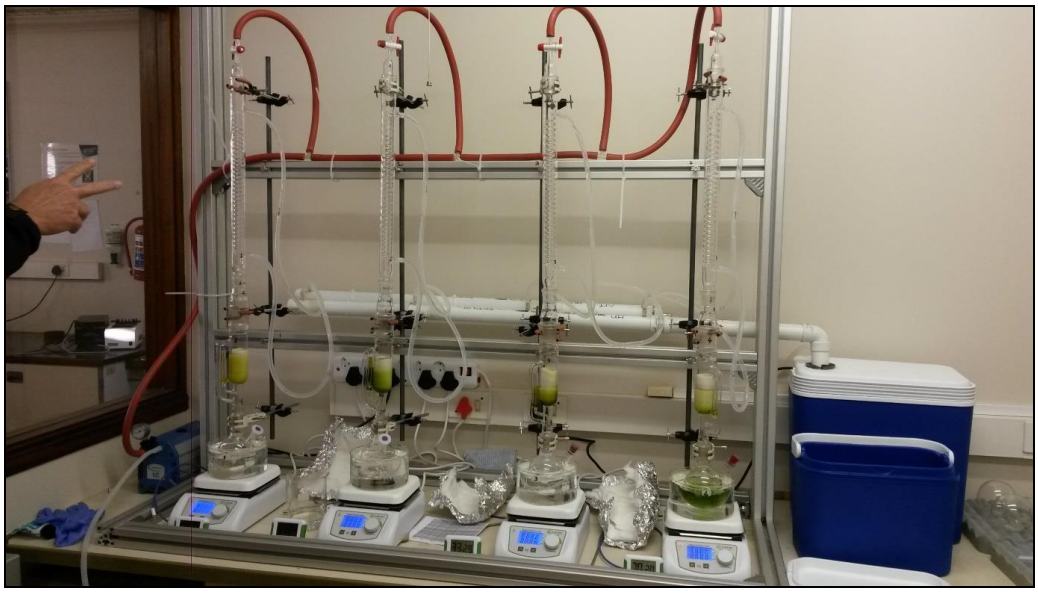


Figure 5.2 Extraction manifold of 4 Soxhlet units connected to vacuum pump and ice bath for circulating cold water through the condensers.

The 20 runs of the experimental design in Table 5.8 were performed over 5 days with 4 extractions per day. For each run, approximately 15g of freeze-dried pomace sample (which included small skin and stone particles) was crushed in a pestle and mortar to obtain a more even particle size, weighed into a cellulose thimble (33 x 80 mm, Whatman) and placed in the Soxhlet apparatus. A total volume of 150 ml of each solvent blend in Table 5.9 was heated on a Lasec digital hotplate stirrer with temperature probe placed in the solvent and set to a temperature of 60°C. The whole extraction apparatus, which included round-bottomed flask, Soxhlet and condenser was placed under vacuum at 400 mbar (Vacuubrand 1C) for the indicated period and the solvent blend magnetically stirred at 400 rpm to ensure adequate mixing. In consideration of the thermolability of most of the bioactive compounds, Cold water from an ice bath was circulated through the condensers to aid condensation of solvents.

After each extraction time had elapsed, the Soxhlet apparatus was isolated from the vacuum manifold and the extract was removed, decanted into a separation funnel and in the case where water was present, each phase collected separately in a pre-weighed round-bottomed flask. The extracts were only filtered through a Whatman No.1 filter paper if pomace particles were observed. Sample extracts were concentrated by rotary evaporation on a Buchi R-210 Rotavapor System (Germany) at approximately 55–60°C, the residues weighed prior to reconstitution with the indicated solvent and made up to volume in 25 ml volumetric flasks. HPLC grade methanol was used for hydrophilic bioactive compounds and single phase extracts and HPLC grade IPA for the lipophilic bioactive compounds. Samples were filtered through sterile

membrane syringe filters (0.45 µm, 47 mm) from Pall and then analysed by HPLC-DAD. The HPLC mobile phase mixtures were degassed by filtering through a sterilised 0.45 µm GHP membrane (Pall).

5.5.4 HPLC analysis of bioactives

Phenolic polar compounds hydroxytyrosol, tyrosol, oleuropein, and non-polar α-tocopherol and squalene were qualitative and quantitatively analysed using the optimised HPLC methods determined in chapter 3 for the hydrophilic and lipophilic compounds respectively. The impact of the solvent blend ratio and extraction time on the yield of each compound was evaluated. Analysis of variance was applied to evaluate the statistical significance of the model.

A high performance liquid chromatograph coupled to a Waters 2487 Dual λ Absorbance Detector, (two-channel, tuneable, ultraviolet/visible) was used for the detection of the bioactive compounds of the different extracts using a Waters-Alliance 2695 Separations Module (Milford, USA). HPLC analysis followed the methods described by Obied et al. (17) and Sagratini et al. (101) and modified as discussed in Chapter 3 for the hydrophilic and lipophilic compounds respectively. The reference standard stock solutions were prepared by dissolving hydrophilic standards in methanol, as a 100 ppm (10 mg/100 ml) concentration and lipophilic standards in IPA as a 1000 ppm (100 mg/100 ml) concentration).

A reverse-phase Zorbax Extend C18 column (Agilent, USA) (250 mm x 4.6 mm x 5 µm d.i.) was used for the chromatographic separation of hydroxytyrosol, tyrosol and oleuropein at a temperature of 25°C, with absorbance detection at 280 nm. The chromatic analysis were: mobile phase A – water/acetic acid (100:1) and mobile phase B – acetonitrile/ methanol/ acetic acid (90:10:1). A five-step gradient as shown in Table 5.10 was applied for a total run time of 50 minutes, as follows: starting from 90% solvent A and 10% solvent B decreasing to 50% solvent B over 20 minutes; then isocratic elution for 15 minutes; then decrease to 10% solvent B over 5 minutes and isocratic elution for 10 minutes. The sample quantity injected was 10 µl and the flow-rate was 1.0 ml/min. Quantitative analysis was performed with calibration curves obtained using pure standards namely hydroxytyrosol, tyrosol and oleuropein prepared in methanol. Five-point calibration curves were used (1 – 100 ppm) and the regression coefficients were in the range of 0.98 – 1.00.

The stationary phase for lipophilic α-tocopherol and squalene separation was a reverse-phase Luna C18 column (Phenomex, Torrance, USA) with the temperature

controlled at 30°C. The eluents were: Mobile phase A - acetonitrile/ methanol (70:30) and Mobile phase B - isopropyl alcohol. A six-step gradient as shown in Table 5.11 was applied for a total run time of 30 minutes, as follows: starting with 90% solvent A and 10% solvent B, isocratic elution for 6 minutes, increasing to 30% solvent B over 2 minutes, then isocratic elution for 9 minutes, then decreasing to 10% solvent B over 3 min and isocratic elution for 10 minutes. The absorbance was detected at 210 nm using a sample quantity of 20 µl and a flow-rate of 1.0 ml/min. Quantitative analysis was performed with calibration curves obtained using pure standards, namely, α-tocopherol and squalene prepared in IPA. Five-point calibration curves were used (1 – 100 ppm) with the regression coefficients in the range of 0.98 – 1.00.

The stock RS solutions were prepared prior to analysis, namely 1000 ppm α-tocopherol and 1000 ppm squalene in amber glass 25 ml volumetric flasks with HPLC grade IPA. Both stock RS solutions were further diluted to 100 ppm α-tocopherol and 100 ppm squalene in amber glass 10 ml volumetric flasks with HPLC grade IPA. Mixed RS concentrations of 1, 5, 10, 25 and 100 ppm which included both α-tocopherol and squalene were prepared from the stock solutions. All these RS solutions and stock solutions were stored in the refrigerator. Although the lipophilic RS and bioactive compounds would have been analysed using IPA as solvent only, methanol was also employed as diluent of both α-tocopherol and squalene RS stock solutions and dilutions for analysis of single phase extracts reconstituted with methanol. The methanolic lipophilic RS solution concentrations ranged from 0.1 mg/100 ml to 10 mg/100 ml and all the single phase extract samples (from run 1, 2, 4, 5 and 10) were analysed for both hydrophilic and lipophilic compounds using both the HPLC methods described. The data was processed on “Empower 6” software.

Table 5.10 HPLC gradients for separation of the value-added bioactive compounds in the olive pomace.

Hydrophilic compounds			**Lipophilic compounds**		
Time (min)	**Solvent A (%)**	**Solvent B (%)**	**Time (min)**	**Solvent A (%)**	**Solvent B (%)**
0	90	10	0	90	10
20	50	50	6	90	10
35	50	50	8	70	30
40	90	10	17	70	30
50	90	10	20	90	10
			30	90	10

5.5.5 Observations during extraction

The first observation was that solvent volumes of between 25 – 70 ml were lost in each extraction. This was due mainly to retention of the solvents by both the cellulose thimble and the pomace, and a little due to loss during transfer of the extract to the separation funnel. In cases where a lot of solvent was lost, this was attributed to very vigorous boiling of some solvents during extraction as a result of a reflux temperature lower than the fixed 60°C of the solvent which meant that at 60°C temperature, the more volatile components of the solvent blend evaporated before they could be condensed.

A second observation was that the extract presented as either one phase or two phases depending on the solvent combination. The non-polar *d*-limonene and *n*-heptane separated from the polar water/ethanol and a dark green or olive green top phase as well as a yellow, yellow-green to brownish-yellow bottom phase developed in these cases. The colour of the single phase extract varied between grass green, olive green and yellow.

Thirdly, in two of the extractions (Run 15 and 19), after an extraction time of 5 hours and 45 hours respectively, the green coloured extract did not syphon back into the solvent flask although the Soxhlet extraction chamber filled. This could be due to the higher reflux temperatures of those blends for which the temperature of 60°C was not sufficient to cause reflux. The extract was thus mixed with the remaining solvent in the Soxhlet flask at the end of the predetermined extraction period prior to rotary evaporation.

D-limonene was not able to evaporate at the end of the extraction. Thus, the extracts containing *d*-limonene were either used “as is” if the volume was 25 ml or more, or in the case where the extract volume was less than 25 ml, IPA was added to a total volume of 25 ml.

Finally, the solvent combinations containing only *n*-heptane and ethanol [run 1 (15:85) & 10 (30:70)] produced a single phase extract which was a grass-green colour and slightly murky. After rotary evaporation, a dark olive-green slightly runny residue formed which was initially dissolved in methanol but did not dissolve completely. It seemed that two phases developed with a clearer upper phase and a thick darker oily phase or globules at the bottom. The methanol was concentrated to dryness and the residue reconstituted with IPA instead. The residue did not dissolve in IPA at all. This

could be associated with a mismatch in the polarities of the solvents and the bioactive compounds. The IPA was evaporated and the residue once again reconstituted with methanol and filtered prior to HPLC analysis through a Whatman no. 1 filter paper.

5.5.6 Results and discussion

The bioactive content of the extracts obtained from each run of the experimental design are shown in Table 5.11. The concentrations obtained were converted to mg per gram dry weight pomace using the mass of the freeze-dried pomace sample (15 g).

Table 5.11 Yields (mg/g dry pomace) of each of the aqueous and organic bioactive compounds for each run in the D-Optimal mixture design with varying time and solvent combinations.

	Solvent blend ratio					**Concentration** (mg/g dry pomace)					
Run	**Volume** (%)				**Time** (hr)	**R1**	**R2**	**R3**	**R 4**	**R 5**	**SP** ($MPa^{½}$)
	***n*-Hep**	***d*-Lim**	**EtOH**	**Water**		**Htyr**	**Tyr**	**Oleu**	**a-Toco**	**Squal**	
1	15	0	85	0	2	0.3960	0.0692	0.0275	0.0505	0.4374	24.76
2	0	0	100	0	4	1.0475	0.1625	0.1685	0.0503	0.0728	26.50
3	0	0	75	25	3	0.9388	0,1487	0,1968	0.0063	0.0131	31.88
4	30	10	60	0	2	0.3445	0.0830	0.0092	0.0184	0.1702	22.10
5	0	0	100	0	4	0.9918	0.1180	0.1350	0.0200	0.0457	26.50
6	0	20	80	0	3	0.0307	0.0468	0.0008	0.0015	0.3861	24.66
7	15	20	65	0	2	0.0080	0.0265	0.0007	0.0000	0.3209	22.92
8	0	20	50	30	2	0.7542	0.1195	0.2100	0.0010	0.0619	31.11
9	30	20	50	0	5	0.0012	0.0203	0.0000	0.0060	0.3684	21.18
10	30	0	70	0	5	0.5680	0.0888	0.0437	0.0365	0.0366	23.02
11	30	0	50	20	4	0.8621	0.1141	0.1676	0.0691	0.2372	27.32
12	0	0	50	50	3	0.7987	0.1175	0.1254	0.0000	0.0000	37.25
13	30	0	50	20	5	0.9762	0.0953	0.0953	0.0817	0.3439	27.32
14	15	0	68	18	4	0.9882	0.0877	0.0822	0.0756	0.2492	28.52
15	30	20	50	0	5	0.0053	0.0002	0.0002	0.0122	0.5090	21.18
16	15	10	50	25	3	0.2852	0.0395	0.0383	0.0036	0.1739	29.22
17	0	20	50	30	2	0.7078	0.1413	0.1267	0.0155	0.0295	31.11
18	0	0	50	50	3	0.3900	0.0877	0.0753	0.0186	0.0254	37.25
19	6	4	57	32	4	0.4145	0.1008	0.0885	0.0159	0.0179	32.27
20	0	20	65	15	5	0.7832	0.1162	0.1217	0.0141	0.6205	27.89

n-Hep: *n*-Heptane *d*-Lim: *d*-Limonene EtOH: Ethanol SP: Solubility parameter
HTyr: Hydroxytyrosol Tyr: Tyrosol Oleu: Oleuropein α-Toco: α-Tocopherol Squal: Squalene

The maximum yields for hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene are summarised in Table 5.12.

Table 5.12 Maximum concentrations of bioactives observed.

Bioactive compounds	**Maximum yield** (mg/g dry pomace)
Hydroxytyrosol	1.05
Tyrosol	0.16
Oleuropein	0.21
α-Tocopherol	0.08
Squalene	0.62

Excel displays a Solver tool that can search for optimal solutions to fairly complex problems. Using this tool, the optimum solvent blend and extraction time which produced the maximum value of each individual response, namely, R1 (hydroxytyrosol), R2 (tyrosol), R3 (oleuropein), R4 (α-tocopherol) and R5 (squalene) was statistically predicted. The Solver tool was also used to determine the optimum solvent blend and extraction time which produced simultaneous maximum responses for all five compounds. The predicted solvent blends and extraction times for the individual maximum responses and the combined maximum response are shown in Table 5.13.

Table 5.13 Predicted maximum response and conditions for each bioactive during combination extractions of hydrophilic and lipophilic compounds.

Blend no	**Pred Htyr (SP 30.96)**	**Pred Tyr (SP 27.82)**	**Pred Oleu (SP 30.58)**	**Pred α-Toco (SP 19.9)**	**Pred Squal (SP 17.01)**	**Time**	**Predicted blend *n*-Hep /*d*-Lim / EtOH/ Water**	**SP of solvent blend**
	mg/g					**(hr)**	**(vol percent ratio)**	**($MPa^{½}$)**
1	**1.62***	0.15	0.24	0.05	0.06	5	0 / 0 / 68.5 / 31.5	33.27
2	1.35	**0.16***	0.16	0.03	0.14	5	0 / 0 / 100 / 0	26.5
3	1.38	0.14	**0.25***	0.05	0.62	2	30 / 0 / 50 / 20	27.32
4	0.83	0.10	0.11	**0.08***	0.34	5	30 / 0 / 50 / 20	27.32
5	0.44	0.1	0.05	0.00	**0.66***	5	0 / 20 / 80 / 0	24.66
6	1.38$	0.14$	0.25$	0.05$	0.62$	2	30 / 0 / 50 / 20	27.32

n-Hep: *n*-Heptane *d*-Lim: *d*-Limonene EtOH: Ethanol SP: Solubility parameter
HTyr: Hydroxytyrosol Tyr: Tyrosol Oleu: Oleuropein α-Toco: α-Tocopherol Squal: Squalene
* Predicted maximum response individual bioactives
$ Predicted maximum response of all bioactives

From the table it can be seen that *d*-limonene was not included in any of the solvent blends except blend 5 which predicted a maximum response for squalene. In this blend, there is no *n*-heptane and a 20:80 *d*-limonene/ethanol mixture is predicted to best extract squalene. Blend 3 predicts a similar amount of squalene and in this blend there is no *d*-limonene but a 30:50:20 *n*-heptane /ethanol/water mixture. This indicates that this blend is as effective as the *d*-limonene/ethanol mixture in extracting squalene, with the additional advantage of also extracting more polar polyphenols due to the presence of water. The responses of Blend 3 coincide with the responses of Blend 6 in which all the desired compounds are optimally extracted. Blend 3 also has the same composition as Blend 6 which is also the predicted blend for optimal extraction of all 5 compounds. This blend consists of 30% heptane, 50% ethanol and 20% water and has a calculated solubility parameter of 27.32 MPa½ which corresponds closely to the average SP (26.64 MPa½) for the 5 compounds. This is a confirmation that the predicted model fits the data. Although the 100% ethanol solvent (Blend 2) has the closest matching SP (26.5 MPa½) to the average value of the compounds, this solvent

is not as effective in extracting squalene. The lower SP value of *n*-heptane together with the very high SP value of water in combination with ethanol contributes to the optimal extraction of squalene as well as the other four compounds. Moreover, the predicted optimum extraction time of 2 hours is better than the 5 hours because increased degradation of the bioactive compounds increases during extended periods. Many phenolic compounds are easily hydrolysed and oxidised and increase the risk for degradation. A recent investigation showed that the most advantageous values of phenol recovery were obtained after a 3 hour extraction period (158).

Table 5.14 shows the calculated volume percentage of each solvent in a condensed phase that is in contact with the pomace for the predicted optimum blend. The condensed phase composition is 24% *n*-heptane, 65% ethanol and 11% water at a temperature of 60°C and a pressure of 400 mbar.

Table 5.14 Determination of percentage solvent vapour in the predicted optimum solvent blend

Extraction temp at 60 °C Pressure: 400 mbar	***n*-Heptane**	**Ethanol**	**Water**
Vol (ml) and percent:	45 (30%)	75 (50%)	30 (20%)
Vapour press (Torr)	293	467.5	198.7
Mass (g)	30.58	59.185	29.40
Total mass (g)	119.693		
Millimoles	0.3052	1.285	1.663
Mole fraction of liquid	0.0938	0.3949	0.5113
Partial press (Torr)	27.49	184.6	101.6
Total vapour press (Torr)	313.7		
Mole fraction of vapour	0.0876	0.5885	0.3239
Mass (g) of 1 mol	8.7801	27.11	5.8301
Vol (ml) of 1 mol	12.92	34.36	5.842
Total vol of 1 mol (ml)	53.12		
Vol fraction of vapour	0.2432	0.6468	0.1100
Vol % of vapour	24.32	64.68	11.00

5.6 The effect of temperature and time

The variability obtained in the results of the d-optimal design can be attributed to the fact that the pomace is a plant material with natural variability. Samples of pomace consisted of pips, skin and pulp in varying proportions which could add to the variability of results. Other factors influencing results are temperature, pressure and time. Reflux temperatures of the solvent blend are determined by the pressure, and will decrease with decreasing pressure or vice versa.

In order to confirm results obtained in the d-optimal design and to optimise extraction conditions of reflux temperature and extraction time, a new experimental design was done for extraction using the optimum solvent blend.

5.6.1 Materials

The same solvents and reference standards as specified in 5.5.1 were used with for the exclusion of the oleuropein reference standard as it was unavailable. Standard stock solutions were prepared by dissolving hydroxytyrosol and tyrosol standards in methanol HPLC grade as a 10 mg/100 ml concentration, and α-tocopherol and squalene in isopropyl alcohol HPLC grade as a 100 mg/100 ml concentration.

5.6.2 Sample preparation

This study included two-phase system olive pomace samples produced from *Frantoio* olive, grown in Stilbaai (Western Cape coastal region) as mentioned in 5.5.2. Freeze-dried pomace was ground in a mortar and pestle, 15 g filled into a Whatman 30 x 80 mm cellulose thimble and extracted using 150 ml optimum solvent blend of *n*-heptane, ethanol and water (30:50:20 vol. %).

5.6.3 Extraction methodology

In order to determine the optimum extraction time and temperature with the optimal solvent blend previously determined, three solvent temperature conditions of 40°C, 50°C and 60°C and three time periods of 2, 3 and 4 hours were considered. Five hours could negatively affect the quality of the bioactive compounds with the development of unwanted degradation products during extended exposure using a quantity of 150 ml extraction solvent, thus 5 hours was not considered in this study. The pressure was reduced in each case to the required value to ensure reflux at the specified temperatures. The experimental design is shown in Table 5.15. Factors evaluated during the temperature/pressure and time study were: content of hydroxytyrosol, tyrosol, α-tocopherol and squalene, total phenolic content and percentage anti-oxidant activity of the extracts.

Table 5.15 Experimental design for determination of optimal temperature/pressure and time.

	Variable 1		Variable 2
Run no.	**Reflux Temperature** (°C)	**Pressure** (mbar)	**Time** (hrs)
1	60	300	2
2	60	300	3
3	60	300	4
4	60	300	2
5	50	250	3
6	50	250	4
7	50	250	3
8	50	250	2
9	40	150	3
10	40	150	4
11	40	150	2
12	40	150	4

5.6.4 HPLC analysis of bioactives

Quantitative determination of the high-value bioactive compounds in the *Frantoio* olive pomace was performed by HPLC analyses according to the previously described methods in 5.5.4.

5.6.5 Determination of total phenolic content

The assay for the determination of total phenolic content in *Frantoio* was performed according to the Folin-Ciocalteu method of Costa *et al*. (138) with some modifications. Gallic acid was used as phenolic compound standard for the calibration curve and the contents of total phenolic compounds in the olive pomace were expressed as gallic acid equivalents in milligram per gram (mg GAE/g dry weight). Total phenols (mgGAE/g) were determined by reading of the absorbance at 760 nm against a standard curve of gallic acid using a Biotek Power Wave X5 spectrophotometer microtiter plate reader and Gen5 software. All samples were analysed in triplicate. The Folin-Ciocalteu method gives a crude estimation of the total phenolic compounds present in the sample and is not specific to polyphenols. Many interfering compounds may also react with the Folin-Ciocalteu reagent and introduce elevated phenolic concentrations which may be the case in this study as the bioactive compounds are comprised in a complex plant matrix (159).

5.6.5.1 Chemical reagents

The following reagents were used for analyses of total olive phenolics: Folin-Ciocalteu reagent (2 M), analytical grade gallic acid as the reference standard, and HPLC grade methanol were purchased from Sigma-Aldrich; and analytical grade sodium carbonate

(Na_2CO_3) from Merck Chemicals. A standard curve for linear regression was constructed using gallic acid (1000 µg/ml) concentrations of 0.05, 0.10, 0.125, 0.25, 0.50, 0.75, 1 mg/ml in absolute methanol. The calibration curve of concentration against the absorbance was plotted. Stock solutions of 0.2 N Folin-Ciocalteu reagent (di water) and 1000 µg/ml gallic acid (in methanol) were prepared.

5.6.5.2 Sample

Methanolic solutions of the aqueous extracts from the *Frantoio* cultivar were prepared as 1000 µg/ml concentrations.

5.6.5.3 Methodology

Amounts of 100 µl of Folin-Ciocalteu reagent, diluted to 0.2 N, was mixed with 20 µl portions of either the sample or standard gallic acid in 96-well microtiter plate. The plate was kept in the dark for 5 minutes. Then, 80 µl of a sodium carbonate solution (75 g/l) was added to each well containing solutions, mixed and the plate kept in the dark for 1 hour. Absorbance was determined at 760 nm and the total phenols were calculated as milligrams of gallic acid equivalents per gram of dry matter (DM).

5.6.6 Determination of percentage antioxidant concentration

The antioxidant activities of olive mill waste waters and olive pomace have been studied and demonstrated by several antioxidant assays including 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity, superoxide anion scavenging and LDL oxidation (54, 160). In this study, the antioxidant activity of the aqueous phenol extracts was evaluated by using the stable organic nitrogen radical (DPPH), according to a modification of the method by Leouifoudi *et al*. (161). The spectrophotometric technique used measures the relative abilities of the antioxidants to scavenge DPPH in comparison with the antioxidant activity of the positive control, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Antioxidant assays are based on measurement of the loss of DPPH colour at 540 nm after reaction with test samples. The advantage of this DPPH assay is that this method is simple and rapid and based on an electron transfer reaction.

5.6.6.1 Chemical reagents

DPPH radical was purchased from Alfa Aesar and Trolox from Otic. All chemicals used were of analytical grade. DPPH was prepared as a 0.004% solution in methanol.

5.6.6.2 Sample

Methanolic solutions of the aqueous extracts from the *Frantoio* cultivar, as well as Trolox were prepared as 1000 µg/ml concentrations.

5.6.6.3 Method

The DPPH radical assay was carried out in a 96-well microtiter plate. The samples and positive control, Trolox, were further diluted with absolute methanol to prepare sample concentrations equivalent to 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg of dried sample/ml solutions. A volume of 150 µl of 0.004% DPPH solution was pipetted into each well of 96-well plate followed by 8 µl of the Trolox or sample solutions. DPPH was used as control. The plates were incubated at 37°C for 30 min and the absorbance was measured at 540 nm, using Biotek Power Wave X5 spectrophotometer microtiter plate reader and Gen5 software. The experiment was performed in triplicate and percentage scavenging activity by inhibition of DPPH, was calculated using the following equation:

$$\% \text{ Inhibition} = (1 - A_{sample}/A_{control}) \times 100 \qquad \text{Equation 5.13}$$

where,

$A_{control}$ = absorbance of the control at 540 nm

A_{sample} = absorbance of the test sample at 540 nm

5.6.7 Observations during extraction

During extraction at 60°C and 300 mbar, the water bath temperature was approximately 90°C while the solvent blend reflux temperatures varied between 48 – 60°C. The lower temperature was observed every time the cooled extracted content syphoned from the Soxhlet apparatus back into the solvent flask and then heated up again to the set temperature. During runs 1 and 4 the solvent syphoned 38 and 36 times respectively during the 2 hour extraction period while the solvent in run 2 syphoned 61 times during the 3 hour extraction period (i.e. approximately every 3 minutes). However, only 21 syphonings were observed for run 3 since the solvent stopped syphoning after 82 minutes. The extraction was thus discontinued after 3 hours. It also became apparent that a pressure of 300 mbar caused too rapid refluxing of solvent which lead to spilling of the sample from the thimble into the Soxhlet chamber and subsequent blockage of the syphon tube.

During extraction at 50°C and 250 mbar, an average of 4.6 minutes per syphoning cycle was observed during one of the 3 hour extractions (run 7). For the other three extractions, the average syphoning period was approximately 8.5 minutes. However, for three of the extractions (runs 5,6 and 8) after 56, 92 and 74 minutes respectively, the solvent temperature had to be adjusted to 55°C as the solvent has stopped syphoning because the temperature was too low to maintain reflux. This adjustment was required twice while run 5 had to be adjusted more frequently to maintain reflux. For each temperature adjustment, after 8 – 10 minutes, the solvent temperature was reduced back to 50°C once reflux continued. Although the more frequent temperature adjustment of run 5 could be related to a hotplate problem, the temperature adjustments required for runs 6 and 8 could be an indication that the proposed conditions might not be sufficient for continuous extraction and syphoning.

During extraction at 40°C and 150 mbar, the syphoning frequency varied between 5.7 minutes and 7.8 minutes for the 2 hour (run 9) and 3 hour (run 11) extractions respectively and 13.3 – 21.8 minutes for the 4 hour period (run 10). The water bath temperatures were approximately 60 – 65°C. For run 12 after an extraction period of 92 minutes, the solvent had only syphoned 7 times and a water bath temperature of 53°C was observed. A further 4 syphons took place after the hotplate was exchanged with another hotplate and the water bath temperature remained below 60°C.

5.6.8 Results and discussion

The results from the temperature/time study are shown in Table 5.16. The variables were solvent temperature and extraction time while the responses measured included: percentage antioxidant activity (%AO), total phenols (TP), hydroxytyrosol, tyrosol, α-tocopherol and squalene. Percentage antioxidant activity results were obtained from the 200 µg/ml sample concentration.

Table 5.16 Summary of results obtained from the temperature/time experimental design using *Frantoio* pomace and solvent blend of heptane/ethanol/water (30/50/20 volume %)

Run	Press (mbar)	Solvent temp (°C)	Extract time (hrs)	%AO	TP mg GAE/g	Htyr (mg/g)	Tyr (mg/g)	α-Toco (mg/g)	Squal (mg/g)	No of extraction cycles
A1	300	60	2	17.87	1.37	0.85	0.115	0.036	0.314	38
A2	300	60	3	15.87	2.06	0.844	0,.92	0.037	0.325	61
A3	300	60	3	16.56	2.73	0.839	0.071	ND	ND	21
A4	300	60	2	10.55	2.42	0.897	0.067	0.033	0303	36
B5	250	50	3	13.95	0.55	0.926	0.135	0.04	0,318	23
B6	250	50	4	9.12	2.74	0.857	0.113	0.01	0.073	30
B7	250	50	3	9.67	2.76	0.88	0.14	0.052	0.315	39
B8	250	50	2	9.49	2.88	0.903	0.086	0.016	0.13	15
C9	150	40	2	9.25	3.79	0.874	0.102	0.006	0.073	23
C10	150	40	4	2.83	4.52	0.884	0.107	0.028	0.28	19
C11	150	40	3	3.30	2.97	0.896	0.097	0.025	0.252	22
C12	150	40	4	2.36	5.38	0.951	0.095	0.022	0.073	11

%AO: % antioxidant activity TP: Total phenols GAE: gallic acid equivalents ND: not detected HTyr: Hydroxytyrosol Tyr: Tyrosol α-Toco: α-Tocopherol Squal: Squalene

5.6.8.1 Percentage antioxidant activity (%AO)

After stepwise regression, the model in Equation 5.14 and Table 5.17 was obtained. This model explains 74.5% of the variation in the observed percentage antioxidant activity. Temperature had a positive influence on the percentage antioxidant activity and was the only variable which showed an effect (P = 0.0003). According to Figure 5.3, the percentage antioxidant activity increases with increased temperature. The validation of the model can be found in Appendix 5.3.

$y = b_0 + b_1 Temp$ Equation 5.14

where,

y: percentage antioxidant activity

bi: i = 0, 1 are the estimated regression coefficient

Table 5.17 Final regression model obtained for the percentage antioxidant activity

	b_i	Std Err	t	p-value
Intercept	-16.2804	4.953439	-3.28669	0.008196
Temp	0.528625	0.097774	5.406617	0,000299

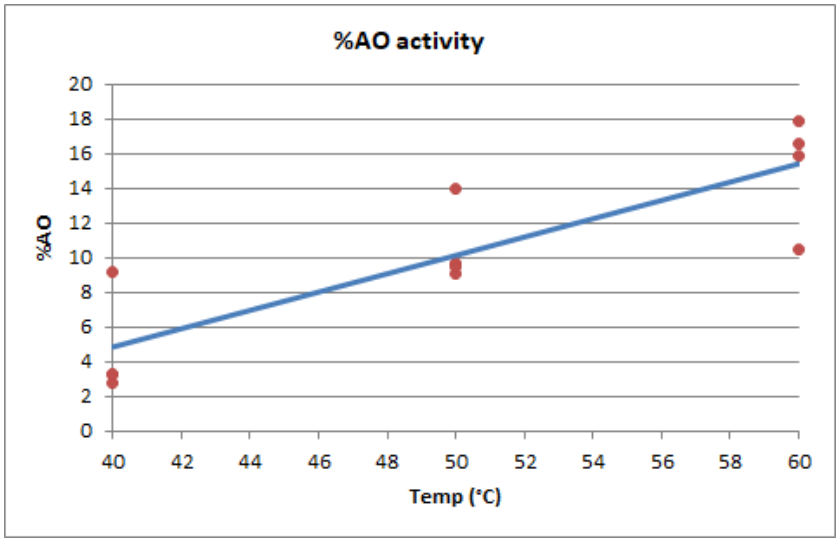


Figure 5.3 Graphic representation of the effect of temperature on the percentage antioxidant activity

5.6.8.2 Total phenol content (TP)

After stepwise regression, the model in Equation 5.15 and Table 5.18 was obtained. This model explains 44.3% of the variation in the observed total olive phenol content. The correlation is weak and temperature was the only parameter which had an effect on the TP (p = 0.0181). Temperature had an negative influence on the total phenol content. Figure 5.4 shows the decrease in total phenol content with increase in temperature. The validation of the model can be found in Appendix 5.3.

$y = b_0 + b_1 Temp$ Equation 5.15

where,

y: total phenol content

bi: i = 0, 1 are the estimated regression coefficient

Table 5.18 Final regression model obtained for the total phenol content

	b_i	Std Err	t	p-value
Intercept	7.898532	1.814218	4.353684	0.001435
Temp	-0.10103	0.03581	-2.82133	0.018119

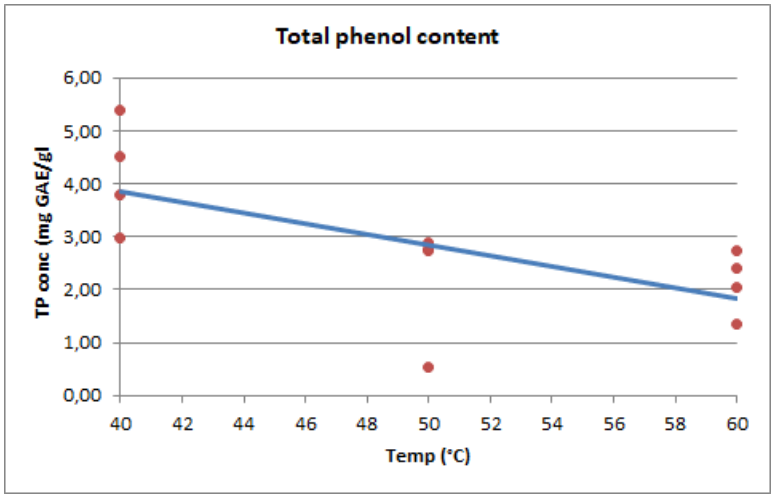


Figure 5.4 Graphic representation of the effect of temperature on the total phenol content

5.6.8.3 Hydroxytyrosol content

After stepwise regression, the model in Equation 5.16 and Table 5.19 was obtained. This model explains 30.6% of the variation in the observed hydroxytyrosol content. The p-value of 0.0621 for temperature is borderline, indicating that there is weak evidence that temperature may play a role, the model showed no time dependence. However, the model correlation is very weak. The validation of the model can be found in Appendix 5.3.

$y = b_0 + b_1 Temp$ Equation 5.16

where,

y: hydroxytyrosol content

bi: i = 0, 1 are the estimated regression coefficients

Table 5.19 Final regression model obtained for the hydroxytyrosol content

	b_i	Std Err	t	p-value
Intercept	590.5875	3.180039	18.73798	4.05E-09
Temp	-0.3175	0.062769	-2.09895	0.062187

5.6.8.4 Tyrosol content

After stepwise regression, the model in Equation 5.17 and Table 5.20 was obtained. This model explains 38.4% of the variation in the observed tyrosol content. The p-values of 0.066 and 0.060 for temperature and Temp^2 respectively are borderline. The model showed a quadratic dependence on temperature and no time dependence. As seen in the results, temperature increases from 40°C to approximately 50°C, tyrosol content increases but declines with further increase to 60°C. However, the correlation

of the model to the data is weak. The validation of the model can be found in Appendix 5.3.

$y = b_0 + b_1Temp + b_2Temp^{\wedge 2}$ Equation 5.17

where,

y: tyrosol content

bi: i = 0, 1, 2 are the estimated regression coefficients

Table 5.20 Final regression model obtained for the tyrosol content

	b_i	Std Err	t	p-value
Intercept	-28.96	17.40744	-1.66366	0.130541
Temp	1.484	0.711444	2.085899	0.066625
$Temp^{\wedge 2}$	-0.01525	0.007103	-2.1471	0.060321

5.6.8.5 α-Tocopherol

After stepwise regression, the model in Equation 5.18 and Table 5.21 was obtained. This model explains 80.7 % of the variation in the observed α-tocopherol content. The validation of the model can be found in Appendix 5.3.

$y = b_0 + b_1Temp + b_2Time + b_3Time^{\wedge 2} + b_4Temp*Time$ Equation 5.18

where,

y: α-tocopherol content

bi: i = 0, 1, 2, 3, 4 are the estimated regression coefficients

Table 5.21 Final regression model obtained for the α-tocopherol content

	b_i	Std Err	t	p-value
Intercept	-22.009	5.558353	-3.95962	0.007455
Temp	0.25036	0.078816	3.176517	0.01916
Time	11.9309	2.895881	4.119956	0.006217
Time^2	-1.36888	0.33792	-04.0509	0.006719
Temp*Time	-0.07774	0.029125	-2.66902	0.037075

All four the factors shown in Table 5.21 are significant. Temperature has a linear effect with a consequent increased α-tocopherol content. Time however has a quadratic effect; a too long extraction time has a negative influence on α-tocopherol and secondly, the effect of temperature on time negatively affects α-tocopherol content and vice versa. The content of α-tocopherol increases with temperature escalation while the optimum extraction time is around 3 hours.

5.6.8.6 Squalene

After stepwise regression, the model in Equation 5.19 and Table 5.22 was obtained. This model explains 79.1 % of the variation in the observed α-tocopherol content. The validation of the model can be found in Appendix 5.3.

$y = b_0 + b_1Temp + b_2Time + b_3Time^{\wedge 2} + b_4Temp*Time$ Equation 5.19

where,

y: squalene content

bi: i = 0, 1, 2, 3, 4 are the estimated regression coefficients

Table 5.22 Final regression model obtained for the squalene content

	b_i	Std Err	t	p-value
Intercept	-169.09	46.33039	-3.64966	0.010711
Temp	2.03456	0.656952	3.096969	0.021198
Time	91.1894	24.13795	3.777843	0.009204
Time^2	-10.3592	2.816658	-3.67784	0.010357
Temp*Time	-0.63221	0.242765	-2.6042	0.040434

All four of the factors in Table 5.22 are significant. Temperature has a positive linear effect with a consequent increased squalene content. Time however has a quadratic effect; a too long extraction time had a negative influence on squalene and secondly, the effect of temperature on time negatively affects squalene content and vice versa. Increase in temperature improves the extraction of squalene while the optimum time is between 2 and 3 hours.

5.6.9 Conclusion

Temperature has a positive linear effect on the antioxidant activity (p=0.0014), and content of both α-tocopherol and squalene (p=0.019 and p=0.021 respectively) while it has a negative effect total phenol and hydroxytyrosol content. The effect of temperature on tyrosol is quadratic with maximum content around 50°C.There is no evidence that extraction time has a significant effect on %AO, total phenol content, and both hydrophilic bioactives content. Both lipophilic bioactives showed a quadratic effect with time; α-tocopherol content increased as extraction time increased from 2 to 3 hours but decreased when extended to 4 hours. Squalene content showed a slight increase initially with time up to 2.5 hours but rapidly declined during extended extraction. In summary, the results indicated that extraction will be beneficial at 60°C to maximise %AO activity and tyrosol content and at 2 hours to maximise α-tocopherol and squalene content.

5.7 Confirmation of the optimum temperature/pressure

In light of the results obtained in 5.6 and to confirm the optimum temperature/pressure setting, it was decided to repeat the experiment at the highest and lowest temperatures namely, 60°C, but at 350 mbar and 40°C at 150 mbar for 2 hours. During the previous Soxhlet extractions, the temperature probe was placed within the solvent flask to control the solvent temperature while the thermometer was positioned in the water-bath to monitor water temperature. However, as the solvent volume in the flask reduces during reflux and condensation into the Soxhlet chamber, the reflux temperature of the remaining solvent increases. Thus, heat is no longer supplied to the probe resulting in cooling of the hotplate with subsequent cooling of the water bath. The reflux and siphoning of the solvent are thus not sustained. Therefore, it was decided to control the water bath temperature with the probe and to measure the solvent reflux temperature with a thermometer. See Figure 5.5. From previous experiments, it was observed that the water bath temperature varied between 75 – 85 °C during extraction at 400 mbar when the reflux temperature was 60°C. The water-bath temperature was therefore set at 80°C for a solvent reflux temperature of 60°C (at 350 mbar) and at 60°C for a solvent reflux temperature of 40°C (at 150 mbar) ensuring a constant water bath temperature and a more controlled extraction process. Four replicates of each extraction condition were carried out. Deviations observed are inherent of the system.

Figure 5.5 Extraction manifold of four Soxhlet units with thermometers inserted into the solvent flasks and probes in the water-bath.

5.7.1 Results and discussion

The average results of the repeat experiments are shown in Table 5.23 while the full set of results are shown in Table A in Appendix 5.4. Only the concentrations of hydroxytyrosol, tyrosol, α-tocopherol and squalene were determined and used as responses.

Table 5.23 Summary of the average bioactive content observed at two different temperatures/pressure conditions after 2 hours extraction

Recovery	**A: 350 mbar, 60°C solvent temp**	**B: 350 mbar, 80°C bath temp**	**C: 150 mbar, 65°C bath temp**
Hydroxytyrosol (mg/g DW)	0.6833	0.7788	0.5684
Tyrosol (mg/g DW)	0.0352	0.0687	0.0335
α-Tocopherol (mg/g DW)	0.0227	0.0309	0.03633
Squalene (mg/g DW)	0.1910	0.2757	0.2850

Statistical analysis by way of t-tests was performed in order to determine significant differences in the concentrations of the bioactive compounds between the three extraction conditions, A,B and C as indicated in Table 5.23. Before t-tests could be applied, the data had to be analysed for equal variances using f-tests. The results of the f-tests between pairs A-B, B-C and A-C for each bioactive concentration are shown in Table B in the Appendix 5.4. T-tests (assuming equal variance) were then performed on all the pairs which showed equal variance while the unequal variance pairs were analysed using the Mann-Whitney test (an alternative to the t-test). The p-values obtained from these tests are shown in Table C in Appendix 5.4. From the bioactive responses obtained when evaluating Soxhlet extraction conditions where the temperature probe was placed within the solvent flask to control the solvent temperature (A) and alternatively when the thermometer was positioned in the water bath to monitor water temperature (B), extraction condition B provided a greater recovery of tyrosol than condition A. ($p = 0.0017$). There were no significant differences observed for the other three bioactives. Comparing the highest (B) and lowest (C) temperature/pressure conditions where in both cases the temperature probe was in the water bath, both the hydroxytyrosol content ($p = 0.00014$) and the tyrosol content ($p = 0,0304$) were significantly higher at the higher bath temperature (B) while no significant differences were observed for α-tocopherol and squalene. Thus, it can be concluded that for both hydroxytyrosol and tyrosol (i.e. the hydrolytic antioxidants) extraction at a bath temperature of 80°C at 350 mbar to give a reflux temperature of around 60°C is best, while the lipophilic antioxidants, are less sensitive to extraction temperature and can be extracted equally well under any of the conditions investigated in Table B in the Appendix 5.4

5.8 Conclusion

Among the different solvent combinations, an optimum solvent blend which includes *n*-heptane, ethanol and water (30:50:20 % volume) for simultaneous extraction of the hydrophilic and lipophilic bioactive compounds from freeze-dried two-phase olive pomace was obtained. The mixture excludes both d-limonene (as a non-polar solvent for α-tocopherol and squalene) and glycerol (polar solvent for hydroxytyrosol, tyrosol and oleuropein) as they demonstrated no role in the extraction of the maximum content of all five bioactives during a two to five hour extraction period.

During optimisation of the extraction conditions, namely, extraction temperature and associated pressure, as well as time, the percent antioxidant activity, α-tocopherol and squalene significantly increased with increased temperature. There is no evidence that extraction time has a significant effect on percentage antioxidant activity, total phenol content, and hydrophilic bioactives content while the content of the lipophilic bioactives showed a quadratic decline with time. It was concluded from the results that a 2 hour extraction time and a reflux temperature of 60°C was optimal.

A constant water bath temperature of 80°C to maintain a reflux temperature of around 60°C at 350 mbar was found to be the optimal extraction set-up.

In conclusion, the optimised conditions proposed for further extractions on olive pomace, for example, a comparison of the content of different olive cultivars, are a solvent blend of *n*-heptane, ethanol and water (30:50:20 % volume), an extraction period of two hours and a water bath temperature set at 80°C to obtain a reflux temperature of around 60°C at a pressure of 350 mbar.

CHAPTER 6

COMPARISON OF OLIVE CULTIVARS USING OPTIMUM SOLVENT BLEND AND EXTRACTION CONDITIONS

6.1 Introduction

The optimum solvent blend and extraction conditions as identified during the combined extraction method development were implemented for analysis of pomace obtained from two different olive cultivars with the following objectives:

a. To characterise and compare the pomace from the two cultivars
b. To compare the bioactive composition of the extracts obtained from the two olive cultivars.
c. To compare the total phenolic content, antioxidant capacity and antimicrobial activity of the extracts obtained from the two olive cultivars.
d. To determine a correlation, if any, between the bioactive compositions of the cultivars and their total phenolic content, antioxidant capacities and antimicrobial activities.

Antioxidant activities of olive oil processing waste which includes olive mill waste (or pomace) and olive mill waste waters, have been well described in literature (49). In addition, these therapeutic activities, Obied et al. (17) reported that the olive phenolic fraction exhibits antibacterial activities against a broad spectrum of gram positive and gram negative bacteria species e.g. *Staphylococcus aureus, Bacillus subtillis, Escherichia coli and Pseudomonas aeruginosa*. Yangui et al. (162) confirmed the fungicidal activities and have linked both the bactericidal and fungicidal activities to the hydroxytyrosol and tyrosol which are the simple phenol alcohols. The secoiridoid, oleuropein, has been shown to inhibit or delay the growth rate of several fungi.

In consideration of the above, the concentrations of hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene, total phenol content, antioxidant capacity and antimicrobial activity of the extracts from the two cultivars were therefore determined and compared. The antimicrobial activity was tested against four microorganisms: *Staphylococcus aureus* (Gram-positive) *and Escherichia coli* (Gram-negative) bacteria and the fungal strains *Aspergillus niger* and *Penicilium notatum (161).*

6.2 Materials

6.2.1 Pomace samples

Fresh olive oil processing pomace of both *Frantoio* and *Coratina* cultivars from a two-phase system was collected from the olive grove of De Rustica, (De Rust, South Africa) in the harvest season of March, 2017. These olive fruit cultivars were picked in the following ratio of green to ripe: *Frantoio* 70:30 and *Coratina* 80:20. (Refer Figure 6.1)

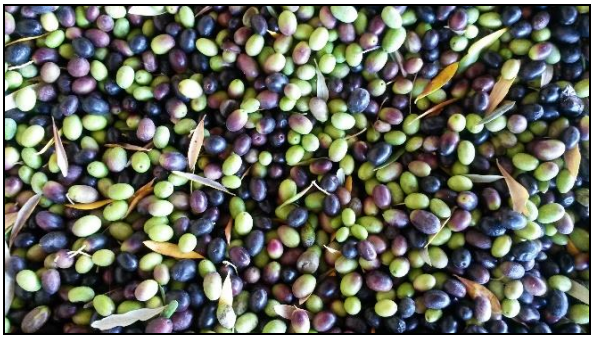


Figure 6.1 Ripening stages of *Frantoio* at harvesting time

All drupes were harvested predominantly at a maturity index of Class 1 (yellowish-green skin) to Class 3 (reddish or purple skin over more than half the fruit which represents the end of ripening). See Figure 6.1. The *Coratina* drupe is medium to large-sized (4 g) with a high oil content. The skin turns dark red during maturation while *Frantoio* becomes black-purple when ripe. *Frantoio* is the main Italian variety and produces a medium-sized fruit (2.5 – 3 g) with medium to high oil content. Both exhibit excellent organoleptic characteristics and stability (163).

The pomace collected at the end of centrifugation, was filled into foil plates, sealed in polyethylene bags after removing most of the air and immediately frozen before being subjected to freeze-drying (Vacutec freeze-drier) for preservation.

6.2.2 Solvents and reference standards

Extraction solvents and chemicals used were: absolute ethanol from Emplura, deionised water (d.i.) purified on a Millipore Milli Q Plus Ultra-pure water system, *n*-heptane from Merck. Hydroxytyrosol, tyrosol, α-tocopherol and squalene HPLC grade reference standards were from Sigma-Aldrich Co. while oleuropein HPLC grade reference standard was from Chromadex. Cellulose extraction thimbles (33 x 80 mm) were obtained from Whatman Int.

Stock solutions (10 mg/100 ml) of the hydrophilic reference standards were prepared pure in methanol (HPLC grade from Alfa Aesar) while stock solutions of the lipophilic standards (100 mg/100 ml) were prepared in isopropyl alcohol (HPLC grade from Rankem). These stock solutions were diluted to prepare six point calibration curves for hydroxytyrosol, tyrosol and oleuropein which ranged from 0.0001 – 0.01 mg/ml and for α-tocopherol and squalene which ranged from 0.0005- 0.05 mg/ml.

6.3 Methods

6.3.1 Characterisation of olive pomace

The moisture content of the pomace from each cultivar was determined by weighing the pomace before and after freeze-drying. The pH of the pomace was obtained using an Accsen portable pH meter with pH spear electrode while the pH and conductivity of extracts obtained from each pomace cultivar were measured using thea Crison MultiMeter MM 41 (Crison Instruments, Barcelona, Spain) with pH electrode and conductivity cell.

6.3.2 Extraction

Freeze-dried pomace was ground in a mortar and pestle to a homogenous powder which included broken stone and skin particles. For each extraction set-up 15 g of ground pomace was filled into a cellulose thimble and placed in the Soxhlet apparatus. The Soxhlet apparatus was placed onto a round-bottomed flask in which a total volume of 150 ml of the optimum solvent blend of n-heptane, ethanol and water (30:50:20) had been placed. The condenser with circulating water from a chiller unit (Labotec) kept at between 4 –10 °C was placed on top of the Soxhlet apparatus. The solvent flask was heated in a water bath on a Lasec digital hotplate stirrer using a temperature probe to maintain the water bath at a temperature of 80°C. The flask, Soxhlet apparatus and condenser were placed under reduced pressure of 350 mbar while the solvent was magnetically stirred at 350 rpm to ensure mixing of the polar and non-polar solvents. These settings provided a reflux temperature of 58 – 60°C. Reflux was continued for 2 hours, after which the solvent blend containing the extract was poured into a separating funnel for separation of the two phases, viz heptane and ethanol/water. The separated aqueous/ethanolic and organic phases were filtered through a Whatman filter no. 1 before being evaporated under reduced pressure at 50 – 60°C on a rotary evaporator (Buchi R-210).

6.3.3 HPLC analysis of bioactive compounds

The concentration of hydrophilic and lipophilic bioactive compounds in the different cultivars was determined by HPLC analysis on a Waters-Alliance 2695 Separations Module (USA) with reversed-phase columns (Zorbax Extend C18 and Phenomex, 250 mm x 4.6 mm x 5 µm d.i. respectively). Binary gradient elution methods with detection at 280 nm for the hydrophilic and 210 nm for the lipophilic bioactives were implemented. The same conditions and mobile phases as described in Chapter 5, section 5.5.4 were implemented. Flow rate was 1 ml/min, the injection volume was 10 µl for the hydrophilic extracts and 20 µl for the lipophilic extracts while the column temperature was maintained at 25°C for the hydrophilic and 30°C for the lipophilic respectively. The data was processed using a Waters Empower program and the quantification of the bioactives was obtained by external standard calibration using authentic (HPLC) standards.

6.3.4 Statistical analysis

Means, standard deviations and R values were determined from 8 replicate extractions of *Coratina* and 4 replicate extractions of *Frantoio* pomace. Significant differences were statistically evaluated using multiple regression with dummy variables (for cultivars) and t-tests at a confidence level of 95% (p values < 0.05) performed by means of the statistical software package STATISTICA and Excel.

6.3.5 Total olive phenol content

The assay for the determination of total phenolic content in *Coratina* and *Frantoio* was performed according to the Folin-Ciocalteu method as described in Chapter 5, section 5.6.5.

6.3.6 Antioxidant activity

Antioxidant capacity of olive phenols in the phenolic extracts obtained from *Coratina* and *Frantoio* pomace were measured using DPPH radical scavenging assay as described in Chapter 5, section 5.6.6.

6.3.7 Antimicrobial activity

The antimicrobial activity of phenolic extracts was determined using the methods employed by the Department of Biochemistry and Microbiology at NMU, Port Elizabeth, South Africa.

6.3.7.1 Chemical reagents and source of micro organisms

PDA (potato dextrose agar) and nutrient agar medium petri plates were prepared by the Department of Biochemistry and Microbiology. Bacteria strains *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) as well as the fungi strains *Aspergillis niger* and *Penicillium notartum* were obtained from the Department of Biochemistry and Microbiology. The bacteria were individually cultured in a nutrition broth for 24 hours at 37°C. The fungal cultures were grown on PDA for a period of 5 days at 28°C.

6.3.7.2 Extract samples

Phenolic extracts from two olive cultivars, *Coratina* and *Frantoio* stored at three different storage conditions for 28 days (-20°C, 5°C and 25°C) were prepared with absolute methanol as a 1000 µg/ml dilution. Sterile water was used as control for each bacterial and fungal strain.

6.3.7.3 Methodology

A Bunsen burner was used to produce a sterilised environment. Before inoculation the fungal spores of *Aspergillus niger and Penicillium notatum* were separately dissolved in a 1% aqueous Tween solution because of their hydrophobicity. The suspensions of fungi were inoculated (100 µl) on plates containing the PDA, while the suspensions of bacteria in nutrient broth were inoculated on nutrient agar plates. The inoculum was immediately and evenly distributed with a sterilised glass spreader and left for approximately 30 minutes at 25°C. Three small wells (5 mm in diameter) were made with the back of a sterile glass Pasteur pipette and clearly marked on the plate before each well was inoculated with 50 µl extract sample. The plate was immediately sealed with a lid before filling the next plate, and incubated at 30°C for 48 hours for fungi, *Aspergillus niger and Penicillium notatum*, and 37°C for 24 hours for bacteria, *Staphylococcus aureus and Escherichia coli*. After this period, the plates were inspected for the presence of inhibition zones and the diameter of the zones measured with a ruler (in mm).

Nine extract samples were analysed: six replicates from *Coratina* and three replicates from *Frantoio*. Control plates for each of the bacteria and fungi strains were prepared using sterile water.

6.4 Results and discussion

6.4.1 Characterisation of pomace from two olive cultivars

The results of the characterisation of pomace and comparison of the two olive cultivars are shown in Table 6.1.

Table 6.1 Characteristics of fresh pomace and extracts of pomace from two olive cultivars originating from a two-phase olive oil processing system

Parameters	***Coratina***	***Frantoio***
Appearance	**Pomace:** Light olive green paste/pulp which includes broken stone and skin particles. **Aqueous extract:** Yellow, clear solution **Concentrated aqua residue:** Viscous, auburn brown residue. **Organic extract:** Bright green clear solution. **Concentrated organic residue:** Viscous dark green liquid	**Pomace**: Light violet paste/pulp which includes broken stone and skin particles. **Aqueous extract:** Yellow, clear solution **Concentrated aqua residue:** Viscous, auburn brown residue. **Organic extract:** Light olive green clear solution. **Concentrated organic residue:** Viscous dark green liquid
pH[a]	**Pomace:** 4.8 **Aqueous extract**: 5.4	**Pomace:** 3.9 **Aqueous extract**: 5.3
Moisture content	64.57 %	53.45 %
Conductivity[a]	Aqueous extract: 782 µS/cm	Aqueous extract: 754 µS/cm
Mass of residue obtained/ 15 g freeze-dried pomace (DW) (% recovery)	**Aqueous extract:** 2.38 ± 0.18 g (15.7 ± 1 %) **Organic extract**: 2.05 ± 0.15 g (13.7 ± 1 %)	**Aqueous extract**: 2.10 ± 0.14 g (14.0 ± 1 %) **Organic extract:** 1.87 ± 0.14 g (12.5 ± 1 %)

[a] Values are means of duplicate analyses and did not differ by more than 5 %

pH

The pH value of *Coratina* pomace (4.8) is higher than that of *Frantoio* (3.9), however the pH of their aqueous extracts were similar, namely, 5.4 and 5.3 respectively. With the exception of *Frantoio* pomace, the pH values fall within the range of values reported in literature for Spanish OMW (4.8 – 6.5) (53, 164).

Conductivity

Both aqueous extracts have a very low electrical conductivity at 25°C compared to the values found in the literature where Dermeche et al. (7) and Toscana et al. (53) reported values of 1.78-5.24 dS/m. and 0.9 – 4.7 dS/m respectively, for pomace. The

very low conductivity obtained is most probably due to the low dielectric constant of the relatively nonpolar solvent blend with which the compounds were extracted compared with water, making the extraction of ionic species very unlikely.

Moisture content

The moisture content of *Coratina* pomace was higher than that of *Frantoio*.

Yield of residue

The percentage recovery (12.5 – 14 %) obtained from the extractions was comparable with results reported by Obied et al. (17) for Soxhlet extraction (8.6 – 13.0 %) although the solvent systems varied. Lesage-Meessen et al. (140) found that the dry residue extracted for pomace from the two-phase system was 14.2 %.

6.4.2 Bioactive compounds comparison of the two cultivars

Results confirmed the efficiency of the ethanol, water and *n*-heptane combination for recovery of both low- and high- molecular weight bioactive compounds. The concentrations of the hydrophilic and lipophilic bioactive compounds obtained from the integrated Soxhlet extraction of the *Coratina* and *Frantoio* two-phase pomace are summarised in Figure 6.2. Oleuropein and squalene presented as the most prominent bioactive compounds in c*oratina* while hydroxytyrosol and squalene were equally prominent in *Frantoio*.

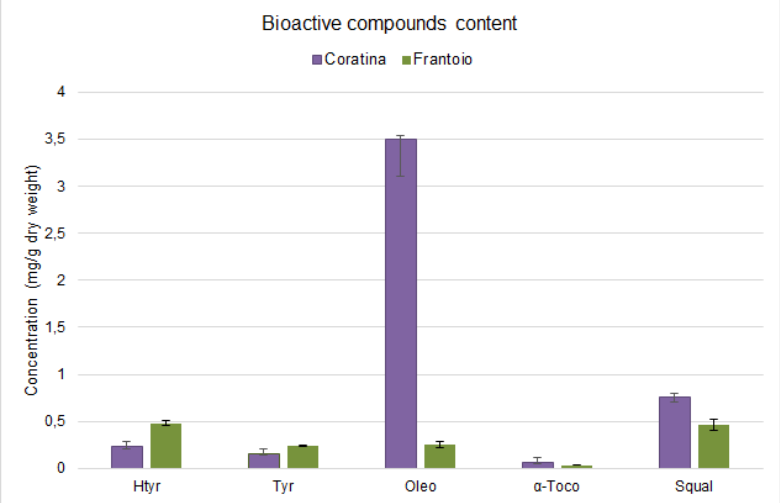


Figure 6.2 Quantification of bioactive compounds in both aqueous and organic extracts of two-phase system olive oil pomace from *Coratina* and *Frantoio*.

A complex mixture of hydrophilic and lipophilic bioactive compounds, in a wide range of polarities, which was difficult to resolve, was revealed by HPLC analysis of the integrated solvent blend extracts from the olive mill pomace.

Phenolic compounds in the aqueous phase, viz, hydroxytyrosol, tyrosol and oleuropein, and lipophylic compounds in the organic phase, viz, α-tocopherol and squalene, which are representative of the diverse structural types present in olives, were qualitatively identified by their retention times. In order to quantify each compound, peak areas in the chromatograms were determined and concentrations obtained from 6-point calibration curves of the relevant reference standards. The equations obtained from the linear regressions of each reference standard's calibration curve relating concentration (x) and peak area (y) were: y = 102574x + 2383.1 for hydroxytyrosol; y = 63077x + 2524.9 for tyrosol; y = 25018x – 1239.4 for oleuropein; y = 468403x + 122144 for α-tocopherol; and y = 591475x + 56808 for squalene. In all cases $r^2 > 0.99$.

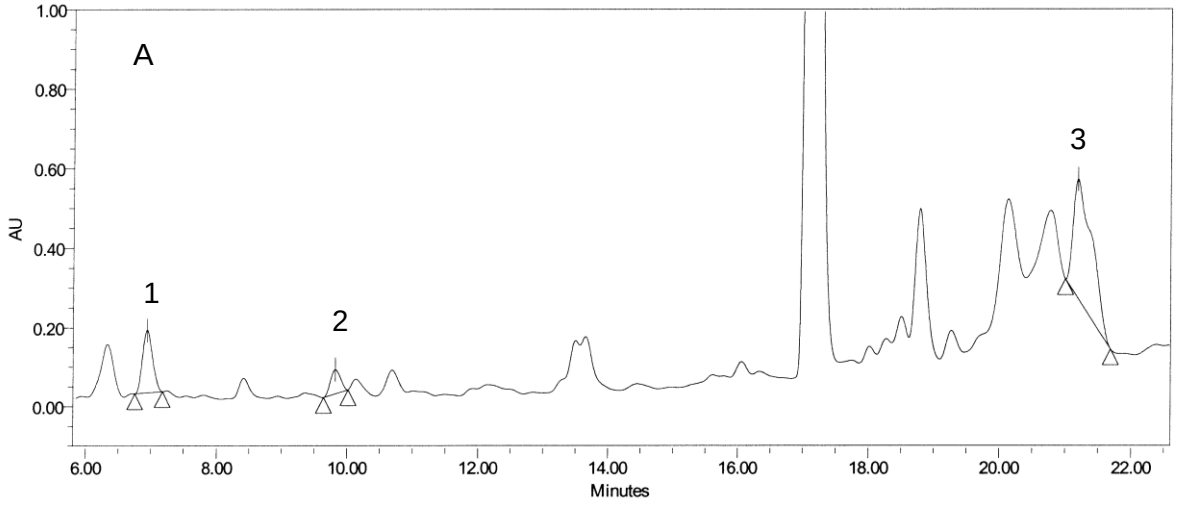
6.4.2.1 Hydrophilic bioactive compounds content

The average concentrations (in mg per gram dry pomace) of the hydrophilic and lipophilic bioactive compounds are compared for the two olive cultivars in Table 6.2.

Table 6.2 Retention times of hydrophilic phenols and average content (mg/g DW) in the early harvested two-phase system olive pomace.

			Coratina		***Frantoio***		
No	**Bioactive compound**	**R_t (min)**	**Ave content (mg/g DW)**	**Std dev**	**Ave content (mg/g DW)**	**Std dev**	**p-Value**
1	Hydroxytyrosol	6.9	0.240	0.028	0.482	0.022	3.16E-08
2	Tyrosol	9.8	0.153	0.015	0.237	0.008	9.44E-07
3	Oleuropein	21.2	3.495	0.385	0.258	0.034	1.48E-08

As can be observed, hydroxytyrosol, tyrosol and oleuropein content differ significantly ($p<0.05$) between *Coratina* and *Frantoio* two-phase olive pomace. Figure 6.3 shows typical HPLC UV-vis chromatograms of the optimum solvent blend-extracted *Coratina cv.* and *Frantoio cv*. aqueous extracts acquired at 280 nm.



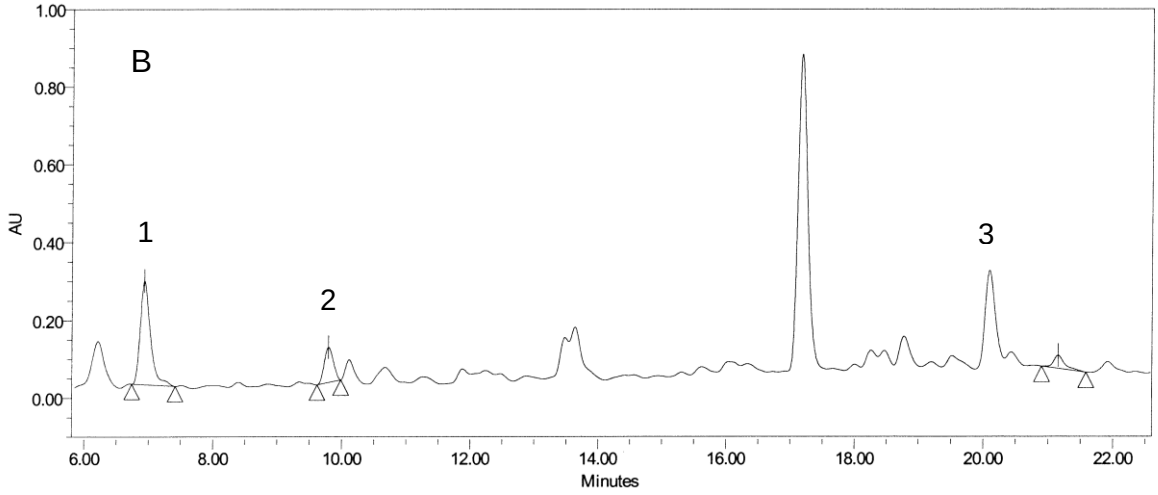


Figure 6.3 Representative HPLC chromatograms of (A) *Coratina* and (B) *Frantoio* olive pomace extract phenolic compounds profile acquired at 280 nm: (1) hydroxytyrosol, (2) tyrosol, (3) oleuropein.

The peculiarity of the *Coratina* chromatogram (A) was the larger peaks of other compounds or suspended material compared with the *Frantoio* extract (B) and seems to be an interesting source of polyphenols. Many other phenolic compounds represented by peaks observed due to the complex nature of the phenolic extracts, have not been identified in this study but could be confirmed during comparison with literature reports. The first part of each chromatogram is characterised by the presence of a series of simple phenols, i.e. hydroxytyrosol (peak 1), and tyrosol (peak 2) which elute within the first ten minutes (6.91 ± 0.03 min and 9.79 ± 0.05 min respectively) of the run due to their strong hydrophilic nature. The second part of the chromatogram contains numerous peaks corresponding to phenols with higher molecular weight, i.e. in this study oleuropein (peak 3) which eluted at 21.14 ± 0.05 min. Other higher molecular weight phenols described in literature include the dialdehydic forms of elenolic acid linked either to hydroxytyrosol (3,4-DHPE-EDA) or to tyrosol (p-HPEA-EDA) which elutes before oleuropein (between peaks 2 and 3) while lignan, an isomer of the oleuropein aglycon (3,4-DHPEA-EA) and a hydroxytyrosol derivative, namely, hydroxytyrosol acetate (3,4-DHPEA-AC) elutes after the major secoiridoid in *Olea europaea* L (after peak 3) (165).

Hydroxytyrosol was present in different amounts (refer to Table 6.2) between the two cultivars, namely 0.482 mg/g for *Frantoio* compared to 0.240 mg/g for *Coratin*a. Tyrosol concentration of 0.236 mg/g was also higher in *Frantoio* than the 0.153 mg/g in *Coratina*. Generally, it is accepted that hydroxytrosol is a hydrolysis product of oleuropein, a process which is enhanced by ripening. The greatest difference was

observed for oleuropein which was very abundant, with a concentration of 3.502 mg/g in *Coratina,* compared to 0.244 mg/g in *Frantoio.* Alagna et al. (35) have qualified the *Coratina* as a high phenolics cultivar while Esti et al. (166) and Sivakumar et al. (167) found oleuropein was more abundant than hydroxytyrosol during harvesting of small unripe fruit (1.44 mg/g oleuropein versus 0.30 mg/g hydroxytyrosol of pulp). The results obtained in this study were in agreement with these literature findings.

The phenolic profile of olive fruits is significantly modified during fruit development and ripening due to enzymatic activity and degradation of secoiridoids (i.e. oleuropein) in a manner closely related to cultivar characteristics realising an increase of total hydroxytyrosol, total tyrosol and oleuropein aglycone and a rapid decrease of oleuropein (168, 169). It is important to note that the malaxation process during olive oil processing/extraction furthermore seems to greatly affect the levels of the bioactive compounds between the concentrations present in the drupe and in the paste. The accumulation of simple phenol alcohols and aglycones can be attributed to the increased enzymatic function of β-glucosidase activity for hydrolysis of the glucosides, namely, oleuropein and ligstroside (170).

6.4.2.2 Lipophilic bioactive compounds

Both α-tocopherol and squalene are antioxidants present in the unsaponifiable fraction of the extracts. Results of the simultaneous extraction and determination of these two bioactive compounds in Table 6.3 show an average content for α-tocopherol of 0.061 mg/g dry weight in *Coratina* and 0.033 mg/g dry weight in *Frantoio* while significantly higher levels of squalene were observed in both the C*oratina* and *Frantoio* extracts (0.75 mg/g and 0.46 mg/g dry weight, respectively). α-Tocopherol concentration is approximately 46% higher and squalene concentration 38% higher in *Coratina* than in *Frantoio*. It has been found in literature that the cultivar significantly influences the content of lipophilic compounds (171).

Table 6.3 Retention times of lipophilic bioactives and average content (mg/g DW) in the early harvested two-phase system olive pomace.

		Coratina		***Frantoio***		
Bioactive compound	**R_t (min)**	**Ave content (mg/g DW)**	**Std dev**	**Ave content (mg/g DW)**	**Std dev**	**p-value**
α-Tocopherol	12.5	0.061	0.005	0.033	0.002	1.93E-06
Squalene	17.6	0.753	0.050	0.464	0.055	3.63E-06

Figure 6.4 shows a comparison of the HPLC chromatograms of the lipophilic compounds obtained from the *Coratina* and *Frantoio* extracts. Identification retention times were 12.54 ± 0.01 min and 17.61 ± 0.01 min for α-tocopherol (peak 4) and squalene (peak 5), respectively. Although the peak shapes were similar, the peak areas for α-tocopherol and squalene were nearly double in the *Coratina* extract and this is supported with literature findings (172).

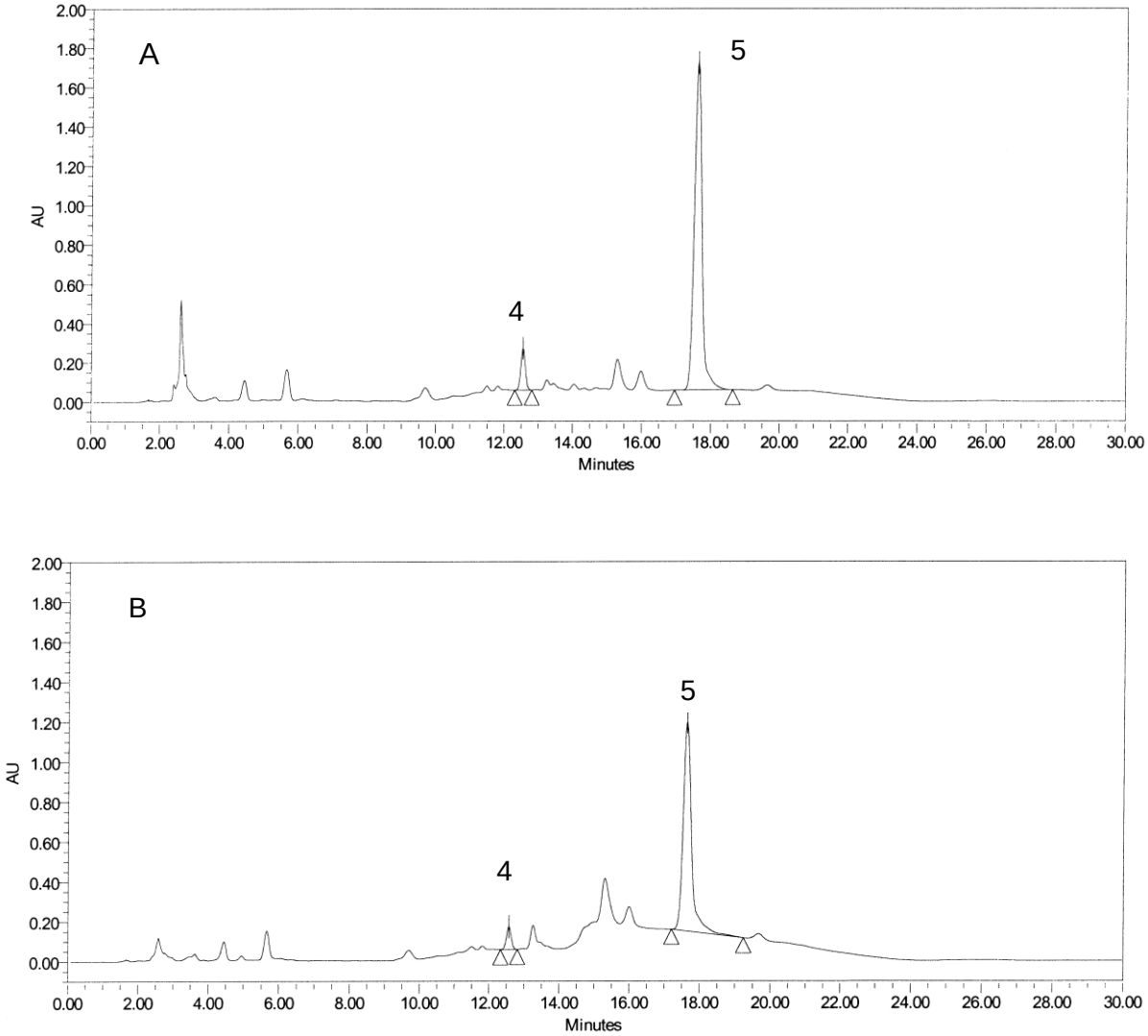


Figure 6.4 HPLC lipid bioactives profiles of (A) *Coratina* and (B) *Frantoio* olive pomace extract acquired at 210 nm: (4) α-tocopherol and (5) squalene.

6.4.3 Total phenolic content comparison of the two cultivars

The results of the total phenol content after statistical evaluation of the replicates are shown in Table 6.4.

Table 6.4 Average total phenol content in the early harvested two-phase system olive pomace

	Coratina	***Frantoio***	**P-value**
Total phenols (mg GAE/g DW)	20.41	8.07	3.05493E-06

Coratina contains a significantly higher amount of total phenols, (20.41 mg gallic acid equivalent or mg GAE/g dry weight) compared with *Frantoio* (8.07 mg GAE/g dry weight of pomace). This agrees with Mulinacci et al. (108) who reported the amounts of phenolic compounds in a sample of ripe *Frantoio* olives added up to approximately 8.10 g/kg. According to Boskou et al. (33), the degree of ripeness was the variable which has the greatest impact on phenolic content followed by the extraction system, variety and place of growing. Results from this study are supported by literature where the highest total phenol content was found in extra virgin oils from *Coratina* olives obtained from early harvesting (26, 173). Total phenolic content of the olive fruit is therefore affected by the cultivar as well as the degree of ripeness as has been observed in the current study.

6.4.4 Antioxidant capacity comparison of the two cultivars

To model the % antioxidant activity results of the aqueous extracts from the eight *Coratina* and four *Frantoio* cultivars, a multiple regression model was used where the response variable is (ŷ).

After stepwise regression, the model in Equation 6.1 which explains 82% of the variation in the observed % antioxidant activity, was obtained. The coefficients and the p-values are shown in Table 6.5.

$$\hat{y} = b_0 + b_1 Conc + b_2 Conc.C + b_3 C \qquad \text{Equation 6.1}$$

where,

ŷ: Predicted % antioxidant activity

b_i: i = 0, 1, 2, 3 are the estimated regression coefficients

Conc: concentration of extract solution (µg/ml)

C – Cultivar: Coratina = 0; Frantoio = 1

Table 6.5 Regression model obtained for the % antioxidant activity for *Coratina* and *Frantoio* pomace.

	B	Std Err	T	p-value
Intercept	2.24692	0.347667	6.46286	0.000000
Conc	0.1962	0.003952	3.27233	0.000000
Conc*C	-0,.15499	0.06830	-8.05017	0.000000
C	-1.23405	0.599386	-2.05885	0.040570

All of the terms in Table 6.5 are significant ($p < 0.05$) which means that both the cultivar and the concentration of the bioactives influences the antioxidant capacity. The interaction term Conc*C indicates that the effect of concentration on the antioxidant capacity is influenced by the cultivar and vice versa. This can be clearly seen in Figure 6.5 which shows the profile plots obtained from the regression model for the two olive cultivars and compares the percentage antioxidant activity with increasing concentration.

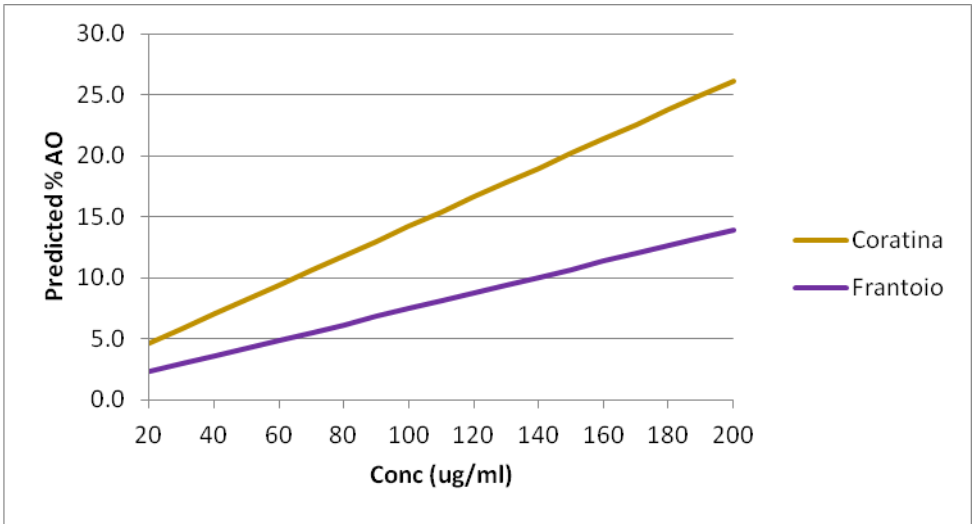


Figure 6.5 Profile plots for % antioxidant activity as a function of concentration for the two olive cultivars.

From the profile plots in Figure 6.5, it is clear that the *Coratina* cultivar extract displays a greater antioxidant activity than the *Frantoio* extract at all concentrations investigated. Both cultivars showed concentration-dependent DPPH radical scavenging activity between 20 µg/ml and 200 µg/mL ($R^2 = 0.82$). Furthermore, the average increase of the % AO activity for every unit increase in concentration for the cultivar *Coratina* is significantly higher than for *Frantoio*. This result is most probably attributed to the higher concentrations of oleuropein in the *Coratina* extract compared with the *Frantoio* extract (3.495 mg/g DW and 0.258 mg/g DW respectively) as well as higher total phenolic content (20.41 mg GAE/g DW and 8.07 mg GAE/g DW respectively). Extracts with the highest levels of secoiridoids and derivatives, mainly oleuropein, verbascoside, hydroxytyrosol glucoside, and oleuropein aglycone have also shown significant antiradical potential (17).

A positive linear correlation coefficient ($R^2 = 0.954$) was found by Leouifoudi (161) between total phenolic content and antioxidant capacity in olive cake extracts

measured using the DPPH method indicating that 95% of the antioxidant activity was due to the contribution of the phenolic compounds.

Olive phenolic compounds have been clearly proven to be antioxidants and free radical scavengers *in vitro*, *and in vivo* and both oleuropein and its hydrolysed product, hydroxytyrosol, have been designated as the major contributors exerting protection against oxidative damage in skin cells (174). These key phenolic compounds with their catechol (ortho-diphenolic) group, are able to scavenge the peroxyl radicals and break propagation chain reactions producing very stable resonance structures as they become non-reactive radicals (175). Having this catecholic structure in their molecular assembly, both hydroxytyrosol and oleuropein can donate a hydrogen to the reactive oxygen species formed during oxidation and neutralise the free radical. Once the phenolic compound donates the hydrogen atom, it becomes a free radical which is essentially non-reactive because the aromatic ring stabilises the newly formed radical through resonance stabilisation. In addition, hydroxytyrosol and oleuropein, having two hydroxyl groups, have superior antioxidant properties compared to compounds lacking this catechol moiety, e.g. tyrosol, which has only one hydroxyl group. This can be explained by the high electron donating effect of the second hydroxyl group. According to a study conducted by Galano et al. (176), the main mechanism for radical scavenging activity determined between hydroxytyrosol and tyrosol was found instigated predominantly by hydroxytyrosol.

As shown in Figure 6.6, the antioxidant properties of the *o*-diphenols (hydroxytyrosol and oleuropein) are associated with their ability to form intramolecular hydrogen bonds between the hydroxyl group and the phenoxyl radical; therefore, the catechol avoids the chain propagation by donating a hydrogen radical to alkylperoxyl radicals (ROO·) formed in the initiation step of lipid oxidation (72).

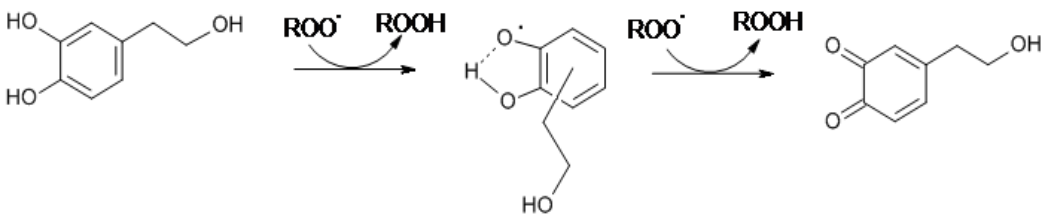


Figure 6.6 Schematic description of radical scavenging activity of hydroxytyrosol.

A study conducted by Hussain et al. (177) indicated that co-administration of hydroxytyrosol and hydrocortisone provide additional anti-inflammatory and antioxidant

benefits in atopic dermatitis treatment whereas oleuropein stimulates re-epithelialisation *in vivo* (178).

Moreover, hydroxytyrosol has an amphiphilic structure where the partition coefficient value is approximately 1 ($P_{o/w}$ ~1) (179). This means that its concentration in cytosol and membranes is practically the same and that hydroxytyrosol will readily cross membranes to provide protection in both lipid and aqueous cellular compartments (water-lipid interface). The hydrophilic glucoside (sugar moiety) in oleuropein probably prevents oleuropein from crossing membranes, realising its poor bioavailability. However, *in vivo* oleuropein can be metabolised into the aglycone hydroxytyrosol in the intestine or the liver; and *in vitro*, oleuropein is transformed to the corresponding aglycone due to enzymatic hydrolysis with *β*-glucosidase which subsequently increases its lipid solubility for inclusion into the olive oil droplets (cytoplasmic inclusion) **(180)**. Hydroxytyrosol is a potent scavenger of several reactive oxygen species, i.e. superoxide radical ($O_2^{\bullet-}$), hydroxyl radical (OH•) and peroxynitrite (ONOOH) (175, 179). Carrasco-Pancorbo et al. (181) showed that the radical scavenging capacity decreases in the following order: hydroxytyrosol > 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA) > oleuropein > dihydroxyphenylethanol-elenolic acid monoaldehyde (3,4-DHPEA-EA).

6.4.5 Antimicrobial activity

The inhibition zones formed by the 1 mg/g phenolic extract solutions against two fungal strains and two bacterial strains are shown in Figures 6.6 – 6.9 in Appendix 6.1 Table 6.6 shows a summary of the average measured diameters of the inhibition zones.

Table 6.6 Results of average measured inhibition zones for antimicrobial activity of *Coratina* and *Frantoio* extracts.

Microorganisms	**Inhibition zones average (mm)**	
Fungi:	***Coratina***	***Frantoio***
Aspergillus niger	8.6 ± 1.4 mm	13.3 ± 1.7 mm
Penicillium notatum	11.0 ± 1.0 mm	12.0 ± 0.0 mm
Bacteria:		
Staphylococcus aureus (Gram +)	13.4 ± 1.4 mm	13.4 ± 1.0 mm
Escherichia coli (Gram -)	12.0 ± 1.0 mm	14.0 ± 4.0 mm

The inhibition zones obtained suggest antimicrobial activity of the 1 mg/ml extract solutions for both cultivars. The inhibitions zones ranged between 8.6 mm and 14.0 mm. According to the measured inhibition zones of both fungal and bacterial growths in the presence of the two olive cultivar extracts namely, *Coratina* and *Frantoio*, antifungal

and antibacterial (gram-positive and gram-negative) activity presented compared to the control. The *Frantoio* extract generally exhibited a higher antifungal and antimicrobial effect than the *Coratina* extract which had a higher average hydroxytyrosol (0.482 mg/g DW) and similar tyrosol content (0.237 mg/g DW) compared with the *Coratina* extract (0.240 mg/g DW and 0.153 mg/g DW respectively). However, the average oleuropein content of 3.495 mg/g DW was significantly higher in the *Coratina* extracts versus the 0.258 mg/g DW in the *Frantoio* extracts.

Hydroxytyrosol, tyrosol and oleuropein content together with other unidentified phenolic bioactives contribute to the antimicrobial properties of the extracts, however, the results indicate that possibly the antimicrobial effect of the hydroxytyrosol and tyrosol is higher than the oleuropein. According to Dagdelen (182), phenolic extracts from oils of different olive cultivars exert antimicrobial activity against various bacteria (gram positive and gram-negative) and hydroxytyrosol and tyrosol were found to be the major phenolic compounds. A study by Bisignano et al. (183) has shown that oleuropein (the bitter principle of olives) and hydroxytyrosol (derived from oleuropein by enzymatic hydrolysis) inhibit or delay the rate of growth of a range of bacteria and fungi.

Moreover, the anti-microbial activity of phenolic compounds is well known and is related to their ability to denature proteins following penetration of the cell membranes. As antibacterial, they act by causing the leaking of cytoplasmic constituents such as protein, glutamate or potassium and phosphate from the bacteria as a result of cell membrane damage or disruption of cell peptidoglycans (183, 184). Gram-negative bacteria have a lipopolysaccharide component in their outer membrane that makes them more resistant to antibacterial compounds.

6.5 Conclusion

Total phenol content (Folin-Ciocalteu method) and antioxidant capacity (DPPH assay method) were determined and both properties were found to be higher in *Coratina* than in *Frantoio* extracts This result is attributed to the higher concentrations of oleuropein in the *Coratina* extract compared with the *Frantoio* extract (3.495 mg/g DW and 0.258 mg/g DW respectively). However, the hydroxytyrosol content in the *Frantoio* extract was almost double that of the *Coratina* (0.482 mg/g DW and 0.240 mg/g DW respectively) but was present in much smaller amount in the *Coratina* compared with the oleuropein, and tyrosol content was also higher in *Frantoio* than in *Coratina* (0.237 mg/g DW and 0.153 mg/g DW respectively).

This finding indicates that phenolic compounds greatly contribute to the total antioxidant capacity of olives due to their ability to scavenge free radicals. The observed antioxidant activity can also be correlated to the chemical composition of the evaluated extracts, which were rich in hydroxytyrosol (*Frantoio*) and oleuropein (*Coratina*) and their derivatives. The higher antioxidant activity in *Coratina* can be attributed to the high oleuropein content. The aqueous phase extracts in the current study were shown to exhibit inhibitory action against microorganisms such as bacteria and fungi. Although triplicate analysis were not practised during determination of the antimicrobial activity, results obtained showed antifungal and antibacterial (gram-positive and gram-negative) activity compared to the control in the presence of the two olive cultivar extracts namely, *Coratina* and *Frantoio*,

Thus the olive pomace extracts containing phenolic compounds hydroxytyrosol, tyrosol and oleuropein as well as the lipophilic antioxidants, α-tocopherol and squalene, can be utilised for their therapeutic activities.

CHAPTER 7

STABILITY EVALUATION OF POMACE EXTRACTS

7.1 Introduction

The purpose of the stability study was to determine the stability of the hydrophilic and lipophilic extracts obtained from the freeze-dried two-phase olive pomace when stored in suitable containers at different temperature conditions over a period of 12 weeks. Extracts from the two olive cultivars (*Olea europaea* var., *Frantoio* and *Coratina* prepared in Chapter 6) were used in order to identify the impact of the different storage conditions on the degradation of the bioactive compounds, hydroxytyrosol, tyrosol and oleuropein in the aqueous extracts; and α-tocopherol and squalene in the organic extracts. The total phenol content and the percentage antioxidant activity of the aqueous extracts were also monitored over this time period.

7.2 Objectives

The objectives of the stability evaluation were:

a. To evaluate the influence of temperature and time on the stability of the hydrophilic and lipophilic bioactive compounds, total phenol content and antioxidant activity in the olive pomace extracts
b. To compare the extract stability of the two different olive cultivars namely, *Coratina* and *Frantoio*;
c. To determine the optimum storage conditions.

7.3 Experimental methodology

Olive pomace extracts were obtained using the combined Soxhlet extraction and optimum extraction conditions as previously determined and described in Chapter 5. The separated aqueous and organic extracts were concentrated to dryness using an R-210 Rotavapor System (Buchi, Germany) at a temperature of approximately 50°C to 60°C respectively. The extracts were reconstituted to 25 ml with the relevant solvents (both HPLC grade) namely, pure methanol for the hydrophilic compounds and pure isopropyl alcohol for the lipophilic compounds. Each of these solutions was transferred into 30 ml amber glass bottles, sealed with a screw top and clearly labelled. HPLC analysis, total phenols and percentage antioxidant activity were performed as described in Chapter 6.

7.4 Storage conditions

A total of 24 samples in 30 ml amber glass bottles were placed at four different storage conditions for stability evaluation as shown in Table 7.1. Aliquots of each sample were taken and analysed at the indicated time intervals. The grouping of the 24 samples were as follows: 6 bottles (2 replicates of *Coratina* aqueous phase (AqC) and 2 replicates of *Coratina* organic phase (LpC) plus 1 *Frantoio* aqueous phase (AqF) and 1 *Frantoio* organic phase (LpF)). Each of the samples were stored at temperatures of: -20°C ± 2°C, 5°C ± 2°C, 25°C ± 2°C and 40°C ± 2°C.

Table 7.1 Stability storage conditions and sampling plan for testing.

Storage condition	Time intervals			
	T0	**T1**	**T2**	**T3**
-20 °C ± 2 °C	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF
5 °C ± 2 °C	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF
25 °C ± 2 °C	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 AqC, 2 LpC 1 x AqF, 1x LpF
40 °C ± 2 °C	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF	2 x AqC, 2 x LpC 1 x AqF, 1x LpF

T0: Initial T1: one month (4 weeks) T2: two months (8 weeks) T3: 3 months (12 weeks)

7.5 Results and discussion

During storage, a colour change was observed for both the aqueous and organic extracts at ambient (25°C) and accelerated (40°C) conditions as seen in Figure 7.1. Samples became either yellow brown or dark brown when stored at ambient and 40°C respectively. The browning of the aqueous extract solutions during storage is suggestive of oxidation and polymerisation reactions. The polyphenoloxidase (PPO) enzyme activity leads exclusively to the formation of oxidation products responsible for the typical brown colouration (185)

For the organic samples (Figure 7.2), the extracts turned a darker green when stored at 40°C as a result of oxidation or photo-oxidation over time as the headspace increases during sampling. According to a study by Rastrelli et al. (186) on the rate of degradation of α-tocopherol, squalene, phenolics, and polyunsaturated fatty acids in olive oil at different storage conditions, the main changes in the concentrations of these compounds were associated with the oxygen level in the half-empty glass bottles. Periodic measurements of peroxide values provided evidence on the extent of oxidation or photo-oxidation and the rated or degradation of α-tocopherol, *o*-diphenols, squalene and polyunsaturated fatty acids. The quantitative analysis of the constituents

was performed by HPLC-DAD, HPLC-MS and GC-MS. According to the study, α-tocopherol was the first molecule to be oxidised (-20% after 2 months, -92% after 12 months). Squalene and *o*-diphenols were protected in the first months by the presence of α-tocopherol, and their content decreased significantly after 6 and 8 months, respectively, in the half-empty bottles.

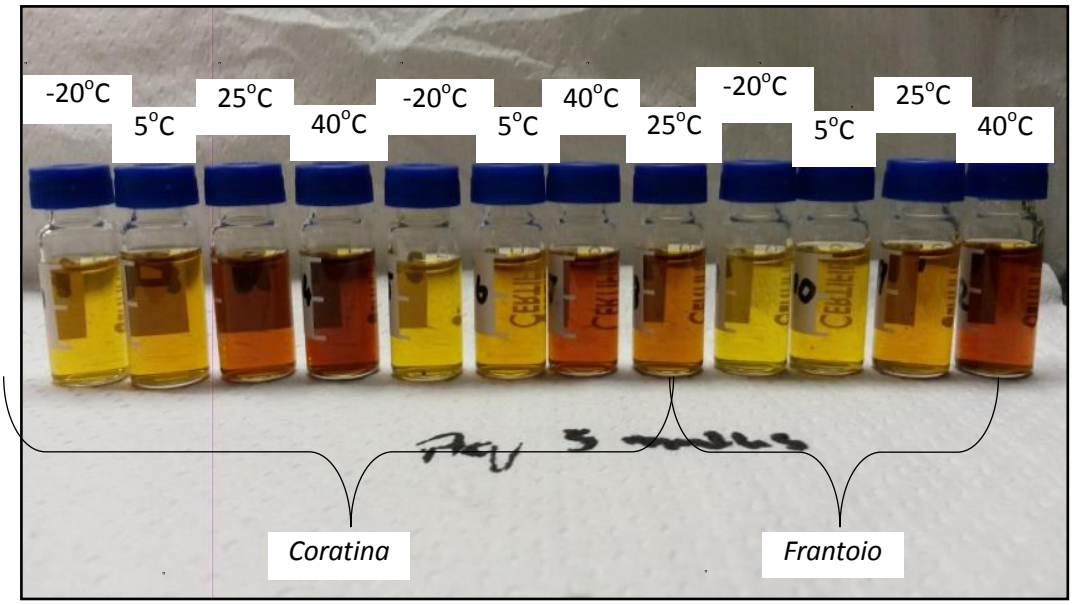


Figure 7.1 Colour changes of aqueous extracts filled into HPLC vials for analysis after a 12 week storage period.

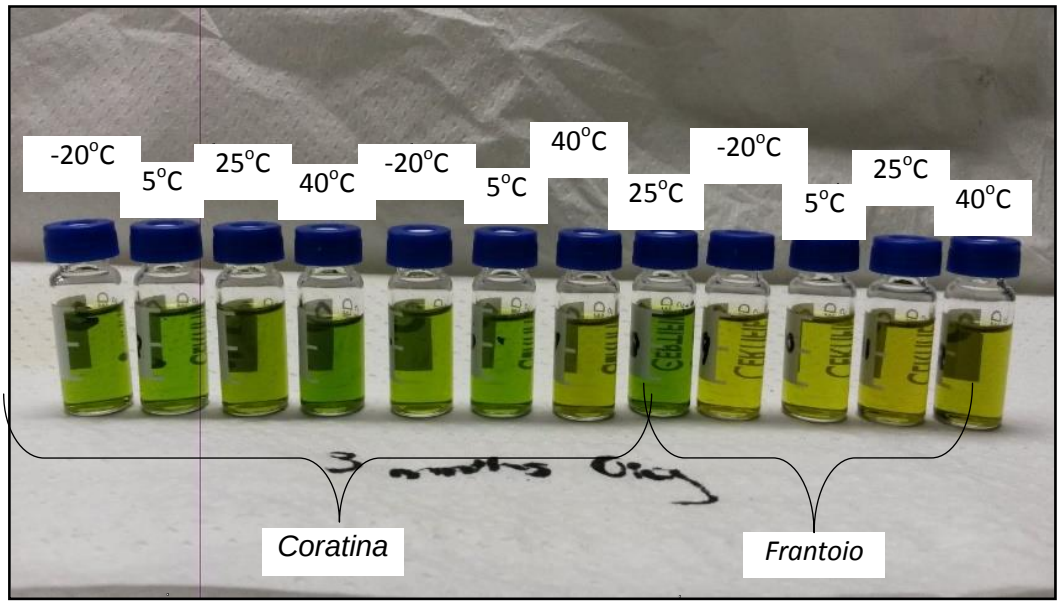


Figure 7.2 Colour changes of organic extracts filled into HPLC vials for analysis after a 12 week storage period.

Multiple regression models were used to analyse all the data obtained from the various storage conditions at the different time intervals using the content of each bioactive

compound, the total phenolic content and the percentage antioxidant capacity as responses in each model. Variability at T0 was due to difference in the different batches of extract recovered. The variables in each multiple regression model were storage temperature, time, olive cultivar and in the case of percent antioxidant capacity, an additional variable was concentration of extract. Each model started with the assumption of a full set of terms including quadratic terms and variable interactions and after stepwise regression to eliminate insignificant terms, a final model was obtained for each response.

The full model is:

$$\hat{y} = b_0 + b_1C + b_2Time + b_3Temp + b_4Time^{\wedge 2} + b_5Temp^{\wedge 2} + b_6C.Time + b_7C.Temp + b_8Time.Temp \quad \text{Equation 7.1}$$

where,

$\hat{y}$: predicted response

b_i: i = 0, 1, 2, …, 8 are the estimated regression coefficients

C – Cultivar: *Coratina* = 0; *Frantoio* = 1

Time: 4 – 12 weeks

Temp: -20°C to 40°C

The profile plots for each response (bioactives content, total phenols and % antioxidant activity) generated from the regression models show the predicted average response within the experimental domain.

7.5.1 Bioactives content (mg/g dry weight)

7.5.1.1 Hydroxytyrosol content

The results of the hydroxytyrosol content as obtained by HPLC analysis over the 12 week storage time period are shown in Table 7.2.

Table 7.2 Hydroxytyrosol content in pomace from two olive cultivars measured over a period of 12 weeks at four storage temperature conditions.

		Time intervals			
		T0	**T1**	**T2**	**T3**
Temp	***Coratina* samples**	mg/g dry weight			
-20°C	AqC1	0.197	0.232	0.260	0.261
	AqC5	0.204	0.226	0.244	0.251
5°C	AqC2	0.235	0.343	0.399	0.499
	AqC6	0.246	0.340	0.401	0.483
25°C	AqC3	0.237	0.581	0.900	1.301
	AqC8	0.261	0.574	0.853	1.156
40°C	AqC4	0.274	1.270	1.944	2.823
	AqC7	0.265	1.079	1.649	2.356
Temp	***Frantoio* samples**	mg/g dry weight			
-20°C	AqF9	0.500	0.519	0.526	0.577
5°C	AqF10	0.483	0.495	0.555	0.748
25°C	AqF11	0.452	0.554	0.712	0.976
40°C	AqF12	0.495	0.850	1.226	1.566

To model the data, a multiple regression model where the response variable (ŷ) is logged, i.e. Ln(ŷ), was used. This was necessary to improve the fit of the model. After stepwise regression, the model in equation 7.2 and Table 7.3 was obtained. This model explains 99.2% of the variation in the observed hydroxytyrosol. The validation of the model can be found in Appendix 7.1A.

$$Ln(\hat{y}) = b_0 + b_1C + b_2Time + b_3Temp + b_4Temp^{\wedge 2} + b_5C.Temp + b_6Time.Temp$$

Equation 7.2

where,

ŷ: Predicted hydroxytyrosol

bi: i = 0, 1, 2, …, 6 are the estimated regression coefficients

C – Cultivar: *Coratina* = 0; *Frantoio* = 1

Time: 4 – 12 weeks

Temp: -20°C to 40°C

R-Sq = 99.2%

Table 7.3 Final regression model obtained for the concentration of hydroxytyrosol.

	b_i	**Std Err**	**T**	**p-value**
Intercept	-1,36687	0,036649	-37,2959	0,000000
C	0,40663	0,026843	15,1484	0,000000
Time	0,04242	0,003875	10,9493	0,000000
Temp	0,01500	0,001449	10,3529	0,000000
Time$^{\wedge 2}$	0,00034	0,000028	12,1487	0,000000
Time*Temp	0,00138	0,000151	9,1573	0,000000
C*Temp	-0,02045	0,001043	-19,6089	0,000000

All of the terms in Table 7.3 are significant ($p < 0.05$). Figure 7.3 shows the profile plots obtained from the regression model for the two olive cultivars at four different storage temperatures over the 12 week period while Figure 7.4 compares the effect of storage temperature for the two cultivars after 12 weeks of storage.

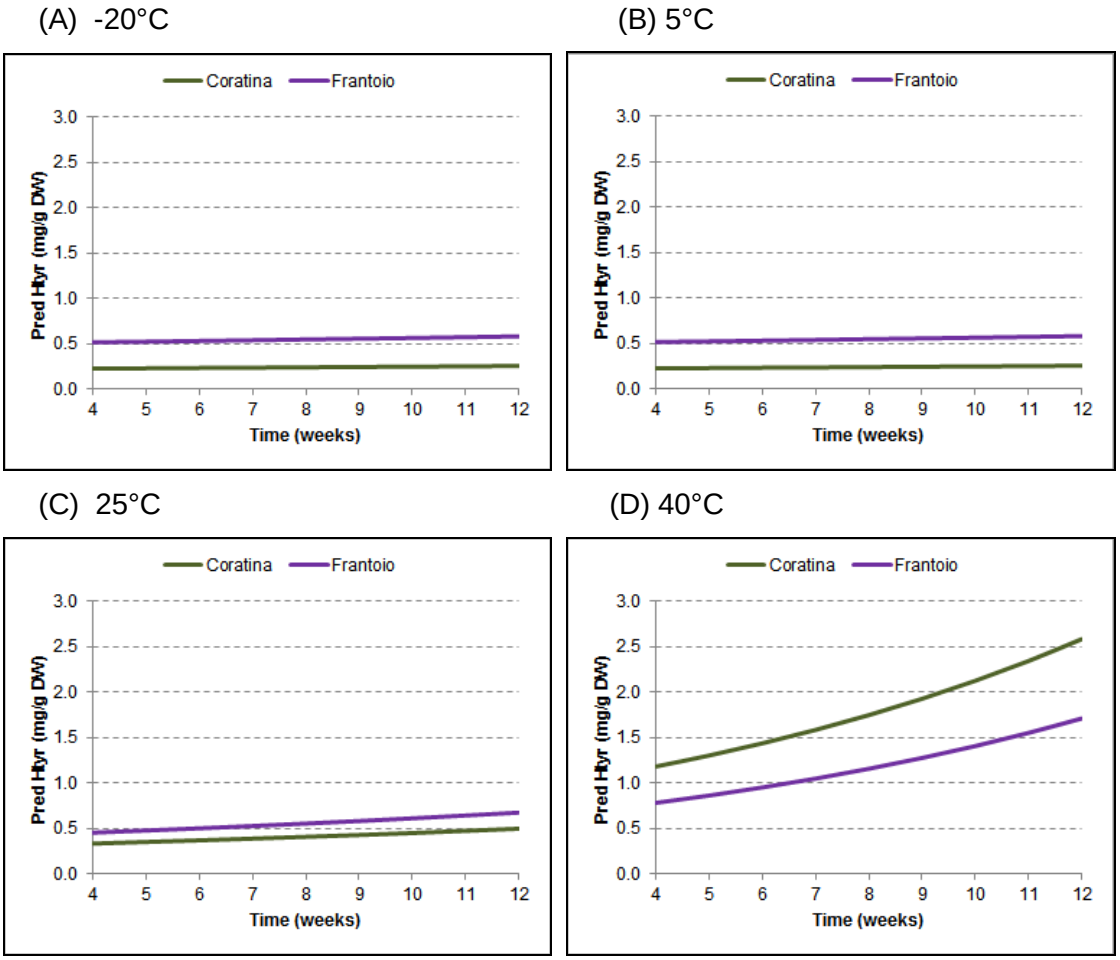


Figure 7.3 Profile plots showing the effect of time on the hydroxytyrosol content in *Coratina* and *Frantoio* extracts at four different storage temperatures.

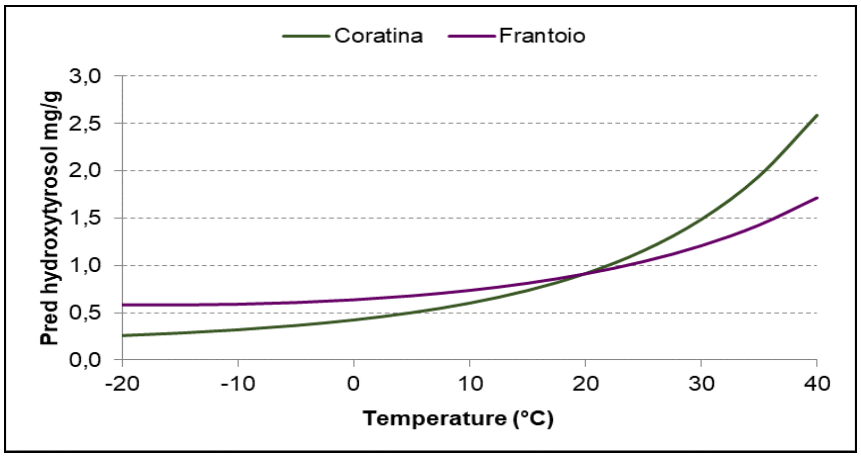


Figure 7.4 Profile plots of the effect of temperature on the hydroxytyrosol content in *Coratina* and *Frantoio* extracts after 12 weeks storage.

The impact of temperature and time on the hydroxytyrosol content was investigated at temperatures ranging from -20°C to 40°C and a time period from 4 weeks (T1) to 12 weeks (T3). The model is slightly exponential. According to Figures 7.3 and 7.4, the influence of temperature on the hydroxytyrosol content is positively influenced by time and vice versa since an increase in temperature and storage time improved the concentration thereof in both *Coratina* and *Frantoio* aqueous extracts. This is due to enhanced oleuropein degradation, both as a result of hydrolysis by endogenous glucosidase of the complex phenol and increased activity of oxidative enzymes such as polyphenol oxidase, lipoxygenase and peroxidase (17, 187). However, the effect of temperature is lower for *Frantoio* than for *Coratina* as seen in Figure 7.4.

7.5.1.2 Tyrosol

The results of the tyrosol content as obtained by HPLC analysis over the 12 week storage time period are shown in Table 7.4.

Table 7.4 The tyrosol content in pomace from two olive cultivars measured over a period of 12 weeks when stored at four temperature conditions.

		Time intervals			
		T0	**T1**	**T2**	**T3**
Temp	**Coratina samples**	mg/g dry weight			
-20°C	AqC1	0.133	0.116	0.105	0.254
	AqC5	0.131	0.106	0.087	0.245
5°C	AqC2	0.157	0.134	0.140	0.319
	AqC6	0.159	0.162	0.127	0.322
25°C	AqC3	0.154	0.171	0.207	0.484
	AqC8	0.169	0.196	0.234	0.430
40°C	AqC4	0.170	0.307	0.623	0.750
	AqC7	0.153	0.280	0.337	0.640
Temp	**Frantoio samples**	mg/g dry weight			
-20°C	AqF9	0.243	0.102	0.127	0.241
5°C	AqF10	0.234	0.144	0.133	0.232
25°C	AqF11	0.227	0.162	0.172	0.383
40°C	AqF12	0.242	0.257	0.251	0.663

To model the data, a multiple regression model where the response variable (ŷ) is logged, i.e. Ln(ŷ), was used to improve the fit of the model. After stepwise regression, the model in equation 7.3 and Table 7.5 was obtained. This model explains 97% of the variation in the observed tyrosol. The validation of the model can be found in Appendix 7.1 B.

$$Ln(\hat{y}) = b_0 + b_1C + b_2Time + b_3Temp + b_4Time^{\wedge 2} + b_5C.Temp^{\wedge 2} + b_6C.Temp$$

Equation 7.3

where,

ŷ: predicted tyrosol

bi: i = 0, 1, 2, …, 6 are the estimated regression coefficients

C: Cultivar *Coratina* = 0; *Frantoio* = 1

Time: 4 – 12 weeks

Temp: -20°C to 40°C

R-Sq = 97%

Table 7.5 Final regression model obtained for the concentration of tyrosol.

	b_i	Std Err	t	p-value
Intercept	-1,31628	0,136529	-9,64107	0,000000
C	-0,05210	0,041765	-1,24738	0,222590
Time	-0,28717	0,038081	-7,54106	0,000000
Temp	0,01305	0,001258	10,38083	0,000000
Time^2	0,02437	0,002357	10,33977	0,000000
Temp ^2	0,00023	0,000045	5,22581	0,000015
C*Temp	-0,00356	0,001642	-2,16980	0,038662

All of the terms in Table 7.5 are significant ($p < 0.05$). Figure 7.5 shows the profile plots obtained from the regression model for the two olive cultivars at four different storage temperatures over the 12 week period while Figure 7.6 compares the effect of storage temperature for the two cultivars after 12 weeks of storage.

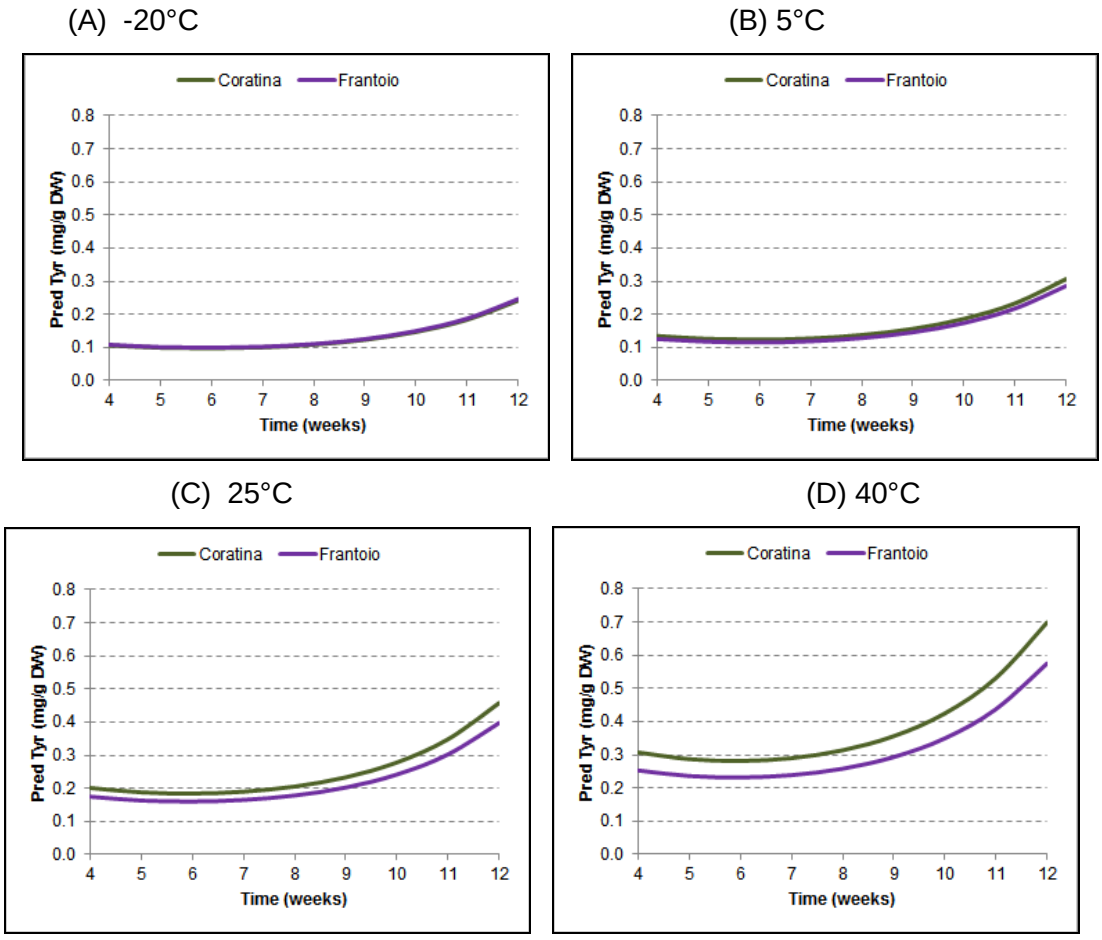


Figure 7.5 Profile plots showing the effect of time on the tyrosol content in *Coratina* and *Frantoio* extracts at four different storage temperatures.

The impact of temperature and time on the tyrosol content was investigated at temperatures ranging from -20°C to 40°C and time from 4 weeks (T1) to 12 weeks (T3). According to Figures 7.5, for the first 8 weeks, both cultivars showed reasonable stability at all temperatures. After 8 weeks, tyrosol content increased exponentially in both cultivars with a very similar trend.

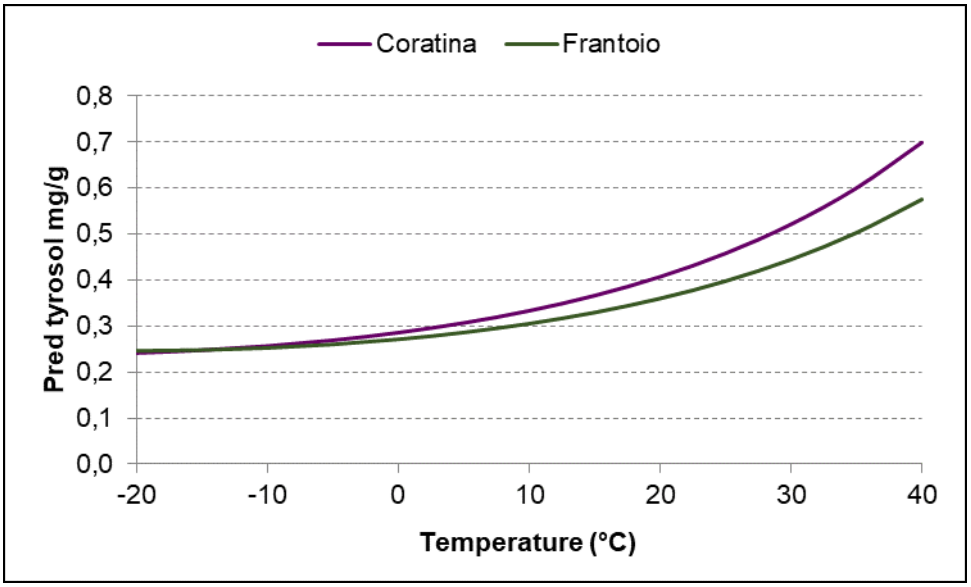


Figure 7.6 Profile plots of the effect of temperature on the tyrosol content in *Coratina* and *Frantoio* extracts after 12 weeks storage.

As the temperature increases, tyrosol content in *Coratina* showed a higher increase compared to *Frantoio* (Figure 7.6). The increase in temperature and storage time improved the yields of tyrosol in both *Coratina* and *Frantoio* aqueous extracts due to enhanced ligstroside (an ester consisting of tyrosol and elenolic acid) degradation as a result of hydrolysis by glucosidase and esterase of the complex phenol (71).

7.5.1.3 Oleuropein

The results of the oleuropein content as obtained by HPLC analysis over the 12 week storage time period is shown in Table 7.6.

Table 7.6 The oleuropein content in pomace from two olive cultivars measured over a period of 2 weeks when stored at four temperature conditions.

		Time intervals			
		T0	**T1**	**T2**	**T3**
Temp	***Coratina* samples**	mg/g dry weight			
-20°C	AqC1	3.069	2.791	3.267	3.223
	AqC5	3.089	3.108	2.917	2.960
5°C	AqC2	3.550	3.266	3.126	2.855
	AqC6	3.673	3.561	3.134	2.915
25°C	AqC3	3.556	2.655	2.117	1.774
	AqC8	3.094	1.769	1.413	1.044
40°C	AqC4	4.124	2.071	1.776	1.375
	AqC7	3.803	2.672	2.008	1.603
Temp	***Frantoio* samples**	mg/g dry weight			
-20°C	AqF9	0.288	0.151	0.189	0.091
5°C	AqF10	0.267	0.104	0.158	0.101
25°C	AqF11	0.209	0.098	0.130	0.088
40°C	AqF12	0.268	0.104	0.130	0.088

To model the data, a multiple regression model where the response variable (ŷ) is logged, i.e. Ln(ŷ), was used to improve the fit of the model. After stepwise regression, the model in equation 7.4 and Table 7.7 was obtained. This model explains 98.9% of the variation in the observed oleuropein. The validation of the model can be found in Appendix 7.1 C.

$$Ln(\hat{y}) = b_0 + b_1C + b_2Time + b_3Temp + b_4Time^{\wedge 2} + b_5Temp^{\wedge 2} + b_6C.Temp \quad \text{Equation 7.4}$$

where,

ŷ: predicted oleuropein

bi: i = 0, 1, 2, …, 6 are the estimated regression coefficients

C – Cultivar: *Coratina* = 0; *Frantoio* = 1

Time: 4 – 12 weeks

Temp: -20°C to 40°C

R-Sq = 98.9%

Table 7.7 Final regression model obtained for the concentration of oleuropein.

	b_i	**Std Err**	**t**	**p-value**
Intercept	0,87125	0,219159	3,9754	0,000428
C	-3,09965	0,069166	-44,8150	0,000000
Time	0,10542	0,061088	1,7257	0,095050
Temp	-0,00809	0,002078	-3,8922	0,000536
$Time^{\wedge 2}$	-0,00848	0,003779	-2,2440	0,032632
$Temp^{\wedge 2}$	-0,00019	0,000072	-2,5791	0,015244
C*Temp	0,00692	0,002687	2,5768	0,015328

All of the terms in Table 7.7 are significant ($p < 0.05$) except for time ($p = 0.095$). Time could not be eliminated as Time^2 contributed to the exponential decline of oleuropein during the storage period ($p = 0.032$).

Figure 7.7 shows the profile plots obtained from the regression model for the two olive cultivars at four different storage temperatures over the 12 week period while Figure 7.8 compares the effect of storage temperature for the two cultivars after 12 weeks of storage.

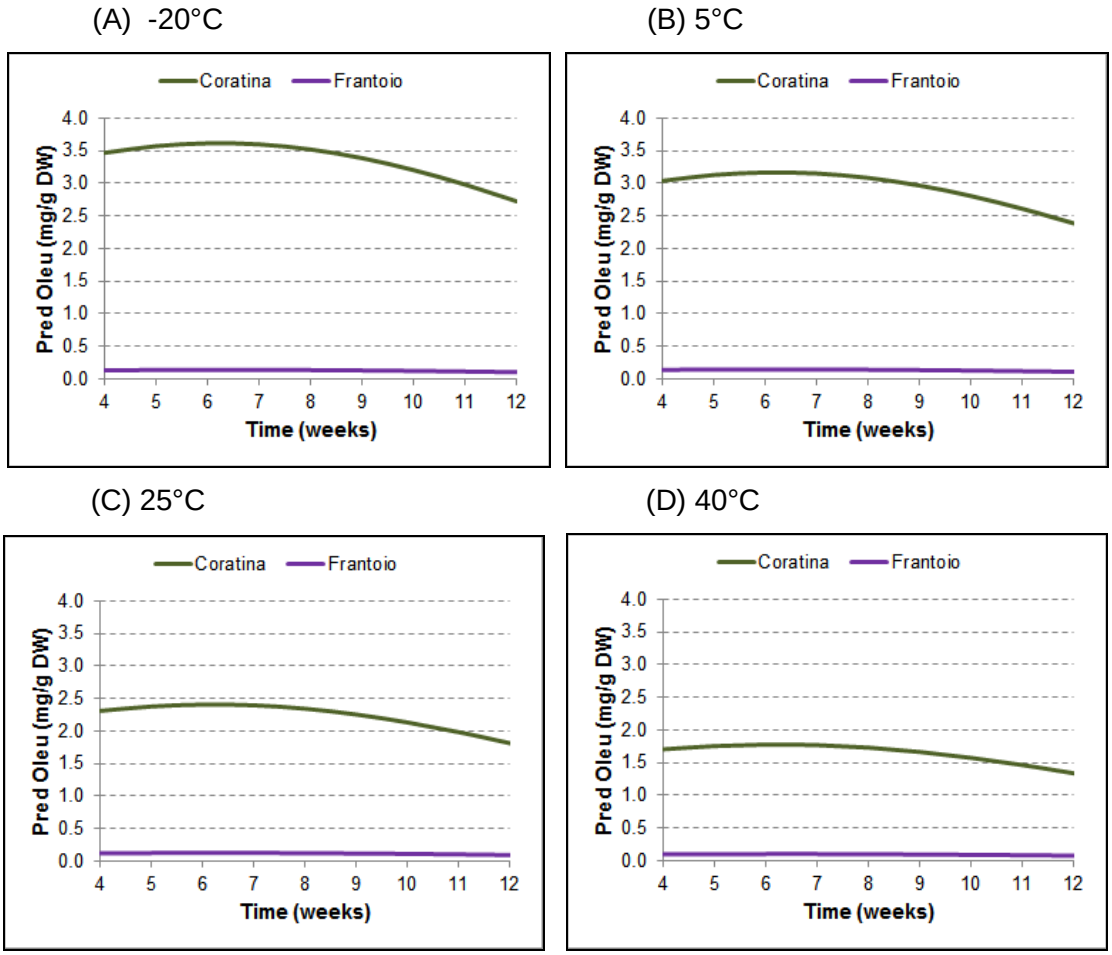


Figure 7.7 Profile plots showing the effect of time on the oleuropein content in *Coratina* and *Frantoio* extracts at four different storage temperatures.

Over time and increased temperature, the oleuropein content remained constant for the *Frantoio* cultivar whereas in the *Coratina* cultivar a significant decrease occurred after 8 weeks.

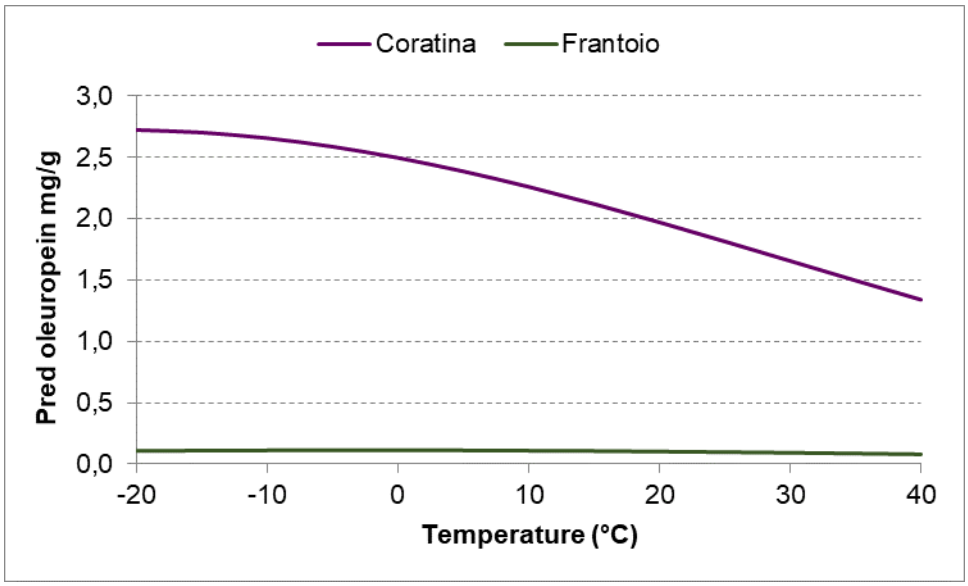


Figure 7.8 Profile plots of the effect of temperature on the oleuropein content in *Coratina* and *Frantoio* extracts after 12 weeks storage.

As seen in Figure 7.8, as the temperature increases, the oleuropein content in the *Coratina* extract decreases significantly ($p < 0.05$) while oleuropein remains fairly constant in the *Frantoio* extract. When these extracts are stored below -10°C, the content of oleuropein remains relatively constant. The changes in bioactive concentration at various temperatures and time intervals predicted by the models can be explained by the biodegradation process of complex phenols (i.e. oleuropein and ligstroside) to simple phenols (i.e. hydroxytyrosol, elenolic acids, tyrosol, and more) as shown in Figure 7.9.

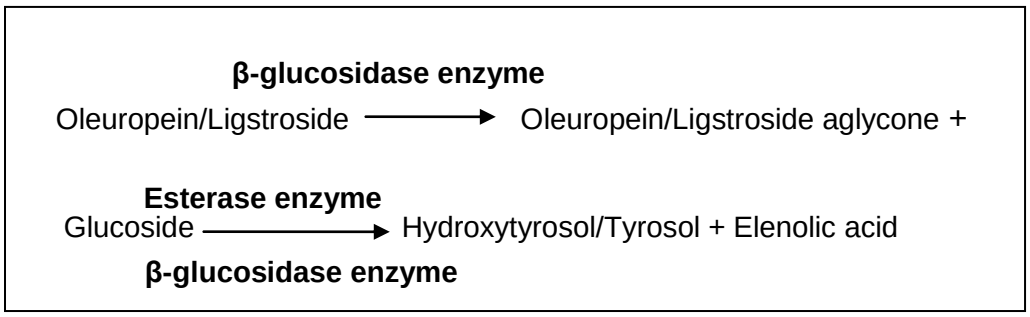


Figure 7.9 Glucosidase catalyses hydrolysis of the glucosidic bonds.

From the HPLC chromatograms (Figure 7.10), the decrease of the oleuropein content (Peak 3) and increase of both hydroxytyrosol (Peak 1) and tyrosol (Peak 2) content can be seen when comparing the peak areas of samples stored at 5°C and those stored at 40°C.

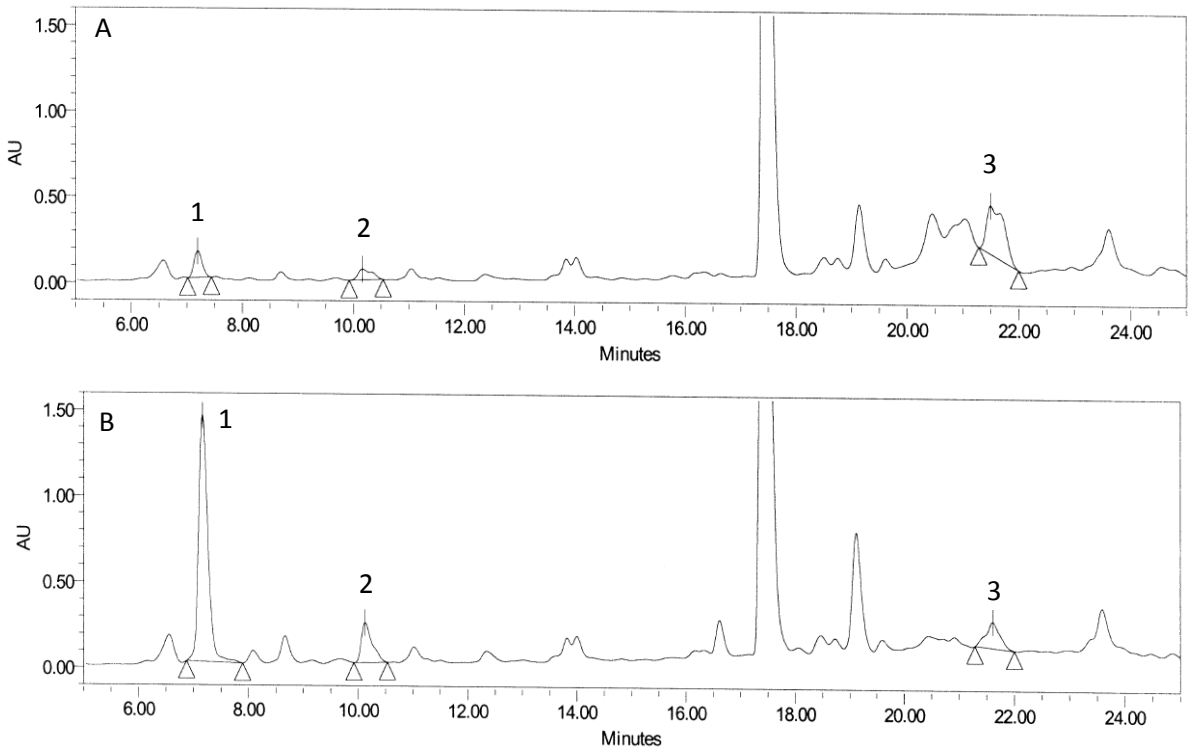


Figure 7.10 HPLC chromatograms for aqueous samples from *Coratina* stored (A) at 5°C and (B) at 40°C: hydroxytyrosol (peak 1 at 7.1 min) and tyrosol (peak 2 at 10.0 min) peak areas increase while oleuropein (peak 3 at 21.5 min) peak area decreases.

In summary, the concentration of oleuropein progressively decreased with increase of temperature and time for *Coratina* whereas these relevant metabolites (hydroxytyrosol and tyrosol) exhibited the opposite trend for both *Coratina* and *Frantoio*. The decline of oleuropein and increase of hydroxytyrosol and tyrosol can be described by two chemical reactions namely, hydrolysis and oxidation during storage in this study. The enzymes involved in degradation are β-glucosidase and polyphenyl oxidase (PPO). Oxygen, a co-substrate for PPO, is required for both enzymatic and non-enzymatic oxidation and could have been introduced during handling of the samples and consequent headspace increase during sampling over the 12 week period (17). Ryan et al. (188) proposed that demethyloleuropein derived from esterase activity (breaking down the ester and giving rise to either elenolic acid glucoside or demethyloleuropein), may also act as a substrate for the β-glucosidase enzyme.

7.5.1.4 α-Tocopherol

The results of the α-tocopherol content as obtained by HPLC analysis over the 12 week storage time period are shown in Table 7.8.

Table 7.8 The α-tocopherol content in pomace from two olive cultivars measured over a period of 12 weeks when stored at four temperature conditions.

		Time interval			
		T0	**T1**	**T2**	**T3**
Temp	***Coratina* samples**	mg/g dry weight			
-20°C	OC1	0.066	0.063	0.058	0.064
	OC5	0.057	0.054	0.041	0.050
5°C	OC2	0.058	0.055	0.051	0.051
	OC6	0.064	0.061	0.055	0.056
25°C	OC3	0.060	0.058	0.054	0.053
	OC8	0.052	0.050	0.042	0.045
40°C	OC4	0.066	0.062	0.055	0.057
	OC7	0.067	0.062	0.056	0.063
Temp	***Frantoio* samples**	mg/g dry weight			
-20°C	OF9	0.035	0.035	0.029	0.030
5°C	OF10	0.034	0.034	0.030	0.030
25°C	OF11	0.030	0.031	0.024	0.027
40°C	OF12	0.034	0.036	0.030	0.033

To model the data, a multiple regression model where the response variable (ŷ) is logged, i.e. Ln(ŷ), was used to improve the fit of the model. After stepwise regression, the model in Equation 7.5 and Table 7.9 was obtained. This model explains 89.3% of the variation in the observed α-tocopherol. The validation of the model can be found in Appendix 7.1 D.

$\hat{y} = b_0 + b_1C + b_2Time + b_3Time^{\wedge 2}$ Equation 7.5

where,

ŷ: predicted α-tocopherol

b_i: i = 0, 1, 2, 3 are the estimated regression coefficients

C – Cultivar: *Coratina* = 0; *Frantoio* = 1

Time: 4 – 12 weeks

R-Sq = 89.3%

Table 7.9 Final regression model obtained for the concentration of α-tocopherol.

	b_i	**Std Err**	**t**	**p-value**
Intercept	-2,48311	0,128750	-19,2862	0,000000
C	-0,57583	0,036018	-15,9872	0,000000
Time	-0,11379	0,036391	-3,1270	0,003746
$Time^{\wedge 2}$	0,00647	0,002251	2,8734	0,007156

All of the terms in Table 7.9 are significant ($p < 0.05$). Figure 7.11 shows the profile plots obtained from the regression model for the two olive cultivars and compares the effect of storage time on the α-tocopherol content.

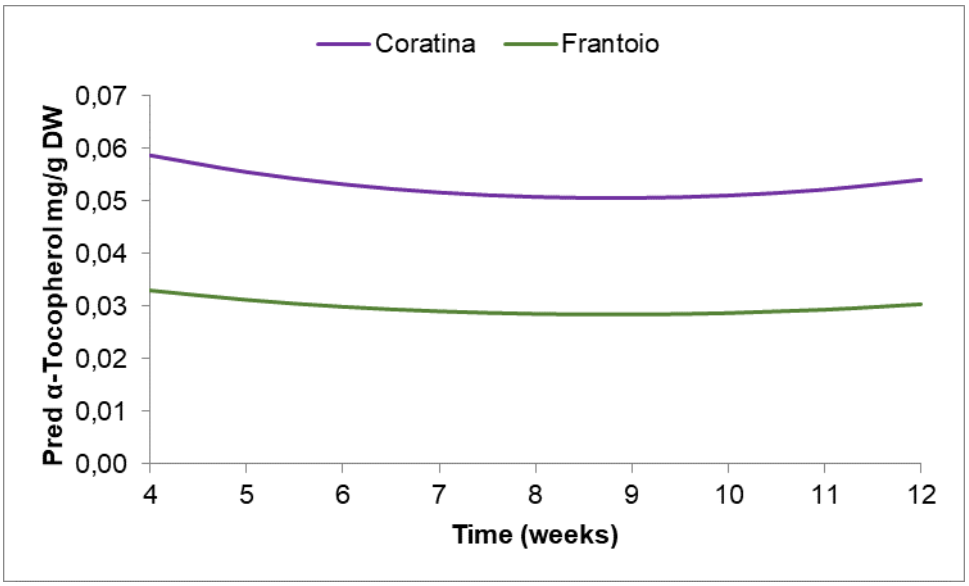


Figure 7.11 Profile plot showing the effect of time on the α-tocopherol content in *Coratina* and *Frantoio* extracts.

The regression model shows that the α-tocopherol content only depends on the cultivar and the storage time. A slight but significant exponential decrease in the α-tocopherol content was observed for both *Coratina* and *Frantoio* extracts ($p < 0.05$) over the 12 week storage period and the slopes indicate a similar rate of decline for both cultivars. This decline could be related to α-tocopherol exerting its antioxidant activity during storage as previously discussed. The different temperatures had no effect on the content. A slight upwards curvature could be explained by analytical error since the correlation is only 89%.

7.5.1.5 Squalene

The results of the squalene content as obtained by HPLC analysis over the 12 week storage time period are shown in Table 7.10.

Table 7.10 The squalene content in pomace from two olive cultivars measured over a period of 12 weeks when stored at four temperature conditions.

		Time interval			
		T0	**T1**	**T2**	**T3**
Temp	***Coratina* samples**	mg/g dry weight			
-20°C	OC1	0.731	0.708	0.689	0.778
	OC5	0.731	0.618	0.482	0.642
5°C	OC2	0.770	0.645	0.646	0.652
	OC6	0.799	0.706	0.665	0.697
25°C	OC3	0.749	0.646	0.591	0.664
	OC8	0.671	0.562	0.527	0.578
40°C	OC4	0.839	0.738	0.672	0.757
	OC7	0.737	0.645	0.659	0.687
Temp	***Frantoio* samples**	mg/g dry weight			
-20°C	OF9	0.527	0.426	0.381	0.421
5°C	OF10	0.435	0.403	0.389	0.408
25°C	OF11	0.403	0.395	0.361	0.402
40°C	OF12	0.489	0.453	0.429	0.469

To model the data, a multiple regression model where the response variable (ŷ) is logged, i.e. Ln(ŷ), was used to improve the fit of the model. After stepwise regression, the model in Equation 7.6 and Table 7.11 was obtained. This model explains 86.3% of the variation in the observed squalene. The validation of the model can be found in Appendix 7.1 E.

$\hat{y} = b_0 + b_1C + b_2Time + b_3Time^2$ Equation 7.6

where,

ŷ: predicted squalene

bi: i = 0, 1, 2, 3 are the estimated regression coefficient

C – Cultivar: *Coratina* = 0; *Frantoio* = 1

Time: 4 – 12 weeks

R-Sq = 86.3%

Table 7.11 Final regression model obtained for the concentration of squalene.

	b_i	**Std Err**	**t**	**p-value**
Intercept	-0,178492	0,117442	-1,5198	0,138371
Cultivar	-0,457677	0,032854	-13,9304	0,000000
Time	-0,081089	0,033195	-2,4428	0,020275
Time^2	0,005280	0,002053	2,5713	0,014984

All of the terms in Table 7.11 are significant ($p < 0.05$). Figure 7.12 shows the profile plots obtained from the regression model for the two olive cultivars and compare the effect of storage time on the squalene content.

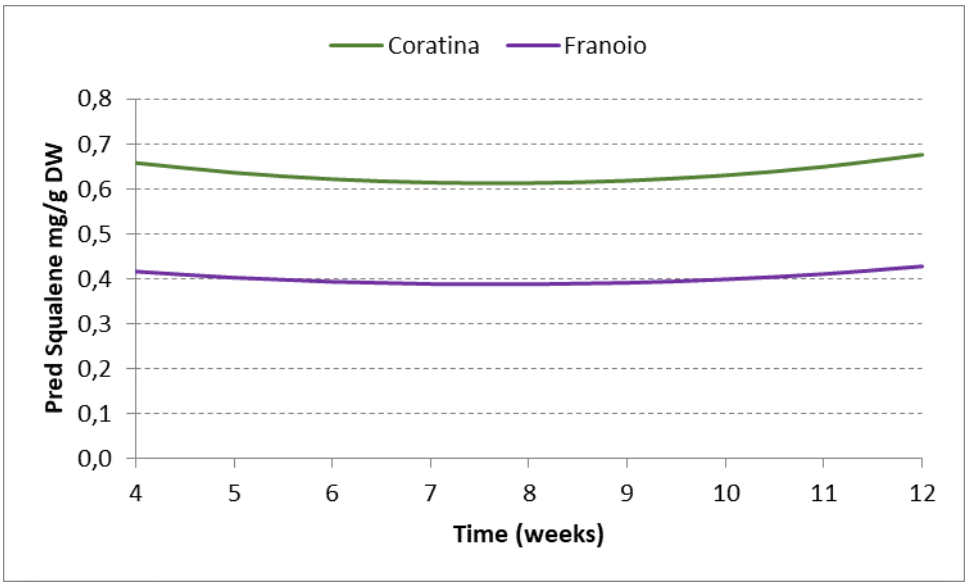


Figure 7.12 The effect of storage time on the squalene content in *Coratina* and *Frantoio* extracts.

Both *Coratina* and *Frantoio* extracts showed a slight decline in squalene content during the stability storage period of 12 weeks. This trend is independent of temperature. The trendline for squalene shows a slight deviation from linearity with an upwards curvature. Since the correlation is only 86%, this could be explained by analytical error.

In summary, the lipophilic compounds for the *Coratina* and *Frantoio* extract samples showed a slight decline in both α-tocopherol and squalene content over the 12 week storage period, independent of temperature. These compounds are oxidized and according to literature, α-tocopherol should be the compound initially degrading as it protects squalene in the first weeks acting as an antioxidant, before a significant decrease will be observed (186).

From the HPLC chromatograms obtained (Figure 7.13), it can be seen that both α-tocopherol (Peak 4) and squalene (Peak 5) peak areas retained their shape but showed a slight decrease in peak areas.

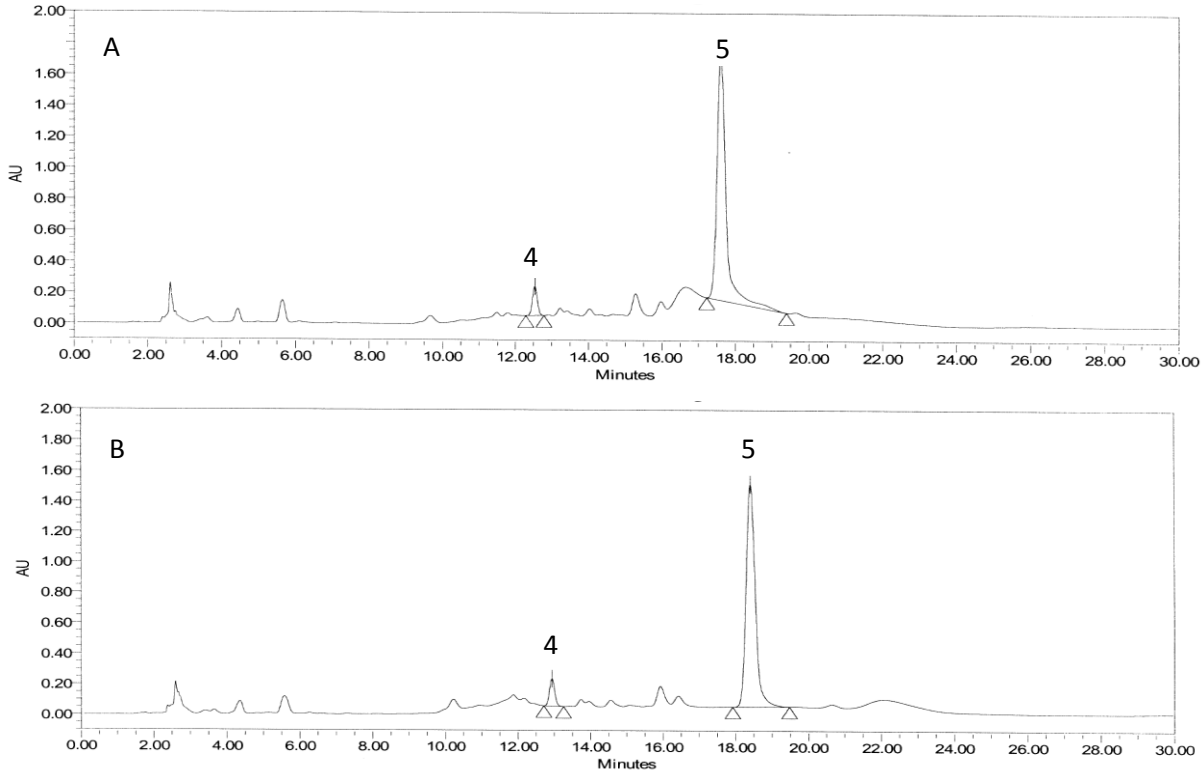


Figure 7.13 HPLC chromatograms of organic samples from *Coratina* showing peak area 4 of α-tocopherol at 12.5 min and peak area 5 of squalene at 18.5 min at (A) the initial analysis time (T0) and (B) after 12 weeks (T3).

7.5.2 Total phenolic content

The results of the total phenolic content as obtained by spectrophotometric analysis over the 12 week storage time period are shown in Table 7.12.

Table 7.12 Results of the total phenolic content (mg GAE/g dry weight) for the aqueous extracts from two olive cultivars stored at various temperature conditions over a period of 12 weeks.

		Time interval			
Temp	**Sample**	**T0**	**T1**	**T2**	**T3**
-20°C	**AqC1**	18.33	17.28	18.62	16.11
	AqC5	22.78	23.56	21.72	20.20
	AqF9	6.64	7.95	5.44	4.65
5°C	**AqC2**	21.06	22.24	22.88	19.55
	AqC6	22.92	19.94	19.78	17.32
	AqF10	8.05	8.34	3.89	7.07
25°C	**AqC3**	22.89	19.18	17.84	23.62
	AqC8	15.99	18.38	18.62	16.32
	AqF11	8.71	7.03	6.99	6.86
40°C	**AqC4**	19.96	17.80	16.68	19.11
	AqC7	19.33	20.64	19.40	19.78
	AqF12	8.88	7.93	5.05	5.95

After stepwise regression of the initial model, the final quadratic model in Equation 7.7 and Table 7.13 was obtained. This model explains 89.0% of the variation in the observed total phenolic content. The validation of the model can be found in Appendix 7.2.

$\hat{y} = b_0 + b_1C + b_2Time + b_3Time^2 + b_4C.Time$ Equation 7.7

where,

$\hat{y}$: predicted total phenolic content

b_i: i = 0, 1, 2, …, 4 are the estimated regression coefficients

C – Cultivar: *Coratina* = 0; *Frantoio* = 1

Time: 0 – 12 weeks

R-Sq = 89.0%

Table 7.13 Final regression model obtained for the total phenolic content.

	b_i	Std Err	t	p-value
Intercept	20.5356	0.422588	48.5948	0.000000
C	-13.2739	0.658008	-20.1728	0.000000
Time	0.2883	0.148724	1.9383	0.054648
Time^2	-0.0812	0.011649	-6.9736	0.000000
C*Time	0.2943	0.087839	3.3503	0.001043

All of the terms in Table 7.13 are significant ($p < 0.05$). The term related to time has a borderline p-value (0.0546) but was retained in the model because its quadratic term was significant. Figure 7.14 shows the profile plots obtained from the regression model for the two olive cultivars and compares the effect of storage time on the total phenolic content.

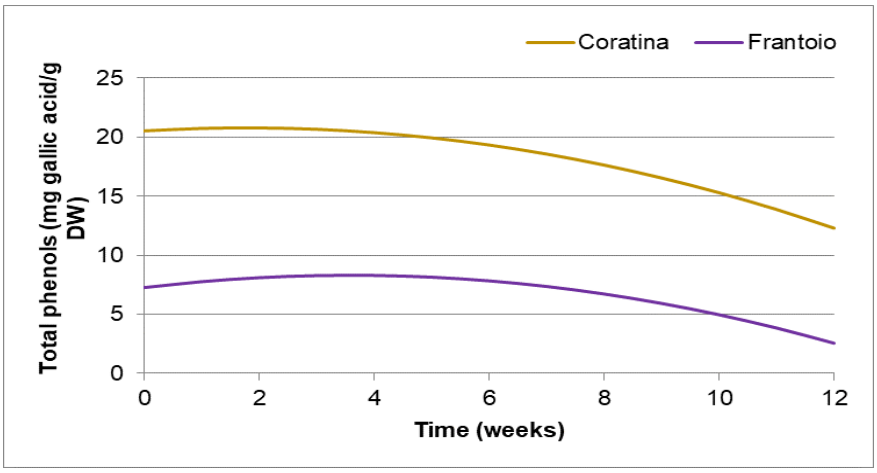


Figure 7.14 Profile plots showing the change in total phenolic content for *Coratina* and *Frantoio* as a function of storage time.

The results show that at any particular time period there was no significant change in total phenol content before and after storage at different temperatures ($p > 0.05$) for both the *Coratina* and *Frantoio* pomace extracts. However, a significant loss in total phenol content was observed after a period of 12 weeks for both cultivars ($p < 0.05$), which was independent of temperature as reflected in Figure 7.13. The rate of degradation of phenolic compounds was similar for both cultivars. The slight increase of total phenol content in *Frantoio* between 2 and 4 weeks storage could be due to analytical error.

7.5.3 % Antioxidant activity

To model the % antioxidant activity of the aqueous extracts from the eight *Coratina* and four *Frantoio* cultivars obtained over a 12 week period storage (Results in Appendix 7.4), a multiple regression model was used where the response variable is ŷ. Refer to Appendix 7.3 for validations of the % antioxidant activity model.

After stepwise regression, the final model in Equation 7.8 and Table 7.8, which explains 86.9% of the variation in the observed % antioxidant activity, was obtained.

$$\hat{y} = b_0 + b_1C + b_2Time + b_3Temp + b_4C + b_5Time^{\wedge 2} + b_6Temp^{\wedge 2} + b_7Time.Conc + b_8C.Time + b_9C.Temp \quad \text{Equation 7.8}$$

where,

ŷ: Predicted % antioxidant activity

bi: i = 0, 1, 2, …, 9 are the estimated regression coefficient

C - Cultivar: *Coratina* = 0; *Frantoio* = 1;

Time: 0 – 12 weeks

Temp: -20°C to 40°C

Table 7.14 Final regression model obtained for the concentration % antioxidant activity.

	B	**Std Err**	**t**	**p-value**
Intercept	4.71766	0.468361	10.0727	0.000000
C	-2.06533	0.609177	-3.3904	0.000725
Time	-0.34961	0.132026	-2.6481	0.008224
Temp	-0.01739	0.011581	-1.5014	0.133565
Conc	0.11721	0.004189	27.9770	0.000000
Time^2	-0.02314	0.009863	-2.3467	0.019135
Temp^2	-0.00113	0.000447	-2.5238	0.011766
Time*Conc	0.00184	0.000516	3.5628	0.000384
C*Time	0.57893	0.072480	7.9874	0.000000
C*Conc	-0.08200	0.004901	-16.7312	0.000000

The Pareto chart below (Figure 7.15) clearly illustrates which variables have the greatest cumulative effect on the percentage antioxidant activity of the extracts and the two major contributors are: concentration of the extract and the effect of cultivar on the concentration.

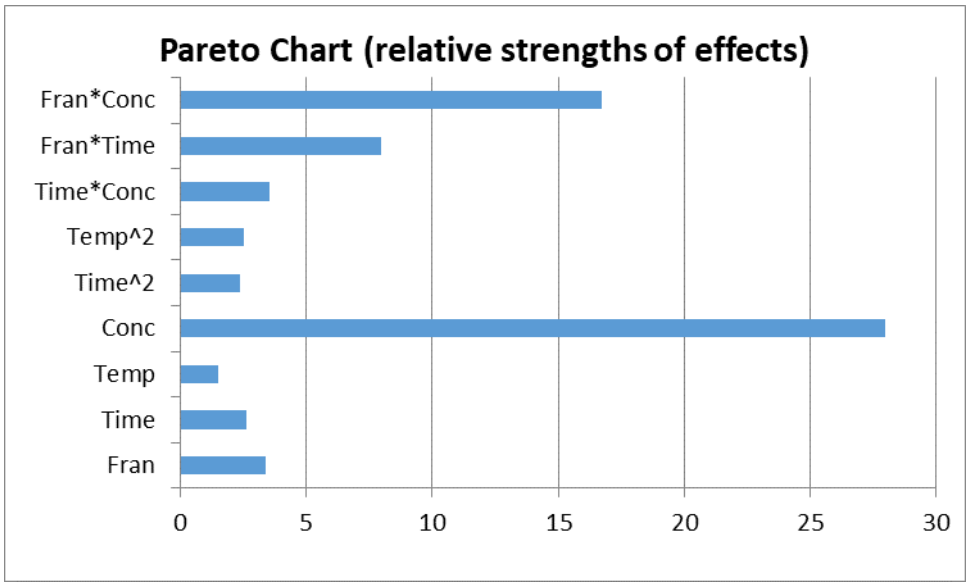


Figure 7.15 Pareto chart

The profile plots of percentage antioxidant activity as a function of temperature, time and concentration are shown in Figure 7.16, Figure 7.17 and Figure 7.18 respectively.

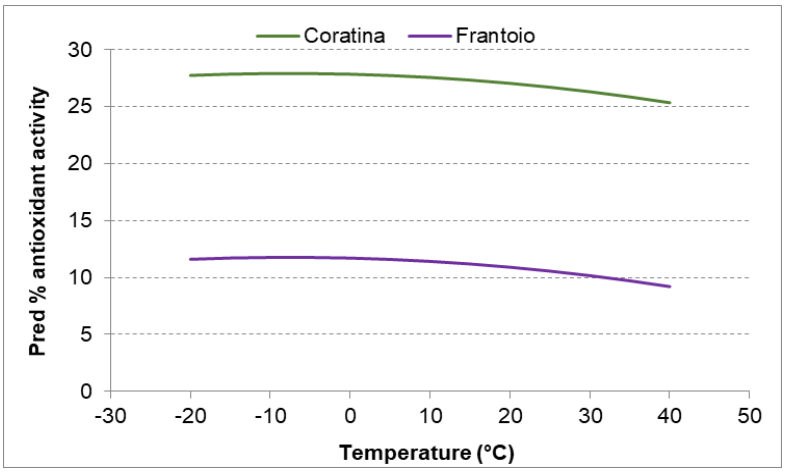


Figure 7.16 Profile plot for % antioxidant activity as a function of temperature at a concentration of 200 µg and after 12 weeks storage.

Figures 7.16 shows the predicted percentage antioxidant activity of the olive extracts as a function of temperature for both cultivars. As can be seen in the profile plot, the antioxidant capacity shows a slight decline for both olive pomace extracts with increase in temperature at 12 weeks storage. The enzymatic activities of polyphenoloxidase and β-glucosidase occurred simultaneously during the degradation of endogenous oleuropein causing oleuropein depletion with the formation of other bioactive compounds such as hydroxytyrosol, oleuropein aglycon (3,4-DHPEA-EA), and elenolic acid. The change in predicted percentage antioxidant activity is similar for both cultivars.

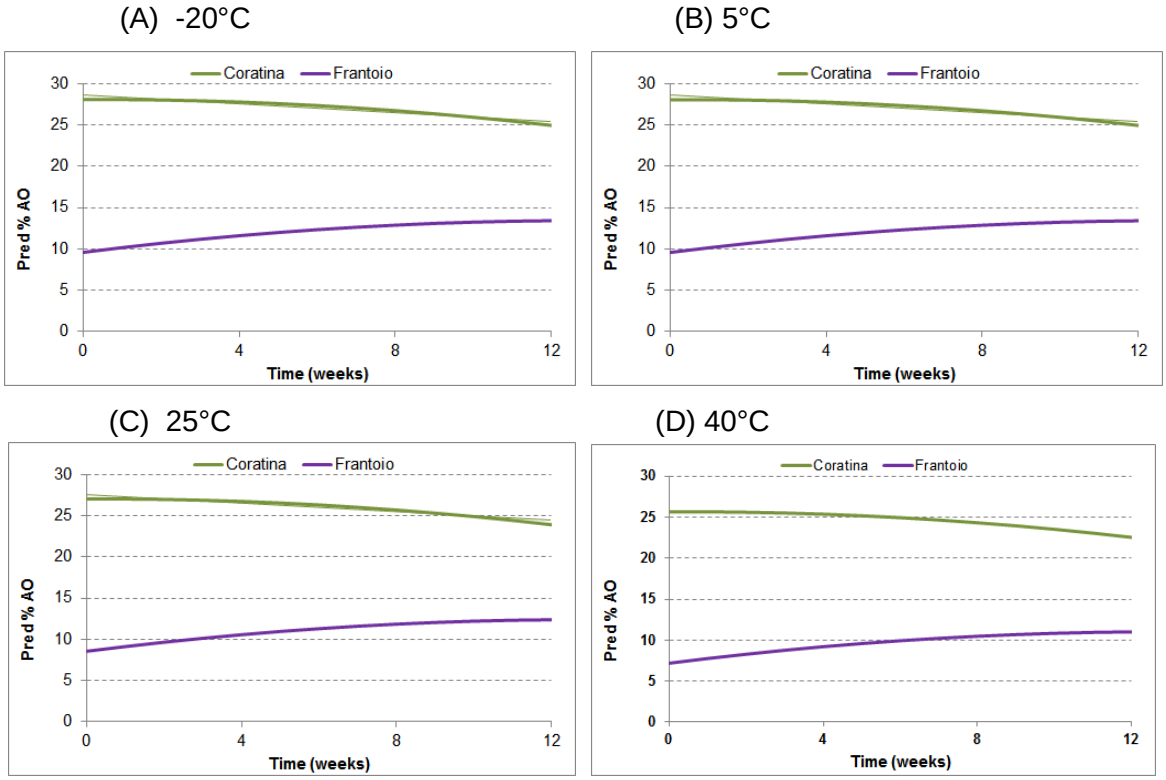


Figure 7.17 Profile plots for % antioxidant activity as a function of time and storage temperatures of (A) -20°C, (B) 5°C, (C) 25°C, (D) 40°C.

Figure 7.17 shows the predicted percentage antioxidant activity of the olive extracts as a function of time at all four storage temperatures for both cultivars. Acordning to the profile plots of the antioxidant capacity, *Coratina* shows a decline over the 12 week period while the *Frantoio* shows a slight increase in the percentage antioxidant activity. According to the results obtained in Chapter 6, the phenolic composition of *Frantoio* differs from that of *Coratina*. *Frantoio* initially has a higher concentration of hydroxytyrosol than *Coratina* and over time, the oleuropein in both cultivars breaks down to form hydroxytyrosol as previously mentioned. However, hydroxytyrosol has a

higher percentage antioxidant activity compared to oleuropein which could contribute to the observed slight increase in antioxidant activity in the *Frantoio*.

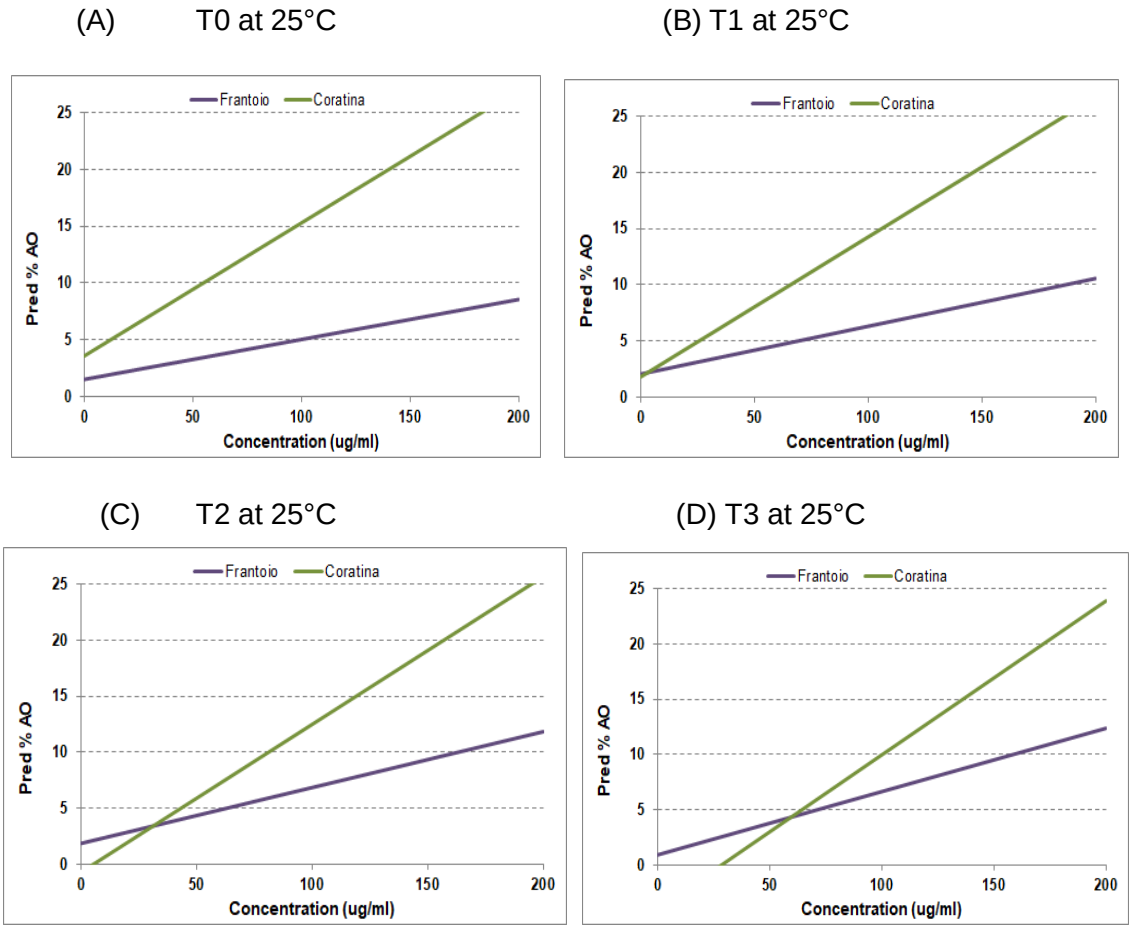


Figure 7.18 Predicted antioxidant activity as a function of concentration for both extracts at the initial analysis (T0) and each test stage until 12 weeks at 25°C.

Figures 7.18 shows the predicted % antioxidant activity of the olive extracts as a function of concentration (0 – 200 µg/ml) at specified time intervals and 25°C for both *Coratina* and *Frantoio* cultivar. Over time, the antioxidant activity of the *Coratina* is predicted to show a greater increase in % antioxidant capacity per unit concentration than Frantoio at each temperature. Moure et al. (189) suggested that the antioxidant activity depends on the extract concentration. In this study, the extract concentrations ranged from 3.125 µg/ml to 200 µg/ml. As a general trend, increased antioxidant activity was found with increasing extract concentration, and the concentration progressing to maximum antioxidant capacity is closely reliant on the antioxidant activity assay used.

7.6 Conclusion

The darkening of the aqueous extracts after 12 weeks storage especially at accelerated temperature (40°C) is indicative of degradation. However, the colour changes in the oil extracts were not as pronounced.

The decline or increase of the bioactives can be attributed to two degradation reactions during storage viz, hydrolysis and oxidation. Oleuropein content in *Coratina* decreased over time and was significantly affected by temperature whereas for *Frantoio*, only a slight decrease in oleuropein was observed. (Fig 7.7) Hydroxytyrosol and tyrosol content increased at a similar rate with time for both the *Coratina* and *Frantoio* while the effect of temperature on both bioactives was more pronounced for *Coratina*. (Fig. 7.3 – 7.6) α-Tocopherol and squalene content were equally stable in both *Coratina* and *Frantoio* extracts over the 12 week storage period, and their concentrations were independent of temperature.

A significant loss in total phenol content was observed after a period of 12 weeks for both cultivars which was independent of temperature and the rate of degradation of phenolic compounds was similar for both cultivars.

Both olive pomace extracts showed a slight decline in antioxidant activity with increase in temperature at 12 weeks storage. The change in percentage antioxidant activity is similar for both cultivars. However, a significant decrease in percentage antioxidant during storage at 25° and 40°C was observed for the *Coratina* extract after 12 weeks whereas a slight increase showed for *Frantoio.* The percentage antioxidant activity is concentration dependent and over time, the activity of the *Coratina* showed a greater increase in percentage antioxidant capacity per unit concentration increase than *Frantoio* at each temperature.

A storage temperature below 5°C is recommended for these hydrophilic and lipophilic extracts. Results show that *Coratina* is richer in high-value bioactives than *Frantoio.*

CHAPTER 8

STABILITY OF COSMETIC FORMULATIONS

8.1 Introduction and rationale

The aqueous and organic phase extracts prepared were incorporated into two cosmetic formulations, namely a cream and water-based gel to assess the stability of the extracts in a vehicle/carrier when stored at the different temperature conditions. Natural raw materials were selected for the two formulations as these are normally considered to be more unstable than synthetic ingredients and would represent the worst case scenario for stability assessment.

8.2 Experimental methods

8.2.1 Materials

Table 8.1 summarises the materials used for the cream and gel formulations.

Table 8.1 Raw materials, suppliers and grades

Raw materials	**Grade**	**Supplier**
Shea butter	100% raw	Natural moisture
Coconut oil	Refined	Wilsons
Olivem 1000	n/a	Millchem
Glycerol	Analytical reagent	Merck
Olive oil	Extra virgin	Greenleaf olive Co
Citric acid monohydrate	uniLAB	Merck

8.2.2 Preparation of formulations

Table 8.2 shows the composition of the cream and the gel formulations. The aqueous and organic extracts from the *Coratina* olive pomace to be incorporated into the cream and gel formulations, were prepared as follows:

Aqueous extract: 4.6 g aqueous extract residue was mixed with 34.4 g glycerin to give a total mass of 40 g.

Organic extract: 2.0 g organic extract residue was mixed with 5.58 g olive oil to give a total mass of 10 g.

Table 8.2 Formulations of the cream and gel.

Cream				Gel			
Aqueous phase	**Function**	**Mass (g)**	**%**	**Aqueous phase**	**Function**	**(Mass) g**	**%**
Water	Solvent	200		Water	Solvent	372.4	93.10
Aqua extract	Active	20	4.98	Aqua extract	Active	20	5.00
Citric acid	Preservative	1.6	0.40	Xanthan gum	Thickener	6	1.50
				Citric acid	Preservative	1.6	0.40
Oil phase							
Coconut oil	Emollient	80	19.92				
Shea butter	Emollient	48	9.96				
Olive oil	Carrier oil/ emollient	30	9.96				
Olivem	Emulsifier	10	2.49				
Org extract	Active	10	2.49				
TOTAL		400	100	**TOTAL**		400	100

Method for cream formulation:

a. An amount of 20 g aqueous extract in glycerine was added to water of the aqueous phase.

b. The aqueous phase was then filtered to obtain a clear solution.

c. A quantity of 1.6 g citric acid was added to the filtered aqueous phase and mixed.

d. In a separate beaker, 10 g organic extract in olive oil was added to the oil phase ingredients of the cream formulation and mixed.

e. Both phases were heated to 70°C, then the water phase was slowly added to the oil phase while blending with an Ultra Turrax homogeniser first at low speed, then at high speed, and then at 10 000 rpm for 10 min until a smooth emulsion was formed.

Method for gel formulation:

a. The water was heated to 40 °C.

b. A quantity of 20 g aqueous extract dissolved in glycerine was added to the hot water aided with stirring (magnetic stirring bar).

c. Xanthan gum and citric acid were slowly added to the mixture while stirring.

d. The mixture was then homogenised with an Ultra Turrax homogeniser for 10 minutes at 10 000 rpm.

8.2.3 Physical analysis

The prepared cream and gel formulations were subjected to a set of organoleptic (colour, look, texture, skin-feel) and physical (pH and viscosity) analyses. pH was measured using a Accsen waterproof pH meter with pH spear electrode, and the viscosity, at various shear rates, was measured using a Haake Viscotester with spindle E100.

8.2.4 Stability tests

Eight plastic containers with screw tops were each filled with 80 g of cream or gel from the pilot batches formulated and placed at four different storage conditions for 3 months. Storage conditions comprised: freezer at -20 °C ± 2 °C, fridge at 5 °C ± 2 °C, ambient temperature at 25 °C ± 2 °C and an oven at 40 °C ± 2 °C. Samples were tested as described in section 8.2.3. every 4 weeks for 12 weeks.

8.3 Results and discussion

8.3.1 Initial characterisation of the cream and gel formulation

Figure 8.1 shows the initial appearance of both the cream and gel preparations while the characteristics of each formulation have been summarised in Table 8.3. Figure 8.2 shows the viscosity profiles of these two formulations.

Figure 8.1 Cream formulation (on the left) and gel formulation (on the right)

Table 8.3 Characteristics of the formulations at initial analysis (T0)

Formulation parameters	**Results**	
	Cream	**Gel**
Colour	Cream-coloured, opaque emulsion	Slightly yellow, slightly opaque
Odour	Soft olive oil smell	Fruity, olive, citrusy smell
Texture	Smooth, creamy	Jelly-like texture
pH	2.56	3.08
Viscosity range	804-39900 mPa.s	541-35500 mPa.s
Skin-feel	Smooth, spreads easily, non-greasy, slight sheen	Smooth, non-greasy, spreads easily, cooling effect

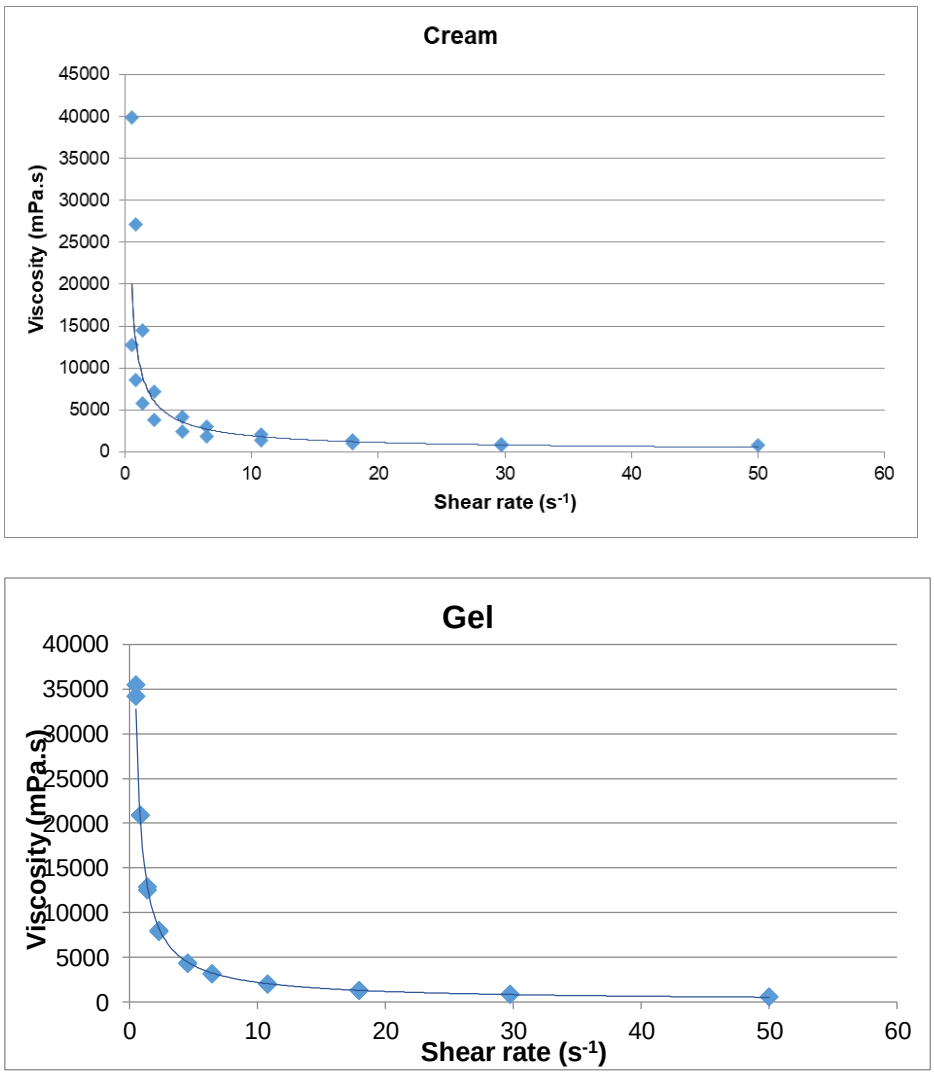


Figure 8.2 Typical viscosity profiles for the cream and the gel.

For both formulations, a low pH was measured due to the 0.4% citric acid used as preservative. Citric acid was chosen since preservative activity is more effective at low pH. Although this ingredient is used primarily as a preservative since, it exhibits antimicrobial activity against bacteria and fungi. , it is likewise include in cosmetics as an anti-aging component. Citric acid is an alpha-hydroxy acid which eradicates dead skin cells and consequently affords a more youthful complexion. The natural pH of the skin normally ranges from 4.5 to 6.0 (190) so the low pH of the formulations provides an opportunity for using these formulations as a skin peel.

8.3.2 Stability of the cream

The viscosity profile of the cream (Figure 8.2) shows that it has slightly thixotropic behaviour, viz, the viscosity shows a time dependency at each shear rate. This is possibly due to the large lipid molecules present in the coconut oil and shea butter that contribute to a semi-solid structure which takes time to rebuild after it has been sheared. The viscosity also shows a decrease with increasing shear rate indicating pseudoplastic behaviour which is an ideal rheology for skin creams as this indicates good spreadability of the cream upon application to the skin,

The gel shows only pseudoplastic rheology and no thixotropy and has a very similar viscosity range to the cream.

As can be seen by the results reflected in Table 8.4 and Figure 8.3, the cream retained its characteristic features when stored at low as well as ambient temperatures.

Table 8.4 Characteristics of the cream formulation after 4, 8 and 12 weeks storage at four different temperatures.

Parameters	**Storage time**			
-20°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Cream, opaque emulsion	Cream, opaque emulsion	Cream, opaque emulsion	Cream, opaque emulsion
Odour	Soft olive oil smell	Soft olive oil smell	Soft olive oil smell	Fruity smell
Texture	Smooth, creamy	Smooth, creamy (frozen) Once thawed, very thick	Smooth, creamy (frozen), once thawed was very thick	Smooth, creamy, thick
pH	2.56	2.59	2.68	2,66
Viscosity range	804 – 39 900 mPa.s	158 - 2850 mPa.s	Not tested	1270 – 25 600 mPa.s
Skin-feel	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen
5°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Cream, opaque emulsion	Cream, opaque emulsion	Cream, opaque emulsion	Cream, opaque emulsion
Odour	Soft olive oil smell	Soft olive oil smell	Soft olive oil smell	Fruity smell
Texture	Smooth, creamy	Smooth, creamy (buttery)	Smooth, creamy (buttery)	Smooth, creamy (buttery)
pH	2.56	2.46	2.64	2.64
Viscosity range	804 – 39 900 mPa.s	592 – 18 800 mPa.s	Not tested	1260 – 15 900 mPa.s
Skin-feel	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen
25°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Cream, opaque emulsion	Light beige, opaque emulsion	Light beige, opaque emulsion	Beige, opaque emulsion
Odour	Soft olive oil smell	Soft olive oil smell	Less intense olive oil smell	Fruity smell
Texture	Smooth, creamy	Smooth, creamy	Smooth, creamy	Smooth, creamy, thinner
pH	2.56	2.89	2.95	2.66
Viscosity range	804 – 39 900 mPa.s	547 – 31 900 mPa.s	Not tested	14.75 - 87.25 mPa.s
Skin-feel	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen
40°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Cream, opaque emulsion	Dark beige, opaque emulsion	Light brown, opaque emulsion	Not tested – sample removed from stability at T2 test stage
Odour	Soft olive oil smell	Pertinent olive oil smell	Pertinent oily smell, less fruity	
Texture	Smooth, creamy	Smooth, runny	Phases separated, watery and lumpy	
pH	2.56	2.86	4.55	
Viscosity range	804 – 39 900 mPa.s	126 - 317 mPa.s	Not tested	
Skin-feel	Smooth, spreads easily, non-greasy, slight sheen	Smooth, spreads easily, non-greasy, slight sheen	Not tested	

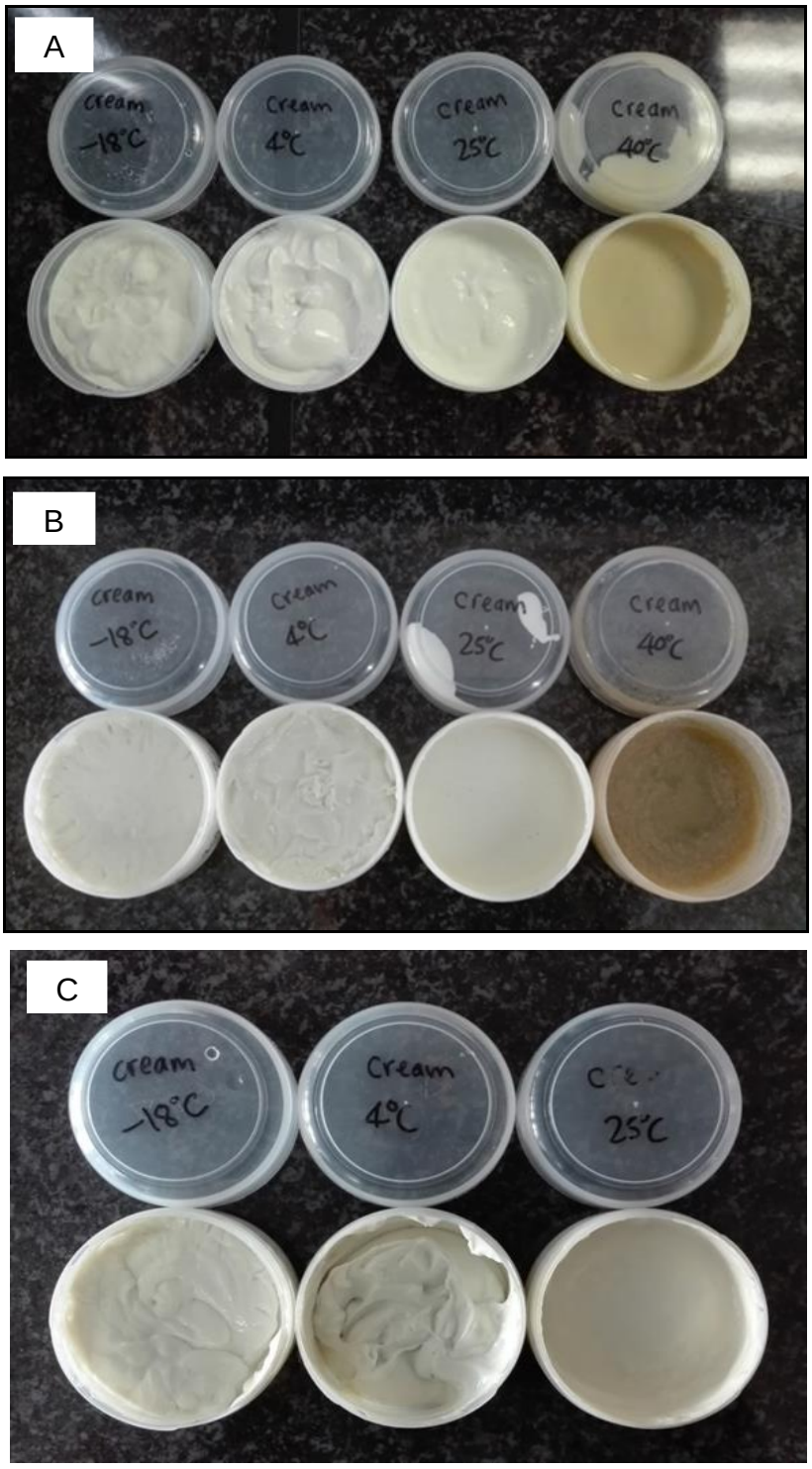


Figure 8.3 Photos of the creams stored in freezer, fridge, at room and in the oven (A) 4 weeks, (B) 8 weeks and (C) 12 weeks storage

Colour

The freshly prepared formulation was a cream, opaque emulsion. The results show that no change in colour was observed for the emulsions stored at -20°C, and 5°C over the 12 week storage period. A slight darkening of the colour occurred from 4 weeks onwards for the emulsion stored at 25°C. A more pronounced darkening in colour was

observed for the emulsion stored at 40°C from 4 weeks onwards. This change was most likely due to degradation of the actives at higher temperature.

Odour Odour remained a soft olive oil smell for samples stored at -20, 5 and 25°C ± 2°C, but changed to a fruity smell at 12 weeks. The sample at 40°C developed a change in odour from 4 to 8 weeks and had a pertinent less fruity, oily smell. The change, again, was due to degradation at higher temperature.

Texture

Temperature influences the texture of the cream. Formulations stored at low temperatures retained a smooth, creamy texture throughout the 12 weeks but became thinner for the sample stored at 25 ± 2°C. At 40°C, at 4 weeks storage, the sample became runny and at 8 weeks, phase separation was observed and the sample was watery and lumpy. It was consequently discarded.

pH

Monitoring the pH value is crucial for determining an emulsion's stability. pH changes indicate the occurrence of chemical reactions that can give an indication of the quality of the final product. The pH of the various emulsion samples stored at various storage conditions, i.e. -20, 5 and 25 ± 2°C, remained fairly constant indicating good product stability. However, at 40°C, a significant increase in pH of the emulsion to 4.55 was observed at 8 weeks. The high temperature contributes to the destabilisation of the emulsion by hydrolysis.

Viscosity

The results show that the viscosity decreased over time for all the emulsions, however, the viscosity decrease was the least at -20°C and showed the largest decrease at 40°C. The large decrease in viscosity of the emulsion stored in the oven can be correlated to the separation of the phases observed.

Skin-feel

No change was observed in the skin feel of the emulsions over time at different temperatures indicating that the emulsions, even though there was a slight colour and odour change remained stable and usable.

Conclusion and recommendation

At 40°C, the cream turned a darker beige to light brown, developed a more pertinent oil smell and became runnier. The water and oil phases eventually separated as a result of the decreased viscosity while the pH increased as a result of degradation products formed at high temperatures.

It is therefore proposed that the cream formulation should be stored in a fridge for maximum durability but can be stored at room temperature for 3 months without loss of functionality.

8.3.3 Physical stability of the gel formulation

The results of the stability tests for the gel are shown in Table 8.5 while Figure 8.5 shows photographs of the gel stored at various temperatures over 12 weeks.

Table 8.5 Characteristics of the gel formulation after 4, 8 and 12 weeks storage at four different temperatures.

Parameters	**Storage time**			
-20°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Slightly yellow, slightly opaque	Slightly yellow, slightly opaque (frozen)	Slightly yellow, slightly opaque (frozen)	Slightly yellow, opaque (frozen)
Odour	Fruity olive citrusy smell	Fruity olive citrusy smell	Fruity olive citrusy smell	Fruity olive citrusy smell
Texture	Petroleum gel texture	Petroleum gel texture (frozen), once thawed had bubbles in it	Jelly-like	Jelly-like
pH	3.08	3,1	3.16	3.12
Viscosity range	541-35 500 mPa.s	171 - 1710 mPa.s	Not tested	Not tested
Skin-feel	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect
5°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Slightly yellow, slightly opaque	Slightly yellow, slightly opaque	Slightly yellow, slightly opaque	Not tested – sample removed from stability at T2 test stage due to microbial growth
Odour	Fruity olive citrusy smell	Fruity olive citrusy smell	Less fruity olive citrusy smell	
Texture	Petroleum gel texture	Slightly thinner petroleum gel texture	Jelly-like	
pH	3.08	3,05	3.13	
Viscosity range	541-35 500 mPa.s	188 - 1370 mPa.s	Not tested	
Skin-feel	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect	
25°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Slightly yellow, slightly opaque	Slightly yellow, slightly opaque	Dark yellow, slightly opaque	Not tested – sample removed from stability at T2 test stage due to microbial growth
Odour	Fruity olive citrusy smell	Fruity olive citrusy smell	No smell	
Texture	Petroleum gel texture	Thinner petroleum gel texture	Jelly-like	
pH	3.08	2,93	3.16	
Viscosity range	541-35 500 mPa.s	62.9 - 264 mPa.s	Not tested	
Skin-feel	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, cooling effect	
40°C	**T0 (initial)**	**T1 (4 weeks)**	**T2 (8 weeks)**	**T3 (12 weeks)**
Colour	Slightly yellow, slightly opaque	Dark beige yellow, opaque	Dark yellow, opaque	Brown opaque
Odour	Fruity olive citrusy smell	Soft acidic, fruity smell	Very slight smell	Very slight fruity smell
Texture	Petroleum gel texture	Runny petroleum gel texture	Thinner gel	Thinner gel
pH	3.08	2,87	3.06	3.03
Viscosity range	541-35 500 mPa.s	125 - 206 mPa.s	Not tested	Not tested
Skin-feel	Smooth, non-greasy, spreads easily, cooling effect	Smooth, non-greasy, spreads easily, non-cooling effect	Smooth, non-greasy, spreads easily, non-cooling effect	Smooth, non-greasy, spreads easily, non-cooling effect

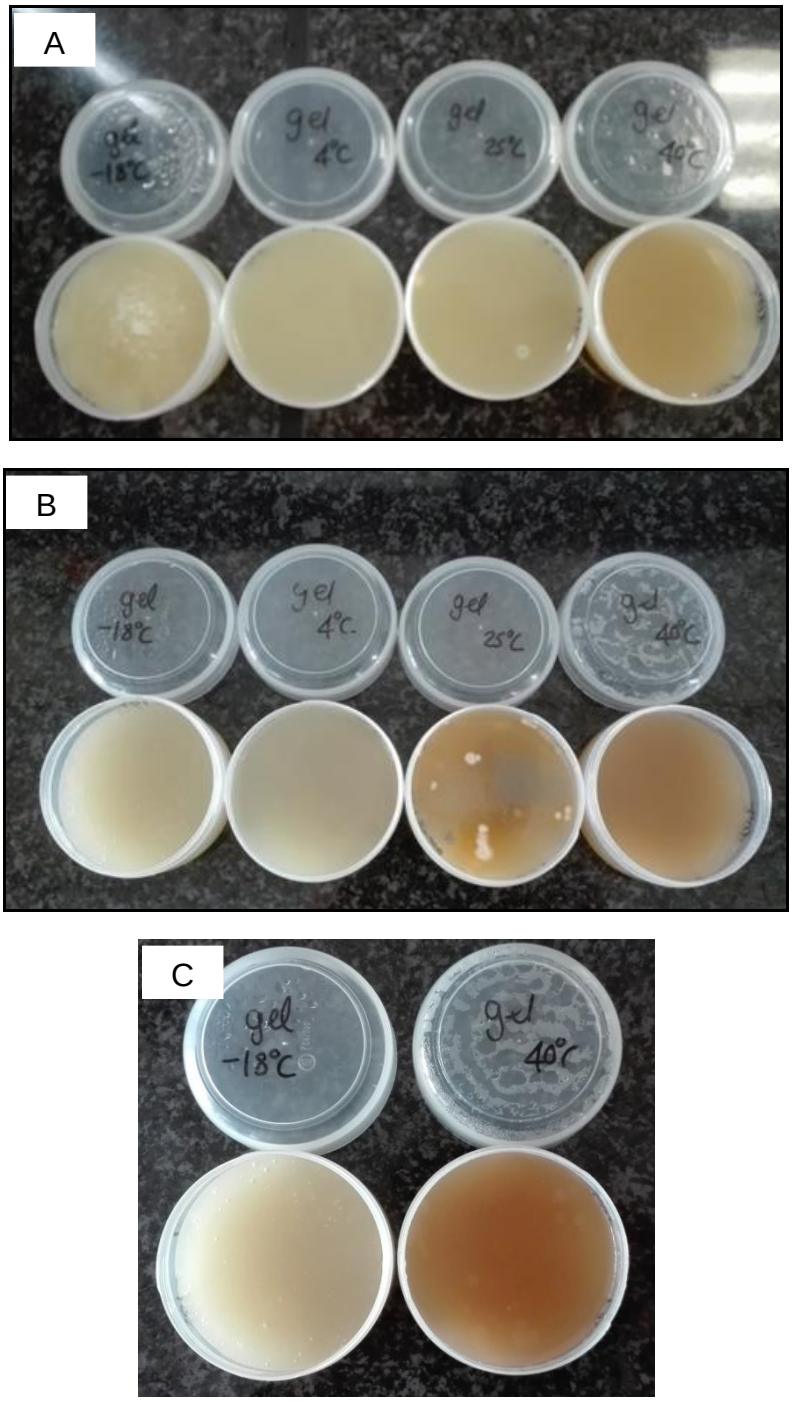


Figure 8.4 Photos of the gel stored in the freezer, fridge, at room temperature and in the oven after (A) 4 weeks, (B) 8 weeks and (C) 12 weeks storage.

Colour

The freshly prepared gel was slightly yellow and slightly opaque. No change in colour was observed for gel samples at -20°C and 5°C over the 12 week storage period. A slight darkening in colour was observed for the gel stored at 25°C after 8 weeks while at 40°C much more pronounced darkening in colour was observed from 4 weeks. This is most likely due to oxidation reactions.

Odour

No change in odour was observed for the gel samples stored at -20°C, however, for the gel at 5°C a slight loss of the fruity odour occurred at 8 weeks while there was a total loss of the fruity odour at 8 weeks for the 25°C sample. For the oven-stored gel, there was a change in odour from 4 weeks onwards.

Texture

Temperature influenced the texture of the gel. Formulations stored at lower temperatures became slightly thinner over time whereas the sample at 40°C became runnier over time.

pH

The pH of the gel remained fairly constant over the 12 week period for samples stored at all the temperatures. This indicates chemical stability of the formulation.

Viscosity

The results show that the viscosity decreased significantly after 4 weeks for all the emulsions, and the decrease was more pronounced as the storage temperature increased.

Skin-feel

Samples stored at -20, 5 and 25°C retained their smooth, non-greasy, easily spreading, cooling effect throughout the 12 weeks whereas the sample at 40°C had no cooling effect from 4 weeks onwards.

Conclusion and recommendation

The major changes in the gel occurred at 40°C with a darkening of the colour, loss of fruity odour and a dramatic decrease in viscosity. The room temperature gel, because of its high water content was also subject to some microbial growth due to contamination of the sample during handling. It is proposed that the gel is stored at temperatures below ambient, namely in the fridge for maximum stability. The gel can be stored at room temperature if the preservative system is improved.

8.4 Comparison of the gel and cream stability

Both the gel and the cream formulations stored at low temperatures namely -20° and 5°C showed no to very little changes during the 12 weeks storage period while both the gel and the cream stored at 40°C showed definite changes from 4 weeks onwards. The gel formulation experienced a greater loss in viscosity than the cream formulation and a loss in cooling effect at high temperatures while the cream formulation retained its

skin-feel characteristics at all temperatures. A storage temperature of 5°C is recommended for both these formulations.

Furthermore, the cream and the gel formulations prepared in this study had low pH values of 2.65 and 3.08 respectively, due to the concentration of citric acid used. When a similar cream was made up with a lower concentration of citric acid, the efficacy against fungal activity was reduced. It is proposed that the preservative system used in both formulations be reevaluated. A more effective natural preservative, e.g. sodium benzoate or phenoxyethanol, in combination with citric acid could be used since natural preservatives are more organism specific than their broader spectrum synthetic counterparts. This would also avoid the very low pH values while still providing a mild acidity for effective preservative action.

CHAPTER 9

CONCLUSION AND RECOMMENDATIONS

OMW or pomace generated from olive oil extraction is an abundant and affordable source of biologically active hydrophilic and lipophilic compounds that hold promising health-beneficial potential. The recovery of both these bioactive compounds from two-phase system olive pomace or *alperujo* was achieved by Soxhlet extraction using solvents of low toxicity, i.e. “green solvents”. The feasibility of this extraction process for industrial use and eventual scale-up capability involved the search for the optimum conditions to maximise the efficiency of the process.

9.1 Preliminary extraction investigations

In the preliminary study, conventional extraction methods, namely, percolation, stir/blend and Soxhlet, pomace type and solvents such as water, polar and non-polar organic solvents, were evaluated to identify optimum extraction conditions for the hydrophilic and lipophilic bioactives. It was found that the extraction of polyphenols was favoured by the continuous Soxhlet extraction since the application of heat renders the cell walls permeable and increases the solubility and diffusion coefficients of the compounds to be extracted. The freeze-dried sample delivered higher yield of when compared to the wet pomace sample which can be explained by the higher moisture content in the fresh sample which reduces the expandability of the matrix and consequent extractability of both hydrophilic and lipophilic constituents. It was also determined that a hydro-ethanolic mixture was most efficient for extraction of hydrophilic compounds of interest. For extraction of the lipophilic squalene and α-tocopherol, hydrocarbon chemical solvent *n*-heptane was found to be the most appropriate solvent to replace the more toxic *n*-hexane, which is normally used.

9.2 Optimisation of combined extraction

Among the different non-toxic organic solvent combinations investigated during the combined extraction, an optimum solvent blend *n*-heptane, ethanol and water in a volume ratio of 30:50:20 for extraction of both hydrophilic and lipophilic bioactive compounds investigated in this study, namely, hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene from freeze-dried two-phase olive pomace, was obtained. The predicted blend for optimal dissolution of the five bioactive compounds, had a

calculated solubility parameter (SP) of 27.32 which corresponded closely to the average SP of 26.64 for these five compounds.

During optimisation of the extraction conditions, reduced pressure with associated reflux temperatures of solvent blends, as well as extraction time was considered. The percent antioxidant activity, α-tocopherol concentration and squalene concentration significantly increased with increased temperature ($p=0.0014$, $p=0.019$ and $p=0.02$ respectively) while there was no evidence that the extraction temperature had an influence on the hydroxytyrosol and tyrosol concentration. There was no evidence that extraction time has a significant effect on percentage antioxidant activity, total phenol content, and hydrophilic bioactives content while the content of the lipophilic bioactives showed a quadratic decline with time. It was concluded from the results that a two hour extraction time at a reflux temperature of 60°C (bath temperature of 80°C) at 350 mbar was optimal.

9.3 Comparison of olive cultivars

Two olive cultivars, viz *Coratina* and *Frantoio*, were compared for their hydrophilic and lipophilic bioactive content using the optimised extraction method and solvent blend. In the *Frantoio* pomace extract, hydroxytyrosol was the major phenolic compound present whereas in *Coratina* it was oleuropein. The results obtained are in line with the findings by Sivakumar et al. (167) who also found that *Coratina* olives contain the highest levels of oleuropein vs *Frantoio*. The content of lipophilic compounds, α-tocopherol and squalene, were nearly double in the *Coratina* extract vs *Frantoio* and is supported by other literature findings (172). to be explored for the use in the food, cosmetics and pharmaceutical industries.

Furthermore, *Coratina* contained a significantly higher amount of total phenols, (20.41 mg GAE/g dry weight) compared with *Frantoio* (8.07 mg galic acid equivalent or GAE/g dry weight of pomace) and was in keeping with published reports. The average increase of the percentage antioxidant activity for every unit increase in concentration for the *Coratina* cultivar was significantly higher than for *Frantoio*. This result is most probably attributed to the higher concentrations of oleuropein in the *Coratina* extract compared with the *Frantoio* extract (3.495 mg/g DW and 0.258 mg/g DW respectively) as well as higher total phenol content. Results show that *Coratina* is richer in high-value bioactives than *Frantoio*.

Both olive cultivars showed some antifungal and antibacterial (gram-positive and gram-negative) activity compared to the control. The highest antimicrobial activity was seen against the gram-negative bacteria *E. coli* by the *Frantoio* extract.

9.4 Stability studies

Stability of the pomace extracts were evaluated for both *Coratina* and *Frantoio* cultivars. The evaluation included the total phenol content, antioxidant activity and hydrophilic and lipophilic bioactive concentrations during 3 months of storage in closed amber glass bottles with a screw on lid, protected from light at four different storage conditions. The bioactive concentrations, total phenol content and antioxidant activity differed among these extracts during storage conditions at freezing (-20°C), fridge (5°C), room (25°C), and accelerated (40°C) temperatures over the 12 week period. Hydroxytyrosol and tyrosol concentrations in both *Coratina* and *Frantoio* aqueous extracts increased over time and with higher temperature due to degradation of oleuropein and ligstroside into hydroxytyrosol and tyrosol respectively. The oleuropein content remained relatively constant at all temperatures over the 3 months storage period for the *Frantoio* cultivar whereas in the *Coratina* cultivar a significant decrease of this polyphenol ($p < 0.05$) occurred.

In both the *Coratina* and *Frantoio* organic extracts, a slight decline in α-tocopherol and squalene content over the 12 week storage period, independent of temperature. A significant loss in total phenol content was observed at the end of the 12 week period for both cultivars ($p < 0.05$), which was independent of temperature.

Temperature significantly decreased ($p < 0.05$) the antioxidant activity of both cultivar extracts. At all temperatures, *Coratina* showed a decline in antioxidant capacity over time whereas *Frantoio* showed a slight increase over time as a result of oleuropein degradation into hydroxytyrosol, a strong antioxidant.

Both the gel and the cream formulations stored at low temperatures, namely, -20° and 5°C, showed no to very little changes during the 12 weeks storage period, however a storage temperature of 5°C is recommended for long-term stability to retain their organoleptic (colour, look, texture, skin-feel) and physical (pH and viscosity) characteristics.

9.5 Recommendations

Based on the findings in this study, the recommendations are given as follows:

- **Olive cultivar and degree of ripeness**

Degree of ripeness and cultivar, are two important variables which affect the contents of antioxidants in olives. Both cultivars studied were picked at early ripeness, however *Coratina* had a slightly higher ratio of green olives. Since oleuropein degrades with olive ripening into hydroxytyrosol, it is recommended that olive pomace from olives harvested at an early ripeness stage be used to maintain high oleuropein content. However, if higher content of hydroxytyrosol is required, pomace from a more mature olive is recommended. *Coratina* has higher levels of α-tocopherol and squalene and is thus recommended if higher levels are required for therapeutically use.

- **Treatment and storage of pomace samples**

Freeze-drying two-phase system olive pomace is highly recommended before extraction in order to decrease the moisture content and thus water activity which could negatively impact on the sample quality. In addition, freeze-drying reduce enzymatic destruction of some polyphenols as a result of enzymatic degradation. The freeze-dried samples should be kept in sealed opaque or amber containers (glass) with a tightly fitted lid and stored in a dark, cool place to avoid oxidative degradation.

- **Extraction method**

Simultaneous extraction of both hydrophilic and lipophilic compounds during a single, integrated Soxhlet process requires a mixture of polar and non-polar solvents. These solvents are stirred and heated under vacuum in order to lower the reflux temperature of the solvent blend in consideration of the thermolability of most of the bioactive compounds. In this study, possible oxidation of antioxidants by polyphenol oxidase activity during extraction was reduced considering by using a Soxhlet system.

The optimised conditions to obtain a maximum content of hydroxytyrsol, tyrosol, oleuropein, α-tocopherol and squalene are: a solvent blend of *n*-heptane, ethanol and water (30:50:20 volume ratio), an extraction period of two hours and a water bath temperature of 80°C to obtain a reflux temperature of around 60°C at a pressure of 350 mbar. However, to obtain a different ratio of the five bioactives, various combinations of

the solvents can be used according to the predicative model obtained. (Refer Chapter 5)

❖ **Quantification of bioactives**

The recommended analysis method is by HPLC equipped with a binary gradient pump and a DAD using authentic reference standards. Chromatographic separation is achieved by gradient elution on a C18 reverse-phase column (250 mm x 4.6 mm, id 5 µm) column. Recommended mobile phases for the hydrophilic bioactives are A: H_2O / acetic acid (100:1), B: MeOH / acetonitrile / acetic acid (90:10:1) and for the lipophilic compounds are: A: acetonitrile /methanol (7:3 v/v), B: IPA.

❖ **Storage conditions for extracts**

The two-phase olive pomace aqueous and organic extracts are recommended to be stored in the absence of oxygen and light and at a temperature below 5°C for long-term storage. Alternative forms of these extracts can be considered such as encapsulation with the aid of hydrocolloids in a capsule form for prolonged shelf-life of the product.

9.6 Further research

Several further research opportunities have been identified and propose investigation to:

- Sieve the freeze-dried olive pomace and evaluate the bioactive content of each fraction thereby obtained, namely, skin, pips and pulp.
- Evaluate the effect of a uniform particle size versus a non-uniform particle size which could influence the bioactive content.
- Quantify the content of α-tocopherol in the aqueous extract.
- Re-investigate the solvents isopropyl alcohol and ethyl acetate in an optimum solvent blend for the combined extraction of hydrophilic and lipophilic bioactives.
- Separate and isolate the individual bioactives, namely, hydroxytyrosol, tyrosol, oleuropein, α-tocopherol and squalene by thin-layer chromatography.
- Scale up the Soxhlet extraction method to produce larger quantities of extract for possible commercialisation.
- Perform cytotoxicity trials to establish safety of the extracts.
- Perform efficacy tests of cosmetic products containing the extracts.

9.7 Impact of research

Two-phase olive pomace is a promising source for beneficiation in terms of bioactive content for use in the pharmaceutical (medicinal and therapeutic), nutraceutical, cosmetic and food industry. Two main advantages of this beneficiation are: to extract valuable phytochemical compounds with beneficial properties, and the rendering of olive oil processing pomace harmless to be used as soil fertilizer or fuel after drying. The advantages of an extract with a mixture of antioxidants is that there is a synergistic interaction between various antioxidants in both the aqueous and lipid composition, increasing their functional, nutritional and therapeutic value. Recent investigations (191) have demonstrated an antioxidant synergism between α-tocopherol, a monophenolic compound and derivative of chromanol, and complex polyphenol oleuropein.

The results of this study could mean the first step for the implementation of a single, integrated continuous extraction process on a large scale to obtain both water-soluble hydroxytyrosol, tyrosol and oleuropein and lipid-soluble α-tocopherol and squalene compounds from two-phase olive processing pomace, which is of major interest from an economical, industrial and environmental point of view. Extraction of biologically active compounds from olive pomace may turn a polluting residue into a source of natural antioxidants and antimicrobials.

Figure 9.1 shows a schematic representation of the beneficiation of olive pomace and the contribution to knowledge that has resulted from this research.

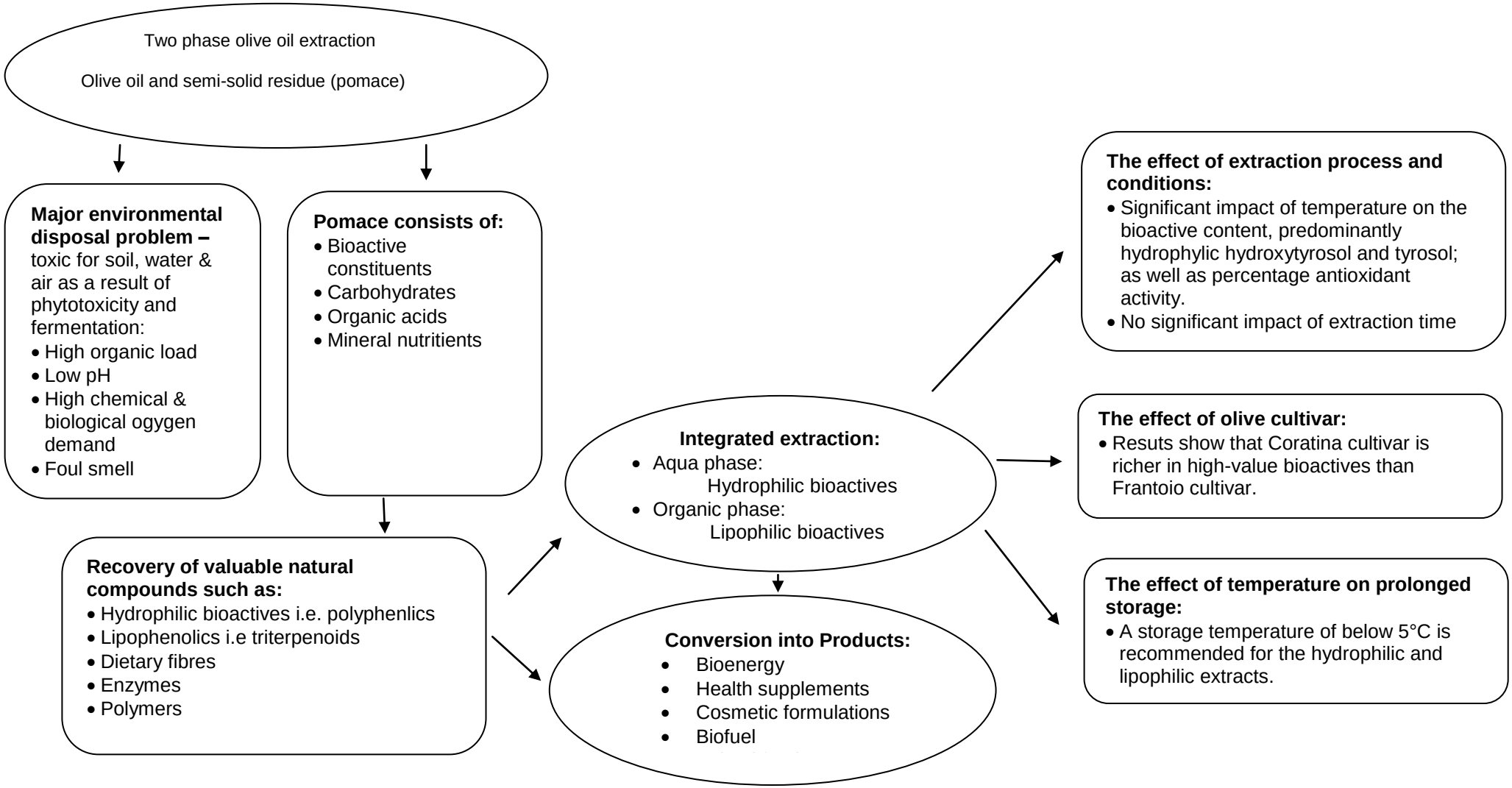


Figure 9.1 Conceptual framework depicting the beneficiation of two-phase system olive pomace

Bibliography

1. Benaiges A GP. 8.5. Botanical Extracts. Analysis of Cosmetic Products2011. 345 p.
2. Tsagaraki E, Lazarides H, Petrotos K. Olive mill wastewater treatment. Utilization of By-products and Treatment of Waste in the Food Industry. 2007:133-57.
3. D'Andria R DSA, Petillo O, Sorrentino G, Peluso G, editors. Nutraceutical, cosmetic, health products derived from olive. . Present and Future of the Mediterranean Olive Sector Options méditerranéennes Options Méditerranéennes, CIHEAM; 2013. p. 153-61.
4. Frankel EN. Nutritional and biological properties of extra virgin olive oil. Journal of Agricultural and Food Chemistry. 2011;59(3):785-92.
5. Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro G, Morozzi G. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. Journal of Chromatography A. 2004;1054(1):113-27.
6. Eurostats. Statistics explained: Agricultural production - crops: European Commission; 2017 [cited 2017 30 Aug]. Available from: http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production_-crops.
7. Dermeche S, Nadour M, Larroche C, Moulti-Mati F, Michaud P. Olive mill wastes: biochemical characterizations and valorization strategies. Process Biochemistry. 2013;48(10):1532-52.
8. Directorate Plant Production in collaboration with the ARC. Olives, production guidelines Pretoria: Department of Agriculture, Forestry and Fisheries; 2010 [cited 2017 22 Sept]. Available from: http://www.daff.gov.za/Daffweb3/Portals/0/Brochures%20and%20Production%20guidelines/olives.pdf.
9. SA Olive Industry Association (SA Olive). Follow The Olive: Harvesting Paarl2017 [cited 2017 22 Sept]. Available from: https://www.saolive.co.za/2017/03/17/follow-olive-harvesting/.
10. International Olive Council. World Olive Oil Figures 2017 [cited 2017 22 Sept]. Available from: http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures.
11. Lafka T-I, Lazou AE, Sinanoglou VJ, Lazos ES. Phenolic and antioxidant potential of olive oil mill wastes. Food Chemistry. 2011;125(1):92-8.

12. Abdalla KZ, Hammam G. Correlation between biochemical oxygen demand and chemical oxygen demand for various wastewater treatment plants in Egypt to obtain the biodegradability indices. International Journal of Sciences: Basic and Applied Research. 2014;13(1):42-8.
13. Azbar N, Bayram A, Filibeli A, Muezzinoglu A, Sengul F, Ozer A. A review of waste management options in olive oil production. Critical Reviews in Environmental Science and Technology. 2004;34(3):209-47.
14. Cardoso SM, Falcão SI, Peres AM, Domingues MR. Oleuropein/ligstroside isomers and their derivatives in Portuguese olive mill wastewaters. Food Chemistry. 2011;129(2):291-6.
15. Matthaus B, Özcan MM. Fatty acid, tocopherol and squalene contents of Rosaceae seed oils. Botanical Studies. 2014;55(1):48.
16. Mulinacci N, Innocenti M, La Marca G, Mercalli E, Giaccherini C, Romani A, et al. Solid olive residues: Insight into their phenolic composition. Journal of Agricultural and Food Chemistry. 2005;53(23):8963-9.
17. Obied HK, Allen MS, Bedgood DR, Prenzler PD, Robards K. Investigation of Australian olive mill waste for recovery of biophenols. Journal of Agricultural and Food Chemistry. 2005;53(26):9911-20.
18. Seidel V. Initial and bulk extraction of natural products isolation. Natural Products Isolation. 2012:27-41.
19. Dejoye Tanzi C, Abert Vian M, Ginies C, Elmaataoui M, Chemat F. Terpenes as green solvents for extraction of oil from microalgae. Molecules. 2012;17(7):8196-205.
20. Joana Gil-Chávez G, Villa JA, Fernando Ayala-Zavala J, Basilio Heredia J, Sepulveda D, Yahia EM, et al. Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: an overview. Comprehensive Reviews in Food Science and Food Safety. 2013;12(1):5-23.
21. Saxena M, Saxena J, Nema R, Singh D, Gupta A. Phytochemistry of medicinal plants. Journal of Pharmacognosy and Phytochemistry. 2013;1(6).
22. Doughari JH. Phytochemicals: extraction methods, basic structures and mode of action as potential chemotherapeutic agents. Phytochemicals-A global perspective of their role in nutrition and health: InTech; 2012.
23. Chemat F, Vian MA, Cravotto G. Green extraction of natural products: concept and principles. International Journal of Molecular Sciences. 2012;13(7):8615-27.
24. Vossen P. Olive oil: history, production, and characteristics of the world's classic oils. HortScience. 2007;42(5):1093-100.

25. Úrbez-Torres J, Peduto F, Vossen P, Krueger W, Gubler W. Olive twig and branch dieback: etiology, incidence, and distribution in California. Plant Disease. 2013;97(2):231-44.
26. Kalogeropoulos N, Kaliora AC. Effect of Fruit Maturity on Olive Oil Phenolic Composition and Antioxidant Capacity. Olive and Olive Oil Bioactive Constituents AOCS Press, Urbana, IL. 2015:123-47.
27. Silva S, Gomes L, Leitao F, Coelho A, Boas LV. Phenolic compounds and antioxidant activity of Olea europaea L. fruits and leaves. Revista de Agaroquimica y Tecnologia de Alimentos. 2006;12(5):385-95.
28. Costa C. Olive production in South Africa: a handbook for Olive growers: ARC-Infruitec; 1998.
29. Rapoport H. Botany and morphology2010. 33-57 p.
30. Mora F, Tapia F, Alberto C, Nunes E. Vegetative growth and early production of six olive cultivars, in southern Atacama desert, Chile. Journal of Central European Agriculture. 2007;8(3):269-76.
31. Yada S, Harris L. Olives: Safe Methods for Home Pickling: UCANR Publications; 2007.
32. Kiritsakis A, Shahidi F. Olives and Olive Oil as Functional Foods: Bioactivity, Chemistry and Processing: John Wiley & Sons; 2017.
33. Boskou D. Olive oil: chemistry and technology: Elsevier; 2015.
34. Ryan D, Robards K. Critical Review. Phenolic compounds in olives. Analyst. 1998;123(5):31R-44R.
35. Alagna F, Mariotti R, Panara F, Caporali S, Urbani S, Veneziani G, et al. Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. BMC Plant Biology. 2012;12(1):162.
36. Caporaso N. Virgin Olive Oils: Environmental Conditions, Agronomical Factors and Processing Technology Affecting the Chemistry of Flavor Profile. Journal of Food Chemistry and Nanotechnology. 2016;2(1):21-31.
37. Caponio F, Gomes T, Summo C, Pasqualone A. Influence of the type of olive-crusher used on the quality of extra virgin olive oils. European Journal of Lipid Science and Technology. 2003;105(5):201-6.
38. Leone A, Romaniello R, Zagaria R, Sabella E, De Bellis L, Tamborrino A. Machining effects of different mechanical crushers on pit particle size and oil drop distribution in olive paste. European Journal of Lipid Science and Technology. 2015;117(8):1271-9.

39. Preziuso SM, Di Serio MG, Biasone A, Vito R, Mucciarella MR, Di Giovacchino L. Influence of olive crushing methods on the yields and oil characteristics. European Journal of Lipid Science and Technology. 2010;112(12):1345-55.
40. Veillet S, Tomao V, Bornard I, Ruiz K, Chemat F. Chemical changes in virgin olive oils as a function of crushing systems: Stone mill and hammer crusher. Comptes Rendus Chimie. 2009;12(8):895-904.
41. Stefanoudaki E, Koutsaftakis A, Harwood JL. Influence of malaxation conditions on characteristic qualities of olive oil. Food Chemistry. 2011;127(4):1481-6.
42. Angerosa F, Mostallino R, Basti C, Vito R. Influence of malaxation temperature and time on the quality of virgin olive oils. Food Chemistry. 2001;72(1):19-28.
43. Clodoveo ML. Malaxation: Influence on virgin olive oil quality. Past, present and future–An overview. Trends in Food Science & Technology. 2012;25(1):13-23.
44. Gómez-Rico A, Inarejos-García AM, Salvador MD, 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(9):3587-95.
45. Parenti A, Spugnoli P, Masella P, Calamai L. The effect of malaxation temperature on the virgin olive oil phenolic profile under laboratory-scale conditions. European Journal of Lipid Science and Technology. 2008;110(8):735-41.
46. Servili M, Taticchi A, Esposto S, Sordini B, Urbani S. Technological aspects of olive oil production. Olive Germplasm-The Olive Cultivation, Table Olive and Olive Oil Industry in Italy: InTech; 2012.
47. Di Giovacchino L, Sestili S, Di Vincenzo D. Influence of olive processing on virgin olive oil quality. European Journal of Lipid Science and Technology. 2002;104(9-10):587-601.
48. Vossen P. Olive Oil Processing Technology Influences on Quality. 2009.
49. Niaounakis M, Halvadakis CP. Olive processing waste management: literature review and patent survey: Elsevier; 2006.
50. Borja R, Raposo F, Rincón B. Treatment technologies of liquid and solid wastes from two-phase olive oil mills. Grasas y Aceites. 2006;57(1):32-46.
51. Gimeno E, Castellote A, Lamuela-Raventós R, De la Torre M, López-Sabater M. The effects of harvest and extraction methods on the antioxidant content (phenolics, α-tocopherol, and β-carotene) in virgin olive oil. Food Chemistry. 2002;78(2):207-11.
52. Frankel E, Bakhouche A, Lozano-Sánchez Js, 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(22):5179-88.

53. Toscano P, Montemurro F. Olive mill by-products management. Olive Germplasm-The Olive Cultivation, Table Olive and Olive Oil Industry in Italy: InTech; 2012.
54. Uribe E, Lemus-Mondaca R, Pasten A, Astudillo S, Vega-Gálvez A, Puente-Díaz L, et al. Dehydrated olive-waste cake as a source of high value-added bioproduct: Drying kinetics, physicochemical properties, and bioactive compounds. Chilean Journal of Agricultural Research. 2014;74(3):293-301.
55. Cicerale S, Conlan XA, Sinclair AJ, Keast RS. Chemistry and health of olive oil phenolics. Critical Reviews in Food Science and Nutrition. 2008;49(3):218-36.
56. Han J, Talorete TP, Yamada P, Isoda H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Cytotechnology. 2009;59(1):45-53.
57. Federici F, Fava F, Kalogerakis N, Mantzavinos D. Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters. Journal of Chemical Technology and Biotechnology. 2009;84(6):895-900.
58. Prosodol. Integrated Strategy of actions, measures and means suitable for Mediterranean Countries : Analysis of national and European legislative frameworks for Oil Olive Waste and Soil Protection: European Commision,,; 2012 [cited 2017 22 Sept]. Available from: http://www.prosodol.gr/sites/prosodol.gr/files/ACT15_1.pdf.
59. Akula R, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior. 2011;6(11):1720-31.
60. Giada MdLR. Food phenolic compounds: main classes, sources and their antioxidant power. Oxidative Stress and Chronic Degenerative Diseases-A role for Antioxidants: InTech; 2013.
61. Paiva-Martins F, Santos V, Mangericão H, Gordon MH. Effects of copper on the antioxidant activity of olive polyphenols in bulk oil and oil-in-water emulsions. Journal of Agricultural and Food Chemistry. 2006;54(10):3738-43.
62. Rodríguez-Morató J, Xicota L, Fito M, Farre M, Dierssen M, de la Torre R. Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases. Molecules. 2015;20(3):4655-80.
63. Talhaoui N, Gómez-Caravaca AM, León L, De la Rosa R, Fernández-Gutiérrez A, Segura-Carretero A. From olive fruits to olive oil: Phenolic compound transfer

in six different olive cultivars grown under the same agronomical conditions. International Journal of Molecular Sciences. 2016;17(3):337.

64. Servili M, Sordini B, Esposto S, Urbani S, Veneziani G, Di Maio I, et al. Biological activities of phenolic compounds of extra virgin olive oil. Antioxidants. 2013;3(1):1-23.
65. Stiti N, Triki S, Hartmann M-A. Formation of triterpenoids throughout Olea europaea fruit ontogeny. Lipids. 2007;42(1):55-67.
66. Fernández-Bolaños J, Rodríguez G, Rodríguez R, Guillén R, Jiménez A. Extraction of interesting organic compounds from olive oil waste. Grasas y Aceites. 2006;57(1):95-106.
67. Rietjens SJ, Bast A, Haenen GR. New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. Journal of Agricultural and Food Chemistry. 2007;55(18):7609-14.
68. Valavanidis A, Nisiotou C, Papageorgiou Y, Kremli I, Satravelas N, Zinieris N, et al. Comparison of the radical scavenging potential of polar and lipidic fractions of olive oil and other vegetable oils under normal conditions and after thermal treatment. Journal of Agricultural and Food Chemistry. 2004;52(8):2358-65.
69. Barbulova A, Colucci G, Apone F. New trends in cosmetics: By-products of plant origin and their potential use as cosmetic active ingredients. Cosmetics. 2015;2(2):82-92.
70. Miralles P, Chisvert A, Salvador A. Determination of hydroxytyrosol and tyrosol by liquid chromatography for the quality control of cosmetic products based on olive extracts. Journal of Pharmaceutical and Biomedical Analysis. 2015;102:157-61.
71. Napolitano A, De Lucia M, Panzella L, d'Ischia M. The chemistry of tyrosol and hydroxytyrosol: implications for oxidative stress. Olives and Olive Oil In Health and Disease Prevention 1st ed Academic Press is an imprint of Elsevier. 2010:1225-32.
72. Fernández-Bolaños JG, López Ó, López-García MÁ, Marset A. Biological properties of hydroxytyrosol and its derivatives. Olive Oil-Constituents, Quality, Health Properties and Bioconversions: InTech; 2012.
73. Puel C, Mardon J, Agalias A, Davicco M-J, Lebecque P, Mazur A, et al. Major phenolic compounds in olive oil modulate bone loss in an ovariectomy/inflammation experimental model. Journal of Agricultural and Food Chemistry. 2008;56(20):9417-22.
74. Richard N, Arnold S, Hoeller U, Kilpert C, Wertz K, Schwager J. Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs

cytokine and chemokine production in macrophages. Planta Medica. 2011;77(17):1890-7.

75. Bu Y, Rho S, Kim J, Kim MY, Lee DH, Kim SY, et al. Neuroprotective effect of tyrosol on transient focal cerebral ischemia in rats. Neuroscience Letters. 2007;414(3):218-21.
76. De Stefano D, Maiuri MC, Simeon V, Grassia G, Soscia A, Cinelli MP, et al. Lycopene, quercetin and tyrosol prevent macrophage activation induced by gliadin and IFN-γ. European Journal of Pharmacology. 2007;566(1):192-9.
77. St-Laurent-Thibault C, Arseneault M, Longpre F, Ramassamy C. Tyrosol and Hydroxytyrosol Two Main Components of Olive Oil, Protect N2a Cells Against Amyloid-β-Induced Toxicity. Involvement of the NF-κB Signaling. Current Alzheimer research. 2011;8(5):543-51.
78. Chandramohan R, Saravanan S, Pari L. Beneficial effects of tyrosol on altered glycoprotein components in streptozotocin-induced diabetic rats. Pharmaceutical Biology. 2017;55(1):1631-7.
79. Ahn E-Y, Jiang Y, Zhang Y, Son EM, You S, Kang S-W, et al. Cytotoxicity of p-tyrosol and its derivatives may correlate with the inhibition of DNA replication initiation. Oncology Reports. 2008;19(2):527-34.
80. Koudounas K, Banilas G, Michaelidis C, Demoliou C, Rigas S, Hatzopoulos P. A defence-related Olea europaea β-glucosidase hydrolyses and activates oleuropein into a potent protein cross-linking agent. Journal of Experimental Botany. 2015;66(7):2093-106.
81. Khoufi S, Hamza M, Sayadi S. Enzymatic hydrolysis of olive wastewater for hydroxytyrosol enrichment. Bioresource technology. 2011;102(19):9050-8.
82. European Medicine Agency. Assessment report on Olea europaea L., folium. In: (HMPC) CoHMP, editor. London: EMA; 2012. p. 1-33.
83. Omar SH. Oleuropein in olive and its pharmacological effects. Scientia Pharmaceutica. 2010;78(2):133-54.
84. 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(1):98-112.
85. Sweetman SC. Nutritional agents and vitamins: Vitamin E substances. Martindale, the Complete Drug Reference. 35th. London: Pharmaceutical Press; 2007. p. 1828-9.
86. Azzi A, Breyer I, Feher M, Pastori M, Ricciarelli R, Spycher S, et al. Specific cellular responses to α-tocopherol. The Journal of Nutrition. 2000;130(7):1649-52.

87. Engin KN. Alpha-tocopherol: looking beyond an antioxidant. Molecular Vision. 2009;15:855.
88. Singh U, Devaraj S, Jialal I. Vitamin E, oxidative stress, and inflammation. Annual Review of Nutrition. 2005;25:151-74.
89. Du Preeze H. Squalene–antioxidant of the future? Medicine. 2007;33:106-12.
90. Das B, Antoon R, Tsuchida R, Lotfi S, Morozova O, Farhat W, et al. Squalene selectively protects mouse bone marrow progenitors against cisplatin and carboplatin-induced cytotoxicity in vivo without protecting tumor growth. Neoplasia. 2008;10(10):1105IN4-19.
91. Kohno Y, Egawa Y, Itoh S, Nagaoka S-i, Takahashi M, Mukai K. Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in n-butanol. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 1995;1256(1):52-6.
92. Kalogeropoulos N, Chiou A, Gavala E, Christea M, Andrikopoulos NK. Nutritional evaluation and bioactive microconstituents (carotenoids, tocopherols, sterols and squalene) of raw and roasted chicken fed on DHA-rich microalgae. Food research international. 2010;43(8):2006-13.
93. Gabás-Rivera C, Barranquero C, Martínez-Beamonte R, Navarro MA, Surra JC, Osada J. Dietary squalene increases high density lipoprotein-cholesterol and paraoxonase 1 and decreases oxidative stress in mice. PloS One. 2014;9(8):e104224.
94. Kelly GS. Squalene and its potential clinical uses. Alternative Medicine Review: A Journal of Clinical Therapeutic. 1999;4(1):29-36.
95. Huang Z-R, Lin Y-K, Fang J-Y. Biological and pharmacological activities of squalene and related compounds: potential uses in cosmetic dermatology. Molecules. 2009;14(1):540-54.
96. Fox CB. Squalene emulsions for parenteral vaccine and drug delivery. Molecules. 2009;14(9):3286-312.
97. Medicines Control Council. Registration of medicine. In: Health, editor. Pretoria: Medicines Control Council; 2013. p. 2-8.
98. Ortega-García F, Peragón J. HPLC analysis of oleuropein, hydroxytyrosol, and tyrosol in stems and roots of Olea europaea L. cv. Picual during ripening. Journal of the Science of Food and Agriculture. 2010;90(13):2295-300.
99. Edison B. Analysis of tocopherols by high performance liquid chromatography. Journal of Chemistry. 2009;6(2):395-8.

100. Popa O, Băbeanu NE, Popa I, Niță S, Dinu-Pârvu CE. Methods for obtaining and determination of squalene from natural sources. BioMed Research International. 2015;2015.
101. Sagratini G, Allegrini M, Caprioli G, Cristalli G, Giardina D, Maggi F, et al. Simultaneous determination of squalene, α-tocopherol and β-carotene in table olives by solid phase extraction and high-performance liquid chromatography with diode array detection. Food Analytical Methods. 2013;6(1):54-60.
102. Snyder LR, Kirkland JJ, Dolan JW. Introduction to modern liquid chromatography: John Wiley & Sons; 2011.
103. Kupiec T. Quality-control analytical methods: High-performance liquid chromatography. International Journal of Pharmaceutical Compounding. 2004;8:223-7.
104. Unites States Pharmacopoeia and National Formulary. <621> Chromatography. Tests, Physical: US Pharmacopeia/National Formulary (USP): Rockville, MD, USP36-NF21.
105. CHROMacademy e-lftacc, ,. The Theory of HPLC. Chromatographic Parameters: Crawford Scientific; [cited 2017 22 Sept]. Available from: http://chemistry.unt.edu/~tgolden/courses/Theory_Of_HPLC_Chromatographic_Parameters.pdf.
106. Agilent Technologies. Agilent J&W GC Column Selection Guide USA: Agilent Technologies Inc.; 2012 [cited 2016 19 Jul]. Available from: https://www.agilent.com/cs/library/catalogs/public/5990-9867EN_GC_CSG.pdf.
107. Kalogerakis N, Politi M, Foteinis S, Chatzisymeon E, Mantzavinos D. Recovery of antioxidants from olive mill wastewaters: a viable solution that promotes their overall sustainable management. Journal of Environmental Management. 2013;128:749-58.
108. Mulinacci N, Romani A, Galardi C, Pinelli P, Giaccherini C, Vincieri F. Polyphenolic content in olive oil waste waters and related olive samples. Journal of Agricultural and Food Chemistry. 2001;49(8):3509-14.
109. Fernández-Bolaños J, Rodríguez G, Rodríguez R, Heredia A, Guillén R, Jiménez A. Production in large quantities of highly purified hydroxytyrosol from liquid−solid waste of two-phase olive oil processing or “Alperujo”. Journal of Agricultural and Food Chemistry. 2002;50(23):6804-11.
110. Lampi A-M. Analysis of tocopherols and tocotrienols by HPLC. The AOCS (American Oil Chemists' Society) Lipid Library,(Aug 3, 2011)(US) available online at http://lipidlibrary aocs org/topics/tocopherols/index htm. 2011.

111. Gimeno E, Castellote A, Lamuela-Raventos R, De La Torre M, Lopez-Sabater M. Rapid determination of vitamin E in vegetable oils by reversed-phase high-performance liquid chromatography. Journal of Chromatography A. 2000;881(1):251-4.
112. Buddrick O, Jones OA, Morrison PD, Small DM. Heptane as a less toxic option than hexane for the separation of vitamin E from food products using normal phase HPLC. RSC Advances. 2013;3(46):24063-8.
113. Czaplicki S, Ogrodowska D, Zadernowski R, Derewiaka D. Characteristics of biologically-active substances of amaranth oil obtained by various techniques. Polish Journal of Food and Nutrition Sciences. 2012;62(4):235-9.
114. Schneider Sonja. Quality Analysis of Extra Virgin Olive Oils – Part 5 Nutritive Benefits – Determination of Squalene in Virgin Olive Oil USA: Agilent Technologies; 2016 [cited 2015 26 Oct]. Available from: https://www.agilent.com/cs/library/applications/5991-2774EN.pdf.
115. Shah MV, Minal R. Novel techniques for isolation and extraction of phyto-constituents from herbal plants. American Journal of Phytomedicine and Clinical Therapeutics. 2013;1(3).
116. Bucar F, Wube A, Schmid M. Natural product isolation–how to get from biological material to pure compounds. Natural Product Reports. 2013;30(4):525-45.
117. Busia K. Fundamentals of Herbal Medicine: History, Phytopharmacology and Phytotherapeutics: Xlibris Corporation; 2016.
118. Sasidharan S, Chen Y, Saravanan D, Sundram K, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. African Journal of Traditional, Complementary and Alternative Medicines. 2011;8(1).
119. Segneanu A-E, Cziple F, Vlazan P, Sfirloaga P, Grozescu I, Gherman VD. Biomass extraction methods. Biomass Now-Sustainable Growth and Use: Intech; 2013.
120. Smith R. Chemical process: design and integration: John Wiley & Sons; 2005.
121. Sambamurthy K. Pharmaceutical engineering: New Age International; 2007.
122. Singh J. Maceration, percolation and infusion techniques for the extraction of medicinal and aromatic plants. Extraction Technologies for Medicinal and Aromatic Plants. 2008;67:32-5.
123. Vinatoru M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. Ultrasonics Sonochemistry. 2001;8(3):303-13.
124. List PH, Schmidt CC. Phytopharmaceutical Technology: Wiley; 1991.
125. Jensen WB. The origin of the Soxhlet extractor. J Chem Educ. 2007;84(12):1913.

126. Wang L, Weller CL. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology. 2006;17(6):300-12.
127. Alupului A, Calinescu I, Lavric V, editors. Ultrasonic vs. microwave extraction intensification of active principles from medicinal plants. AIDIC Conference Series; 2009.
128. Herrero M, Cifuentes A, Ibañez E. Sub-and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. Food Chemistry. 2006;98(1):136-48.
129. Mandal V, Mohan Y, Hemalatha S. Microwave assisted extraction–an innovative and promising extraction tool for medicinal plant research. Pharmacognosy Reviews. 2007;1(1):7-18.
130. Kaufmann B, Christen P. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. Phytochemical Analysis. 2002;13(2):105-13.
131. Kettle A. Use of Accelerated Solvent Extraction to Improve Laboratory Workflow. Thermo Fisher Scientific. 2013:5.
132. Shams K, Abdel-Azim N, Saleh I, Hegazy M, El-Missiry M, Hammouda F. Green technology: economically and environmentally innovative methods for extraction of medicinal & aromatic plants (MAP) in Egypt. J Chem Pharm Res. 2015;7:1050-74.
133. Wesolowski Paul. Analysis of moisture content is critical to ensuring material quality Nanikon, Switzerland: Scientist Live; 2013 [cited 2017 20 Feb]. Available from: http://www.scientistlive.com/content/9298.
134. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: a review. Internationale Pharmaceutica Sciencia. 2011;1(1):98-106.
135. Nawaz H, Shi J, Mittal GS, Kakuda Y. Extraction of polyphenols from grape seeds and concentration by ultrafiltration. Separation and Purification Technology. 2006;48(2):176-81.
136. Unites States Pharmacopoeia and National Formulary. <467> Residual solvents. Tests, Chemical: US Pharmacopeia/National Formulary (USP): Rockville, MD, USP38-NF28
137. Tercan S, Seker M. Comparison of polyphenol extractions from olive pomace and solid fraction of olive mill waste water. Natural Product Research. 2012;26(19):1837-41.
138. Costa AS, Alves RC, Vinha AF, Barreira SV, Nunes MA, Cunha LM, et al. Optimization of antioxidants extraction from coffee silverskin, a roasting by-

product, having in view a sustainable process. Industrial Crops and Products. 2014;53:350-7.

139. Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules. 2010;15(10):7313-52.
140. Lesage-Meessen L, Navarro D, Maunier S, Sigoillot J, Lorquin J, Delattre M, et al. Simple phenolic content in olive oil residues as a function of extraction systems. Food Chemistry. 2001;75(4):501-7.
141. Hwang E-S, Do Thi N. Effects of extraction and processing methods on antioxidant compound contents and radical scavenging activities of laver (Porphyra tenera). Preventive Nutrition and Food Science. 2014;19(1):40.
142. Pagliaro M, Rossi M. The future of glycerol2008.
143. Garcia-Fuentes AR, Wirtz S, Vos E, Verhagen H. Short Review of Sulphites as Food Additives. 2015.
144. Manousaki A, Jancheva M, Grigorakis S, Makris DP. Extraction of antioxidant phenolics from agri-food waste biomass using a newly designed glycerol-based natural low-transition temperature mixture: A comparison with conventional eco-friendly solvents. Recycling. 2016;1(1):194-204.
145. Agency EUSEP. Health and Environmental Effects of Hazardous Air Pollutants Washington, USA: EPA; 2017 [cited 2016 5 May]. Available from: https://www.epa.gov/haps/health-and-environmental-effects-hazardous-air-pollutants.
146. McConnell B. Kinetics of Lipid Extraction From Microalgae. 2013.
147. Waldebäck M, Señoráns FJ, Fridström A, Markides KE. Pressurized fluid extraction of squalene from olive biomass. ACS Publications; 2006.
148. Gunawan S, Kasim NS, Ju Y-H. Separation and purification of squalene from soybean oil deodorizer distillate. Separation and Purification Technology. 2008;60(2):128-35.
149. Lagos JB, Gemin CAB, Dias JdFG, Miguel MD, Campos R, de Souza Carvalho JL, et al. Contribution to the Study of Phytosterols and Phenolics from the Barks and Leaves of the Trichilia catigua A. Juss., Meliaceae. Journal of Analytical Sciences, Methods and Instrumentation. 2012;2(01):37.
150. Sicaire A-G, Vian M, Fine F, Joffre F, Carré P, Tostain S, et al. Alternative bio-based solvents for extraction of fat and oils: solubility prediction, global yield, extraction kinetics, chemical composition and cost of manufacturing. International Journal of Molecular Sciences. 2015;16(4):8430-53.
151. Barton AF. Handbook of Solubility Parameters and other Cohesion Parameters, . 2nd, ed. Florida,: CRC Press,; 1991. 64 p.

152. Hansen CM. Hansen solubility parameters: a user's handbook: CRC press; 2007.
153. Kitak T, Dumičić A, Planinšek O, Šibanc R, Srčič S. Determination of solubility parameters of ibuprofen and ibuprofen lysinate. Molecules. 2015;20(12):21549-68.
154. Mollet H, Grubenmann A. Formulation technology: emulsions, suspensions, solid forms: John Wiley & Sons; 2008.
155. Aissou M, Chemat-Djenni Z, Yara-Varón E, Fabiano-Tixier A-S, Chemat F. Limonene as an agro-chemical building block for the synthesis and extraction of bioactive compounds. Comptes Rendus Chimie. 2017;20(4):346-58.
156. Clark J. Raoult's law and ideal mixtures of liquids United Kingdom2009 [cited 2015 7 July]. Available from: http://www.chemguide.co.uk/physical/phaseeqia/idealpd.html#top.
157. Clark J. Non-ideal mixtures of liquids United Kingdom2009 [cited 2015 7 July]. Available from: http://www.chemguide.co.uk/physical/phaseeqia/nonideal.html#top.
158. Meireles MAA. Extracting bioactive compounds for food products: theory and applications: CRC press; 2008.
159. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry. 2005;53(10):4290-302.
160. De Marco E, Savarese M, Paduano A, Sacchi R. Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. Food Chemistry. 2007;104(2):858-67.
161. Leouifoudi I, Harnafi H, Zyad A. Olive mill waste extracts: polyphenols content, antioxidant, and antimicrobial activities. Advances in Pharmacological Sciences. 2015;2015.
162. Yangui T, Sayadi S, Gargoubi A, Dhouib A. Fungicidal effect of hydroxytyrosol-rich preparations from olive mill wastewater against Verticillium dahliae. Crop Protection. 2010;29(10):1208-13.
163. Barranco D. Varieties and rootstocks. 5th ed. Australia: RIRDC; 2010 September. 756 p.
164. Alburquerque J, Gonzálvez J, Garcıa D, Cegarra J. Agrochemical characterisation of “alperujo”, a solid by-product of the two-phase centrifugation method for olive oil extraction. Bioresource Technology. 2004;91(2):195-200.
165. Trombetta D, Smeriglio A, Marcoccia D, Giofrè SV, Toscano G, Mazzotti F, et al. Analytical Evaluation and Antioxidant Properties of Some Secondary Metabolites in Northern Italian Mono-and Multi-Varietal Extra Virgin Olive Oils (EVOOs) from

Early and Late Harvested Olives. International Journal of Molecular Sciences. 2017;18(4):797.

166. Esti M, Cinquanta L, La Notte E. Phenolic compounds in different olive varieties. Journal of Agricultural and Food Chemistry. 1998;46(1):32-5.
167. Sivakumar G, Bati CB, Uccella N. HPLC-MS screening of the antioxidant profile of Italian olive cultivars. Chemistry of Natural Compounds. 2005;41(5):588-91.
168. Alagna F, Geu-Flores F, Kries H, Panara F, Baldoni L, O'Connor SE, et al. Identification and characterization of the iridoid synthase involved in oleuropein biosynthesis in olive (Olea europaea) fruits. Journal of Biological Chemistry. 2016;291(11):5542-54.
169. Kalogeropoulos N, Tsimidou MZ. Antioxidants in Greek virgin olive oils. Antioxidants. 2014;3(2):387-413.
170. Gutierrez-Rosales F, Romero MaP, Casanovas Ma, Motilva MaJ, Mínguez-Mosquera MaI. Metabolites involved in oleuropein accumulation and degradation in fruits of Olea europaea L.: Hojiblanca and Arbequina varieties. Journal of Agricultural and Food Chemistry. 2010;58(24):12924-33.
171. Ambra R, Natella F, Lucchetti S, Forte V, Pastore G. α-Tocopherol, β-carotene, lutein, squalene and secoiridoids in seven monocultivar Italian extra-virgin olive oils. International Journal of Food Sciences and Nutrition. 2017;68(5):538-45.
172. Escuderos M, Sayago A, Morales M, Aparicio R. Evaluation of α-tocopherol in virgin olive oil by a luminescent method. Grasas y aceites. 2009;60(4):336-42.
173. Gambacorta G, Faccia M, Previtali M, Pati S, Notte EL, Baiano A. Effects of olive maturation and stoning on quality indices and antioxidant content of extra virgin oils (cv. Coratina) during storage. Journal of Food Science. 2010;75(3).
174. Arbonés-Mainar J. Olive oil phenolics as potential therapeutical agents: Nova Science Publishers; 2009.
175. Morales JC, Lucas R. Structure–Activity Relationship of Phenolic Antioxidants and Olive Components. Olives and Olive Oil in Health and Disease Prevention: Academic Press Tokyo; 2010. p. 905-14.
176. Galano A, Alvarez-Idaboy JR, Francisco-Márquez M, Medina ME. A quantum chemical study on the free radical scavenging activity of tyrosol and hydroxytyrosol. Theoretical Chemistry Accounts. 2012;131(3):1173.
177. Hussain Z, Katas H, Amin MCIM, Kumolosasi E, Buang F, Sahudin S. Self-assembled polymeric nanoparticles for percutaneous co-delivery of hydrocortisone/hydroxytyrosol: an ex vivo and in vivo study using an NC/Nga mouse model. International Journal of Pharmaceutics. 2013;444(1):109-19.

178. Mehraein F, Sarbishegi M, Aslani A. Evaluation of effect of oleuropein on skin wound healing in aged male BALB/c mice. Cell Journal (Yakhteh). 2014;16(1):25.
179. Rietjens SJ. Hydroxytyrosol. A versatile antioxidant from olive oil: Maastricht university; 2008.
180. Montedoro G, Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A. Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterizations of the secoiridoid derivatives. Journal of Agricultural and Food Chemistry. 1993;41(11):2228-34.
181. Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Del Carlo M, Gallina-Toschi T, et al. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. Journal of Agricultural and Food Chemistry. 2005;53(23):8918-25.
182. Dağdelen A. Identifying Antioxidant and Antimicrobial Activities of the Phenolic Extracts and Mineral Contents of Virgin Olive Oils (Olea europaea L. cv. Edincik Su) from Different Regions in Turkey. Journal of Chemistry. 2016;2016.
183. Bisignano G, Tomaino A, Cascio RL, Crisafi G, Uccella N, Saija A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. Journal of Pharmacy and Pharmacology. 1999;51(8):971-4.
184. Sousa A, Ferreira IC, Calhelha R, Andrade PB, Valentão P, Seabra R, et al. Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra'. Bioorganic & Medicinal Chemistry. 2006;14(24):8533-8.
185. De Leonardis A, Macciola V, Cuomo F, Lopez F. Evidence of oleuropein degradation by olive leaf protein extract. Food Chemistry. 2015;175:568-74.
186. Rastrelli L, Passi S, Ippolito F, Vacca G, De Simone F. Rate of degradation of α-tocopherol, squalene, phenolics, and polyunsaturated fatty acids in olive oil during different storage conditions. Journal of Agricultural and Food Chemistry. 2002;50(20):5566-70.
187. Luo H. Extraction of antioxidant compounds from olive (Olea europaea) leaf: a thesis present [ie presented] in partial fulfilment of the requirements for the degree of Master of Technology in Food Technology at Massey University, Albany, New Zealand: Massey University; 2011.
188. Ryan D, Antolovich M, Prenzler P, Robards K, Lavee S. Biotransformations of phenolic compounds in Olea europaea L. Scientia Horticulturae. 2002;92(2):147-76.
189. Moure A, Cruz JM, Franco D, Domínguez JM, Sineiro J, Domínguez H, et al. Natural antioxidants from residual sources. Food Chemistry. 2001;72(2):145-71.

190. Lambers H, Piessens S, Bloem A, Pronk H, Finkel P. Natural skin surface pH is on average below 5, which is beneficial for its resident flora. International journal of cosmetic science. 2006;28(5):359-70.
191. Baiano A, Gambacorta G, Terracone C, Previtali M, Lamacchia C, La Notte E. Changes in Phenolic Content and Antioxidant Activity of Italian Extra-Virgin Olive Oils during Storage. Journal of Food Science. 2009;74(2).

APPENDICES

Appendix 4.1 Extraction trials to determine optimum method, sample type and solvent mixture for hydrophilic compounds

A. Extraction Method:	**Soxhlet A(SA)**	**Soxhlet B(SB)**	**Soxhlet C(SC)**	**Soxhlet D(SD)**	**Soxhlet E2(SE2)**	**Soxhlet E1(SE1)**	**Percolation 1(P1)**	**Percolation 2(P2)**	**Blend/stir in beaker(SB)**
Solvent	**H_2O (filtered)**	**H_2O + acetic acid**	**H_2O + acetic acid + 2% mbs**	**H_2O + 2% mbs**	**60% MeOH + 2% mbs**	**60% MeOH + 2% mbs**	**60% MeOH + 2% mbs**	**H_2O (unfiltered)**	**60% MeOH + 2% mbs**
pH of solvent	7.70	2.00	2.53	3.84	4.63	4.63	4.63	6.27	4.63
Solvent cond.	0.055 µS/cm	-	25.9 mS/cm	21.2 mS/cm	6.67 mS/cm	6.67 mS/cm	6.67 mS/cm	0.055 µS/cm	6.67 mS/cm
Solvent vol.	150 ml	150 ml	150 ml	150 ml	150 ml	150 ml	3 x 50 ml	150 ml	50 ml
Pomace type*	fresh	fresh	fresh	fresh	fresh	freeze-dried	freeze-dried	freeze-dried	freeze-dried
Pomace mass	50.41 g	50.32 g	50.05 g	50.02 g	50.09 g	21.45 g**	21.42 g	21.29 g	5.02 g
Stir setting	4	4	4	4	4	4	na	na	4
Temp setting	7	9	9	9	7-9	7	na	na	na
Reflux temp	98 ^oC	98 ^oC	101 ^oC	99 ^oC	82 ^oC	82 ^oC	RT	RT	RT
Extraction time	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr	10 min per 50ml portion	not measured #	30 min + 15 min
Extract pH	4.65	3.03	4.51	5.28	6.43	6.71	5.51	4.73	5.38
Extract cond.	3.64 mS/cm	3.24 mS/cm	15.67 mS/cm	16.27 mS/cm	7.41 mS/cm	7.67 mS/cm	6.64 mS/cm	4.42 mS/cm	6.72 mS/cm
Extract vol.	-	-	-	-	±140 ml	±125 ml	20 + 50 + 50 = 120 ml	±110 ml	±40 ml
Extract colour	dark brown	red-brown	amber	gold	light yellow	yellow-brown	red-brown	dark brown	amber
Extract turb.	clear	clear	clear	clear	**clear	**foamy, murky, thick	clear	murky	clear
Extract BRIX	2.3%	3.1%	4.4%	5.1%	9.8%	13.5%	13.1%	4.7%	11.3%
B. Defat extracts:	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 10 ml hexane, filtered

Appendix 4.1 Extraction trials to determine optimum method, sample type and solvent mixture for hydrophilic compounds (continued)

Extraction Method:	**Soxhlet A (SA)**	**Soxhlet B (SB)**	**Soxhlet C (SC)**	**Soxhlet D (SD)**	**Soxhlet E2 (SE2)**	**Soxhlet E1 (SE1)**	**Percolation 1 (P1)**	**Percolation 2 (P2)**	**Blend/stir in beaker (SB)**
Solvent	**H_2O (filtered)**	**H_2O + acetic acid**	**H_2O + acetic acid +2% mbs**	**H_2O + 2% mbs**	**60% MeOH + 2% mbs**	**60% MeOH + 2% mbs**	**60% MeOH + 2% mbs**	**H_2O (unfiltered)**	**60% MeOH + 2% mbs**
C. Rota-dry: Mass RBF	147.28 g	142.16 g	150.09 g	132.88 g	106.08 g	106.08 g	106.11 g	61.55 g	61.55 g
Mass residue + RBF	150.73 g	145.57 g	156.80 g	139.01 g	112.07 g	113.61 g	110.48 g	64.79 g	63.60 g
Mass residue	3.45 g	3.41 g	6.71 g	6.13 g	5.99 g	7.53 g	4.37 g	3.24 g	2.05 g
Extractable matter (mg/g pomace)	**3.45 /50.41 =68.4 mg/g *172.5 mg/g$***	**3.41/50.32 = 67.8 mg/g *170.5 mg/g$***	**6.71/50.05 = 35.0 mg/g 134 *mg/g$***	**6.13/50.02 122.6 mg/g *299.5 mg/g$***	**5.99/50.09 =119.6mg/g**	**7.53/21.45 =351.0mg/g**	**4.37/21.42 =204.0mg/g**	**3.24/21.09 =152.2 mg/g**	**2.05/5.0 =408.4 mg/g**
Temp of waterbath:	-	-	-	-	actual 55 ^oC	Approx. 70^oC	actual 55 ^oC	actual 55 ^oC	actual 55 ^oC
Observations:	Very sticky residue, difficult to dissolve in HPLC solvent	Very sticky residue, effort to dissolve in HPLC solvent	Very sticky residue, effort to dissolve in HPLC solvent	Very sticky residue, effort to dissolve in HPLC solvent	Syrupy liq residue	Timeous vapo-drying process solvent > 4 hr	Liquid boiled over, some lost, residue syrupy	Very sticky residue, added heat to dissolve	Syrupy liquid residue
Sample prep for HPLC analysis	Dissolved residue in 25 ml 50% aqua methanol	Dissolved residue in 25 ml 50% aqua meth	Dissolved residue in 25 ml 50% aqua methanol	Dissolved residue in 25 ml 50% aqua methanol	Dissolved residue in 25 ml 50% aqua meth	Dissolved residue in 25 ml 50% aqua methanol	Dissolved residue in 25 ml 50% aqua methanol	Dissolved residue in 25 ml 50% aqua methanol	Dissolved residue in 10 ml 50% aqua methanol
D. Quantificaion: Tyrosol T Oleuropein O	T: 0.034 mg/g* T: 0.082 mg/g$ O: 0.024 mg/g*	T: 0.80 mg/g* T: 1.91 mg/g$ O: ND	T: 0.13 mg/g* T:0.32 mg/g$ O: ND	T: 0.087 mg/g* T: 0.21 mg/g$ O: 0.14 mg/g*	T: 0.049 mg/g* T: 0.12 mg/g$ O: ND	T: 0.44 mg/g$ O: ND	T: 0.016 mg/g$ O: 0.26 mg/g$	T: 0.06 mg/g$ O: 0.60 mg/g$	T: 0.31 mg/g$ O: 0.20 mg/g$
E. Total phenols F-C:	10.45 mg GAE /g dry pomace	7.27 mg GAE /g dry pomace	10.45 mg GAE /g dry pomace	20.83 mg GAE /g dry pomace	35.49 mg GAE /g dry pomace	34.06 mg GAE /g dry pomace	Not determined	Not determined	Not determined

* Two-phase processing olive pomace from *Frantoio* cultivar – fresh pomace; $ - 2nd yield value: freeze-dried pomace (dry weight - ± 60% water loss during freeze-drying)
** In both extracts a white precipitation was formed upon storage in fridge but SE1 was repeated. 2nd extract also formed white precipitation, it was thus filtered before being defatted.
# Drainage of eluting solvent was very slow thus system was allowed to stand overnight in order to percolate through sample and drain into beaker.
ND: Not detected

*

Appendix 4.2 Extraction trials to determine repeatability and optimum aqueous/alcohol extraction solvent

A. Extraction Method:	***Soxhlet 1H (S1H)***	***Soxhlet 2GH (S2GH)***	***Soxhlet 3M (S3M)***	***Soxhlet 4E (S4E)***	***Soxhlet 5A (S5A)***	***Soxhlet 6MBS (S6mbs)***	***Soxhlet 7HR***	***Soxhlet 8M***
Solvent	**H_2O (filtered)**	**Glycerol/H_2O 50:50**	**Methanol/H_2O 50:50 €**	**Ethanol/H_2O 50:50 €**	**H_2O/acetic acid**	**60% MeOH + 2% mbs**	**H_2O repeat (filtered)**	**Methanol 100%**
pH of solvent	5.94	3.96	6.67	7.24	2.00	4.02	5.52	6.23
Solvent cond.	-	-	-	-	-	-	unstable	0.72 µS/cm
Solvent vol.	150 ml	150 ml	150 ml	150 ml	150 ml	150 ml	150 ml	150 ml
Pomace type	**freeze-dried**	**freeze-dried**	**freeze-dried**	**freeze-dried**	**freeze-dried**	**freeze-dried**	**freeze-dried**	**freeze-dried**
Pomace mass	**20.07 g**	**20.10 g**	**20.09 g**	**20.04 g**	**20.07 g**	**20.06 g**	**20.00 g**	**20.03 g**
Stir setting	4	4	4	4	4	4	4	4
Temp setting	7	9	9	9	9	9	9	9
Reflux temp	100 ^oC	130 ^oC	85 ^oC	92 ^oC	99 ^oC	84 ^oC	100 ^oC	65 ^oC
Extraction time	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr	2 hr
*Extract pH***	4.64 – 4.70	4.95	5.34	5.01	2.97	6.24	-	-
*Extract cond.***	2.13 mS/cm	55.5 µS/cm	2.44 mS/cm	1239 µS/cm	2.26 mS/cm	10.0 mS/cm	-	-
Extract vol.	92 ml post filt	60 ml (spilled)	70 ml post filt	70 ml post filt	95 ml pre-filt 85 ml post filt	95 ml pre-filt 85 ml post filt	-	-
Extract colour	yellow brown	Light yellow	Murky light yellow brown	Murky red brown with ring	Light yellow	Amber	Red brown	Amber
Extract turb.	Clear after defat + filter twice	Turbid pre-filter Clear post-filter	Murky ppt + ring pre-filter Murky – post filter #	Yellow ppt at bottom – clear after filtration but slightly murky after cooling ##	Slightly turbid pre-filter pre-defat Clear post-filter post-defat	Slightly turbid pre-filter pre-defat Clear post-filter post-defat	Slightly turbid	murky
Extract BRIX	1.1%	53.8%	6.6%	15.8%	1.9%	12.2%	-	-
B. Defat extracts:	3 x 50 ml hexane, filtered	3 x 30 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml hexane, filtered	3 x 50 ml ¥ hexane, filtered

MeOH – Methanol
* 2-Phase processed Frantoio pomace from Stilbaai
** post-filter and defatting

Mbs – metabisulfite ($Na_2O_5S_2$)
filtered 2x before measuring conductivity, pH and Brix – still slightly unclear
filtered 1x before measuring conductivity, pH and Brix

Appendix 4.2 Extraction trials to determine repeatability and optimum aqueous/alcohol extraction solvent (continued)

Extraction Method:	***Soxhlet 1H (S1H)***	***Soxhlet 2GH (S2GH)***	***Soxhlet 3M (S3M)***	***Soxhlet 4E (S4E)***	***Soxhlet 5A (S5A)***	***Soxhlet 6MBS (S6mbs)***	***Soxhlet 7HR***	***Soxhlet 8M***
Solvent	**H_2O (filtered)**	**Glycerol/H_2O 50:50**	**Methanol/H_2O 50:50 €**	**Ethanol/H_2O 50:50 €**	**H_2O/acetic acid**	**60% MeOH + 2% mbs**	**H_2O repeat (filtered)**	**Methanol 100%**
Residue	1.08 g	ND	2.23 g	2.54 g	0.92 g	5.45 g	ND	ND
Extractable matter	**53.8 mg/g**	**-**	**111 mg/g**	**126.7 mg/g**	**45.8 mg/g**	**271.7 mg/g**	**ND**	**ND**
Temp of waterbath:	60°C	80°C	60-70°C	60-70°C	60°C	60°C	60°C	60°C
Observations:	Evaporated to dryness Light brown residue	Syrupy amber coloured residue – couldn't evaporate glycerol	Methanol evaporated easily – dark brown murky residue with ppt	Ethanol evaporated easily – dark brown residue	Evaporated to dryness but left a yellowish residue	Bubble formed during evaporation, residue was syrupy	Evaporated to dryness Light brown residue	Methanol evaporated easily – dark brown murky residue with ppt
Sample prep for HPLC analysis (filtered thru 0.45 µm syringe filter before analysis)	Dissolved residue in 25 ml 50% aq methanol	Dissolved residue in 25 ml 50% aq meth	Dissolved residue in 25 ml 50% aq methanol	Dissolved residue in 25 ml 50% aq methanol	Dissolved residue in 25 ml 50% aq meth	Dissolved residue in 25 ml 50% aq methanol	Dissolved residue in 25 ml 50% aq meth	Dissolved residue in 25 ml 50% aq methanol
D. Total phenols F-C: mg GAE/g	ND	ND	**10.93**	**12.11**	**6.2**	**20.17**	**7.65**	**13.01**

ND: Not determined MeOH – Methanol Mbs – metabisulfite ($Na_2O_5S_2$)

€ In both extracts a yellowish edge formed in the round bottom flask while the extraction made soapy bubble during extraction. A slight white precipitation formed in both extracts and were thus filtered before being defatted. After the last defat, the still murky extracts filtered very slowly through the Whatman no.1 filter paper.

¥ During defatting of the 100% MeOH extract with the 1st 50 ml hexane, a blackish precipitate formed that blocked the separation funnel while the hexane portion changed colour to light green. The black precipitate had to be removed for collection of the 1st defatted extract. A black edge formed in the separation funnel. The hexane portion remained light green in the remaining defatting.

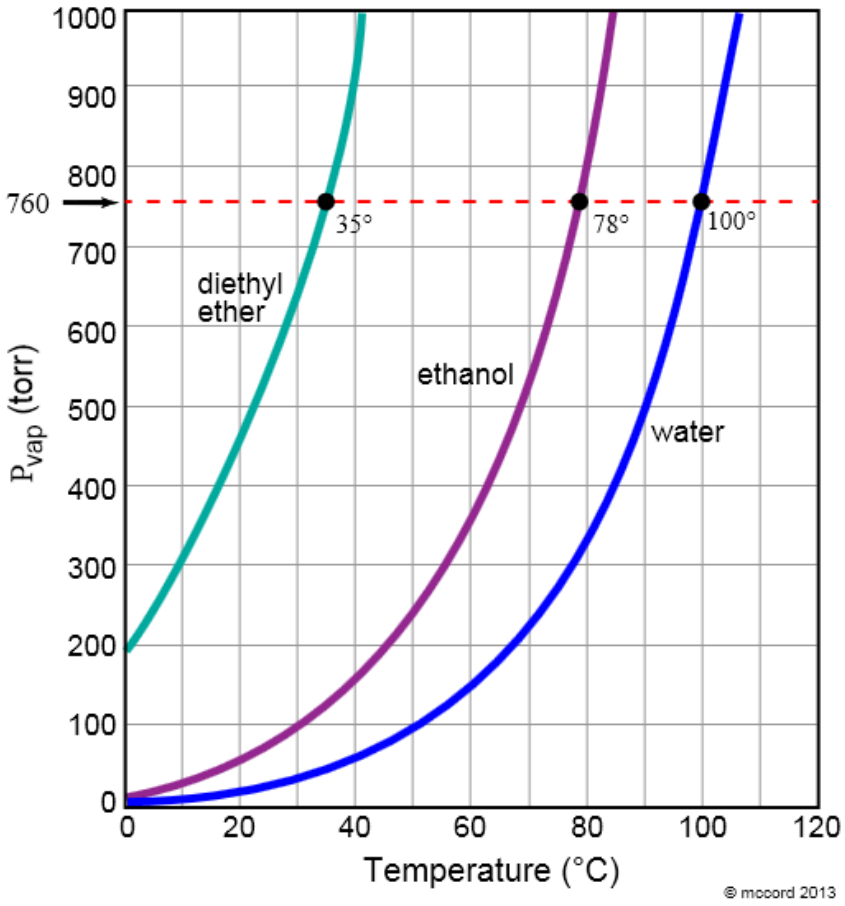
Appendix 4.3 Extraction trials to determine solvent mixture for lipophilic compounds

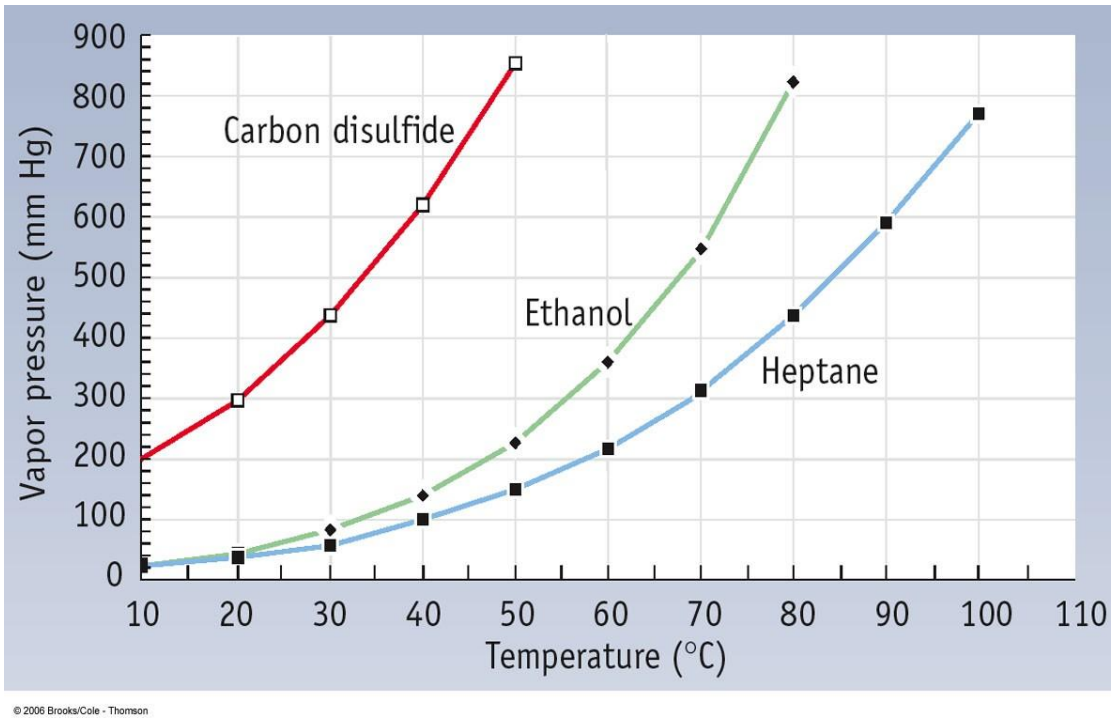
A. Extraction Method:	**Soxhlet**	**Soxhlet**	**Soxhlet**	**Soxhlet**
Solvent	***n*-Hexane + ethyl acetate 50:50**	***n*-Heptane**	***n*-Heptane + ethyl acetate 50:50**	**Isopropyl alcohol**
Boiling point of sol (°C)	68 77.1	98.42	98.42 77.1	82.6
Solvent polarity	0.1 : 4.4	0.1	0.1 : 4.4	3.9
Solvent vol.* (ml)	100	100	100	100
Pomace type	freeze-dried	freeze-dried	freeze-dried	freeze-dried
Pomace mass	10.07 g	10.02 g	10.02 g	10.06 g
Stir setting	4	4	4	4
Temp setting	9	8	9	9
Reflux temp (^{o}C)	64	95	96	84
Extraction time (min)	60	60	60	60
Extract vol. (ml)	65	75	80	80
Extract colour	Light, olive green	Yellow	Light green	Light green
Extract turb.	turbid	murky	clear	clear
Extract BRIX %	25.2	39.9	30.1	27.7
B. Filtration of extracts:	Not identified	Filtered normally	Filtered well	Filtered slowly
C. Rota-dry: mass RBF + lid (g)	158.86	152.54	158.86	202.11
mass residue + RBF + lid (g)	160.59	154.20	160.10	160.85
mass residue (g)	1.71	1.66	1.24	1.99
Extractable matter (mg/g)	169.8	165.7	123.7	197.8
Temp of waterbath (°C)	60	60	60	60
Observations	Yellow/green residue – fairly clear/slightly murky	Yellow/green residue – fairly clear	Yellow/green residue - murky	Brown/yellow residue with brown precipitate
D. Sample prep for HPLC analysis	Dissolved residue in 10 ml IPA	Dissolved residue in 10 ml IPA	Dissolved residue in 10 ml IPA	Dissolved residue in 10 ml IPA
Observations:	Slightly murky yellow/green solution	Slightly murky yellow/green solution	Slightly murky yellow/green solution	Brown precipitate did not dissolve in IPA even after heating (water soluble)

IPA – Isopropyl alcohol RBF – Round bottom flask

* 100 ml solvent was too little for Soxhlet apparatus – liquid did not flow back into RB flask

Appendix 5.1 Graphs for determination of the solvent vapour pressure at a specific temperature





Appendix 5.2 D-Optimal Mixture design – bioactive concentrations obtained

Table A Responses (concentrations) of the five bioactive

D-Optimal Mixture design

Run	Vol %				Time (h)	Conc (ppm)				
	n-Heptane	Limonene	EtOH	Water		R1 Htyr	R2 Tyr	R3 Oleu	R 4 a-Toco	R 5 Squal
1	15	0	85	0	2	23,76	4,15	1,65	3,03	26,24
2	0	0	100	0	4	**62,85**	**9,75**	**10,11**	**3,02**	**4,37**
3	0	0	75	25	3	56,33	8,92	11,81	0,38	0,78
4	30	10	60	0	2	20,67	4,98	0,55	1,10	10,21
5	0	0	100	0	4	59,51	7,08	8,10	1,20	2,74
6	0	20	80	0	3	**1,84**	**2,81**	**0,05**	**0,09**	**23,17**
7	15	20	65	0	2	**0,48**	**1,59**	**0,04**	**0,00**	**19,26**
8	0	20	50	30	2	45,25	7,17	12,60	0,06	3,72
9	30	20	50	0	5	**0,07**	**1,22**	**0,00**	**0,36**	**22,10**
10	30	0	70	0	5	34,08	5,33	2,62	2,19	2,19
11	30	0	50	20	4	51,73	6,85	10,06	4,15	14,23
12	0	0	50	50	3	47,92	7,05	7,52	0,00	0,00
13	30	0	50	20	5	**58,57**	**5,72**	**5,72**	**4,90**	**20,63**
14	15	0	68	18	4	**59,29**	**5,26**	**4,93**	**4,54**	**14,95**
15	30	20	50	0	5	**0,32**	**0,01**	**0,01**	**0,73**	**30,54**
16	15	10	50	25	3	**17,11**	**2,37**	**2,30**	**0,21**	**10,43**
17	0	20	50	30	2	**42,47**	**8,48**	**7,60**	**0,93**	**1,77**
18	0	0	50	50	3	**23,40**	**5,26**	**4,52**	**1,12**	**1,53**
19	6	4	57	32	4	**24,87**	**6,05**	**5,31**	**0,95**	**1,07**
20	0	20	65	15	5	**46,99**	**6,97**	**7,30**	**0,84**	**37,23**

Appendix 5.2 D-Optimal Mixture design – bioactive concentrations obtained (continued)

Table B Example of the observed concentrations and the predicted concentrations for hydroxytyrosol using the proportions of a solvent in the solvent blend.

	Actual					Conc (ppm)						Proportions								
	a	b	c	d	T	OBS	R2	R3	R 4	R 5		a/c	b/c	d/c	T	Pred Htryr			Intercept	-18,720
Run	n-Heptane	Limonene	EtOH	Water	Time	Htyr	Tyr	Oleu	a-Toco	Squal		H	L	W	T		Std.Err. Pred.Val	95% uncert	H	82,727
1	15	0	85	0	2	23,76	4,15	1,65	3,03	26,24		0,18	0,00	0,00	2	22,5	7,1	**15,9**	L	-284,809
2	0	0	100	0	4	62,85	9,75	10,11	3,02	4,37		0,00	0,00	0,00	4	60,9	5,1	**11,4**	W	72,266
3	0	0	75	25	3	56,33	8,92	11,81	0,38	0,78		0,00	0,00	0,33	3	56,4	6,0	**13,5**	T	19,911
4	30	10	60	0	2	20,67	4,98	0,55	1,10	10,21		0,50	0,17	0,00	2	18,0	7,7	**17,1**	H^2	158,872
5	0	0	100	0	4	59,51	7,08	8,10	1,20	2,74		0,00	0,00	0,00	4	60,9	5,1	**11,4**	L^2	920,217
6	0	20	80	0	3	1,84	2,81	0,05	0,09	23,17		0,00	0,25	0,00	3	2,9	7,6	**17,0**	W^2	-78,594
7	15	20	65	0	2	0,48	1,59	0,04	0,00	19,26		0,23	0,31	0,00	2	4,3	5,6	**12,5**	H*T	-51,430
8	0	20	50	30	2	45,25	7,17	12,60	0,06	3,72		0,00	0,40	0,60	2	43,4	6,0	**13,4**	L*T	-32,626
9	30	20	50	0	5	0,07	1,22	0,00	0,36	22,10		0,60	0,40	0,00	5	1,4	6,0	**13,4**		
10	30	0	70	0	5	34,08	5,33	2,62	2,19	2,19		0,43	0,00	0,00	5	35,3	6,6	**14,8**		
11	30	0	50	20	4	51,73	6,85	10,06	4,15	14,23		0,60	0,00	0,40	4	60,7	6,1	**13,6**		
12	0	0	50	50	3	47,92	7,05	7,52	0,00	0,00		0,00	0,00	1,00	3	34,7	6,2	**13,8**		
13	30	0	50	20	5	58,57	5,72	5,72	4,90	20,63		0,60	0,00	0,40	5	49,7	6,6	**14,6**		
14	15	0	68	18	4	59,29	5,26	4,93	4,54	14,95		0,22	0,00	0,26	4	54,9	5,5	**12,3**		
15	30	20	50	0	5	0,32	0,01	0,01	0,73	30,54		0,60	0,40	0,00	5	1,4	6,0	**13,4**		
16	15	10	50	25	3	17,11	2,37	2,30	0,21	10,43		0,30	0,20	0,50	3	10,6	6,1	**13,5**		
17	0	20	50	30	2	42,47	8,48	7,60	0,93	1,77		0,00	0,40	0,60	2	43,4	6,0	**13,4**		
18	0	0	50	50	3	23,40	5,26	4,52	1,12	1,53		0,00	0,00	1,00	3	34,7	6,2	**13,8**		
19	6	4	57	32	4	24,87	6,05	5,31	0,95	1,07		0,11	0,07	0,56	4	38,9	4,8	**10,8**		
20	0	20	65	15	5	46,99	6,97	7,30	0,84	37,23		0,00	0,31	0,23	5	42,6	7,3	**16,3**		

Appendix 5.2 D-Optimal Mixture design – bioactive concentrations obtained (continued)

Table C Statistical results of bioactives from the D-Optimal design

	Htyr					Tyr				
R2=	0,92					R^2=	0,86			
F=	12,18	df=9, 10	F(5%)=	3,02		F=	10,98	df=7, 12	F(5%)=	2,91
Se=	8,82					Se=	1,22436			
No outliers						No outliers				
	b	Std.Err.	t(10)	p-value			b	Std.Err.	t(12)	p-value
Intercept	-18,720	19,1107	-0,97956	0,350405		Intercept	5,6381	1,848921	3,04941	0,010096
H	82,727	66,3211	1,24737	0,240682		H	-12,8546	6,357250	-2,02203	0,066049
L	-284,809	85,2812	-3,33965	0,007495		L	-14,6973	2,459039	-5,97683	0,000064
W	72,266	19,1648	3,77078	0,003657		W	-1,6505	1,094069	-1,50856	0,157281
T	19,911	5,1312	3,88040	0,003057		T	0,7724	0,464903	1,66151	0,122492
H^2	158,872	69,8113	2,27573	0,046119		H^2	36,4954	9,311489	3,91939	0,002037
L^2	920,217	210,7654	4,36607	0,001408		L*W	30,0471	6,009133	5,00023	0,000309
W^2	-78,594	21,6825	-3,62479	0,004653		H*T	-2,7865	1,360086	-2,04880	0,063005
H*T	-51,430	13,6985	-3,75440	0,003757						
L*T	-32,626	11,8089	-2,76288	0,020031						

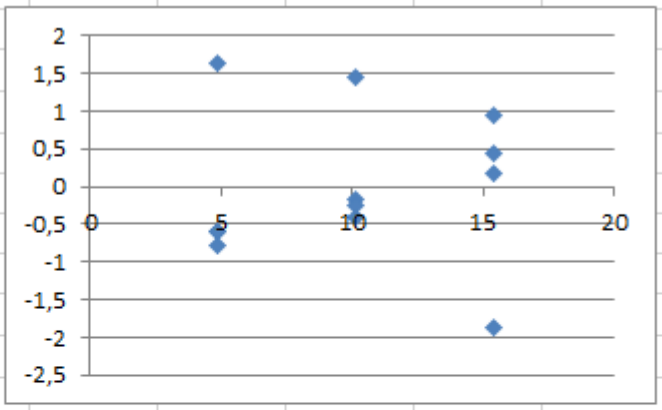
	Oleu					a-Toco				
R^2=	0,88					R^2=	0,8			
F=	9,83	df=8, 11	F(5%)=	2,95		F=	6,98	df=7, 12	F(5%)=	2,91
Se=	1,91000					Se=	0,88600			
No outliers						No outliers				
	b	Std.Err.	t(11)	p-value			b	Std.Err.	t(12)	p-value
Intercept	3,8456	3,24758	1,18413	0,261317		Intercept	2,6315	1,17297	2,24347	0,044518
H	-7,9051	14,21082	-0,55627	0,589160		H	3,6683	1,27305	2,88153	0,013791
L	-62,1837	16,35826	-3,80136	0,002936		L	-20,3283	6,57396	-3,09224	0,009323
W	20,3362	3,78810	5,36844	0,000227		W	-7,5744	3,93899	-1,92293	0,078540
T	1,0920	0,84066	1,29899	0,220514		T	-0,2153	0,30141	-0,71423	0,488743
H^2	45,9605	15,07654	3,04848	0,011078		L^2	51,9441	18,36335	2,82868	0,015213
L^2	149,5002	45,56509	3,28102	0,007320		H*L	-14,1318	4,82919	-2,92632	0,012690
W^2	-21,2758	4,24966	-5,00648	0,000398		W*T	2,0951	1,23676	1,69399	0,116040
H*T	-6,5104	2,89300	-2,25040	0,045858						

	Squal			
R^2=	0,88			
F=	11,2	df=9, 10	F(5%)=	3,02
Se=	4,70000			
Run 14: Stres=2.18				
	b	Std.Err.	t(10)	p-value
Intercept	84,954	24,91153	3,41023	0,006656
H	-10,119	16,32251	-0,61994	0,549162
L	-63,117	32,43206	-1,94614	0,080260
W	-9,977	4,49769	-2,21836	0,050836
T	-39,485	14,35587	-2,75043	0,020463
T^2	4,821	2,07597	2,32232	0,042604
H*L	-110,803	52,20868	-2,12230	0,059791
H*W	92,583	33,24091	2,78522	0,019277
L*W	-91,532	32,01027	-2,85947	0,016972
L*T	37,851	8,69122	4,35512	0,001432

Appendix 5.3 Statistical output for determination of optimum temperature and time during extraction

1. Percentage antioxidant activity (%AO)

SUMMARY OUTPUT



Regression Statistics	
Multiple F	0,863193
R Square	0,745103
Adjusted I	0,719613
Standard E	2,765458
Observati	12

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regressio	1	223,5555	223,5555	29,23151	0,000299
Residual	10	76,47758	7,647758		
Total	11	300,0331			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	-16,2804	4,953439	-3,28669	0,008196	-27,3174	-5,24347	-27,3174	-5,24347
Temp	0,528625	0,097774	5,406617	0,000299	0,310772	0,746478	0,310772	0,746478

ONLY TEMP HAS AN EFFECT ON %AO, NO TIME DEPENDENCE

RESIDUAL OUTPUT

)bservatio	*edicted %A*	*Residuals*	*dard Residuals*
1	15,43708	2,462917	0,934069
2	15,43708	0,462917	0,175563
3	15,43708	1,162917	0,44104
4	15,43708	-4,93708	-1,8724
5	10,15083	3,849167	1,459809
6	10,15083	-1,05083	-0,39853
7	10,15083	-0,45083	-0,17098
8	10,15083	-0,65083	-0,24683
9	4,864583	4,335417	1,644221
10	4,864583	-2,06458	-0,783
11	4,864583	-1,55458	-0,58958
12	4,864583	-1,56458	-0,59337

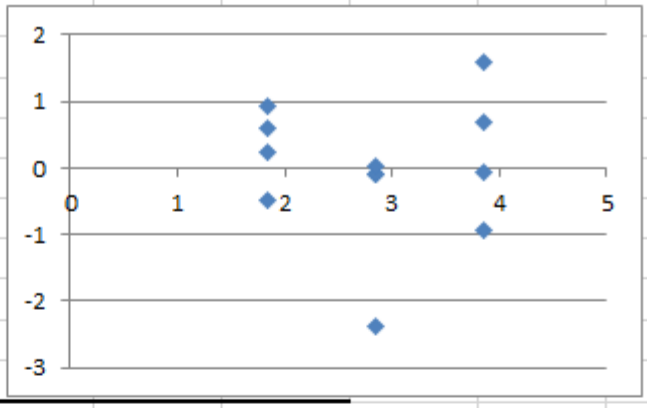
PROBABILITY OUTPUT

Percentile	*%AO*
4,166667	2,8
12,5	3,3
20,83333	3,31
29,16667	9,1
37,5	9,2
45,83333	9,5
54,16667	9,7
62,5	10,5
70,83333	14
79,16667	15,9
87,5	16,6
95,83333	17,9

Appendix 5.3 Statistical output for determination of optimum temperature and time during extraction (continued)

2. Total phenol content

SUMMARY OUTPUT



Regression Statistics	
Multiple F	0,665735
R Square	0,443203
Adjusted I	0,387524
Standard E	1,012861
Observati	12

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regressio	1	8,165931	8,165931	7,959876	0,018119
Residual	10	10,25887	1,025887		
Total	11	18,4248			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	7,898532	1,814218	4,353684	0,001435	3,856202	11,94086	3,856202	11,94086
Temp	-0,10103	0,03581	-2,82133	0,018119	-0,18082	-0,02124	-0,18082	-0,02124

Correlation is weak, and only temp plays a role, NO TIME DEPENDENCE

RESIDUAL OUTPUT

)bservatio	*redicted Tp*	*Residuals*	*dard Residuals*
1	1,836627	-0,47091	-0,48763
2	1,836627	0,221151	0,229
3	1,836627	0,894167	0,925902
4	1,836627	0,583056	0,603749
5	2,846944	-2,29694	-2,37847
6	2,846944	-0,10345	-0,10712
7	2,846944	-0,0844	-0,0874
8	2,846944	0,029881	0,030941
9	3,857262	-0,06615	-0,0685
10	3,857262	0,664008	0,687575
11	3,857262	-0,89155	-0,92319
12	3,857262	1,521151	1,575139

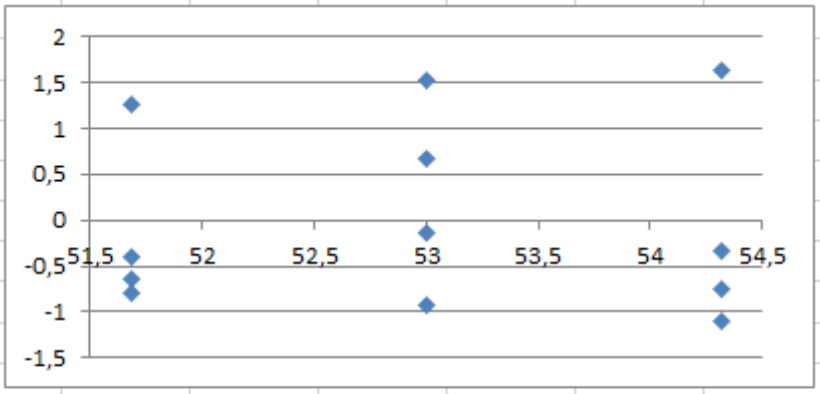
PROBABILITY OUTPUT

Percentile	*Tph*
4,166667	0,55
12,5	1,365714
20,83333	2,057778
29,16667	2,419683
37,5	2,730794
45,83333	2,743492
54,16667	2,76254
62,5	2,876825
70,83333	2,965714
79,16667	3,791111
87,5	4,52127
95,83333	5,378413

Appendix 5.3 Statistical output for determination of optimum temperature and time during extraction (continued)

3. Hydroxytyrosol content

SUMMARY OUTPUT



Regression Statistics	
Multiple F	0,553015
R Square	0,305826
Adjusted I	0,236408
Standard E	1,775386
Observati	12

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regressio	1	13,88645	13,88645	4,405607	0,062187
Residual	10	31,51995	3,151995		
Total	11	45,4064			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	59,5875	3,180039	18,73798	4,05E-09	52,50193	66,67307	52,50193	66,67307
Temp	-0,13175	0,062769	-2,09895	0,062187	-0,27161	0,008109	-0,27161	0,008109

P VALUE IS BORDERLINE, correlation is weak, and there is weak evidence that temp may play a role, NO TIME DEPENDENCE

RESIDUAL OUTPUT

)bservatio	*edicted Ht*	*Residuals*	*dard Residuals*
1	51,6825	-0,6925	-0,40909
2	51,6825	-1,0725	-0,63358
3	51,6825	-1,3425	-0,79308
4	51,6825	2,1475	1,268635
5	53	2,57	1,518227
6	53	-1,58	-0,93338
7	53	-0,22	-0,12996
8	53	1,15	0,679362
9	54,3175	-1,8675	-1,10323
10	54,3175	-1,2675	-0,74878
11	54,3175	-0,5775	-0,34116
12	54,3175	2,7525	1,626039

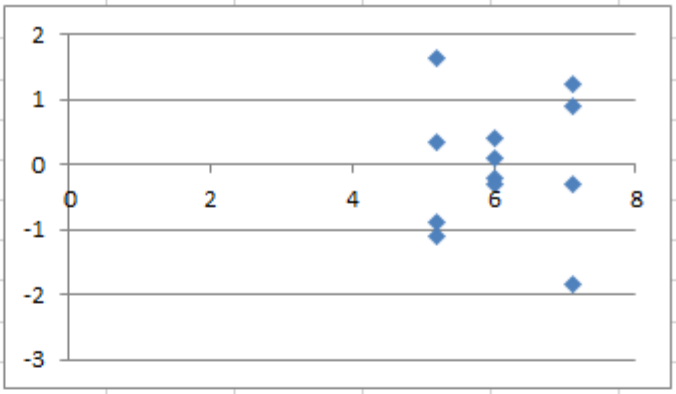
PROBABILITY OUTPUT

Percentile	*Htyr*
4,166667	50,34
12,5	50,61
20,83333	50,99
29,16667	51,42
37,5	52,45
45,83333	52,78
54,16667	53,05
62,5	53,74
70,83333	53,83
79,16667	54,15
87,5	55,57
95,83333	57,07

Appendix 5.3 Statistical output for determination of optimum temperature and time during extraction (continued)

4. Tyrosol content

SUMMARY OUTPUT



Regression Statistics	
Multiple F	0,619654
R Square	0,383971
Adjusted I	0,247075
Standard E	1,159852
Observati	12

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regressio	2	7,546467	3,773233	2,804845	0,113033
Residual	9	12,1073	1,345256		
Total	11	19,65377			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	-28,96	17,40744	-1,66366	0,130541	-68,3384	10,41836	-68,3384	10,41836
Temp	1,484	0,711444	2,085899	0,066625	-0,1254	3,093398	-0,1254	3,093398
Temp^2	-0,01525	0,007103	-2,1471	0,060321	-0,03132	0,000817	-0,03132	0,000817

P VALUES ARE BORDERLINE, correlation is weak, and there is weak evidence that temp may play a role, NO TIME DEPENDENCE

RESIDUAL OUTPUT

)bservatio	*redicted Ty*	*Residuals*	*dard Residuals*
1	5,18	1,73	1,648993
2	5,18	0,35	0,333611
3	5,18	-0,94	-0,89598
4	5,18	-1,14	-1,08662
5	7,115	0,955	0,910282
6	7,115	-0,325	-0,30978
7	7,115	1,305	1,243894
8	7,115	-1,935	-1,84439
9	6	0,12	0,114381
10	6	0,42	0,400334
11	6	-0,21	-0,20017
12	6	-0,33	-0,31455

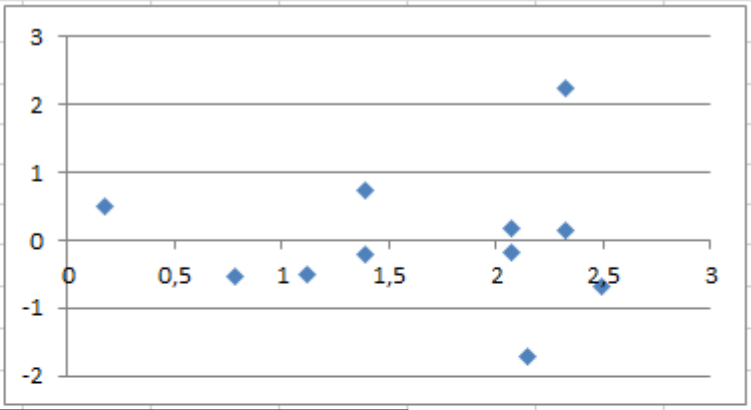
PROBABILITY OUTPUT

Percentile	*Tyr*
4,166667	4,04
12,5	4,24
20,83333	5,18
29,16667	5,53
37,5	5,67
45,83333	5,79
54,16667	6,12
62,5	6,42
70,83333	6,79
79,16667	6,91
87,5	8,07
95,83333	8,42

Appendix 5.3 Statistical output for determination of optimum temperature and time during extraction (continued)

5. α-Tocopherol content

SUMMARY OUTPUT



Regression Statistics	
Multiple R	0,898279
R Square	0,806906
Adjusted R S	0,678176
Standard Erro	0,471015
Observations	11

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regression	4	5,562547	1,390637	6,268219	0,024628
Residual	6	1,331131	0,221855		
Total	10	6,893678			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	-22,009	5,558353	-3,95962	0,007455	-35,6098	-8,40817	-35,6098	-8,40817
Temp	0,25036	0,078816	3,176517	0,01916	0,057504	0,443216	0,057504	0,443216
Time	11,9309	2,895881	4,119956	0,006217	4,844935	19,01686	4,844935	19,01686
Time^2	-1,36888	0,33792	-4,0509	0,006719	-2,19574	-0,54202	-2,19574	-0,54202
Temp*Time	-0,07774	0,029125	-2,66902	0,037075	-0,149	-0,00647	-0,149	-0,00647

RESIDUAL OUTPUT

Observation	*dicted α-To*	*Residuals*	*dard Residuals*
1	2,070671	0,065329	0,17906
2	2,493041	-0,24704	-0,67711
3	2,070671	-0,06967	-0,19096
4	2,3215	0,0545	0,149378
5	0,783459	-0,19146	-0,52477
6	2,3215	0,8225	2,254372
7	1,121776	-0,18278	-0,50097
8	0,172882	0,187118	0,512867
9	1,389271	0,270729	0,742036
10	2,149959	-0,62996	-1,72664
11	1,389271	-0,07927	-0,21727

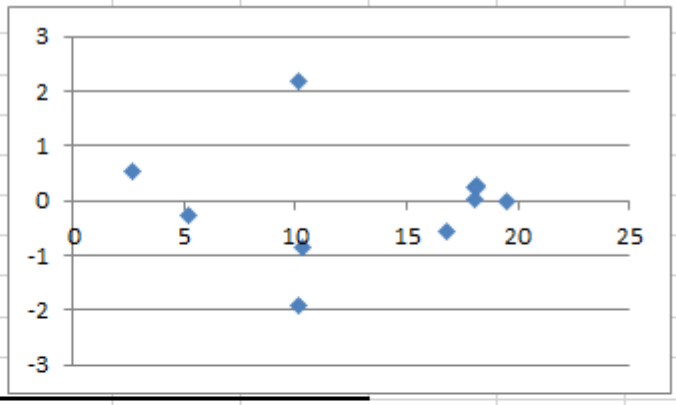
PROBABILITY OUTPUT

Percentile	*α-Toco*
4,545455	0,36
13,63636	0,592
22,72727	0,939
31,81818	1,31
40,90909	1,52
50	1,66
59,09091	2,001
68,18182	2,136
77,27273	2,246
86,36364	2,376
95,45455	3,144

Appendix 5.3 Statistical output for determination of optimum temperature and time during extraction continue

6. Squalene content

SUMMARY OUTPUT



Regression Statistics	
Multiple F	0,889405
R Square	0,791041
Adjusted I	0,651734
Standard E	3,926039
Observati	11

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regressio	4	350,1039	87,52599	5,678425	0,030776
Residual	6	92,48267	15,41378		
Total	10	442,5866			

	Coefficients	*andard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*pper 95.0%*
Intercept	-169,09	46,33039	-3,64966	0,010711	-282,457	-55,7238	-282,457	-55,7238
Temp	2,03456	0,656952	3,096969	0,021198	0,427057	3,642063	0,427057	3,642063
Time	91,1894	24,13795	3,777843	0,009204	32,12595	150,2528	32,12595	150,2528
Time^2	-10,3592	2,816658	-3,67784	0,010357	-17,2513	-3,46711	-17,2513	-3,46711
Temp*Tim	-0,63221	0,242765	-2,6042	0,040434	-1,22623	-0,03818	-1,22623	-0,03818

RESIDUAL OUTPUT

)bservatio	*edicted Squ*	*Residuals*	*dard Residuals*
1	18,06032	0,762682	0,250792
2	19,52109	-0,00809	-0,00266
3	18,06032	0,097682	0,032121
4	18,14175	0,90825	0,298659
5	5,206165	-0,83216	-0,27364
6	18,14175	0,77225	0,253938
7	10,35889	-2,55289	-0,83947
8	2,657471	1,692529	0,556552
9	10,14892	6,631082	2,180491
10	16,76241	-1,67241	-0,54994
11	10,14892	-5,79892	-1,90685

PROBABILITY OUTPUT

Percentile	*Squal*
4,545455	4,35
13,63636	4,35
22,72727	4,374
31,81818	7,806
40,90909	15,09
50	16,78
59,09091	18,158
68,18182	18,823
77,27273	18,914
86,36364	19,05
95,45455	19,513

Appendix 5.4 Results for confirmation of temperature/pressure

Table A Summary of all the reults of the responses

Conditions	**HTyr** (ppm)	**Tyr** (ppm)	**α-Toco** (ppm)	**Squal** (ppm)
A. 350 mbar, 60°C solv temp	0,5744	0,0326	0,02567	0,21333
	0,7442	0,0326	0,03767	0,28100
	0,6232	0,0341	0,02733	0,26950
	0,7914	0,0414	ND	ND
Ave	0,6833	0,0352	0,02267	0,19096
St dev	*0.1014*	*0,00421*	*0,0065*	*2,172*
Rel std dev	*14,85*	*11,96*	*28.68*	*18.96*
B. 350 mbar, 80°C bath temp	0,7578	0,07133	0,03183	0,25683
	0,7970	0,07983	0,02833	0,24483
	0,7658	0,07133	0,03117	0,28817
	0,7945	0,05217	0,03233	0,31283
Ave	0,7788	0,06867	0,03092	0,27567
St dev	*0.0199*	*0,0117*	*0,0018*	*0.0308*
Rel std dev	*2,552*	*17,049*	*5,781*	*11,167*
C. 150 mbar, 65°C bath temp	0,5428	0,03696	0,04700	0,32633
	0,5499	0,03482	0,02750	0,27950
	0,5446	0,02933	0,03817	0,23250
	0,6362	0,03291	0,03267	0,30183
Ave	0,5684	0,03350	0,03633	0,28504
St dev	*0.0453*	*0,0032*	*0,0083*	*0.0399*
Rel std dev	*7,970*	*9,661*	*22,951*	*14,001*

ND: Not determined HTyr: Hydroxytyrosol Tyr: Tyrosol α-Toco: α-Tocopherol Squal: Squalene

Table B Results of the f-tests between pairs A-B, B-C and A-C for each bioactive

Htyr	*p-value*	**Tyr**	*p-value*	**α-Toco**	*p-value*	**Squal**	*p-value*
A-B	0,0119	A-B	*0,0633*	A-B	0,0325	A-B	*0,375*
B-C	*0,1046*	B-C	0,0314	B-C	0,0154	B-C	*0,34*
A-C	*0,109*	A-C	*0,338*	A-C	*0,3996*	A-C	*0,4811*

If p > 0.05 then assume equal variances (italic)

Table C T-tests (assuming equal variance) performed on all the pairs which showed equal variance while the unequal variance pairs were analysed using the Mann-Whitney test (an alternative to the t-test)

Htyr	*p-value*	**Tyr**	*p-value*	**α-Toco**	*p-value*	**Squal**	*p-value*
A-B	**0,1124**	A-B	*0,0017*	A-B	**0,5959**	A-B	0,4424
B-C	*0,0001*	B-C	**0,0304**	B-C	**0,3123**	B-C	0,7227
A-C	0,0841	A-C	0,5467	A-C	0,3439	A-C	0,3478

If p < 0.05, null hypothesis is proved wrong, there is a sign diff between the means. P-Value for T-test (Italic); p-value for Mann-Withney test as alternative to t-test (Bold). The unequal variance pairs were analysed using the Mann-Withney test (alternative to t-test). **Non-equal variances were found for:** R1 between A and B, R2 between B and C, and R3 between A and B as well as B and C.

Appendix 6.1 Inhibition zones as a result of antimicrobial potential

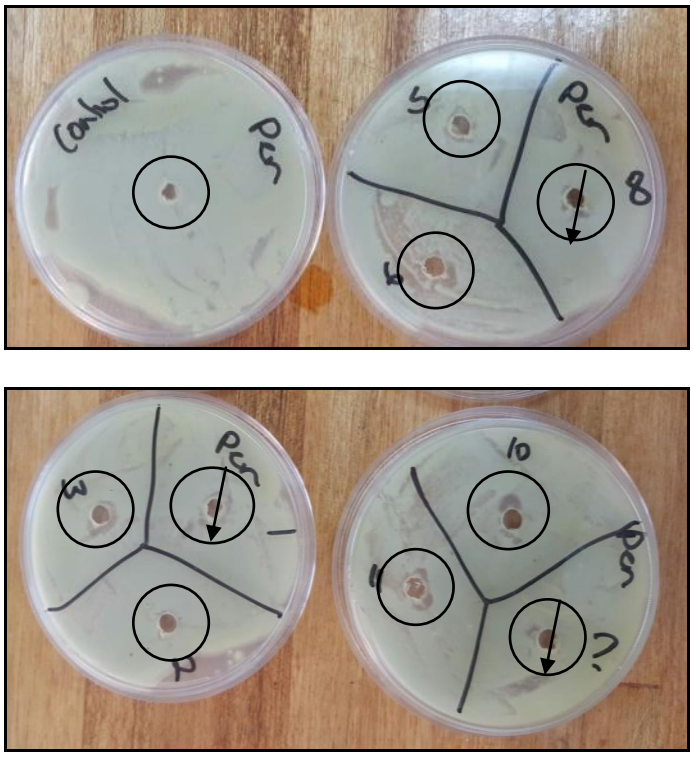


Figure 6.6 Inhibition zones after inoculation with *Penicillium notatum:* samples 1, 2. 3, 5, 6, 8 are *Coratina* extracts; samples 9, 10, 11 are *Frantoio* extracts

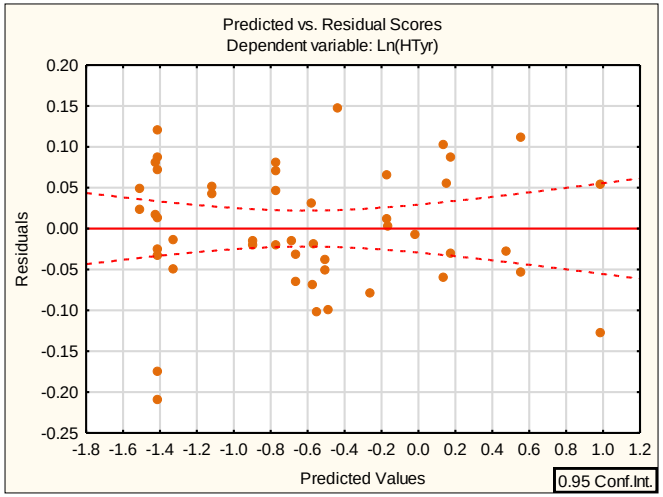
Figure 6.7 Inhibition zones after inoculation with *Escherichia coli:* Samples 1, 2. 3, 5, 6, 8 are *Coratina* extracts; samples 9, 10, 11 are *Frantoio* extracts

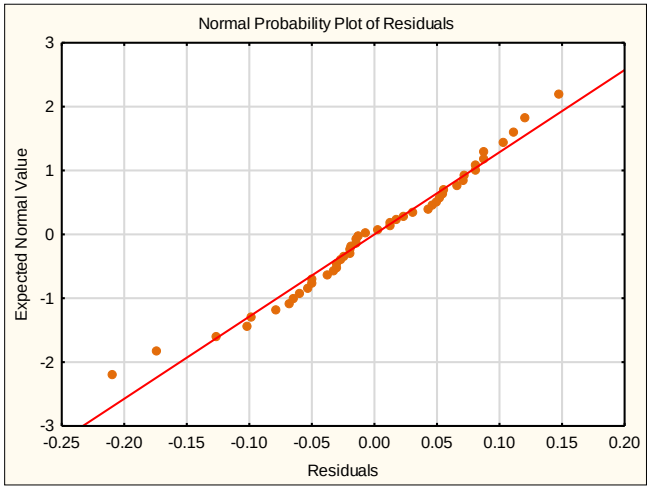
Figure 6.8 Inhibition zones after inoculation with *Aspergillis niger:* Samples 1, 2. 3, 5, 6, 8 are *Coratina* extracts; samples 9, 10, 11 are *Frantoio* extracts

Figure 6.9 Inhibition zones after inoculation with Gram +: *Staph. aureus:* Samples 1, 2. 3, 5, 6, 8 are *Coratina* extracts; samples 9, 10, 11 are *Frantoio* extracts

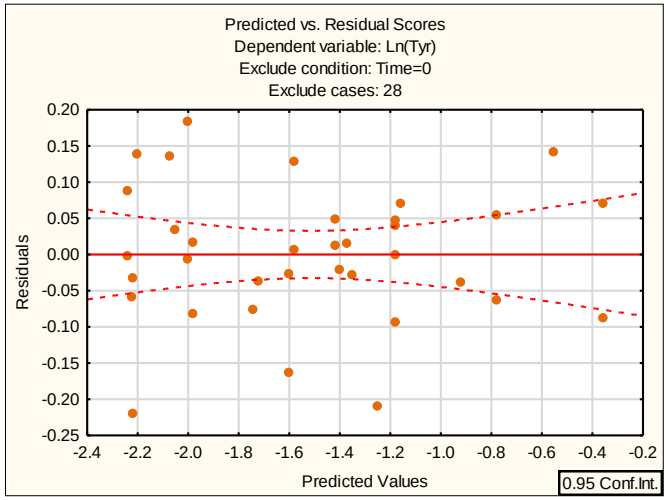
Appendix 7.1 Validation of the multiple regression model for the bioactive compounds content (mg/g dry weight)

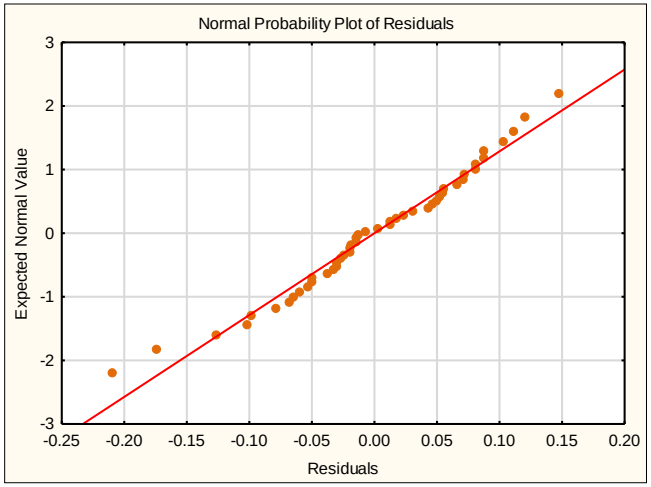
A. Hydroxytyrosol



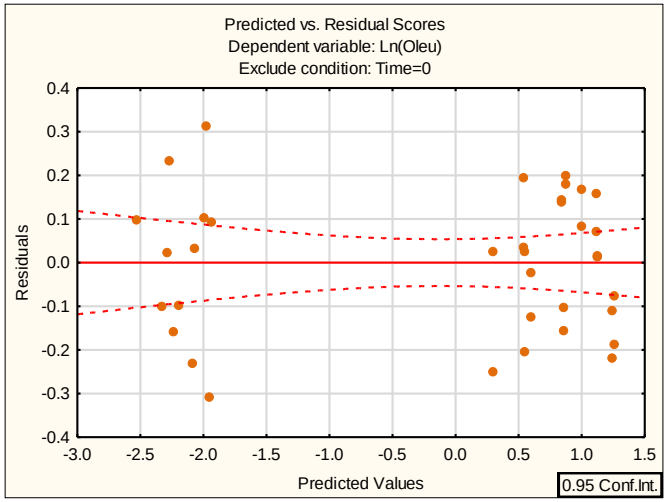


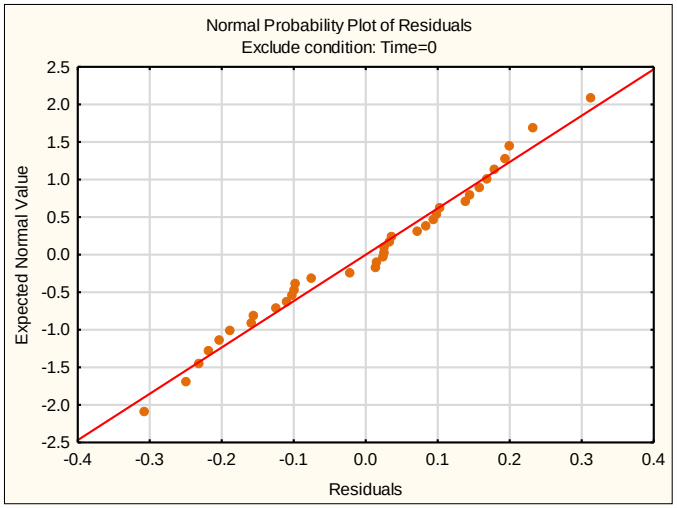
B. Tyrosol



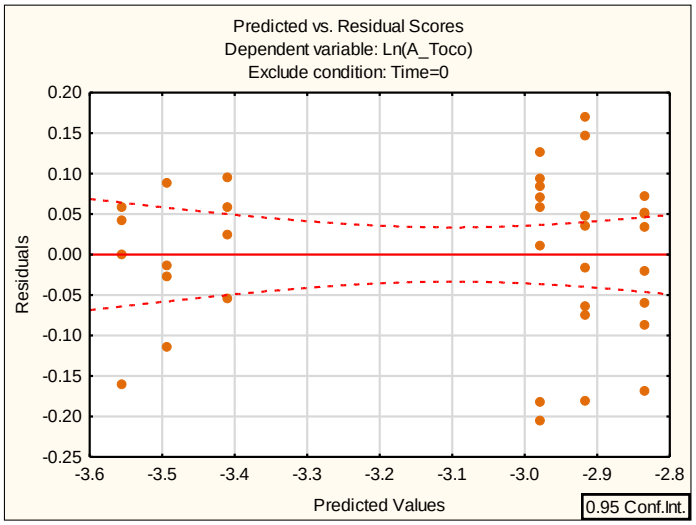


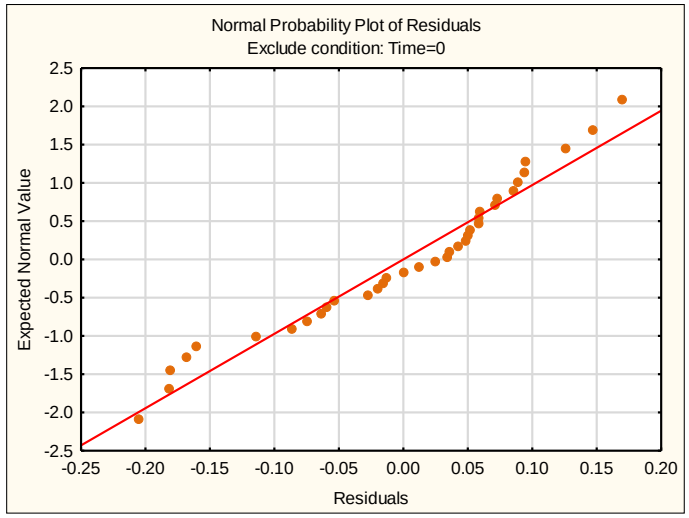
C. Oleuropein



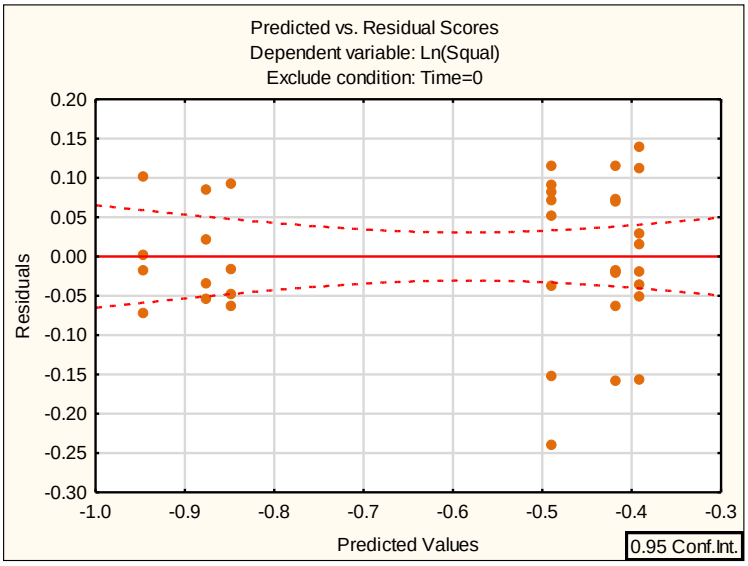


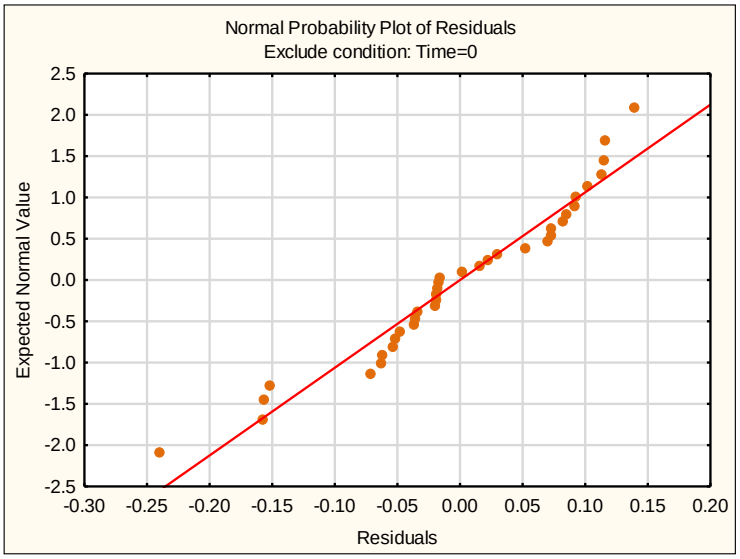
D. α-Tocopherol



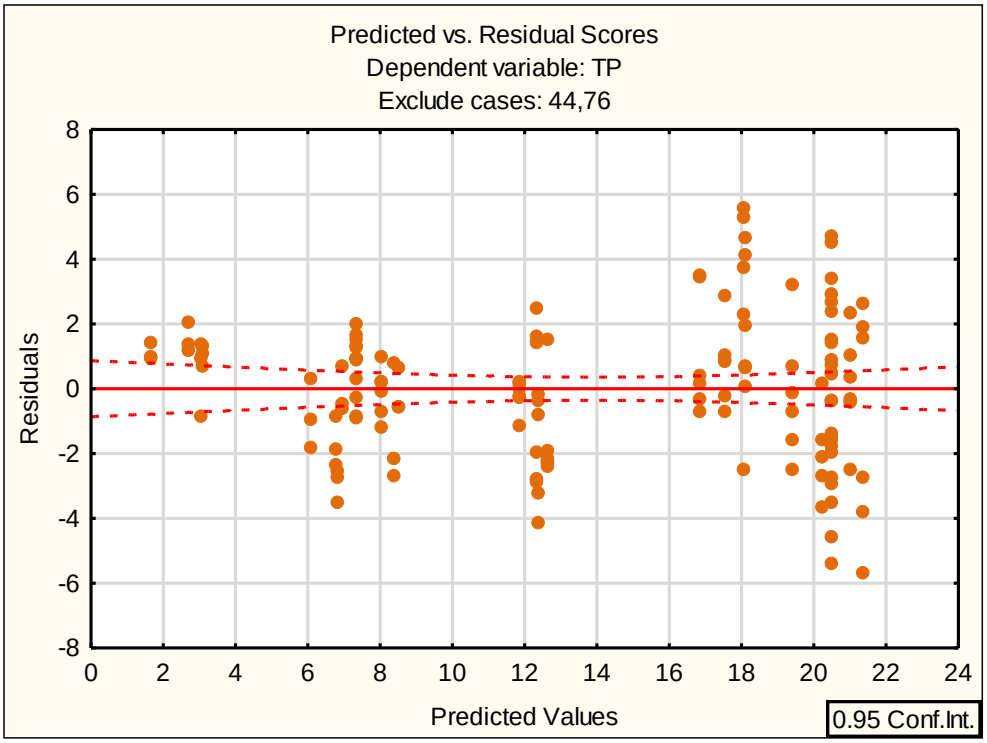


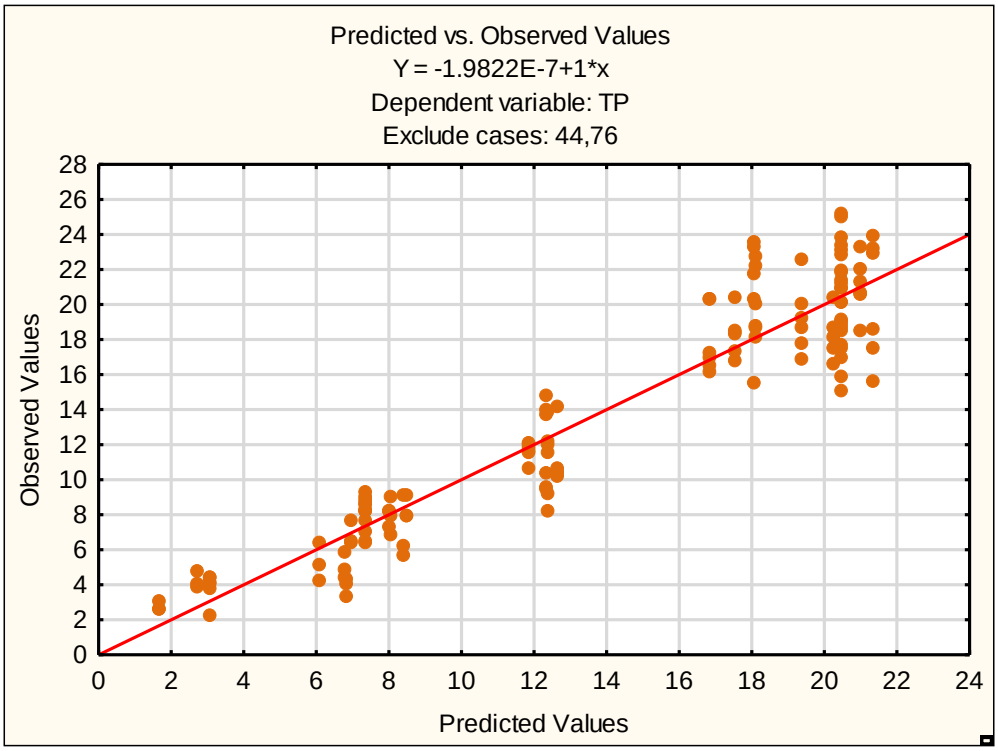
E. Squalene


Predicted vs. Residual Scores
Dependent variable: Ln(Squal)
Exclude condition: Time=0
Residuals
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
-1.0
-0.9
-0.8
-0.7
-0.6
-0.5
-0.4
-0.3
Predicted Values
0.95 Conf.Int.


Normal Probability Plot of Residuals
Exclude condition: Time=0
Expected Normal Value
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-0.30
-0.25
-0.20
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
Residuals

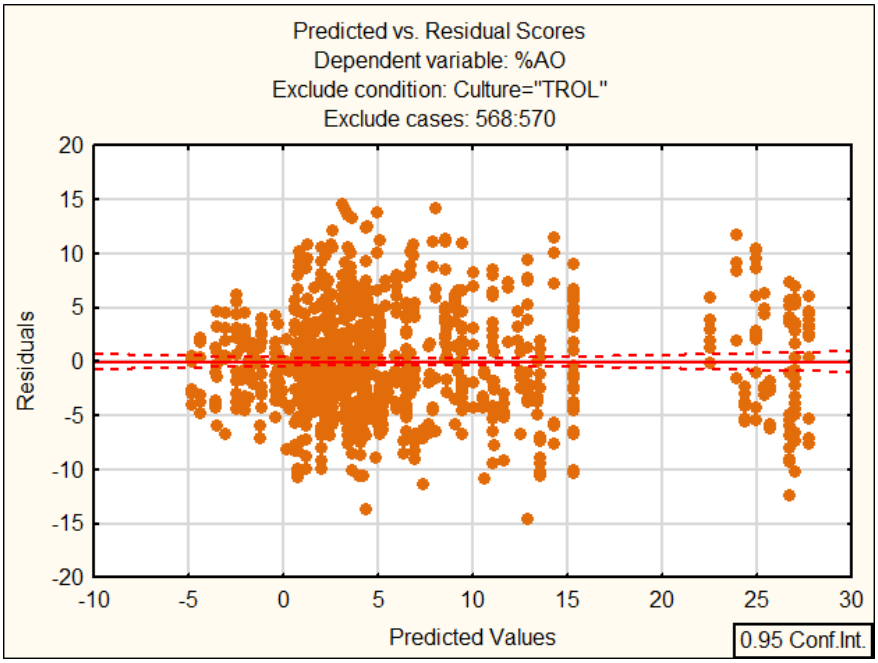
Appendix 7.2 Validation of the total phenol regression model



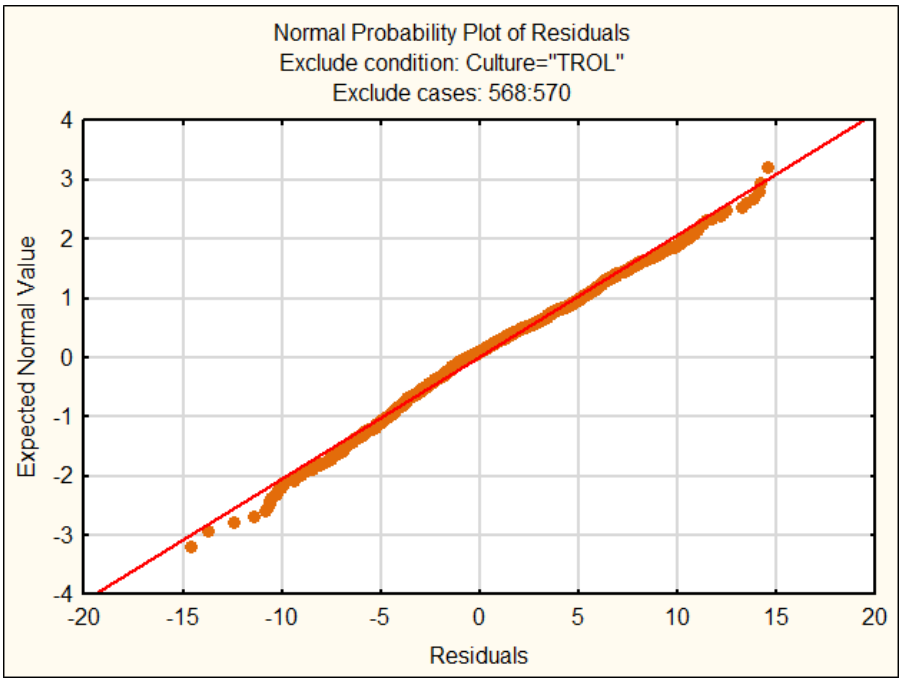


Appendix 7.3 Validation of the % antioxidant activity model

1. Predicted versus residual results: the model fits the observed percentage antioxidant activity data.



2. Normal probability of the residuals: Distribution of results are on the line



Appendix 7.4 Summary of % antioxidant results of *Coratina* and *Frantoio* extracts at different conc. and over a period of 12 weeks

		Coratina								*Frantoio*			
Conc						**T0**							
µg/ml	**Trolox**	**S1C**	**S2C**	**S3C**	**S4C**	**S5C**	**S6C**	**S7C**	**S8C**	**S9F**	**S10F**	**S11F**	**S12F**
200	87.29	30.25	30.11	31.77	27.62	28.87	24.72	21.41	19.75	21.09	19.66	16.28	17.19
100	57.32	18.65	13.12	19.34	12.71	13.26	13.12	11.88	9.81	14.06	13.41	11.59	13.80
50	32.46	12.71	13.26	11.88	7.32	6.49	12.85	5.80	6.08	12.89	9.90	11.33	13.15
25	21.41	11.33	10.36	9.25	5.11	3.04	3.87	3.18	3.04	8.20	7.42	3.39	10.55
12.5	14.64	8.56	9.39	4.97	3.04	0.55	1.10	-0.14	2.90	8.07	5.99	6.25	5.99
6.25	12.29	5.39	6.91	6.91		1.66	-0.28	-5.94	2.21	7.94	5.47	6.38	7.42
3.125	9.81	5.94	7.32	4.28	0.00	-0.55	1.24	-4.83	4.01	8.72	4.43	5.21	7.81
						T1							
	Temp	**-20°C**	**5°C**	**25°C**	**40°C**	**-20°C**	**5°C**	**40°C**	**25°C**	**-20°C**	**5°C**	**25°C**	**40°C**
200	87.53	29.42	30.76	32.02	30.31	32.56	20.81	22.42	17.67	14.98	5.38	5.92	10.76
100	51.57	20.81	21.26	23.59	19.46	19.91	11.75	10.58	7.53	0.27	-1.17	-1.52	4.93
50	30.22	13.36	14.71	16.68	15.43	14.26	6.10	4.66	2.87	0.00	-0.54	-0.36	3.50
25	19.55	10.85	13.18	14.80	12.11	11.03	2.87	2.78	0.00	-0.54	-0.54	0.00	0.81
12.5	15.34	10.13	10.49	13.63	11.03	8.88	1.61	-0.90	-2.60	-0.18	-1.52	1.88	1.79
6.25	11.93	9.51	10.13	11.93	10.85	9.60	0.72	-2.33	-0.09	-3.41	-1.88	-0.63	0.18
3.125	11.75	8.70	10.13	10.04	9.96	7.62	1.70	-1.43	-2.69	-0.45	-0.81	0.72	0.81
						T2							
		-20°C	**5°C**	**25°C**	**40°C**	**-20°C**	**5°C**	**40°C**	**25°C**	**-20°C**	**5°C**	**25°C**	**40°C**
200	82.36	25.35	36.09	26.22	23.50	24.24	19.65	19.04	19.39	19.30	9.04	15.48	4.87
100	54.23	14.37	18.45	14.00	11.41	10.80	10.61	8.09	9.65	16.52	2.43	12.70	1.13
50	27.33	8.70	12.65	8.95	5.98	5.24	5.57	2.26	3.48	10.09	1.13	12.26	1.04
25	13.63	4.13	9.81	4.26	2.53	0.80	3.57	1.04	1.39	8.17	-0.70	9.39	-0.35
12.5	6.48	2.90	4.50	2.78	0.56	-1.30	2.78	-1.91	-0.78	6.78	1.39	6.52	0.17
6.25	5.00	2.04	3.15	3.39	-0.68	-1.67	2.70	-2.96	-2.43	5.13	-0.52	6.26	-1.04
3.125	5.24	2.65	3.76	3.89	3.39	-3.64	4.70	-3.57	-1.30	6.61	1.74	7.74	-0.43
						T3							
		-20°C	**5°C**	**25°C**	**40°C**	**-20°C**	**5°C**	**40°C**	**25°C**	**-20°C**	**5°C**	**25°C**	**40°C**
200	85.61	27.12	34.47	33.59	26.718	31.30	21.44	23.64	23.16	10.26	10.71	15.66	8.63
100	45.76	13.66	18.01	16.03	9.815	14.83	6.60	7.66	8.52	5.68	5.59	10.26	5.64
50	24.12	6.42	7.87	3.93	-1.854	5.45	-2.87	2.58	2.87	3.02	2.29	8.61	3.08
25	11.08	2.48	5.38	-2.18	-5.671	1.42	-6.60	-1.53	-0.48	3.48	1.10	7.23	0.62
12.5	5.90	2.80	1.14	-3.71	-7.634	-1.53	-4.21	-2.97	-2.39	3.66	0.64	4.30	1.06
6.25	5.38	1.24	0.62	-4.25	-8.397	-3.71	-5.55	-2.87	-0.77	3.75	1.19	5.13	1.50
3.125	3.00	1.55	0.31	-5.02	-8.179	-2.94	-5.17	-4.50	-1.63	4.03	0.27	6.50	0.97