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Quality Enhancement of Australian Extra Virgin Olive Oils

A report for the Rural Industries Research and Development Corporation

by Paul Prenzler, Kevin Robards and Dan Bedgood

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Foreword

The Australian Olive Industry experienced rapid growth in the 1990's in response to increasing consumer demand and is currently valued at around $200 million per annum. As production of quality oil is essential for the industry to compete with imports and to develop an export niche, it is important to gain as comprehensive a view as possible of contributors to quality. In this study, which is the first of its kind in Australia, the researchers have principally investigated two classes of compounds (volatiles and phenolics) that are directed related to aroma (volatiles), flavour (volatiles and phenolics) and oil stability (phenolics). The researchers have identified and examined the critical points in the oil production process – from fruit, through extraction, to storage and consumer use – where volatile and phenolic compounds are formed and modified, either to the benefit or detriment of the final product.

Through the use of powerful multivariate statistical techniques, the researchers were able to identify volatile and phenolic compounds that were uniquely associated with each stage of the production process. New analytical methodologies were established and developed, which objectively distinguished oils from different cultivars at different stages of maturity, based on different patterns of volatile and phenolic compounds. Post-harvest low temperature fruit storage was found to be potentially viable to preserve fruit quality prior to processing. Investigation of malaxation time and temperature showed that a malaxation temperature of 30°C has benefits in terms of oil yield, while still maintaining sensory quality. Experiments looking at shelf-life issues showed clearly that once oil is exposed to oxygen, i.e. during domestic consumption, sensory quality rapidly deteriorates. In all cases, the objective measurement of volatile and phenolic compounds (those directly linked to sensory quality) led to new insights into oil chemistry during all stages of production.

This research should provide considerable benefit to the industry. The tools developed and described in this report will enable the industry to measure volatile and phenolic compounds and link them to production and consumer needs. Furthermore, this work presents possibilities for further investigations that look at the relationship between horticultural practices (e.g. pruning and fertilizer regimes) and their effect on fruit (and hence oil) quality. This work also identifies the need for consumer education campaigns emphasising the importance of using olive oil quickly, while it still maintains its positive flavour characteristics.

This project was funded from RIRDC Core Funds which are provided by the Australian Government.

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

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Abbreviations

3,4-DHPEA-DEDA, 3,4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde; DVB-CAR-PDMS, divinylbenzene-carboxen-polydimethylsiloxane; FFA, free fatty acid; GC-MS, gas chromatography – mass spectrometry; HPLC-DAD, high-performance liquid chromatography – diode array detector; IOOC, International Olive Oil Council; LC-ESI-MS, liquid chromatography – electrospray ionization – mass spectrometry; LDA, linear discriminant analysis; MI, maturity index; PCA, principal component analysis; PV, peroxide value; SLDA, stepwise linear discriminant analysis; SPME-GC-FID, solid phase microextraction – gas chromatography – flame ionization detection; SPME-GC-MS, solid phase microextraction-gas chromatography – mass spectrometry; UV, ultra violet.

Contents

Foreword III

Acknowledgments IV

Abbreviations IV

Contents V

Executive Summary VIII

1. Introduction 1

1.1 Background 1
1.2 Grading of olive oil 2
1.3 Volatile and phenolic compounds 2
1.4 Control of volatile and phenolic compounds during production 3
1.5 Aims of this project 4

2. Methodology 6

2.1 Materials 6
2.2 Analysis of volatile compounds 6
2.3 Analysis of phenolic compounds 8
2.4 Free fatty acid (FFA) determination 9
2.5 Peroxide value (PV) determination 9
2.6 Ultraviolet (K_{232}, K_{270} and ΔK) spectrophotometric determinations 10
2.7 Oil yield determination 11
2.8 Olive fruit maturity index (MI) determination 11
2.9 Statistical data analysis 12
2.9.1 Analysis of differences between samples with ANOVA *13*
2.9.2 Sample characterisation with stepwise linear discriminant analysis (SLDA) *13*
2.9.2.1 Pattern recognition *13*
2.9.2.2 Variable selection *14*
2.9.2.3 Variable contribution *14*
2.9.3 Statistical associations with multiple linear regression (MLR) *15*
2.9.4 Optimum processing conditions with response surface curve fitting *16*

3. Discrimination of olive oils and fruits into cultivars and maturity stages based on phenolic and volatile compounds 17

3.1 Introduction 17
3.2 Methodology 18
3.2.1 Materials *18*
3.2.2 Fruit harvest and oil extraction. *18*
3.2.2.1 Samples for phenolic compound characterization *19*
3.2.2.2 Samples for volatile compound characterization *19*
3.2.3 Determination of volatile compounds *19*
3.2.4 Determination of phenolic compounds *19*
3.2.5 Statistical data analysis *19*
3.3 Results and Discussion 20
3.3.1 Phenolic compound characterization *20*
3.3.2 Volatile compound characterization *21*
3.3.3 Multivariate approach towards cultivar and maturity stage discrimination *22*

3.3.4 Cultivar discrimination *23*
3.3.5 Compounds that discriminate cultivars. *24*
3.3.6 Maturity stage discrimination *25*
3.3.7 Compounds that characterize maturity *28*
3.3.8 Maturity stage and cultivar dependence *29*
3.4 Conclusions *29*

4. Effect of Low Temperature Fruit Storage on Virgin Olive Oil Quality 30

4.1 Introduction 30
4.2 Methodology 31
4.2.1 Materials *31*
4.2.2 Low temperature olive fruit storage *32*
4.2.3 Qualitative and quantitative analysis of phenolic compounds *32*
4.2.4 Qualitative and quantitative analysis of volatile compounds *32*
4.2.5 Determination of quality parameters *33*
4.2.6 Statistical data analysis *33*
4.3 Results and Discussion 35
4.3.1 Low temperature fruit storage effect on virgin olive oil quality indices and yield *35*
4.3.2 Trends in levels of volatile compounds during low temperature fruit storage *36*
4.3.3 Trends in levels of phenolic compounds of olive oil during low temperature fruit storage *38*
4.3.4 Trends in phenolic compounds of olive fruit during low temperature storage *39*
4.3.5 Associations of olive minor components with olive oil quality during fruit storage *40*
4.3.6 Associations of phenolic compounds in the fruit *42*
4.3.7 Associations of phenolic compounds in the oil *42*
4.3.8 Associations of volatile compounds *43*
4.3.9 Associations of quality indices *43*

5. Changes in Volatile and Phenolic Compounds with Malaxation Time and Temperature during Virgin Olive Oil Production 44

5.1 Introduction 44
5.2 Methodology 45
5.2.1 Materials *45*
5.2.2 Olive oil extraction *46*
5.2.3 Determination of quality parameters *46*
5.2.4 Qualitative and quantitative analysis of volatile compounds *46*
5.2.5 Qualitative and quantitative analysis of phenolic compounds *46*
5.2.6 Statistical data analysis *47*
5.3 Results and Discussion 47
5.3.1 Malaxation time and temperature discrimination *47*
5.3.2 Parameters that discriminate malaxation times *49*
5.3.3 Parameters that discriminate malaxation temperatures *52*
5.3.4 Effect of malaxation time-temperature combination on virgin olive oil quality and yield .. *52*
5.3.5 Changes in phenolic compounds with processing *53*
5.3.6 Changes in volatile compounds with processing *56*
5.3.7 Industrial and Bench Scale Oil Extraction Comparison *59*
5.4 Conclusions 64

6.0 Discrimination of Storage Conditions and Freshness in Virgin Olive Oil 65

6.1 Introduction 65
6.2 Methodology 66
6.2.1 Materials *66*
6.2.2 Virgin olive oil storage conditions. *68*
6.2.3 Qualitative and quantitative analysis of phenolic compounds *68*
6.2.4 Qualitative and quantitative analysis of volatile compounds *68*

6.2.5 Determination of quality parameters *69*
6.2.6 Statistical data analysis *69*
6.3 Results and Discussion 69
6.3.1 Discrimination of storage conditions relative to freshness *69*
6.3.2 Discrimination of storage conditions in absence of oxygen *70*
6.3.3 Discrimination of storage conditions in presence of oxygen *72*
6.3.4 Parameters that characterize low temperature storage *73*
6.3.5 Parameters that characterize dark storage *75*
6.3.6 Parameters that characterize light storage *76*
6.3.7 Effect of oxygen exposure during virgin olive oil storage *77*
6.3.8 Potential oxidation and freshness markers of virgin olive oil *77*

7. Changes from Olive Fruit to Oil during Virgin Olive Oil Production. 79

7.1 Introduction 79
7.2 Methodology 80
7.2.1 Virgin oive oil production steps and sample selection *80*
7.2.2 Data treatment and analysis. *81*
7.3 Discrimination of Virgin Olive Oil Production Steps 81
7.3.1 Variables characterizing single processes *82*
7.3.2 Variables Characterizing Multiple Processes *83*
7.4 Identification of Critical Steps in Virgin Olive Oil Production. 84
7.4.1 Quality indices – trends from olive fruit to oil. *84*
7.4.2 Volatile compounds – trends from olive fruit to oil *86*
7.4.3 Phenolic compounds – trends from olive fruit to oil *88*
7.5 Conclusions 89

8. Conclusions and Suggestions for Further Work. 90

8.1 Conclusions 90
8.2 Suggestions for Further Work 92
8.2.1 Changes in olive oil quality and composition resulting from fruit storage. *92*
8.2.2 Alternative processing conditions and oil extraction methods. *92*
8.2.3 Effect of Low Temperature Olive Oil Storage on Quality. *93*

9. References 94

Appendix 1 103

Appendix 2 Rapid assessment of oil defects with zNose® technology 106

Executive Summary

What the report is about

The Australian Olive Association has put quality at the heart of its Vision Statement in the 2003-2008 strategic plan:

> "*By 2010 Australia will be globally acknowledged as a producer of high quality and price competitive olive products.*" (RIRDC, 2002)

The quality of olive oil is largely determined by the minor components, especially volatile (aroma, flavour) and phenolic (flavour, antioxidant) compounds. We have devoted a large part of this study in identifying and monitoring these compounds (among others) in fruit, and during processing and oil storage, while exploring how these stages may affect levels of these compounds and hence consumer satisfaction and acceptance. We show in this study that it is possible to have objective, reproducible, reliable measures of many of the compounds that affect the sensory properties of olive oil, and hence consumer satisfaction and acceptance.

All parameters of the production process have been surveyed, including: cultivar, maturity stage, post-harvest fruit storage; processing (malaxation time and temperature); oil storage; and finally oil storage during consumer use. Multivariate statistical analyses have been applied to identify the volatile and phenolic compounds (and other parameters) most characteristic of a certain process. It is these compounds that may be monitored more closely in any follow up studies.

Who is the report targeted at?

We see this report as forming the basis for others to further investigate the complex relationships that emerge during the production of oil from fruit, through to the finished product. It also provides useful advice to alive growers and processors on the maintenance of quality of virgin olive oil.

Aims/Objectives

This project set out to achieve the following:

(i) Statistically identify cultivar differences and determine changes in volatile and phenolic profiles during fruit maturation for the production of premium quality virgin olive oil.

(ii) Systematically identify volatile compounds; phenolic compounds; and quality indices that significantly ($p < 0.01$) change with simultaneous changes in malaxation time and temperature and ultimately predict the optimum processing conditions for the transferring of the best quality attributes of the fruit to the extracted oil.

(iii) Determine changes in virgin olive oil quality due to different storage conditions and identify conditions that best preserve the quality and freshness of virgin olive oil.

(iv) Identify the critical production steps from olive fruit to oil at consumption that can be controlled to produce and maintain premium quality virgin olive oil.

Methods and results

Cultivar/maturity stage. Olive oil and fruit samples from six cultivars sampled at four different maturity stages were discriminated through statistical analysis into cultivars and maturity stages. The variables – volatile and phenolic compounds – that significantly ($p< 0.01$) discriminated cultivars and maturity stage groups were identified. Separation by stepwise linear discriminant analysis revealed that Manzanilla olive cultivar was separated from cultivars Leccino, Barnea, Mission, Corregiola, and Paragon, whereas cultivars Corregiola and Paragon formed a cluster. The volatile compounds hexanol, hexanal, and 1-penten-3-ol were responsible for the discrimination of cultivars. All maturity stages

were discriminated, with the separation of early stages attributed to oil phenolic compounds, tyrosol and oleuropein derivatives, whereas the volatile compounds (*E*)-2-hexenal, hexanol, 1-penten-3-ol, and (*Z*)-2-penten-3-ol characterized the separation of all maturity stages and in particular the late stages. Hexanol and 1-penten-3-ol characterized the separation of both cultivars and maturity stages. These results demonstrate that objective, instrument-based analyses are capable of measuring compounds that distinguish between cultivars and maturity stages and that oils from different fruit give different responses. This knowledge may be utilised in future studies that wish to investigate more fully the relationship between horticultural practices and sensory properties.

Post-harvest fruit storage. *Frantoio* olive fruits were stored at low temperature (4 ± 2°C) for 3 weeks to investigate the effect of post-harvest storage on virgin olive oil quality. Statistical analysis of variables recognized by the IOOC as measures of oil quality (FFA, PV, K_{232} and K_{270}) could not explain changes in sensory quality of oils produced from stored fruit. Volatile and phenolic compounds, however, did account for observed changes in quality. Increase in concentrations of *E*-2-hexenal and hexanal corresponded to positive sensory quality whereas increase in *E*-2-hexenol and (+)-acetoxypinoresinol was associated with negative sensory quality. Volatile and phenolic compounds were also indicative of the period of low temperature fruit storage. Oleuropein and ligstroside derivatives in olive oil decreased with respect to storage time and their significant ($p < 0.05$) change corresponded to changes in bitterness and pungency. *Z*-penten-1-ol increased during low temperature fruit storage whereas 2-pentylfuran decreased.

Total volatile compounds were negatively associated with K_{270} and positively associated with a ketone, 6-methyl-5-hepten-2-one. These associations during low temperature storage show that olive oil quality indices were associated with volatile compounds, which in turn were associated with phenolic compounds in both the fruit and oil. The changes and associations of quality indices, sensory notes, volatile and phenolic compounds indicate that virgin olive oil quality is lost within the first week of low temperature fruit storage and re-gained at two weeks. Our research suggests that low temperature storage of olive fruit may be beneficial to the produced oil, with a possibility of increasing yield and moderating the sensory quality of olive oils. As this was a pilot study, much more work is needed to optimise storage conditions to ensure that high quality oil may be reliably produced from fruit stored prior to processing.

Malaxation time/temperature. Virgin olive oils produced at wide ranges of malaxation temperatures (15, 30, 45 and 60°C) and times (30, 60, 90 and 120 min) in a complete factorial experimental design were discriminated with stepwise linear discriminant analysis (SLDA) revealing differences in volatile and phenolic profiles with processing conditions. Virgin olive oils produced at 15°C and 60°C and malaxed for 30 min showed the most significant ($p < 0.01$) differences. Discrimination was based upon volatile and phenolic compounds detected in olive oils, PV, FFA, UV absorbances and oil yield. There were different discriminating variables for processing conditions illustrating the dependence of virgin olive oil quality on malaxation time and temperature. Volatile compounds were the dominant discriminating variables. Common oxidation indicators of olive oil (PV, K_{232} and K_{270}) were not among the variables that significantly ($p < 0.01$) changed with malaxation time and temperature. Variables that discriminated both malaxation time and temperature were hexanal, 3,4-DHPEA-DEDA and FFA whereas 1-penten-3-ol, *E*-2-hexenal, octane, tyrosol and vanillic acid significantly ($p < 0.01$) changed with temperature only; and *Z*-2-penten-1-ol, (+)-acetoxypinoresinol and oil yield changed with time only. Virgin olive oil quality was significantly influenced by malaxation temperature whereas oil yield discriminated malaxation time. This study demonstrates the two modes - enzymatic and non-enzymatic - of hexanal formationduring virgin olive oil extraction. Results from this study suggest that a malaxation temperature of 30°C has benefits in terms of oil yield, while still maintaining quality. Processors may be encouraged to experiment with different malaxation times and temperatures to modify the sensory properties of their oils.

Oil storage. Virgin olive oil samples stored for 12 months in the light at ambient temperature, in the dark at ambient temperature, and at low temperature in the dark, both with and without headspace (i.e. oxygen), were separated into recognisable patterns with stepwise linear discriminant analysis (SLDA). The discrimination with variables: volatile and phenolic compounds, free fatty acid (FFA), peroxide values (PV), K_{232} and K_{270}; revealed a departure of stored oil from freshness and showed significant ($p < 0.01$) differences between storage conditions. Virgin olive oil stored at low temperature had characteristics closest to fresh oil while oil stored in the light showed the largest departure from freshness. Parameters that exclusively and significantly ($p < 0.01$) discriminated storage conditions were identified as potential markers of the storage condition. In the presence of oxygen, hexanal was a marker of storage in the light, FFA was a marker for dark storage and markers of low temperature storage were acetic acid and pentanal. In the absence of oxygen, octane was the marker for storage in the light whereas tyrosol and hexanol were markers of virgin olive oil stored in the dark, with no marker indicative of low temperature storage. *E*-2-hexenal, K_{232} and K_{270} were identified as markers of virgin olive oil freshness. The pronounced and rapid (< 2 months) departure from virgin status for oils stored with headspace has important implications for consumer use of oil – that once opened a bottle of oil needs to be used quickly to ensure that it remains of extra-virgin quality. A consumer education campaign may need to be devised to alert Australian olive oil users. Storage of oil in colourless glass containers may also be problematical if the oil is likely to be stored on supermarket shelves exposed to continuous visible light.

Implications

This project demonstrates the importance of the combination of objective, instrument-based analyses with statistical methods in the identification and characterisation of compounds and production steps that govern the quality and characteristics of virgin olive oil. The influence on consumed oil of important steps along the oil production process have been examined: fruit (cultivar, maturity, fruit storage); processing (malaxation time and temperature); and oil storage. Fruit cultivars have been separated based upon constituent volatile and phenolic compounds; four maturity stages have been separated by phenolic and volatile components. Storage of fruit at low temperature may be beneficial to the produced oil, with a possibility of increasing yield and moderating the sensory quality of olive oils. Processing conditions affect oil quality and yield - virgin oil quality was significantly influenced by malaxation temperature whereas malaxation time influence oil yield; results from this study suggest that a malaxation temperature of 30°C has benefits in terms of oil yield, while still maintaining quality. The oil used by a consumer is likely no longer the oil produced at the manufacturing plant; this work indicates the sensory quality of virgin olive oil degrades upon exposure to light, and particularly degrades in a few months upon exposure to air.

1. Introduction

1.1 Background

Olive oil enjoys a tradition and mystique dating back thousands of years. Today olive oil is recognised as the healthy oil and is favoured for its unique aroma and flavour. Despite its ancient origins, modern olive oil production is being enhanced by scientific investigations in the major olive producing countries.

The Australian Olive Industry has undergone rapid expansion in the last decade. With several million trees now planted there are many challenges to developing a vibrant and sustainable industry – in growing, processing and marketing olive products. The Australian Olive Association (AOA) has put quality at the heart of its Vision Statement in the 2003-2008 strategic plan:

> "*By 2010 Australia will be globally acknowledged as a producer of high quality and price competitive olive products.*" (RIRDC, 2002)

The term "quality" can be contentious and has been defined in many ways with perhaps no single universal definition that will adequately apply in all situations. For example, most people when asked what they understand by quality would immediately reply "the very best". Contrast this with:

> *"Good quality does not necessarily mean high quality. It means a predictable degree of uniformity and dependability at low cost and suited to the market."*

The latter definition comes from Deming, one of the engineers of the post-war recovery in Japan. Under Deming's definition there is a clear message that "market" preferences must be taken into account. In food and beverage industries this consideration is paramount, but consumer preferences may change over time. Economically it makes sense to educate consumers to appreciate the properties of extra-virgin olive oil (EVOO) since it retails for higher prices than lower grades (see below for definitions), e.g. refined olive oil. However, it is interesting that:

> *"The IOOC appears not to promote one grade of oil (such as extra virgin) over any other type (for example refined). The promotional approach is that all olive products are good for us."* (Miller, 2002)

Combining this thought with Deming's definition of quality it is possible to conceive of a good quality refined olive oil or, for that matter, a poor quality extra virgin olive oil (EVOO). Quality, therefore, can be thought of as being independent of the grade of the oil. In this report, we have been interested in researching quality attributes of virgin oils, since these are of highest value to the Australian Industry. For the purposes of this report, we will define quality as:

> *The presence in an oil of a set of characters that distinguishes it from other oils.*

In order to enhance quality, it is necessary to have a deep, fundamental understanding of how the "distinguishing set of characters" is affected by the various stages in the production chain. It is this understanding that we will be addressing in this report. The "distinguishing set of characters" is really another way of referring to the chemical constituents of the olive oil. As shall be shown below, both the quality and the grade of an oil is determined by a very small percentage of the chemical constituents present. However, these so-called "minor components", especially volatile and phenolic compounds, have a large influence on how an oil is perceived by a consumer. We have devoted a large part of this study in identifying and monitoring these compounds (among others) in fruit, processing and storage, while looking at how these stages may affect levels of these compounds and hence consumer acceptance

1.2 Grading of olive oil

Olive oil is classified by how it was produced, by its chemistry and by its flavour. Oil produced solely from the fruit of the olive tree (*Olea europea sativa*) using solely mechanical or other physical means under conditions that do not lead to alterations in the oil is defined as virgin olive oil (VOO). The classification of olive oil is governed through the "International Olive Oil Council (IOOC)", which holds great influence over global production and sets quality standards for the international market. There are many chemical tests used to grade olive oil as specified by the IOOC, but three appear to be more common in the literature (e.g. Di Giovacchino, 2000): free fatty acids (FFA), peroxide value (PV) and absorbance of light of 270 nm (K_{270}).

Table 1.1 Grading of olive oil according to FFA, PV and K_{270} (Di Giovacchino, 2000).

Parameter	Extra Virgin	Virgin	Ordinary Virgin	Lampante
FFA (% oleic acid)	≤ 0.8	≤ 2.0	≤ 3.3	>3.3
PV (meq O_2/kg)	≤ 20.0	≤ 20.0	≤ 20.0	no limit
K_{270}	≤ 0.25	≤ 0.25	≤ 0.30	no limit

These parameters have been referred to as the "spoilt degree" of an oil, and PV and K_{270} are very closely related to oxidative damage. They relate to the how the environment – temperature, exposure to light, exposure to oxygen – has affected the lipid content of the oil. As we shall show later (see for example Section 4), sometimes these parameters do not indicate unacceptable changes to the oil as judged by sensory perception. It was thus our goal to investigate more thoroughly the types of compounds – volatiles and phenolics – that are more closely linked with flavour.

Flavour and changes to flavour due to the chemistry in the oil plays a major role in dictating standards and market value of olive oil. For instance, premium quality, fresh, virgin olive oil is characterised by a fruity aroma and a peppery finish. For such oil, it is common for consumers to pay high prices. By contrast, the lower grades of olive oil, which retail at low prices are distinctly "flat" in flavour. Surprisingly there is little chemical difference between these oils, being approximately 95 – 98 % similar. There is thus an enormous commercial incentive to understand the 2 – 5 % of minor components that account for these flavour differences and the corresponding price differential.

1.3 Volatile and phenolic compounds

The distinctive flavours of premium quality virgin olive oil are due to small molecules known as volatiles, while the pepperiness (more correctly pungency and bitterness) is attributable to the phenols. There is a subtle and poorly understood relationship between these two classes of compounds and their changes due to olive oil production conditions leading to differences in flavour development, which will be addressed in this report.

Interestingly, the aroma volatiles are not present in significant quantities in fresh olives. They are formed during the processing of the fruit to give oil and might be altered by the time olive oil reaches the consumer. In particular it is the malaxation step where the most significant flavour development occurs and lost thereafter during the distribution/retail supply chain and consumption. During the malaxation step, fatty acids are broken down by enzymatic oxidation to give volatiles. Also present during the malaxation step are phenolic compounds, which are known antioxidants (DelCarlo, Sacchetti, et al. 2004, Baldioli et al., 1996, Servili & Montedoro, 2002), therefore if they are present in too high a concentration, flavour development might be hindered. On the other hand, if oxidation is allowed to proceed for too long, the oil will go rancid. Rancidity is caused by the same broken down of fatty acids forming volatile compounds that change the oil fruity aroma, with a concurrent loss in phenolic compounds, which changes the bitterness and pungency of olive oil. Thus the balance between levels of volatile and phenolic compounds during virgin olive oil production is critical for stable and premium quality oil.

1.4 Control of volatile and phenolic compounds during production

Research into these issues is needed because it is not clear which individual components are important, or how production steps affect levels of individual compounds. Minimal work has been done on the quality of varietal oils in Australia and although considerable attention has been paid to the gross differences between the major production steps (**Figure 1.1**), less attention has been given to the effects of subtle changes in production conditions on oil quality. For instance, several studies on promoting fruit quality for olive oil production have investigated the agronomical factors such as irrigation practices (Patumi, et al., 2002, Tovar, et al., 2001) and cultivar selection and harvest timing (Sweeney, 2003)(Mailer, Conlan, et al. 2005). Although some effort has been invested in agronomic practices and cultivar selection for the production of quality olive fruits, limited research has been published linking the quality of the fruit to the quality of VOO produced. Such a complex interplay of factors require the development of a systematic approach to understand the transfer of quality attributes from the fruit to olive oil - the first step being simply to examine how production conditions affect the levels and changes of the important flavour compounds. Given the significant economic advantage of producing premium quality olive oil it is essential that processors have a thorough knowledge of how phenolic and volatile compounds are affected by the entire olive oil production process (**Figure 1.1**).

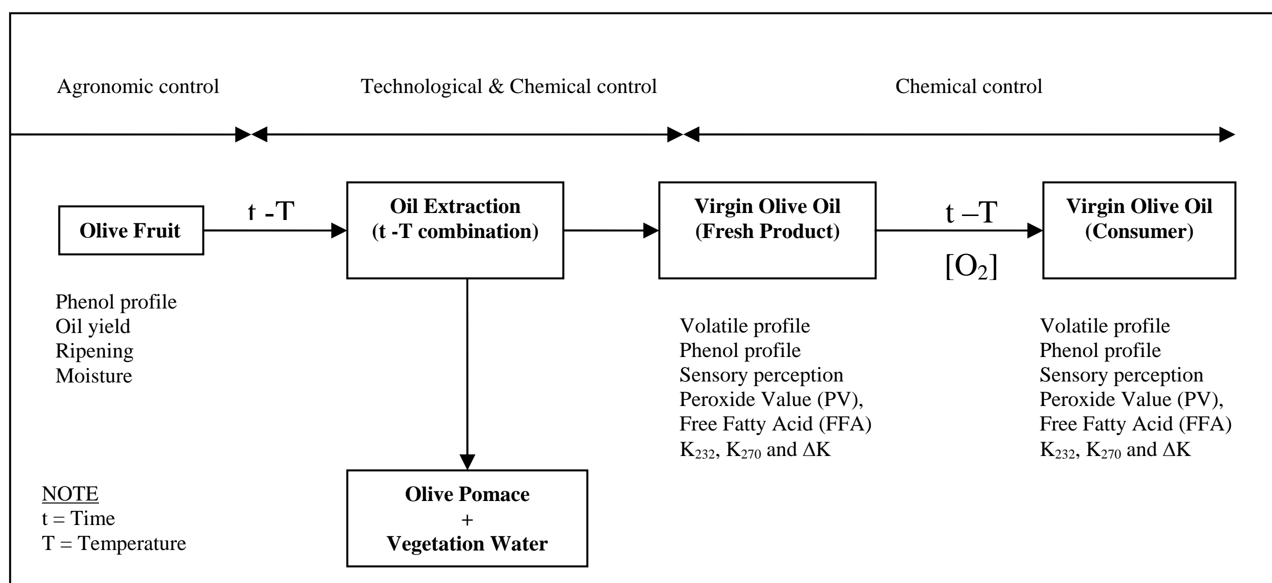

Figure 1. 1 Flow diagram on the possible changes in virgin olive oil along the production line.

Different approaches have been suggested for the transfer of quality attributes from olive fruit to oil and maintenance of olive oil quality until consumption. The IOOC handbook (IOOC, 1990) emphasizes the roles of harvesting, post-harvest fruit handling, and good manufacturing practices in the improvement of olive oil quality with scant consideration on the quality of the fruit from the grove (IOOC, 1990). To ensure good quality virgin olive oil (VOO) at the time it reaches the consumer, there should be a collaborative effort from all stakeholders along the production line of VOO from production to consumption.

The approach in this study followed the changes in olive oil quality that occur from the fruit to the VOO at consumer level (**Figure 1.1**) with emphasis on changes in volatile and phenolic compounds. Quality changes were monitored by looking at the quality indices as specified by the IOOC (2003) and the composition of the minor fraction of the oil, phenolic and volatile compounds in particular, which are important minor components determining VOO quality (Tsimidou, 1998, Servili & Montedoro, 2002, Angerosa et al., 2000z, Angerosa, et al. 2004z).

During production of VOO, quality is controlled through agronomic, technological and chemical means (**Figure 1.1**). Chemical and technological factors affecting VOO quality have been investigated as discrete production steps with minimal inter-relationships with other unit processes along the production line of VOO (**Figure 1.1**). This research focussed on the technological and chemical factors that can change the quality of VOO with emphasis to steps along virgin olive oil production line (**Figure 1.1**), from the olive grove to the consumer as a continuous process in an attempt to identify critical production steps.

Results from this study will ultimately lead to a more fundamental understanding of the chemistry of flavour development while at the same time providing processors with information as to how to optimise production conditions to maximise oil quality. To date, it would appear that the objective of most olive processors has been to maximise oil quantity. This is understandable, yet there is little future in producing high volumes of oil if it only lasts a week on the supermarket shelves, or if it just does not taste good. There is a need to understand how production conditions can be fine-tuned to improve and maintain quality. Among the production conditions that can be manipulated, time and temperature are likely to be important in pulling out more desirable compounds; retarding extraction of less desirable compounds; and influencing chemical changes that can result in off-flavours and less stable oils.

1.5 Aims of this project

This project grew from our previous investigations into the biogenesis of phenolic compounds within the olive tree (Ryan et al. 1999, 2002a, 2003) and preliminary studies on volatile compounds (Prenzler et al. 2002a, Tura et al. 2004). As originally conceived, the project aims were:

> "**Aim 1**: *To determine the effect of processing conditions on the levels of volatile compounds that are the primary contributors to the flavour and aroma of the olive oil;*
> **Aim 2**: *To determine the effect of processing conditions on the levels of phenolic antioxidants*" (Prenzler et al, 2002b).

As we investigated the literature in this area (as above), it became clear that "processing" should be widened to encompass a number of other factors that are important in producing and maintaining the quality of olive oil. Thus our research question became:

"How can we produce and maintain premium quality virgin olive oil that the olive fruit is capable of providing within the constraints of a two-phase centrifugation system[1]?"

Therefore in order to produce and maintain premium quality virgin olive oil, critical control steps and parameters should be identified for the entire production process: from the olive fruit; through oil extraction; and eventually to consumer level oil storage. The corresponding set of aims for this research are to:

(v) Statistically identify cultivar differences and determine changes in volatile and phenolic profiles during fruit maturation for the production of premium quality virgin olive oil.

(vi) Systematically identify volatile compounds; phenolic compounds; and quality indices that significantly ($p < 0.01$) change with simultaneous changes in malaxation time and temperature and ultimately predict the optimum processing conditions for the transferring of the best quality attributes of the fruit to the extracted oil.

[1] the two phase system was chosen because it is the most commonly used extraction system in Australia.

(vii) Determine changes in virgin olive oil quality due to different storage conditions and identify conditions that best preserve the quality and freshness of virgin olive oil.

(viii) Identify the critical production steps from olive fruit to oil at consumption that can be controlled to produce and maintain premium quality virgin olive oil.

This research is the first of its kind to follow the quality of virgin olive oil from the fruit to oil during consumption as a continuous process and to systematically investigate the chemistry of volatile and phenolic compounds and their role in the improvement and enhancement of virgin olive oil quality.

2. Methodology

2.1 Materials

Reagents, phenolic and volatile standards from the indicated sources were used without further purification. The following reagents were used for phenolic compounds analysis: acetic acid (Biolab, Sydney, Australia), hexane and methanol (Mallinckrodt Chemicals, Paris, France), acetonitrile (J.T. Baker, Phillipsburg, USA), formic acid (Sigma, St. Louis, USA). The phenolic standards used were as follows: caffeic acid, *p*-coumaric acid and gallic acid (Sigma, St. Louis, USA), tyrosol (Aldrich, Milwaukee, USA), hydroxytyrosol (Sapphire Bioscience, Sydney, Australia), oleuropein (Extrasynthese, Genay, France). Verbascoside was kindly donated by Prof. Okuyama of Chiba University, Japan. Standards were prepared in methanol + water (50 + 50 v/v) and filtered through 0.45 µm plastic non-sterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for chromatographic preparations.

The volatile standards used were as follows: pentanal, *trans*-2-hexenal and nonanol (Merck, Hohenbrunn, Germany); hexanal, heptanal, *trans*-2-octenal, *trans*-2-nonenal, 1-penten-3-ol, 2-penten-1-ol, heptanol, octanol, hexyl acetate, methyl isobutyl ketone (MIBK) and 2-nonanone (Aldrich, Milwaukee, USA); octanal, octane, nonane, decane, undecane and dodecane (Sigma, St. Louis, USA); benzaldehyde (Ajax chemicals, Auburn, Australia), ethanol and acetic acid (Biolab, Sydney, Australia); ethyl acetate (Mallinckrodt Chemicals, Paris, France), and hexanol (Riedel de Haen, Seelze, Germany).

Reagents used in the determination of peroxide values (PV), UV absorbances (K_{232}, K_{270} and ΔK) and free fatty acid (FFA) were as follows: chloroform, acetic acid, and potassium iodide (Biolab, Sydney, Australia), sodium thiosulphate (Asia Pacific Speciality Chemicals Ltd., Seven Hills, Australia), and starch (Scharlau Chemie S. A., Barcelona, Spain) for PV; cyclohexane spectrophotometric grade (Sigma, St. Louis, USA) for UV absorbances; and propan-2-ol (Mallinckrodt Chemicals, Paris, France), sodium hydroxide (Ajax chemicals, Auburn, Australia), and phenolphthalein indicator (Sigma, St. Louis, USA) for FFA determination.

2.2 Analysis of volatile compounds

2.2.1 Solid Phase Microextraction – Gas Chromatograaphy

Volatile compounds in virgin olive oil were analysed using a developed solid phase microextraction - gas chromatography (SPME-GC) method (Kalua et al., 2006) that was adapted from an earlier method developed in our laboratory (Tura et al., 2004) with reference to other methods (Vichi et al., 2003a, Martos & Pawliszyn, 1997, Servili et al., 2000).

Virgin olive oil (1 g) in reactivials (Supelco, 10 mL) sealed with a teflon-lined septum, was placed in a thermostated oven at 40°C. After thermal equilibration for 15 min the SPME needle (DVB-CAR-PDMS - 50/30 µm fiber, Supelco) was inserted through the septum and left exposed in the headspace for 30 min to extract volatile compounds. The sample was agitated using a magnetic stirrer throughout the equilibration and extraction process. The fiber was withdrawn after 30 min of extraction and the volatile compounds thermally desorbed at the GC injection port at 250°C. The thermal desorption was done in split-less mode for 3 min and thereafter the fiber was cleaned in split mode for 10 min at the injection port prior to re-use.

Solid phase microextraction - gas chromatography – mass spectrometry (SPME-GC-MS) was used to qualitatively analyze volatile compounds using a Varian Star 3400CX gas chromatograph (Varian, Melbourne, Australia) coupled with a Saturn 2000 ion trap mass spectrometer (Varian, Melbourne, Australia). After extraction of the volatile compounds and desorption of the volatile compounds at the injection port, chromatographic separation was achieved under the following column temperature program: 40°C for 8 min, increasing at 5°C/min to 200°C with a final isothermal period of 10 min. Separation was achieved on a SGE BPX5 column (length 30 m, 0.25 mm id, film thickness 0.25 µm) using nitrogen carrier gas at a flow rate of 2 mL/min (pressure 23 psi).

The volatile compounds separated in the column were detected using MS detection in electron impact ionization (EI) mode with automatic gain control (AGC). The electron multiplier voltage for MS was 1850 V, AGC target was 25 000 counts and filament emission current was 15 µA with the axial modulation amplitude at 4.0 V. The ion trap temperature was maintained at 250°C and the manifold temperature was maintained at 60°C. The temperature of the transfer line, interfacing the GC and MS, was set at 250°C. Mass spectral scan time from m/z 35 to 450 was 0.8 sec (using 2 microscans). Background mass was set at 45 m/z.

Volatile compounds were identified by comparison of the retention times with that of authentic standards on GC-FID and confirmed by GC-MS, comparing the mass spectra with the NIST 98 Library. The identity of the compounds was further confirmed by comparing the retention indices obtained with literature values (Acree & Arn, 2004, Reiners & Grosch, 1998, Vichi et al., 2003a). Positive identification was achieved when a volatile compound was identified by both GC-MS and retention time of external standards and also in cases where the volatile compound was positively identified in at least three samples by GC-MS.

Quantitative analysis used the same conditions as above, but detection was done with a flame ionization detector (FID) maintained at 300°C. Quantification was based on two mixed standards – Standard A (ethyl acetate, pentanal, hexanal, *trans*-2-hexenal, heptanal, benzaldehyde, octanal, *trans*-2-octenal, *trans*-2-nonenal, 1-pentene-3-ol, methyl isobutyl ketone (MIBK), 2-nonanone, and dodecane) grouped to eliminate co-elution with volatile compounds in Standard B (ethanol, 2-pentene-1-ol, hexanol, heptanol, octanol, nonanol, hexyl acetate, acetic acid, octane, nonane, decane and undecane). Calibration curves were generated from series of standards (1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0 and 20.0 µg/g), which were prepared from two working standards (50 µg/g). The working standards were prepared from mixed stock standards (2000 µg/g), which were prepared by adding the volatile compounds (0.02 g, about 2 drops) into a known mass (*ca* 10 g) of stripped light olive oil.

Quantitative analysis of individual volatile compounds was achieved using an internal standard, dodecane (0.1 g, 50 µg/g) and calculations made based on the Relative Response Factor (RRF) from the calibration curves (**Section 3.4**) as defined in **Equation 2.1**.

$$[x] = \text{peak area}(x) \times \frac{[IS]}{\text{peak area}(IS)} \times RRF(x) \qquad \textbf{2.1}$$

$$\text{RRF} = \frac{\text{peak area}(IS)}{[IS]} \times \frac{[IS]}{\text{peak area}(x)} = \frac{Slope(IS)}{Slope(x)} \qquad \textbf{2.2}$$

Where:

$[x]$ = concentration (µg /g oil) of the volatile compound (x);

$[IS]$ = concentration (5 µg /g oil) of the Internal Standard (IS);

RRF = ratio of the slope of IS to x from the calibration curves defined as in **Equation 2.2.**

2.3 Analysis of phenolic compounds

Phenolic compounds in virgin olive oil and olive fruit were analysed using a high-performance liquid chromatography (HPLC) method (Kalua et al., 2005) that was adapted from an earlier method developed in our laboratory (Ryan et al., 2003).

Olive fruit (1 g) was crushed in liquid nitrogen and immediately blended with methanol + water (5 mL, 50 + 50 v/v) and gallic acid (0.5 mL, 100 µg /g) as an internal standard using an Ultra Turax blender. The blended sample was left to stand for 30 min at ambient temperature and filtered (GF/F filter paper) using Buchner filtration apparatus. The solid mass was recovered and re-extracted as above but now the blended sample was left to stand for 15 min prior to filtering. The filtrates were combined and washed with hexane (3 x 5 mL). Hexane was discarded and the aqueous phase filtered through 0.45 µm plastic non-sterile filters prior to qualitative and quantitative analysis.

Virgin olive oil (15 g) was dissolved in hexane (15 mL), then gallic acid (0.5 mL, 100 µg /g) was added to the oil as an internal standard and the mixture extracted with 50 + 50 (v/v) methanol + water solutions (3 x 1 mL). The methanolic extract was washed with hexane (3 x 3 mL) and filtered through 0.45 µm plastic non-sterile filter prior to qualitative and quantitative analysis.

Phenolic compounds were identified with a Waters 2695 LC chromatograph with a Waters 2695 LC pump (Waters, Rydalmere, Australia) and a Waters Quattro micro, tandem quadrupole mass spectrometer (Waters, Rydalmere, Australia) by electrospray ionization (ESI). Phenolic compounds were separated on SGE Wakosil C18 column (150 mm x 2.0 mm; 5 µm) with the gradient program described for high-performance liquid chromatography – diode array detector (HPLC-DAD) analysis below except that formic acid (0.1%) replaced acetic acid (1%) in both solvents (A and B). The flow rate of the mobile phase was 0.25 mL /min and sample injection volume was 5 µL. The UV detector (Waters 2487 dual wavelength UV detector) output was monitored at 280 nm and 320 nm by the MassLynx 4.0 Data System for alignment with the mass spectral data. The mass spectral data were acquired at four alternating scans from m/z 80 to 1000 with a scan time of 2 sec using both positive (ES+) and negative (ES–) ion modes at cone voltages of 30 and 70 V.

Characterization of the phenolic compounds with liquid chromatography – electrospray ionization – mass spectrometry (LC-ESI-MS) was reached after comparing results from several samples. Positive characterization was considered to be achieved when a phenolic compound showed the same fragmentation pattern in at least three samples and showed a similar pattern with data from literature (Ryan et al., 2002b)(Bendini et al., 2003)(Perri et al., 1999)(De Nino et al., 2000)(Cardoso et al., 2005). Qualitative analysis was also performed through the comparison of retention times of unknowns with phenolic standards, whenever the standards were commercially available.

HPLC-DAD analysis was performed using a Varian 9012 instrument (Varian, Melbourne, Australia) equipped with a 20 µL sample loop injector. The column eluent was monitored through a Varian 9065 Polychrome Diode Array detector (Varian, Melbourne, Australia) and data collected at 259 nm, 280 nm and 320 nm. Separation was achieved on a Phenomenex C18 column (150 mm x 4.6 mm; 5 µm) with gradient elution. The mobile phase was filtered under vacuum using Alltech Nylon 66 membranes. The flow rate of the mobile phase was 1 mL/min and the solvents for gradient elution were Solvent A (water + acetic acid; 100 + 1 v/v) and Solvent B (methanol + acetonitrile + acetic acid; 95 + 5 + 1 v/v/v). A stepwise linear gradient commencing with 10% solvent B was employed. This was increased to 30% at 10 min, isocratic to 15 min, and then increased to 40% at 25 min, followed by further increases to 50% at 40 min, 75% at 50 min and 95% at 55 min respectively with a final 5 min isocratic run. There was a 5-min equilibration time at the end of the 60-min run.

Quantitative analysis of phenolic compounds was performed using phenolic standards calibration curves (**Section 3.9.3**) based on the internal standard, gallic acid (0.5ml, 100 µg /g) and calculations made based on the Relative Response Factor (RRF) as defined in **Equation 2.3**.

$$[x] = Area(x) \times \frac{[IS]}{Area(IS)} \times \frac{ExtractVolume}{SampleWeight} \times RRF(x) \qquad \textbf{2.3}$$

Where:
$[x]$ = concentration (µg /g oil or fruit) of the phenolic compound (x);
$[IS]$ = concentration (14.29 µg /g oil or 4.76 µg /g fruit) of the Internal Standard (IS);
RRF = ratio of the slope of IS to x from the calibration curves as earlier (**Equation 2.2**) defined.

Direct quantification of some phenolic compounds was not possible because standards were not commercially available. Therefore, the quantification of such compounds was based on oleuropein (for glycosidic phenolic compounds) and hydroxytyrosol (for simple phenols). Phenolic compounds in the fruit were calculated on a dry basis.

2.4 Free fatty acid (FFA) determination

FFA in virgin olive oil was determined using a titrimetric standard method (EC, 1991, IOOC, 2003). The oil sample was dissolved in organic solvent and the free fatty acids present titrated using sodium hydroxide.

Virgin olive oil (5.0 g) was dissolved in neutral propan-2-ol (10 mL) and three drops of phenolphthalein indicator (20 g/L solution in 95 % ethanol) were added and swirled to mix thoroughly. The oil mixture was titrated while stirring with a magnetic stirrer with standardised aqueous sodium hydroxide solution (0.01 mol/L) until the pink colour of phenolphthalein persisted for at least 10 seconds. FFA content was expressed as percentage oleic acid (**Equation 2.4**).

$$\text{FFA (\% oleic acid)} = \frac{V \times c \times M}{10 \times m} \qquad \textbf{2.4}$$

Where:
V = titre volume (mL) of sodium hydroxide solution;
c = exact concentration (mol/L) of sodium hydroxide solution;
M = molar mass of the acid used to express the result (e.g. Oleic acid = 282 g/mol);
m = weight (g) of oil sample.

The mean of duplicate independent determinations was calculated and taken as the result. The calculated FFA value was acceptable when the coefficient of variation was less than 5 %.

2.5 Peroxide value (PV) determination

PV in virgin olive oil was determined using a titrimetric standard method (EC, 1991, IOOC, 2003). PV is the quantity of those substances in the sample, expressed in terms of milli-equivalents of active oxygen per kilogram, which oxidize potassium iodide. PV determination follows the principle of back - titration where a test portion of virgin olive oil is dissolved in chloroform and acetic acid and treated with a solution of potassium iodide. The liberated iodine is titrated with standardized sodium thiosulphate solution.

Virgin olive oil (1.0 g) was accurately weighed into a stoppered flask (200 mL) and rapidly dissolved with stirring in chloroform (10 mL). Acetic acid (15 ml), then saturated potassium iodide solution (1 mL) was quickly added to the oil solution; stopper inserted quickly; shaken for one minute, and left for exactly five minutes away from the light at ambient temperature (preferably 15 – 25°C). Distilled water (approximately 75 mL) was quickly added to quench the oxidation of potassium iodide after incubation in the dark to release iodine. The liberated iodine was titrated with standardised sodium

thiosulphate solution (0.01 N) while stirring with a magnetic stirrer, using starch solution (10 g/L) as indicator until the blue-black colour of starch indicator was decolourised. PV was calculated and expressed in milli-equivalents of active oxygen per kilogram using **Equation 2.5**.

$$PV = \frac{V \times T \times 1000}{m} \qquad \textbf{2.5}$$

Where:
V = titre volume (mL) of the standardized sodium thiosulphate solution;
T = exact normality (N) of the sodium thiosulphate solution;
m = weight (g) of the virgin olive oil test portion.

Simultaneously a blank run was carried out after every ten determinations. If the blank titre volume exceeded 0.05 ml, the reagents were deemed to be contaminated or impure. The impure reagents were replaced before proceeding to the next PV determinations. The mean of duplicate independent determinations (with blank readings less than 0.05 mL) was calculated and taken as the result. The calculated PV was acceptable when the coefficient of variation was less than 5 % for values below 20 milli-equivalents of active oxygen per kilogram and 10 % for PV above 20.

2.6 Ultraviolet (K_{232}, K_{270} and ΔK) spectrophotometric determinations

Spectrophotometric examination in the ultraviolet region can provide information on the quality of a fat, its state of preservation and fat changes from technological processes. The absorption at the wavelengths specified in the method is due to the presence of conjugated diene and triene systems (EC, 1991). The fat is dissolved in the required solvent and the absorbance of the solution is then determined at the specified wavelengths (UV region) with reference to pure solvent. The absorptivity of 1 % solution of the fat in the specified solvent were calculated from the spectrophotometer readings based on a standard method (EC, 1991, IOOC, 2003).

Clear and well settled virgin olive oil (0.25 g) was accurately weighed, dissolved and filled to the mark with spectro-grade cyclohexane in a volumetric flask (25 mL). The virgin olive oil solution was homogenized and where opalescence or turbidity was observed, the solution was discarded and a fresh perfectly clear solution was prepared. Virgin olive oil solution was filled in rectangular quartz cuvettes (optical length = 1 cm) and the absorbances were measured with a spectrophotometer (Cary 50 Conc UV-VIS Spectrophotometer, Varian, Melbourne, Australia) at appropriate wavelengths (232, 266, 270 and 274 nm), using the spectro-grade cyclohexane as a reference. Spectrophotometric analysis of olive oil in accordance with the International Olive Oil Council (IOOC) and the European Union (EU) regulations (IOOC, 2003, EC, 1991) specifies determination of the absorptivity at wavelengths of 232 and 270 nm (**Equation 2.6**) and the determination ΔK (**Equation 2.7**).

$$K_{\lambda} = \frac{E_{\lambda}}{c \times s} \qquad \textbf{2.6}$$

$$\Delta K = K_{270} - 0.5(K_{266} + K_{274}) \qquad \textbf{2.7}$$

Where:
K_{λ} = absorptivity at λ equal to 232 or 270 nm;
E_{λ} = absorbance measured at λ equal 232 or 270 nm;
c = concentration (g/100 mL) of the virgin olive oil solution;
s = path length (cm) of the rectangular quartz cuvette;
K_{266}, K_{270} and K_{274} = absorptivity at 266, 270 and 274 nm respectively.

The absorbance values measured must lie within the range 0.1 to 0.8, the optimum operating range of the spectrophotometer. If not the measurements were repeated using more concentrated or more dilute solutions as appropriate. In addition, each new batch of cyclohexane was tested for spectro-purity. Cyclohexane was spectrophotometrically pure when the transmittance of the solvent was not less than 40 % at 220 nm and not less than 95 % at 250 nm with reference to distilled water.

The mean of duplicate spectrophotometric determinations (K_{232}, K_{270} and ΔK) was calculated to two decimal places. The calculated mean value was acceptable when the coefficient of variation was less than 5 %.

2.7 Oil yield determination

Oil yield was determined by weighing the amount of oil extracted, either at laboratory or industrial scale extraction, and expressing the yield as a dry weight percentage of the olive fruit.

2.8 Olive fruit maturity index (MI) determination

The maturity index (MI) was assessed based on the method of the Instituto Nacional de Investigaciones Agronomicas, Estacion de Jaen (Spain) and described by IOOC (1990). MI was calculated (**Equation 2.8**) after visual colour inspection of both the skin and pulp of 100 olives randomly drawn from a 1 kg olive fruit sample with the maturity score described in **Table 2.1**.

Table 2. 1 Maturity classification of olive fruits (IOOC, 1990).

Maturity Score	Maturity Description
0	Olives with epidermis intense green or dark green
1	Olives with epidermis yellow or yellowish green
2	Olives with epidermis yellowish, with reddish spots or areas
3	Olives with epidermis reddish or light violet
4	Olives with epidermis black and pulp totally white
5	Olives with epidermis black and pulp violet to the midpoint
6	Olives with epidermis black and pulp violet almost to the pit
7	Olives with epidermis black and pulp totally black

Maturity scores 1 – 3 (**Table 2.1**) were assessed through visual inspection of the epidermis only whereas for higher scores the olive fruit was cut open parallel and close to the pit with a sharp knife to assess the colour of the pulp. Once the colour of the epidermis and pulp were assessed, maturity index (MI) was calculated using **Equation 2.8**.

$$\mathrm{MI} = \frac{a*0+b*1+c*2+d*3+e*4+f*5+g*6+h*7}{100} \qquad \mathbf{2.8}$$

Where:
a, b, c, …, h = number of olives fruits in each classification from 0 to 7 (**Table 2.1**) respectively.

The mean of duplicate MI determinations was calculated to two decimal places. The calculated mean MI value was acceptable when the coefficient of variation was less than 5 %.

2.9 Statistical data analysis

Development of modern chemical analytical techniques has led to the generation of vast amounts of data that need statistical analysis to provide maximum relevant information and obtain knowledge about chemical systems and changes within systems (Hopke, 2003). During virgin olive oil production vast amounts of data can be obtained from the analysis of virgin olive oil and olive fruit (**Section 2.2**) at different stages along virgin olive oil production line (**Figure 1.1**). However, it should be noted that not all of this vast data is relevant in the understanding of the changes in virgin olive oil from olive fruit to oil.

To extract the relevant information that describes the virgin olive oil production system from fruit to oil, data generated through chemical analytical techniques should meet certain conditions. The application of parametric statistical analysis, such as ANOVA, regression analysis and discriminant analysis, need to meet certain assumptions for the test to be accurate and robust (Field, 2000). The four basic tests that need to be checked before applying parametric tests are normality, homogeneity of variance, independence and interval of data.

Independence and interval of data are usually taken care of and checked during experimental design and sampling. In order to meet the assumption of independence, sampling and experimental design ensured that the characteristics of one sample did not influence the characteristics of another. Assuring that the data is sampled and generated on an equal scale ensures that the assumption of interval data is met (Field, 2000, Miller & Miller, 2005).

While the assumption of independence and interval data can be checked through experimentation, statistical tests are applied to check homogeneity of variance and normality. Levene's test is a common test that is used to check homogeneity of variances (Field, 2000). To meet the assumption of homogeneity the Levene's test should be non-significant ($p > 0.05$) indicating that for any of the dependent variables the variances are equal.

To check for normality of the data sets, the Kolmogorov-Smirnov (K-S) test (SPSS 12.0, SPSS Inc., Chicago, USA) was used. A non-significant ($p > 0.05$) K-S test indicated that the data set was normally distributed (Field, 2000). In addition, QQ-plots were used to check normality where all points in the QQ-plots should lie more or less on a straight-line to assume normality. Finally to apply parametric tests, the box plots should be symmetric, with the median more or less in the middle of the box to confirm normality of the data set.

In cases where normality and equality of variances assumption were not met, statistical z-scores were calculated by subtracting the mean and dividing by the standard deviation of a distribution. The z-score transformation standardises the original data set into a normal distribution that has a zero mean and a unit standard deviation (Field, 2000, Miller & Miller, 2005). By converting to z-scores it is possible to compare any scores even if they were originally measured in different units (Field, 2000) and this allowed the comparison of the trends for individual unit processes and the entire virgin olive oil production process.

Once the parametric tests assumptions were met, changes in virgin olive oil during production were statistically analysed. Initially, it was important to identify significant differences between samples during virgin olive oil production (**Section 2.3.1**). When significant differences were identified, the data was explored to investigate recognisable patterns and identify parameters that discriminated the patterns during virgin olive oil production (**Section 2.3.2**). The parameters that were extracted from the vast data set and attributed to patterns recognition were investigated for statistical associations with parameters that significantly changed during virgin olive oil production (**Section 2.3.3**). With parameters that discriminate patterns determined, conditions for optimising the discriminating variables were investigated (**Section 2.3.4**) bearing in mind the statistical associations of parameters that showed significant ($p < 0.01$) dependence on the discriminating variables (**Section 2.3.3**). The ultimate aims of these statistical analyses were to identify the changes during virgin olive oil production and predict the optimum conditions for the production of premium virgin olive oil.

2.9.1 Analysis of differences between samples with ANOVA

Differences between samples during virgin olive oil production were identified when the F-test was significant ($p \leq 0.01$). One-way ANOVA post hoc multiple comparison tests were performed for a significant F-test to determine the parameters that were significantly different at an α-value of 0.05 unless otherwise stated.

For the parameters where it was assumed that the variances are equal, Duncan's multiple range test was chosen since it is conservative and hence finds the most statistical differences. In cases where equal variances were not assumed, Games-Howell test was used. Games-Howell test is a pairwise comparison test that is appropriate when the variances are unequal (SPSS 12.0, SPSS Inc., Chicago, USA).

2.9.2 Sample characterisation with stepwise linear discriminant analysis (SLDA)

The significant ($p < 0.05$) differences identified for virgin olive oil samples (**Section 2.3.1**) were characterised with parameters that were extracted from a large data set with stepwise linear discriminant analysis (SLDA). Linear discriminant analysis is a standard statistical technique for projecting data from a high dimensional space onto a perceivable reduced subspace such that the data can be separated by visual inspection (Li et al., 1999). For instance, thirty-one variables with over 4 500 data points (**Chapter 7**) were significantly reduced to fifteen representative variables depicting only data points that identify trends and patterns in the original 4 500 points, which may not be evident from the use of univariate statistics. Therefore, in this research SLDA was used to reduce dimensionality (number of variables) of the data set that discriminated different patterns during virgin olive oil production and extract the useful information while retaining most of the original variability in the data.

Sample characterisation with SLDA was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Unlike other multivariate exploratory procedures, standardizing the variables in linear discriminant analysis has no effect on the outcome but merely re-scales the axes (Miller & Miller, 2005, Marini et al., 2004). However in this study, all variables had an almost normal distribution, so that no transformation was done to the data set. SLDA was used with quality indices and concentrations of volatile and phenolic compounds (**Section 2.2**) as independent variables to recognise patterns that best separated different virgin olive oil production conditions. SLDA involves variable selection and evaluation of variable contribution to discrimination, which explains the recognised pattern.

2.9.2.1 Pattern recognition

The first two linear discriminant functions were used to recognise different patterns during virgin olive oil production; these functions were represented as combined-group scatter plots in two dimensions, x – axis (Function 1) and y – axis (Function 2). The significance of the discriminant functions in the scatter plots was tested with the Wilks' Lambda statistic (SPSS 12.0, SPSS Inc., Chicago, USA) where values close to zero indicate that the group means are different and values close to one indicate that the group means are not different (Field, 2000). Small significance values ($p < 0.05$) indicate that the group means differ and large significance values ($p > 0.05$) indicate that the group means are the same. The group differences explained by the canonical discriminant functions should be significant ($p < 0.05$) to warrant discrimination in the underlying dimension.

A cumulative variance explained of at least 75 per cent in the first two discriminant functions revealed distinct patterns and clustering that were acceptable for the separation of virgin olive oil characteristics. The first two linear discriminant functions had selected variables that determined the location of a particular cluster of virgin olive oil samples in the two-dimensional scatter plot and hence carried relevant information that defined virgin olive oils with similar characteristics.

2.9.2.2 Variable selection

Linear discriminant functions that characterised different patterns during virgin olive oil production (**Section 2.3.2.1**) were defined as multivariate linear equations. Variables in the multivariate linear equations are sequentially entered in stepwise variable selection. The variable considered for entry into the discriminant function is the one with the largest positive or negative correlation that significantly improves the prediction of the outcome. The variable is entered into the discriminant function only if it satisfies the criterion for entry. The variable entry procedure stops when there are no variables that meet the entry criterion (Field, 2000). A stringent criterion ($p = 0.01$) for entry was chosen to select the most likely predictors of patterns during virgin olive oil production, hence eliminating highly correlated and redundant variables (Marini et al., 2004) and subsequently identify markers of virgin olive oil production conditions.

Markers were discriminating variables that exclusively and significantly ($p < 0.01$) characterised different virgin olive oil production conditions. In the case of this study, markers included discriminating variables that positively and negatively correlated with a production condition indicating that markers encompass variables that are both formed and lost during virgin olive oil production.

2.9.2.3 Variable contribution

Once the variables are selected from a vast data set (**Section 2.3.2.2**), the sign of the variable in the linear discriminant equation defines the location of a particular cluster of virgin olive oils and eventually linked to production conditions. The relative contributions of the variables towards discrimination can be explained with the standardized discriminant function coefficients, which are equivalent to the standardized beta in regression and indicates the contribution of each variable to the discriminant functions (Field, 2000). The discriminant functions are the linear combinations of dependent variables that predict which cluster a sample belongs to. These discriminant functions can be described in terms of linear regression equations that are used in calculating scores for discriminating different samples. The magnitude of the discriminant function coefficient is equivalent to the relative contribution of the discriminating variable in the function while the positive or negative sign of the coefficient indicates either a positive or negative contribution respectively (Field, 2000).

In the two-dimensional scatter plots, variables with positive coefficients in the first discriminant function explain virgin olive oil clusters that appear on the positive side of the scatter plot whereas negative coefficients explain virgin olive oil clusters that appear on the negative side of the scatter plot. Similarly, the second discriminant function explains the location of virgin olive oil clusters in relationship with signs of the coefficients of the variables but now on the y-axis of the two-dimensional scatter plots. With the variables defined for both the positive and negative side of the x- and y-axis, variables that defined clusters appearing in different quadrants of the two-dimensional scatter plot were deduced and attributed to the characterisation of virgin olive oils under different production conditions.

2.9.3 Statistical associations with multiple linear regression (MLR)

Statistical associations of parameters that significantly changed during virgin olive oil production followed the reduction of dimensionality with SLDA (**Section 2.3.2**), which extracted useful and relevant information while retaining most of the original variability in the data. The extraction of the data left behind parameters (noisy variables) that did not directly and significantly ($p < 0.01$) characterise virgin olive oil production conditions but might become significantly discriminating during maximisation of desirable quality attributes or minimisation of undesirable attributes associated with the extracted discriminating variables. Multiple linear regression (MLR) is reported (Todeschini et al., 2004) to correlate with noisy variables when a large amount of correlated information is available, making MLR unsuitable for extracting relevant information from a raw data set but might be important in the identification of confounding variables during optimisation of virgin olive oil production conditions from fruit to oil.

MLR with the stepwise method ($p < 0.01$) was therefore applied to identify confounding variables during maximisation of desirable quality attributes or minimisation of undesirable attributes associated with the extracted discriminating variables. Relationships between discriminating variables, as dependent variables, and confounding variables were identified and presented as multiple linear regression models. Model statistics were generated to check the validity and accuracy of the models in predicting statistical associations between discriminating and confounding variables.

Among the statistical measures of model validity, the R^2-value is important in explaining the success of a model in predicting statistical associations and the predictive power of the model when it is extrapolated to a population. The R^2-value of the model is a measure how much of the variability in the outcome is accounted for by the predictors; with values close to 1 representing a good fitting model that explains almost all the variation in the sample. Once the model is generated, it is important to predict the cross-validity of the model, which is indicated with the adjusted R^2-value. Adjusted R^2-value indicates the loss of predictive power or shrinkage. Ideally when the difference between adjusted R^2-value and R^2-value, is zero then there is no variation between the sample and population; in such cases the model derived from the sample accurately represents the population. In other words, cross-validity of the model is very good when the difference between adjusted R^2-value and R^2-value is small. The adjusted R^2-value should therefore be close to or similar to R^2-value with values close to 1 for a good fitting model (Field, 2000).

Once the validity and accuracy of the model in predicting statistical associations are checked, the effect of confounding variables (independent variables) on discriminating variables (dependent variables) during virgin olive oil production optimisation can be determined with certainty. Regression coefficients in the multiple linear model, standardized β-values, indicate the individual contribution of each predictor to the outcome. The magnitude and sign of the β-value predicts the degree of the contribution of the predictor (confounding variable) to the outcome (discriminating variable) if all other predictors are held constant (Field, 2000). For instance, an increase in confounding variables with positive standardized β-values during virgin olive oil production increases the levels of the discriminating variables whereas an increase in confounding variables with negative standardized β-values decrease the levels of the discriminating variables and vice-versa. Identification of confounding variables is important during the optimisation of virgin olive oil production conditions to ensure that the maximisation of desirable quality attributes associated with discriminating variables is not concurrent with the maximisation of undesirable attributes associated with the confounding variables.

2.9.4 Optimum processing conditions with response surface curve fitting

Response surface curve fitting predicted the optimum processing conditions, malaxation time-Temperature (t-T) combinations, through maximisation of discriminating variables associated with desirable virgin olive oil quality attributes while minimising variables associated with undesirable attributes. The data for curve fitting was normalised to obtain statistical z-scores (**Section 2.3**), which was fitted into the Gaussian normal distribution equation (**Equation 2.9**) to predict the equation of best fit using SigmaPlot 8.02 curve fitter (SPSS Inc., 2002) with a running average smoother at a sampling portion of 0.1. The running average smoother averages the values at neighbouring points before predicting the coefficients that give the best fit (SPSS Inc., 2002)

$$Response = F(t,T) = a * \exp * (-0.5[(\frac{t-t_0}{b})^2 + (\frac{T-T_0}{c})^2] \qquad (2.9)$$

Where:

t = malaxation time; t_0 = optimum malaxation time;

T = malaxation temperature; T_0 = optimum malaxation temperature;

a, b and c = constants in the Gaussian normal distribution equation.

SigmaPlot curve fitter uses the Marquardt-Levenberg algorithm to find the coefficients (parameters) of the independent variable(s) that give the "best fit" between the equation and the data (SPSS Inc., 2002). This algorithm uses an iterative process to seek the values of the parameters that minimize the sum of the squared differences between the values of the observed and predicted values of the dependent variable. The equation converges when the differences between the residual sum of squares no longer decreases significantly, which represents the equation of best fit (SPSS Inc., 2002). Curve fitting was considered unsuccessful when the equation failed to converge after 100 iterations and in instances where the predicted processing conditions were out of the experimental range.

In SigmaPlot curve fitting, statistical measures are generated to check the validity and accuracy of the predicted values. Power of regression gives a probability that the fitted equation correctly describes the relationship of the variables. Power of regression with a probability below 0.8000 should be interpreted cautiously, since the fitted equation might not correctly describe the relationship of the variables (SPSS Inc., 2002). In this study, power of regression below 0.8000 is interpreted as lack of correct convergence of the fitted equation. The suitability of the equation of best fit is also shown through the mean squares of residuals (MSR) with lower values of MSR indicating a close fit between experimental data and the predicted equation. MSR is frequently used to compare the suitability of different models in describing a data set and predicting the optimum conditions (SPSS Inc, 2002). The other measures of the model validity are similar to MLR (**Section 2.3.3**). For instance, the coefficient of determination (R^2-value) indicated the percentage of the data that is explained by the generated equation of best fit with R^2-values close to one indicating that the predicted equation explained close to 100 % of the experimental data set (Miller & Miller, 2005).

Once the accuracy and validity of response surface curve fitting were checked, the optimum processing conditions of discriminating variables were obtained from the coefficients that gave the best fit to a data set. Aggregated optimum conditions for individual discriminating variables predicted the range of processing conditions under which there is a high probability of maximising desirable virgin olive oil quality attributes without maximising the undesirable attributes.

3. Discrimination of olive oils and fruits into cultivars and maturity stages based on phenolic and volatile compounds

3.1 Introduction

Olive oil is unique among the high-volume oils in that it is valued for its unique aroma and taste. As the consumption of olive oil increases in non-traditional markets (i.e. those outside the Mediterranean region), consumer preference for oil with particular sensory properties will dictate sales, pricing and market differentiation. To this end objective, quantitative measures of compounds responsible for aroma and taste will be necessary to deliver a consistent product.

Although the precise relationship between chemical composition and sensory properties is yet to be elucidated for olive oil, it is now well established that phenolic compounds (Andrews et al., Angerosa et al 2000z, Gutierrez-Rosales et al., 2003) and volatile compounds (Angerosa, 2002, Angerosa, et al. 2004z, Morales et al., 1995) have a direct influence on the taste and aroma of olive oil. Phenolic and volatile profiles of olive oil originate in the fruit and consequently variations in the chemical and biochemical make-up of olive fruit can have a huge influence on the resultant oil. Many factors may impact on the chemical make-up of olive fruit. For example it has been suggested that cultivar, maturity stage (degree of ripeness), geographic location and agronomic practices (Garcia et al., 1996a, Rotondi et al., 2004, Tovar et al., 2001, Vichi et al., 2003c) may all affect oil properties through effects on fruit. In addition, climate and environmental factors probably have an indirect effect on cultivar characteristics by modifying the degree of ripeness (Angerosa et al., 1999). This leaves olive fruit cultivar and maturity stage as the main factors that explain the variation in the characteristics of olive oil.

The application of multivariate analysis to olive oil has enabled the identification of the variables – geographic location, cultivar, etc. – that explain the variations in samples – phenols/volatiles (Vichi et al., 2003c*,* Aparacio, 2000). It has been shown that multivariate analysis with canonical discriminant analysis, using sensory attributes and chemical compounds as predictors, can efficiently authenticate some olive cultivars (Stefanoudaki et al., 2000). Discrimination of olive oils into varietal and maturity stage groups with stepwise linear discriminant analysis (SLDA) establishes the variables that are the best predictors in separating the groups (Aparacio, 2000). Vichi et al. (2003c) reported the use of linear discriminant analysis (LDA) in distinguishing virgin olive oils by geographic origin and variety according to their volatile composition, with a greater success in the classification of geographic region than cultivar differences.

Identifying volatile and/or phenolic compounds that that explain the variations in olive oil characteristics is a major challenge since the parameters may not be independent. Phenolic and volatile compounds are a characteristic of certain maturity stages (Bonoli et al., 2004, Aparicio & Morales, 1998) and discrimination of cultivars at the same maturity stage introduces bias, further necessitating multivariate analysis. Moreover, not all compounds present in olive oils and fruits at high concentrations characterize cultivar or maturity stages. For instance, lignans are among the main phenols in olive oil (Bonoli et al., 2004) but it was reported (Bonoli et al., 2004, Montedoro et al., 1992a) that the amount of the lignans, (+) – pinoresinol and (+) – acetoxypinoresinol, did not significantly ($p<0.05$) change with ripening. It is therefore imperative to consider a wide spectrum of predictors and not necessarily the major compounds alone in the discrimination of cultivars and maturity stages.

The objective of this study was to identify the phenolic/volatile markers of maturity stages and cultivars in olive fruit and oil. In this work, 20 phenolic compounds from olive fruit and oil and 18 volatile compounds from olive oil were investigated for their ability to predict the discrimination of olive maturity stage and cultivar independent of each other. Both cultivar and maturity stage were discriminated through SLDA and the volatile and phenolic compounds most likely to contribute to discrimination were identified. To the best of our knowledge, this is the first study to examine simultaneously the two major classes of compounds responsible for sensory quality of olive oil in order to identify cultivar and maturity stage markers. Results generated for this section are based on an invitation by Dr Rod Mailer to Dr Paul Prenzler to undertake collaborative research. Project harvest dates and sampling design were based on Dr Mailer's understanding of maturity gained through previous similar studies. The maturity index data in **Table 3.1** were obtained by Mr Jamie Ayton of New South Wales Department of Primary Industries.

3.2 Methodology

3.2.1 Materials

Reagents, phenolic and volatile standards from the indicated sources were used without further purification. The following reagents were used: acetic acid (Biolab, Sydney, Australia), hexane and methanol (Mallinckrodt Chemicals, Paris, France), acetonitrile (J.T. Baker, Phillipsburg, USA), formic acid (Sigma, St. Louis, USA). The phenolic standards used were as follows: caffeic acid, *p*-coumaric acid and gallic acid (Sigma, St. Louis, USA), tyrosol (Aldrich, Milwaukee, USA), hydroxytyrosol (Sapphire Bioscience, Sydney, Australia), oleuropein (Extrasynthese, Genay, France). Verbascoside was kindly donated by Prof. Okuyama of Chiba University, Japan. Standards were prepared in methanol + water (50 + 50 v/v) and filtered through 0.45 µm plastic non-sterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for chromatographic preparations.

The volatile standards used were as follows: pentanal, E-2-hexenal and nonanol (Merck, Hohenbrunn, Germany); hexanal, heptanal, E-2-octenal, E-2-nonenal, 1-penten-3-ol, 2-penten-1-ol, heptanol, octanol, hexyl acetate, methyl isobutyl ketone (MIBK) and 2-nonanone (Aldrich, Milwaukee, USA); octanal, octane, nonane, decane, undecane and dodecane (Sigma, St. Louis, USA); benzaldehyde (Ajax chemicals, Auburn, Australia), ethanol and acetic acid (Biolab, Sydney, Australia); ethyl acetate (Mallinckrodt Chemicals, Paris, France), and hexanol (Riedel de Haen, Seelze, Germany).

3.2.2 Fruit harvest and oil extraction.

Olive fruit samples (3 kg) were hand picked in duplicate from Cookathama farm, near Darlington Point in southwestern New South Wales, Australia during the 2004-harvest season. Forty-eight fruit samples were collected at four maturity stages (**Table 3.1**) from six cultivars (*Leccino*, *Barnea*, *Manzanilla*, *Mission*, *Corregiola* and *Paragon*). The maturity index (MI) was assessed using the method of the Instituto Nacional de Investigaciones Agronomicas, Estacion de Jaen (Spain) and described by IOOC (1990). The color of the olive skin was not very useful in the description of maturity stage since different cultivars showed different rates of change in the skin pigmentation. For instance, the color of *Leccino* fruit remained black and was not significantly different ($p>0.05$) throughout the maturity stages except for fruit at black maturity stage (**Table 3.1**). MI values for *Leccino*, at the same maturity stage, were significantly ($p<0.05$) different at early maturity stages (green and spotted) but were not significantly ($p>0.05$) different at late maturity (**Table 3.1**). *Leccino* was excluded in the calculation of the maturity index (MI) to avoid skewing the maturity description. The maturity stage description was predominantly based on the sampling date in relation to the weeks after flowering (**Table 3.1**) whereas MI indicated the overall range of skin pigmentation.

Oil was extracted from the olive fruit (700 g) using a cold press Abencor extraction unit (Abencor, Spain) according to the manufacturer specifications. The oil was stored (< 1 week) in the dark at room temperature prior to volatile and phenolic compounds analysis.

Table 3.1. Olive fruit sample description

Maturity Stage	**Sampling Date**	**Weeks after flowering**	**Maturity Index (MI) (without *Leccino*)**[a]	**Maturity Index (MI) (*Leccino*)**
Green Olives	13/04/2004	22	2.28 ± 0.68 a	3.98 ± 0.01 c
Spotted Olives	05/05/2004	25	3.06 ± 0.68 b	4.00 ± 0.01 c
Red Olives	31/05/2004	29	4.27 ± 0.41 c	4.10 ± 0.17 c
Black Olives	12/07/2004	35	4.46 ± 0.68 c, d	5.13 ± 0.32 d

[a] Different letters indicate significantly different ($p<0.05$) mean ± standard deviation of at least three replicates.

3.2.2.1 Samples for phenolic compound characterization

Ten olive samples (3 oil, 3 fruit and 4 paste) covering a wide range of phenolic compounds from different cultivars at different maturity stages were used in the characterization of phenolic compounds. The paste sample was an intermediate between the fruit and oil that was obtained after crushing the fruit and malaxing the paste. The paste represented phenolic compounds found in both the fruit and oil.

3.2.2.2 Samples for volatile compound characterization

Characterization of volatile compounds with gas chromatography – mass spectrometry (GC-MS) was performed using *Fusty*, *Rancid* and *Musty* IOOC standard oils, *Leccino* oil sample, *Mission* oil sample and two olive oil samples spiked with volatile standards (ethanol, 2-penten-1-ol, hexanol, heptanol, octanol, nonanol, hexyl acetate, octane, nonane, decane, undecane, acetic acid, ethyl acetate, pentanal, hexanal, *E*-2-hexenal, heptanal, benzaldehyde, octanal, *E*-2-octenal, *E*-2-nonenal, 1-penten-3-ol, methyl isobutyl ketone (MIBK), 2-nonanone, and dodecane).

3.2.3 Determination of volatile compounds

See Section 2.2 for details of the determination of volatile compounds in oil.

3.2.4 Determination of phenolic compounds

See Section 2.3 for details of the determination of phenolic compounds in fruit and oil.

3.2.5 Statistical data analysis

See Section 2.9.2 for details of sample characterisation with stepwise linear discriminant analysis (SLDA)

3.3 Results and Discussion

Discrimination of olive oils into cultivars and maturity stages was studied by initially identifying the volatile and phenolic compounds present in the olive oil and fruit (**Tables 3.2 & 3.3**) then using SLDA with the identified compounds as predictors. Those compounds that significantly ($p<0.01$) separated cultivars and maturity stages into recognizable and mutually exclusive clusters were classified as discriminating compounds (**Tables 4 & 5**), and whether fruit or oil compounds contributed more to the discrimination of olive cultivars and maturity stages was examined. The relative contributions of the predictors were reached after examining the canonical discriminant coefficients. Similarities between groups that were not separated by volatile and phenolic compounds were also recognized.

3.3.1 Phenolic compound characterization

Olive maturity stages and cultivars have been characterized by either the presence or absence of compounds and by a significant increase or reduction of compounds in a sample (Bonoli et al., 2004, Vinha et al, 2005, Romani, et al, 1999, Esti et al, 1998, Amiot et al., 1986). A study of eight olive cultivars (Esti et al., 1998) based on hydroxytyrosol, elenolic acid glucoside, demethyloleuropein, quercetin-3-rutinoside, luteolin-7-glucoside and oleuropein, proposed demethyloleuropein as a varietal marker. The same study proposed hydroxytyrosol as a maturity marker, although the work did not include the black maturation stage. A decrease in secoiridoid concentrations with an increase of olive maturity has been reported (Bonoli et al., 2004) demonstrating that phenolic compounds may be used to identify maturity stages.

The present study used twenty phenolic compounds (**Table 3.2**) as predictors in discrimination of olive oils and fruits into cultivars and maturity stages. As the phenolic profiles of olive fruit and oil are different, separate fruit and oil phenolic groups were used for discriminant analysis.. Glycosylated phenolic compounds found only in olive fruit included hydroxytyrosol glucoside; luteolin-7-rutinoside; verbascoside and oleuropein (**Table 3.2**). These molecules showed fragmentation in both ES- and ES+ modes; formed sodium adducts in the ES+ mode (with the exception of luteolin-7-rutinoside); and gave weaker peaks but more fragmentation in the ES+ mode.

Phenolic compounds detected in olive oil, but absent in the fruit, were derivatives of oleuropein and ligstroside (dialdehydes and hemiacetals), lignans (pinoresinol and acetoxypinoresinol), aglycones such as oleuropein aglycone, and *p*-coumaric acid (**Table 3.2**). Fragmentation of these compounds showed fewer, but more intense, peaks in the ES- mode and in some cases no trace in the ES+ mode, as with *p*-coumaric acid. Sodium adducts were not observed in the ES+ mode for tyrosol, hydroxytyrosol, luteolin, luteolin-7-glucoside and luteolin-7-rutinoside. Apart from luteolin-7-glucoside and luteolin-7-rutinoside, all compounds that did not form sodium adducts were components of the oil suggesting that they may be less polar and preferentially partition into the oil.

For discriminant analysis, the concentrations of tyrosol and hydroxytyrosol were combined with those of their respective glycosides and, for oleuropein and ligstroside, the hemiacetals and dialdehydes were combined and classified as oleuropein and ligstroside derivatives, respectively.

Table 3.2. Characterization of the phenolic compounds used to discriminate olive oil and fruits into cultivars and maturity stages.

Compound	UV[a]	MS[b]	RT[c] (min)	Oil	Fruit	Major ES- peaks	Major ES+ peaks
Hydroxytyrosol glucoside	-	X	6.2 (0.1)	-	Y[d]	315, 153	339, 317, 155, 137
Hydroxytyrosol	X	X	6.76 (0.09)	Y	Y	153, 151, 123	155, 137
Tyrosol glucoside	-	X	8.50 (0.08)	-	Y	399, 299	323, 301, 225
Tyrosol	X	-	9.72 (0.07)	Y	Y	No trace	No trace
Luteolin -7-rutinoside	-	X	11.18 (0.02)	-	Y	593, 285	595, 287
Caffeic acid	X	X	13.0 (0.1)	-	Y	179, 139, 135	165, 151
p-Coumaric acid	X	X	17.9 (0.3)	Y	-	195, 165, 163	No clear trace
3,4-DHPEA-DEDA[e]	-	X	19.1 (0.2)	Y	Y	319, 195, 165	343, 321, 303, 137
Verbascoside	X	X	23.1 (0.3)	-	Y	623, 461, 161	647, 471, 325
Luteolin-7-glucoside	-	X	25.3 (0.5)	-	Y	447, 381	449, 297, 225, 165, 137
Dialdehyde form of ligstroside	-	X	26.4 (0.5)	Y	-	303, 285, 179, 165	327, 297, 225, 165
Hesperidin	-	X	27.1 (0.3)	-	Y	609, 463, 377, 361	633, 611, 465, 433, 303, 137
Hemiacetal of ligstroside	-	X	27.9 (0.2)	Y	-	335, 275, 377	359, 361, 137, 433
Oleuropein	X	X	29.8 (0.5)	-	Y	539, 415, 377	563, 379, 361 137
(+) - pinoresinol	-	X	32.57 (0.02)	Y	-	459, 377, 361, 303, 285, 179	359, 319, 121, 417
(+) - acetoxypinoresinol	-	X	33.2 (0.2)	Y	-	459, 377, 361, 333	811, 439, 417, 357, 233
Ligstroside	-	X	35.8 (0.3)	-	Y	523, 495	547, 417, 363, 345
Oleuropein aglycone	-	X	41.0 (0.7)	Y	-	755, 377, 307, 275	843, 433, 361, 137
Luteolin	-	X	48.9 (0.3)	Y	Y	285, 223	287, 225, 173
Hemiacetal of oleuropein	-	X	49.7 (0.5)	Y	-	409, 377, 361	433, 411, 245, 173, 137

[a] detection by HPLC-DAD denoted by X
[b] characterization by LC-ESI-MS denoted by X
[c] retention time
[d] presence of the compound denoted by Y.
[e] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde

3.3.2 Volatile compound characterization

Olive oil volatile compounds have been used previously to characterize maturity stages and cultivars using multivariate analysis, unlike olive phenolic compounds. Differences in four cultivars were characterized in six European varieties of virgin olive oil using 55 volatile compounds (Morales et al., 1995), and ten C6 volatile compounds have been used to characterize three maturity stages (Aparicio & Morales, 1998).

The current study is based on eighteen volatile compounds (**Table 3.3**) from six cultivars at four different maturity stages over a period of three months (**Table 3.1**). The first ten early eluting volatile compounds in **Table 3.3** (acetic acid, 1-penten-3-one, 1-penten-3-ol, pentanal, Z-2-penten-1-ol, octane, hexanal, E-2-hexenal, E-2-hexen-1-ol and hexanol) are predominantly C5 and C6 compounds and were common in all olive oils except oil from *Manzanilla*, which had C8 compounds (octane, octanal and octanol) as the predominant volatiles. Two volatile compounds, *E*-2-nonen-1-ol and 1-dodecene, were identified by GC-MS only, without either reference retention index or comparison with external standards by GC-FID (**Table 3.3**). Volatile compounds that were positively identified showed a high probability (>70%) when compared with the reference compounds in the NIST 98 Library.

Table 3.3. Characterization of volatile compounds used to discriminate olive oil and fruits into cultivars and maturity stages.

Volatile compounds	FID[a]	MS[b]	RI (Exp)[c]	RI
Acetic acid	X	X	718	710 (Vichi et al., 2003a)
1-penten-3-one	-	X	733	682 (Reiners & Grosch, 1998)
1-penten-3-ol	X	X	733	686 (Acree & Arn, 2004)
Pentanal	X	X	738	732 (Acree & Arn, 2004)
Z-2-penten-1-ol	X	-	771	767 (Acree & Arn, 2004)
Octane	X	X	800	800 (Acree & Arn, 2004)
Hexanal	X	X	794	800 (Reiners & Grosch, 1998)
E-2-hexenal	X	X	855	854 (Acree & Arn, 2004)
E-2-hexen-1-ol	-	X	869	870 (Vichi et al., 2003a)
Hexanol	X	X	874	858 (Vichi et al., 2003a)
6-methyl-5-hepten-2-one	-	X	1011	965 (Vichi et al., 2003a)
5-methyl-5-hepten-2-one	-	X	1012	-
2-pentyl furan	-	X	1012	993 (Acree & Arn, 2004)
Octanal	X	X	1029	1006 (Acree & Arn, 2004)
Hexyl acetate	X	X	1036	1014 (Acree & Arn, 2004)
Octanol	X	X	1089	1072 (Acree & Arn, 2004)
E-2-nonen-1-ol[d]	-	X	1120	-
1-dodecene[d]	-	X	1187	-

[a] detection by GC-FID denoted by X
[b] characterization by GC-MS denoted by X
[c] experimental retention index based on BPX5 column
[d] tentative assignment based on MS

3.3.3 Multivariate approach towards cultivar and maturity stage discrimination

SLDA was used to identify the compounds that predict cultivar and maturity stage patterns. It involves variable selection, evaluation of variable contribution to discrimination, and pattern recognition as outlined in the Methods section. Important to the successful implementation of SLDA are a stringent criterion ($p = 0.01$) for entry of variables, and evaluation of the Wilks' Lambda statistic to indicate the significance of the discriminant functions. The outcome of a discriminant analysis can be visualized in two dimensions by a combined-group scatter plot (e.g. **Figure 3.1**), where the x-axis plots the values of discriminant Function 1 and the y-axis plots the values of discriminant Function 2.

The "% variance explained" indicates the extent to which the discriminant functions explain the patterns (**Tables 3.4 & 3.5**), with higher values indicating a better discrimination. The cumulative % variance explained for the first two functions in the discrimination of olive cultivars in this study ranged from 84.3 - 93.7 % (**Table 3.4**); values higher that those gained through other multivariate statistical analysis methods, such as principle component analysis (PCA) on monovarietal olive oils which gave a cumulative % variance explained on the first two components of 37.2 - 56.8 % (Benincasa et al., 2003).

Table 3.4. Cultivar discrimination by volatile and phenolic compounds from the olive oil and fruit.

Sample (Compounds)	Cultivar Discriminating compounds	% Variance explained (Function 1)	% Variance explained (Function 2)	% Variance explained (Cumulative)
Fruit (Phenols)	Hesperidin	68.0	16.3	84.3
	Verbascoside			
	Tyrosol			
	Luteolin-7-rutinoside			
	Hydroxytyrosol			
Oil (Phenols)	Tyrosol	55.7	29.0	84.7
	DHPEA-DEDA			
	Ligstroside dialdehyde			
	Acetoxy-pinoresinol			
	Oleuropein aglycone			
	Luteolin			
Oil (Volatiles)	Hexanal	80.9	12.8	93.7
	1-penten-3-ol			
	Hexanol			
	E-2-nonen-1-ol			
	Hexyl acetate			
	1-Dodecene			
Oil/Oil (Volatiles/Phenols)	Hexanal	77.7	12.4	90.1
	1-penten-3-ol			
	Hexanol			
	E-2-nonen-1-ol			
	Hexyl acetate			
	1-dodecene			
	Tyrosol			
	Ligstroside dialdehyde			

3.3.4 Cultivar discrimination

Discriminant analysis of cultivars was investigated with the olive fruit phenolic compounds, the oil phenolic compounds, the oil volatile compounds, and combined oil phenolic/volatile compounds (**Table 3.4**). The highest cumulative % variance explained (93.7%) was observed with oil volatile compounds. Discrimination based on phenolic compounds produced a lower % variance explained than volatile compounds (~84% for both fruit and oil phenols, **Table 3.4**). It was observed that scatter plots with higher cumulative % variance explained on the first two functions had a better discrimination of the cultivars (*cf.* maturity stage discrimination, below).

In addition to a better discrimination with volatile compounds, the Wilks' Lambda statistic for the first two canonical discriminant functions was close to zero and significantly different ($p<0.05$), indicating the suitability of the functions to discriminate the cultivar groups. These functions separated the six cultivars into five distinct clusters that were mutually exclusive (**Figure 3.1**).

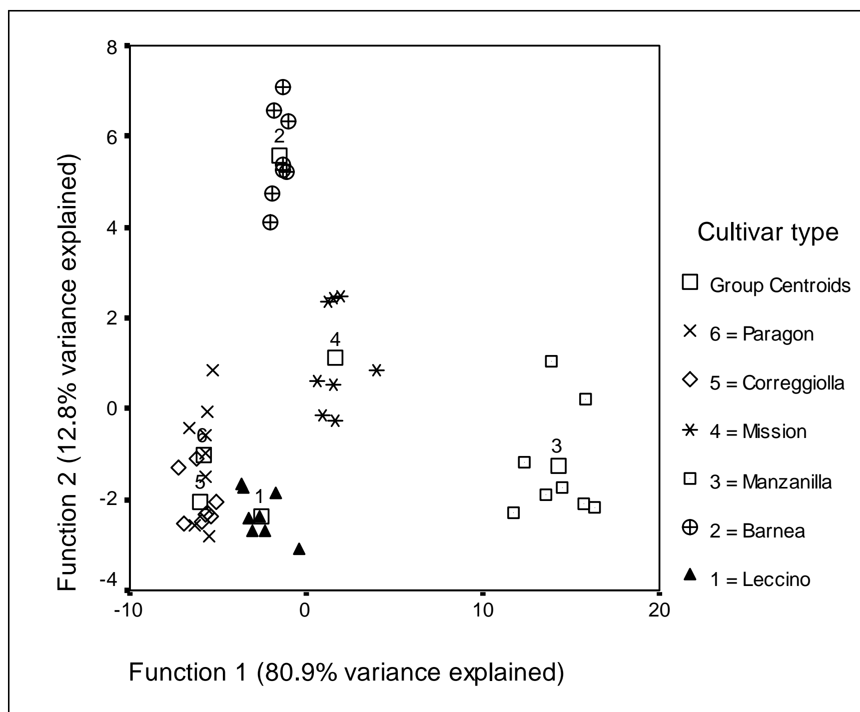

Figure 3.1. Scatter plot for scores of olive oil volatile compounds based on the first two canonical discriminant functions separating cultivars.

In the current study, *Manzanilla* (3) was separated from *Barnea* (2) and *Mission* (4); a cluster was formed for *Corregiola* (5) and *Paragon* (6); and *Leccino* (1) was close to this cluster (**Figure 3.1**).
The best x-axis separation (Function 1, 80.9% variance explained) was observed for *Manzanilla* (**Figure 3.1**) indicating a big difference from the other cultivars. This is consistent with our observation (above) that the C8 compounds, octane, octanal and octanol, were the predominant volatile compounds for *Manzanilla* only. The smallest separation on the x-axis was between *Paragon* and *Corregiola* (**Figure 3.1**) supporting a report (Kailis & Considine, 2002) that the two cultivars might be from the same *Frantoio* family. The closeness of *Leccino* to the *Paragon*/*Corregiola* cluster (**Figure 3.1**) indicates similarities in the volatile profiles of the three cultivars. Function 2 (y-axis, 12.8% variance explained) was successful at discriminating *Barnea* from the rest of the cultivars as shown by the wide separation between the centroids (**Figure 3.1**). This good separation of the cultivars provided by the olive oil volatile compounds is consistent with earlier reports (Campeol et al., 2001) in which three olive cultivars, *Leccino*, *Frantoio* and *Cipressino* were distinguished on the basis of their volatile composition.

3.3.5 Compounds that discriminate cultivars.

To investigate which volatile compounds contribute to the cultivar discrimination in Figure 3.1, it is necessary to examine the "standardized discriminant function coefficients" for the first and second discriminate functions (Function 1, V_1 and Function 2, V_2, respectively). The relative contribution of the volatile compounds towards the discrimination of cultivars along the x-axis of Figure 3.1 is given in the linear discriminant equation (V_1, **1**) below.

V_1 = 0.84[hexanal] – 0.72[1-penten-3-ol] + 0.60[hexanol]	(**1**)
+ 0.76 [*E*-2-nonen-1-ol] – 0.10[hexyl acetate] + 1.18[1-dodecene]	

The contribution of the variables was similar in magnitude but different in the sign. Group centroids for *Mission and Manzanilla* lie on the positive side of the x-axis (**Figure 3.1**), indicating that volatile

compounds with positive coefficients (hexanal, hexanol, *E*-2-nonen-1-ol and 1-dodecene), have a greater contribution than the volatile compounds with negative coefficients (1-penten-3-ol and hexyl acetate). Similarly, it can be deduced that 1-penten-3-ol and hexyl acetate discriminate the cultivars on the negative side of the x-axis in **Figure 3.1** – *Leccino*, *Corregiola* and *Paragon*. This discrimination on V_1 explained more variance (80.9%) than V_2 (12.8%). Of the six cultivars under study, the discrimination of all but one (*Barnea*) was explained by V_1.

Barnea was discriminated on the y-axis of the scatter plot (**Figure 3.1**) by the second discriminant function (V_2, **2**):

$$V_2 = 1.44[\text{hexanal}] - 0.55[\text{1-penten-3-ol}] + 1.33[\text{hexanol}] -0.55[E\text{-2-nonen-1-ol}] + 0.58[\text{hexyl acetate}] - 0.37[\text{1-dodecene}] \quad (\mathbf{2})$$

In fact, group centroids for both *Barnea* and *Mission*, lie on the positive side of the y-axis in **Figure 3.1**, indicating that volatile compounds with positive coefficients (hexanal, hexanol and hexyl acetate) were important in discriminating these cultivars. The volatile compounds with negative coefficients (1-penten-3-ol, *E*-2-nonen-1-ol and 1-dodecene) were important in discriminating the cultivars on the negative side of the y-axis in **Figure 3.1**, *Leccino*, *Corregiola*, *Paragon* and *Manzanilla*.

By combining the effect of both linear discriminant functions (V_1 and V_2), 93.7% of the variance is explained. Thus, it can be concluded that, in this study, pattern recognition in olive cultivars is strongly dependent on volatile compounds. Not all volatile compounds present in the oil are responsible for cultivar discrimination. Of the six compounds listed in **Table 3.4**, the greatest effects were observed with hexanal and hexanol in discrimination of *Mission*, *Barnea* and *Manzanilla*, and with 1-penten-3-ol in discrimination of *Leccino*, *Corregiola* and *Paragon*.

Various volatile compounds have previously been identified as cultivar markers. Morales et al. (1995) reported that *E*-2-hexenal, *E*-3-hexenal, hexanal, butyl acetate and 2-butanone were responsible for olive cultivar differences between *Koroneiki, Koratina, Arbequina* and *Picual*. The variation in the compounds identified by that study and this may be due to the different cultivars studied, although both studies observed that the occurrence of hexanal is cultivar dependent.

Esti et al. (1998) suggested the use of demethyloleuropein as a varietal marker. It was reported in only two cultivars (*Coratina* and *Leccino*) out of eight olive cultivars examined (*Gentile (Larino), Gentile (Colletorto), Gentile (Santacroce), Coratina, Peranzana, Rosciola, Saligna* and *Leccino*). In the current study, however, demethyloleuropein did not significantly ($p<0.01$) discriminate cultivars. Our results (**Table 3.4**) indicate that both fruit and oil phenolic compounds explained a lower variance in cultivar groups than oil volatile compounds did. This suggests that oil volatile compounds are better varietal markers than phenolic compounds.

3.3.6 Maturity stage discrimination

Pattern recognition of maturity stages was done with olive fruit phenolic compounds, oil phenolic compounds, oil volatile compounds, and oil phenolic/volatile compounds (**Table 3.5**). All of these provided a cumulative % variance explained close to 100%, indicating a strong discriminating potential with both volatile and phenolic compounds.

Table 3.5. Maturity discrimination by volatile and phenolic compounds from the olive oil and fruit.

Sample (Compounds)	Maturity Discriminating compounds	% Variance explained (Function 1)	% Variance explained (Function 2)	% Variance explained (Cumulative)
Fruit (Phenols)	Hydroxytyrosol Luteolin-7-rutinoside Ligstroside derivatives	80.6	18.0	98.6
Oil (Phenols)	Oleuropein derivatives Oleuropein aglycone Luteolin Oleuropein hemiacetal	92.8	6.2	99.3
Oil (Volatiles)	*E*-2-hexenal 1-penten-3-ol Z-2-penten-1-ol Hexanol	93.8	6.1	99.9
Oil (Volatiles/Phenols)	*E*-2-hexenal 1-penten-3-ol Z-2-penten-1-ol Hexanol Tyrosol Oleuropein derivative	63.9	34.3	98.2

The maximum cumulative % variance explained (99.9%) was observed with oil volatile compounds. However, the strong influence of the first discriminant function, explaining 93.8% of the variance (**Table 3.5**), limited the ability of the y-axis to discriminate different maturity stages (**Figure 3.2**). Although group centroids were separated on the x-axis, different maturity groups were not mutually separated, except for green and black olives. Points for oil from spotted olives (2) were scattered all over the plot along the y-axis (**Figure 3.2**) indicating that the linear discriminant function 2 was not good at discriminating spotted olives. The Wilks' Lambda statistic for Function 2 was close to one and the means of the scores of the maturity stage groups were not significantly different ($p>0.05$), confirming the unsuitability of using olive oil volatile compounds to discriminate maturity stages. The lack of good separation of the centroids for a large cumulative % variance explained (99.9%), illustrates the importance of considering the loading of the scores on the respective discriminant functions to achieve a recognized pattern in samples.

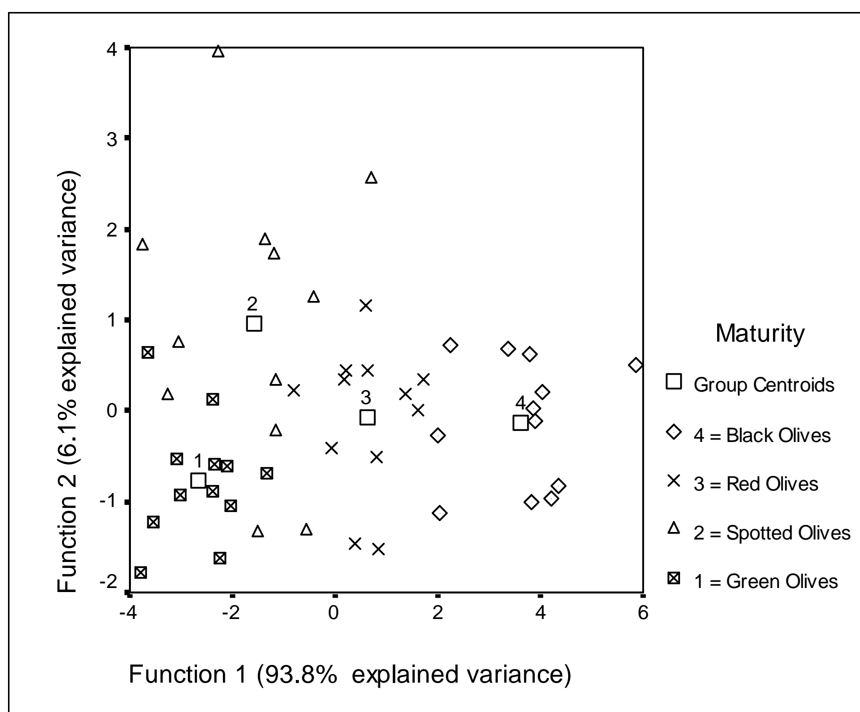

Figure 3.2. Scatter plot for scores of olive oil volatile compounds based on the first two canonical discriminant functions separating maturity stages.

The largest % variance explained for Function 2 was gained when both the volatile and phenolic compounds, from olive oil samples, were included in the analysis (**Figure 3.3**). Moreover, this was achieved without significant loss in the cumulative % variance explained (98.2%). The Wilks' Lambda statistic of both functions was close zero with the means of the maturity stages scores calculated from both functions significantly different ($p<0.05$). The combination of olive oil volatile and phenolic compounds clearly separated the green (1) and spotted (2) fruits on the y-axis (Function 2) and the two maturity stages were further separated from oil of red (3) and black (4) fruits on the x-axis (**Figure 3.3**). Olive oil from black olives (4) had the largest separation with respect to all maturity stage centroids on the x-axis. Quantitative data (**Table 3.6**) supports the significant ($p<0.05$) differences between late (black) and early (green) maturity stages. The two discriminant functions therefore successfully separated the different maturity stages of olives with the green and spotted maturity stages, that were not well separated on Function 1, achieving a good separation on the second function. These results show that oil extracted from late maturity (black olives) has different chemical characteristics from the other maturity stages (**Table 3.6**) and that a combination of volatile and phenolic compounds achieves a reasonable separation of the maturity stages.

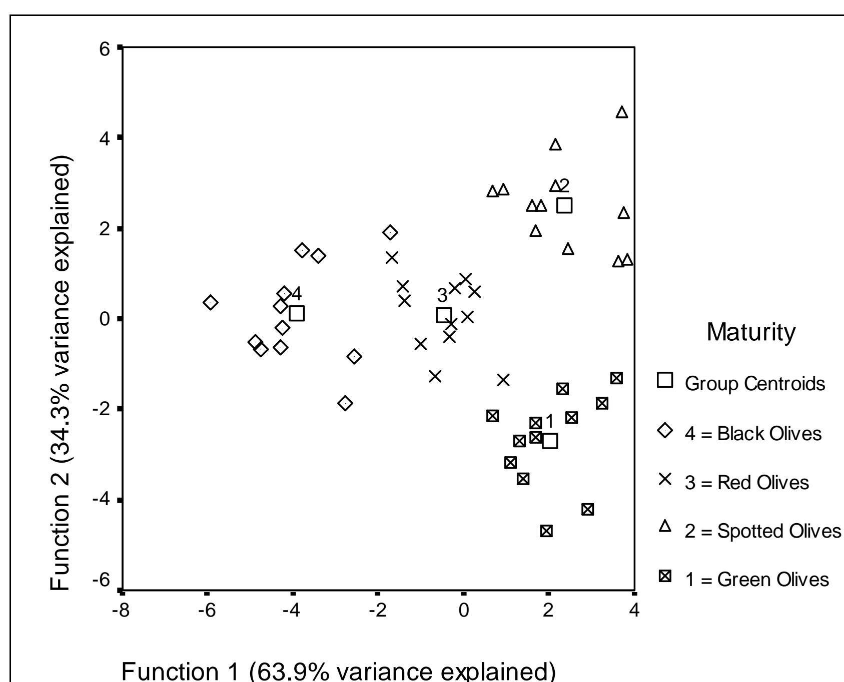

Figure 3.3. Scatter plot for scores of olive oil volatile and phenolic compounds based on the first two canonical discriminant functions separating maturity stages.

Table 3.6. Quantitative data for early (green) and late (black) maturity stages showing the different levels for predictors of maturity stages and cultivars in olive oil.

	E-2-hexenal	**Hexanal**	**Hexanol**	**1-penten-3-ol**	**Z-2-penten-1-ol**	**Tyrosol**	**Oleuropein derivatives**
Green maturity							
Leccino	8.0 ± 1.0[a, b, c, d]	2.86 ± 0.02[a]	<0.03	0.26 ± 0.02[a]	0.17 ± 0.01[d, e, f]	<1.0	<3.0
Barnea	13.0 ± 0.9[d, e, f]	19.44 ± 0.07[d]	<0.03	0.30 ± 0.01[a]	0.155 ± 0.007[c, d, e, f]	<1.0	106 ± 1[a]
Manzanilla	4.7 ± 0.2[a]	18.4 ± 0.9[d]	<0.03	0.14 ± 0.01[a]	0.15 ± 0.01[c, d, e]	<1.0	58 ± 5[a]
Mission	16.7 ± 1.2[e, f]	14.2 ± 0.5[c]	<0.03	1.12 ± 0.07[c]	0.24 ± 0.03[g]	4.0 ± 0.6[a]	246 ± 16[b]
Corregiola	37.4 ± 0.9[g]	3.7 ± 0.4[a]	<0.03	1.1 ± 0.1[c]	0.19 ± 0.03[e, f]	<1.0	286 ± 97[b]
Paragon	38.6 ± 6.3[g]	4.2 ± 0.9[a]	<0.03	1.0 ± 0.2[c]	0.20 ± 0.01[f, g]	<1.0	267 ± 6[b]
Black maturity							
Leccino	6.4 ± 2.2[a, b, c]	3.2 ± 1.3[a]	<0.03	0.26 ± 0.07[a]	0.07 ± 0.02[a]	<1.0	<3.0
Barnea	17.6 ± 0.7[f]	7.3 ± 0.4[b]	0.28 ± 0.03[b]	0.31 ± 0.01[a]	0.095 ± 0.007[a, b]	<1.0	<3.0
Manzanilla	5.4 ± 0.2[a, b]	6.7 ± 0.3[b]	0.215 ± 0.007[a]	0.36 ± 0.02[a]	0.115 ± 0.007[b, c]	<1.0	<3.0
Mission	11.2 ± 1.8[c, d]	6.7 ± 0.7[b]	0.20 ± 0.03[a]	0.70 ± 0.06[b]	0.12 ± 0.04[b, c]	3.3 ± 0.2[a]	<3.0
Corregiola	11.9 ± 0.4 [d, e]	4.3 ± 0.1[a]	<0.03	1.1 ± 0.2[c]	0.125 ± 0.007[b,c, d]	<1.0	<3.0
Paragon	10.4 ± 1.7[b, c, d]	4.1 ± 1.3[a]	<0.03	0.7 ± 0.1[b]	0.065 ± 0.007[a]	<1.0	<3.0

Different superscripts in a column indicate significantly different ($p<0.05$) mean ± standard deviation in μg/g of duplicates.

3.3.7 Compounds that characterize maturity

The relative contribution of the compounds towards the discrimination of cultivars along the x-axis of **Figure 3.3** is given by the coefficients in the linear discriminant equation (**3**) below.

$$V_1 = 0.52[E\text{-2-hexenal}] - 2.06[\text{1-penten-3-ol}] + 2.28[Z\text{-2-penten-1-ol}] - 0.79[\text{hexanol}] - 0.07[\text{tyrosol}] + 0.46[\text{oleuropein derive}] \quad (\mathbf{3})$$

The red (3) and black (4) maturity stages, which are on the negative side of the x-axis on the scatter plot (**Figure 3.3**), are discriminated by compounds with negative coefficients, particularly1-penten-3-ol and hexanol which have larger coefficients than tyrosol. However, the compounds with positive coefficients, *E*-2-hexenal, *Z*-2-penten-1-ol and oleuropein derivatives contributed little to discriminating the green (1) and spotted (2) maturity stages on the positive side of the x-axis (**Figure 3.3**).

The green (1) and spotted (2) maturity stages were discriminated on the y-axis (**Figure 3.3**), and the relative contribution of the compounds that discriminated the maturity stages is given through Function 2 (V_2, **4**) below.

$$V_2 = -1.84[E\text{-2-hexenal}] + 2.13[\text{1-penten-3-ol}] - 0.75[Z\text{-2-penten-1-ol}] + 1.18[\text{hexanol}] - 1.07[\text{tyrosol}] + 1.42[\text{oleuropein derive}] \quad (\mathbf{4})$$

Discrimination of the green (1) maturity stage, on the negative side of the y-axis of the scatter plot (**Figure 3.3**), is influenced by those compounds with negative coefficients, *E*-2-hexenal, *Z*-2-penten-1-ol and tyrosol. The compounds with positive coefficients, 1-penten-3-ol, hexanol and oleuropein derivatives, had an important contribution in discriminating the spotted (2) maturity stage, which appears on the positive side of the y-axis in the scatter plot (**Figure 3.3**).

Not all compounds available in olive oil contributed to the discrimination of maturity stage groups. The results from both linear discriminant functions (V_1 and V_2), discussed above, show that the volatile compounds *E*-2-hexenal and *Z*-2-penten-1-ol characterized olive oils extracted from green fruits whereas 1-penten-3-ol and hexanol discriminated olive oils from spotted, red and black olives. An earlier study (*15*) concluded that the unripe stage was best characterized by C6 volatile compounds and this was attributed to alcohols, which had levels of concentrations far apart in different maturity stages. This was not the case in our study as those volatile compounds failed to separate the green maturity stage from the other stages.

In the current study, phenolic compounds characterized the early maturity stages only, in contrast to volatile compounds, which characterized all olive fruit maturity stages. Tyrosol contributed to the discrimination of oil from green olive fruits while oleuropein derivatives contributed to discrimination of oil from spotted olives. Oil from red and black olives had a slight contribution from tyrosol (coefficient of –0.07) in their separation from the early maturity stages. Our findings, showing that oleuropein derivatives (dialdehydes and hemiacetals) significantly ($p<0.01$) discriminate early from the late maturity stages, are consistent with earlier observations (Bonoli et al., 2004) in which it was reported that the amount of secoiridoids decreased with ripening.

Previously, when fruit phenolic compounds were used as predictors, hydroxytyrosol was reported (Esti et al., 1998) as an indicator of maturation, in agreement with our results when olive fruits were considered (**Table 3.5**). However the low % variance explained for Function 2 of 18.0%, compared to a value of 34.3% for the same Function when using a combination of oil volatile and phenolic compounds (**Table 3.5**), justifies the use of the latter for the discrimination of maturity stages. Interestingly, when using olive oil phenolic and volatile compounds as maturity predictors, hydroxytyrosol was not among the compounds that significantly ($p<0.01$) discriminated the maturity stages (**Table 3.5**).

Just as maturity predictors may differ depending on whether olive fruit or oil is considered as the basis for discrimination, so too maturity markers may change if different discriminating variables or cultivars are used. A study (Aparicio & Morales, 1998) based on ten C6 volatile compounds from *Arbequina, Picual, Koroneiki* and *Coratina* olive cultivars showed that the major indicators of ripeness in olive oil were *E*-3-hexen-1-ol, *Z*-3-hexen-1-ol, *E*-2-hexen-1-ol, hexanal and hexyl acetate. Our results indicate otherwise. This may be due to the different cultivars and volatile compounds studied. The SLDA method used in the present study did not pre-suppose which volatiles should be included in the analysis, whereas the earlier study (Aparicio & Morales, 1998) pre-selected the volatile compounds for consideration; this pre-selection may have influenced the outcome of the analysis. Our results show that the volatile compounds *E*-2-hexenal, hexanol, 1-penten-3-ol and *Z*-2-penten-1-ol (**Table 3.5**) had a significant ($p<0.01$) contribution towards the discrimination of maturity stages. The C5 compounds, 1-penten-3-ol and *Z*-2-penten-1-ol, were not included in the earlier study (Aparicio & Morales, 1998).

3.3.8 Maturity stage and cultivar dependence.

The contributions of compounds to the discrimination of cultivars and maturity stages are not independent of each other. For instance, in our study, the volatile compounds hexanol and 1-penten-3-ol characterized both cultivar and maturity discrimination. An earlier study (Aparicio & Morales, 1998), based on cultivars different from ours, concluded however that hexanol did not contribute to ripeness characterization. Another volatile compound that has shown cultivar and maturity dependence is *E*-2-hexen-1-ol. A study of *Arbequina, Picual, Koroneiki* and *Coratina* reported (Aparicio & Morales, 1998) that *E*-2-hexen-1-ol was one of the major contributors towards ripeness characterization, but significant differences in concentration of this compound were not observed in *Carolea* and *Gentile di Chieti* olive cultivars (Angerosa & Basti, 2001). This dependence of compounds responsible for characterizing both cultivar and maturity calls for careful consideration when identifying maturity and varietal markers.

3.4 Conclusions

The results from this study illustrate the value of multivariate analysis with SLDA in identifying compounds that are responsible for cultivar and maturity stage patterns. Olive cultivar strongly influenced the abundance of volatile compounds, in particular hexanol, hexanal and 1-penten-3-ol. Maturity stage was discriminated best by both volatile and phenolic compounds. This approach may be applied to selectively produce olive oil with particular attributes (sensory or stability) from chosen cultivars at certain maturity stages.

4. Effect of Low Temperature Fruit Storage on Virgin Olive Oil Quality

4.1 Introduction

Most guides to olive processing recommend that oil be extracted as soon as possible after harvesting the fruit. This is to minimise potential defects in olive oil, such as "mustiness" and "fustiness", which result from microbial damage during fruit storage (Morales et al., 2005). On the other hand, if harvesting capacity exceeds processing capacity, some form of fruit storage is inevitable – be it short term, days; or medium term, weeks. Ideally, storage conditions should preserve olive fruit quality and curb deterioration processes without introducing oil defects (IOOC, 1990, Di Giovacchino, 2000).

Variables that affect the potential for storage of olive fruit include: storage temperature, storage time, rate of cooling, relative humidity, maturity, cultivar, storage media (e g. air, water or brine) and modified atmospheres (e.g. reduced ambient oxygen and/or increased carbon dioxide concentration) (Kiritsakis et al., 1998, Koprivnjak et al., 2000, Castellano et al., 1993). Among these variables, low temperature fruit storage with temperatures ranging from 0 to 8°C (Pereira et al., 2002, Agar et al., 1998, Gutierrez et al., 2000, Garcia et al., 1996b) and modified atmosphere storage (Castellano et al., 1993, Kiritsakis et al., 1998, Dourtoglou et al., 2006) have received much attention due to their potential to considerably change olive fruit quality. The effect of storage media on olive oil quality has been reported (Koprivnjak et al., 2000) in a study where olives were kept in sea water (traditional Croatian practice), brine, water and air at 10, 20 and 30 degrees.

Storage of olive fruit at temperatures above 5°C is associated with a fast deterioration in quality and lower temperatures are responsible for chilling injury (Garcia et al., 1996b, Castellano et al., 1993, Agar et al., 1998, Kiritsakis et al., 1998). Low temperatures (3 – 5°C) are usually used to preserve the quality of olive fruit. Low temperatures reduce the rate of chemical reactions and microbial activity that may result in loss of olive fruit quality and subsequent loss of quality of the extracted oil. However, even though low temperature storage reduces the rate of reactions in the fruit, there is an enhancement of mechanical, physicochemical and physiological alterations involved during fruit ripening and senescence, such as softening, respiration, ethylene production and the activity of pectic enzymes (Morello et al., 2003, Pereira et al., 2002).

Low temperature fruit storage may cause cell structure breakdown resulting from mechanical damage due to frozen extracellular water. The freezing of extracellular water causes cellular dehydration and physical membrane destruction by ice crystals resulting in contact between enzymes and their respective substrates (Morello et al., 2003). Softening of fleshy fruit cell wall tissue is characterised by modification and degradation of cell wall components through depolymerisation, deesterification and loss of neutral sugar side chains from the pectic fraction of the cell wall (Jimenez et al., 2001). Decrease in total phenols and quality indices in olive oil produced from fruit after low temperature storage has been reported (Kiritsakis et al., 1998). Even though there is evidence of degradation of olive fruit cell wall components (Jimenez et al., 2001) and decrease of total phenols in the oil (Kiritsakis et al., 1998), studies on effect of phenolic compounds in the fruit during low temperature fruit storage are rare.

Studies on the quality changes in the fruit during storage have focussed on parameters such as firmness, decay incidence, fungus development and visual quality (Agar et al., 1998, Kiritsakis et al., 1998, Castellano et al., 1993, Garcia et al., 1996b), which provide information on external quality changes and little on the changes in specific fruit components that might eventually affect oil quality. The effects of low temperature fruit storage on olive oil quality have been investigated mainly based on quality indices (Peroxide values, free fatty acids, K_{232} and K_{270}) and sensory quality (Garcia et al., 1996b, Agar et al., 1998) with a few studies looking directly at other components of olive oil such as volatile compounds (Koprivnjak et al., 2000), sterol fraction, fatty acid composition and acidity

(Gutierrez et al., 2000). It is interesting to note that the lack of effect of low temperature fruit storage on olive oil quality indices compared with the marked changes in phenolic (Morello et al., 2003) and volatile compounds (Koprivnjak et al., 2000) have not shifted the focus of researchers towards investigating changes in volatile and phenolic compounds during low temperature fruit storage.

The narrow scope of parameters used as indicators in the reported studies and the differences in the time intervals at which olive oil quality is monitored limit the comprehension of investigations on effects of low temperature fruit storage on virgin olive oil. For instance, peroxide value (PV) was not significantly ($p < 0.05$) different during low temperature (5°C) fruit storage after 30 and 60 days (Kiritsakis et al., 1998) whereas monitoring at shorter intervals showed a significant ($p < 0.01$) increase from 0 to 7 days with a decrease in 14 days (Pereira et al., 2002). There are also conflicting reports in literature on the effectiveness of storage of olive fruit prior to oil extraction. For example, García et al (1996) found that fruit storage at 5°C maintained the initial sensorial and chemical qualities of olive oil for 45 days whereas Pereira et al (2002) noticed a decline in oil quality (measured by quality indices) after just 7 days storage at 5°C. Contrary to the general view that oil quality decreases or does not significantly change with fruit storage, olive oil extracted after 30 days of air storage at ambient temperature was characterised by better odour properties than oil extracted after 10 and 20 days. However, these observations did not correspond to qualitative and quantitative changes in volatile compounds (Koprivnjak et al., 2000). This is an interesting observation which calls for further investigation to find out the cause for changes in odour properties that did not correspond to volatile compounds. Phenolic compounds are also related to sensory quality of olive oil (Angerosa et al., 2000z, Andrewes et al., 2003). The concentrations of phenolic compounds decreased in olive oil extracted from frost damaged fruit when no differences in quality indices of olive oil were observed (Morello et al., 2003). This emphasises the importance of monitoring phenolic and volatile compounds in both olive oil and fruit (in addition to quality indices) to detect subtle changes that might have an overall effect on the stability and quality of the oil extracted from stored fruit.

This paper reports on a trial carried out in Australia on the effect on virgin olive oil quality extracted from fruit after periods of low temperature fruit storage. Analyses were based on volatile and phenolic compounds in addition to quality indices. The study was initiated after being approached by a local olive grower and processor to conduct a pilot study on *Frantoio* olive cultivar, which is a popular and widely grown olive cultivar in Australia and other parts of the world such as Italy and Spain. The objective of this study was to investigate the subtle changes in virgin olive oil quality (shown through volatile and phenolic compounds) during low temperature fruit storage. This study is the first of its kind to investigate phenolic compounds in both the fruit and oil simultaneously with volatile compounds in virgin olive oil during low temperature fruit storage.

4.2 Methodology

4.2.1 Materials

Standards and reagents from the indicated sources were used without further purification. Phenolic standards: caffeic acid, *p*-coumaric acid and gallic acid (Sigma, St. Louis, USA), tyrosol (Aldrich, Milwaukee, USA), hydroxytyrosol (Sapphire Bioscience, Sydney, Australia), oleuropein (Extrasynthese, Genay, France). Standards were prepared in methanol + water (50 + 50 v/v) and filtered through 0.45 µm plastic non-sterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for chromatographic preparations.

Volatile standards: pentanal, E-2-hexenal and nonanol (Merck, Hohenbrunn, Germany); hexanal, heptanal, E-2-octenal, E-2-nonenal, 1-penten-3-ol, 2-penten-1-ol, heptanol, octanol, hexyl acetate, methyl isobutyl ketone (MIBK) and 2-nonanone (Aldrich, Milwaukee, USA); octanal, octane, nonane, decane, undecane and dodecane (Sigma, St. Louis, USA); benzaldehyde (Ajax chemicals, Auburn, Australia), ethanol and acetic acid (Biolab, Sydney, Australia); ethyl acetate (Mallinckrodt Chemicals, Paris, France), and hexanol (Riedel de Haen, Seelze, Germany).

Reagents were as follows: chloroform, acetic acid, and potassium iodide (Biolab, Sydney, Australia), sodium thiosulphate (Asia Pacific Speciality Chemicals Ltd., Seven Hills, Australia), and starch (Scharlau Chemie S. A., Barcelona, Spain) for peroxide values (PV); cyclohexane spectrophotometric grade (Sigma, St. Louis, USA) for UV absorbances (K_{232}, K_{270} and ΔK); and propan-2-ol (Mallinckrodt Chemicals, Paris, France), sodium hydroxide (Ajax chemicals, Auburn, Australia), and phenolphthalein indicator (Sigma, St. Louis, USA) for free fatty acid (FFA) determination. Acetic acid (Biolab, Sydney, Australia), hexane and methanol (Mallinckrodt Chemicals, Paris, France), acetonitrile (J.T. Baker, Phillipsburg, USA), formic acid (Sigma, St. Louis, USA) were used in phenolic compounds analysis.

4.2.2 Low temperature olive fruit storage

Frantoio olive fruit (3 x 100 kg) harvested in the 2005 olive harvest season from Riverina region, New South Wales was kept in crates in a cold room (4 ± 2°C) and industrially extracted with a two-phase decanter every week for 3 weeks. Virgin olive oil from the same *Frantoio* batch (as stored fruit) was used as to establish the properties of oil processed from non-stored fruit at zero weeks. The oil extracted from the olive fruit was stored (< 1 week) in the dark at room temperature prior to analysis of quality indices (PV, FFA, K_{232} and K_{270}), volatile and phenolic compounds. Virgin olive oil sensory description and oil yield were provided by the processor. Phenolic compounds in the olive fruit were analysed to monitor changes during low temperature storage.

4.2.3 Qualitative and quantitative analysis of phenolic compounds

See Section 2.3 for details of the determination of phenolic compounds listed in **Table 4.1**.

4.2.4 Qualitative and quantitative analysis of volatile compounds

See Section 2.2 for details of the determination of volatile compounds listed in.

Table 4.1. Variables detected and measured in oil and fruit during low temperature olive fruit storage.

Fruit phenolic compounds	Oil phenolic compounds	Volatile compounds	Quality and yield
Hydroxytyrosol	Hydroxytyrosol	Acetic acid	Free Fatty Acid (FFA)
Tyrosol	Tyrosol	1-penten-3-ol	Peroxide Value (PV)
Luteolin rutinoside	Vanillic acid	Z-2-penten-1-ol	K_{232}
Caffeic acid	3,4-DHPEA-DEDA[A]	Octane	K_{270}
Verbascoscide	Ligstroside dialdehyde	Hexanal	ΔK
Luteolin glucoside	Ligstroside derivatives	E-2-hexenal	Maturity Index (MI)
Ligstroside derivatives	Oleuropein derivatives	E-2-hexen-1-ol	Yield
Oleuropein derivatives	(+) – pinoresinol	Hexanol	
	(+) – acetoxypinoresinol	6-methyl-5-hepten-2-one	
	Oleuropein aglycone	2-pentyl furan	
		E-2-nonen-1-ol	

[A] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde

4.2.5 Determination of quality parameters

Determination of FFA (Section 2.4), PV (Section 2.5) and UV absorbances (K-values, Section 2.6) were performed according to the standard EC and IOOC methods (EC, 1991, IOOC, 2003). These parameters (PV, FFA K_{232}, K_{270} & ΔK) are commonly used to assess the quality of olive oil (IOOC, 2003)and were used to investigate the effect of low temperature fruit storage.
Free fatty acid (FFA), indicates the free fatty acid content of the oil and is an important parameter in the commercial classification of olive oil (IOOC, 2003, EC, 1991). Peroxide value (PV) is used to assess the oxidative deterioration and offers the most direct measure of the primary products of lipid oxidation, the hydroperoxides (Boskou, 1996, IOOC, 2003).

The UV absorbance of samples were measured at four wavelengths (232, 266, 270 & 274 nm) in spectrophotometric grade cyclohexane (Sigma, St. Louis, USA). The parameters K_{232} and K_{270} were calculated from UV absorbance at 232 and 270 nm respectively whereas ΔK was calculated from the absorbances at 266, 270 and 274 nm (IOOC, 2003, EC, 1991). Extinction coefficients measured at 232 and 270 nm (K_{232} and K_{270}) corresponds to the maximum absorption of the conjugated dienes and trienes respectively and indicates an increase in olive oil oxidation levels (Gutierrez & Fernandez, 2002). Extinction coefficients at 232 nm, K_{232}, give a measure of the primary oxidation products (hydroperoxides), whereas K_{270} gives an indication of secondary oxidation products (aldehydes, ketones, alcohols and hydrocarbons) (Gutierrez & Fernandez, 2002).

The maturity index (MI) of the olive fruits (**Table 4.2**) were assessed according to Section 2.8.

4.2.6 Statistical data analysis

Significant ($p < 0.05$) differences for parameters measured at different storage times (**Table 4.2**) were determined using one-way ANOVA post hoc multiple comparison tests (Section 2.9.1) using Duncan's test with SPSS 12.0 (SPSS Inc., Chicago, USA). This statistical test identified parameters that significantly ($p < 0.05$) changed with low temperature fruit storage. Parameters that changed with low temperature fruit storage were of different magnitudes (**Table 4.2**) and to necessitate comparison of trends on a similar reference scale, standardized normal variables (statistical z-values) were used (**Figures 1 – 3**).

Associations of parameters that were identified as significantly ($p < 0.05$) changed with low temperature fruit storage were determined with multiple linear regression (Section 2.9.3) using a stepwise method ($p < 0.01$). Associations between variables were predicted through multiple linear equations. The value of R^2 measured how much of the variability in virgin olive oil quality during low temperature fruit storage was accounted for by the multiple linear equation. Regression coefficients in the multiple linear equations, standardized β-values, predict the degree of the contribution of the predictor to the outcome when all other predictors are held constant i.e. the bigger the β-value, the greater the contribution whereas positive or negative sign of the β-value indicates a positive or negative contribution respectively (Field, 2000).

Table 4.2. Virgin Olive Oil Quality, Yield, Volatile and Phenolic Compounds Changes during Fruit Storage.

Time (weeks)	0	1	2	3	Max Limit [C]
Quality and Yield					
FFA [A]	0.12 ± 0.01 [a]	0.16 ± 0.01 [b]	0.22 ± 0.01 [c]	0.14 ± 0.01 [a, b]	0.8
PV [B]	9.50 ± 0.06 [a]	14.1 ± 0.2 [b]	13 ± 1 [b]	12 ± 1 [a, b]	20
K_{232}	1.53 ± 0.01 [a]	1.84 ± 0.01 [c]	1.74 ± 0.01 [b]	1.72 ± 0.01 [b]	2.50
K_{270}	0.10 ± 0.01 [a]	0.11 ± 0.01 [b]	0.10 ± 0.01 [a]	0.11 ± 0.01 [b]	0.22
Maturity Index	2.65 ± 0.04 [b]	2.56 ± 0.04 [a, b]	2.46 ± 0.05 [a]	2.92 ± 0.06 [c]	NA
Yield (% v/w)	21.58	32.84	34.83	17.17	NA
Sensory Notes	Mild fruity, bitterness, pepper & pungency	Flat, bland oil	Nice mild fruity, bitterness, pepper & pungency	Fatty, no bitterness & no fruitiness	NA
Oil Volatile Compounds [D]					
Z-2-penten-1-ol	< 0.02	<0.02	0.08 ± 0.01 [a]	0.15 ± 0.04 [b]	NA
Hexanal	2.4 ± 0.1 [b]	1.8 ± 0.1 [a, b]	3.2 ± 0.3 [c]	1.3 ± 0.3 [a,]	NA
E-2-hexenal	7.8 ± 0.6 [b]	4.0 ± 0.2 [a]	5.2 ± 0.3 [a]	4.0 ± 0.9 [a]	NA
E-2-hexen-1-ol	<0.03	0.06 ± 0.01 [a, b]	<0.03	0.11 ± 0.02 [b]	NA
2-pentyl furan	0.22 ± 0.02 [a]	0.10 ± 0.01 [b]	0.08 ± 0.01 [b]	<0.02	NA
Total volatiles [E]	10.7 ± 0.1 [a]	6.7 ± 0.4 [b]	8.8 ± 0.6 [a, b]	7 ± 1 [b]	NA
Oil Phenolic Compounds [D]					
Hydroxytyrosol	0.19 ± 0.03 [a, b]	0.36 ± 0.09 [c]	0.30 ± 0.01 [b, c]	0.13 ± 0.01 [a]	NA
Tyrosol	0.56 ± 0.05 [a]	1.3 ± 0.1 [b]	1.3 ± 0.1 [b]	0.8 ± 0.1 [a]	NA
Vanillic acid	0.18 ± 0.01 [a]	0.06 ± 0.01 [b]	<0.05	<0.05	NA
Ligstroside derivatives	24.2 ± 0.2 [a]	23 ± 8 [a]	18 ± 2 [a]	3.2 ± 0.5 [b]	NA
Oleuropein derivatives	27 ± 3 [a]	13.4 ± 0.9 [b]	12 ± 1 [b]	4.7 ± 0.1 [c]	NA
Pinoresinol	17 ± 8 [a]	6 ± 3 [a, b]	3 ± 1 [b]	2.8 ± 0.3 [b]	NA
Acetoxypinoresinol	82 ± 8 [a]	97 ± 2 [b]	77 ± 2 [a]	94 ± 1 [b]	NA
Oleuropein aglycon	15 ± 3 [a]	8 ± 1 [b]	6 ± 3 [b]	5.1 ± 0.7 [b]	NA
Fruit Phenolic Compounds [D]					
Hydroxytyrosol	33 ± 2 [a]	45 ± 15 [a]	43 ± 1 [a]	108 ± 3 [b]	NA
Tyrosol	118 ± 11 [a]	233 ± 42 [b]	123 ± 47 [a,]	189 ± 4 [a, b]	NA
Luteolin rutinoside	146 ± 1 [a]	276 ± 38 [b]	299 ± 63 [b]	638 ± 42 [c]	NA
Luteolin glucoside	138 ± 35 [a]	269 ± 44 [b]	114 ± 7 [a]	530 ± 35 [c]	NA
Ligstroside derivatives	966 ± 81 [a]	1727 ± 69 [b]	726 ± 140 [a]	1530 ± 175 [b]	NA
Oleuropein derivatives	328 ± 54 [a]	113 ± 6 [c]	220 ± 34 [b]	376 ± 27 [a]	NA

Different superscripts in a row indicate significantly different ($p < 0.05$) mean ± standard deviation of duplicate analyses.

[A] Free Fatty Acid as % oleic acid

[B] Peroxide Value expressed as milli-equivalents oxygen per kg oil

[C] Maximum allowable limit as specified by IOOC for extra-virgin olive oil.

[D] Concentrations of phenolic and volatile compounds are expressed in μg/g.

[E] Concentration expressed as μg/g of E-2-hexenal based on total area counts.

4.3 Results and Discussion

Changes of volatile compounds in virgin olive oil, phenolic compounds in both the fruit and oil during low temperature fruit storage were measured to explore the effect on virgin olive oil quality.
Parameters (**Table 4.1**) that significantly ($p < 0.05$) changed during low temperature fruit storage at weeks 0, 1, 2 and 3 were identified and their levels are presented in **Table 4.2**. To explain how these parameters changed with storage time on a relative and comparable scale, statistical z-values were used (**Figures 4.1 – 4.3**), which display the trends in volatile compounds, phenolic compounds in the oil and fruit in relation to virgin olive oil quality. The associations and correlations between the individual changes and trends in the quality indices, volatile and phenolic compounds (**Table 4.3**) were explored with multiple linear regression. Associations established the links from olive fruit to oil (during low temperature fruit storage) and between virgin olive oil quality with volatile and phenolic compounds.

4.3.1 Low temperature fruit storage effect on virgin olive oil quality indices and yield

Low temperature fruit storage changed the overall quality of olive oil and this is illustrated in **Table 4.2** where the common indices used to classify virgin olive oil quality, FFA, PV, K_{232} and K_{270} (IOOC, 2003, Gutierrez & Fernandez, 2002), were significantly ($p < 0.05$) different during the three-week storage period. The quality indices (FFA, PV, K_{232} and K_{270}) of olive oil in this study had minimum values at time zero, which were below the maximum limits for extra-virgin olive oil, and showed positive sensory descriptors (**Table 4.2**).

At one-week of low temperature fruit storage, the sensory quality of olive oil deteriorated to flat and bland (**Table 4.2**), losing all the aroma and taste of the oil extracted from fresh fruit (week zero). There was a gain in yield relative to time zero (**Table 4.2**) despite the loss in quality. A gain in yield was earlier reported (Ranalli et al., 1999) during oil extraction with the aid of enzymes that degraded the cell walls of oil bearing cells. The gain in yield in this study is probably from a similar effect of cell wall degradation due to low temperature fruit storage.

The high yielding olive oil extracted at one week had maximum values for oxidation indicators (PV and K_{232}) but all the quality indices were below the maximum limit for extra-virgin olive oil (**Table 4.2**) with no sensory defects. These quality indices subsequently decreased at two weeks (**Table 4.2**). An increase in PV within the first 7 days and thereafter a decrease at 14 days of storage, similar to the observation in this study, has been earlier observed (Pereira et al., 2002) and was attributed to the probable consumption of minor components, such as phenolic compounds that hinder the formation of peroxides.

Interestingly, good sensory properties were re-gained at 2 weeks storage with the re-emergence of the fruity aroma, bitter taste and pungency (**Table 4.2**). Most of the quality indices improved with respect to oil extracted from fruit stored at low temperature for one week except for FFA (**Table 4.2**). The level of FFA reached a maximum at 2 weeks for the three-week storage period (**Table 4.2**). The maximum FFA value indicated an increased hydrolytic activity and it coincided with maximum yield, which suggests that most of the oil trapped in the cell walls was easily released. Apart from associating cell wall degradation with an increase in yield (Ranalli et al., 1999), the degradation of olive fruit cells during olive oil processing has been reported (Ranalli et al., 2003a) to result in enhanced oil quality with higher hydrolysable phenolic compounds and sensory scores. Modification and degradation of cell wall components through depolymerisation, deesterification and loss of neutral sugar side chains of the pectic fraction has been reported (Jimenez et al., 2001) during aging of olive fruits, resulting in tissue softening. During olive oil extraction with the aid of enzymes, the cell softening process is accelerated. By contrast, low temperature storage allows for a slow natural

degradation, which can have a negative impact on hydrolysing triglycerides leading to high FFA values.

In our case, the cell wall porosity probably increased with low temperature fruit storage and culminated into enhanced interactions between intra- and extra-cellular components. Hence, the improved sensory quality may be explained by the fact that fatty acid substrates were in contact with enzymes for longer. The re-emergence of good sensory attributes for oil extracted from olive fruit stored for two weeks can also be evidenced from the higher concentrations of E-2-hexenal and total volatiles than in oil extracted from one-week low temperature stored fruit (**Table 4.2**).

At three weeks of low temperature fruit storage, sensory quality and yield decreased and coincided with a significant ($p < 0.05$) increase in Maturity Index (**Table 4.2**). The low yield at three weeks could indicate advanced stages of hydrolysis where the hydrolytic products further interacted with triglycerides forming emulsions. The evidence of advanced stages of hydrolysis is further illustrated with fruit phenolic compounds below, where phenolic compounds rise in the fruit but drop in the oil suggesting that they might be ending up in the waste stream.

The sensory notes indicate that the oil extracted from olive fruit at one and three weeks of low temperature storage was of a low quality whereas the oil extracted from fresh fruits and olive fruits stored for 2 weeks had acceptable, positive sensory properties and quality attributes (**Table 4.2**). Our observation on the re-gaining of sensory quality is consistent with a report (Koprivnjak et al., 2000) where olive oil extracted after 30 days of fruit storage in air atmosphere at ambient temperature had better odour properties than samples extracted after 10 and 20 days.

The quality indices (FFA, PV, K_{232} and K_{270}) did not correspond to the above changes observed for sensory quality. For instance, K_{232}, which is associated with hydroperoxides (Garcia et al., 1996a), significantly ($p < 0.05$) changed with post-harvest fruit storage (**Table 4.2**) while K_{270}, which is associated with volatile compounds from oxidative rancidity (Garcia et al., 1996a, Gutierrez & Fernandez, 2002), did not change with oil sensory quality. Studies (Pereira et al., 2002, Kiritsakis et al., 1998, Garcia et al., 1996b) on post-harvest fruit storage have reported minimal changes in K_{232} and K_{270} in olive oil extracted from stored fruit, which was consistent with our observation on K_{270} but not for K_{232}. The observed changes in the sensory quality of olive oil, which were not explained by quality indices, are however explained through levels of volatile and phenolic compounds below.

4.3.2 Trends in levels of volatile compounds during low temperature fruit storage

Volatile compounds are important contributors to aroma associated with olive oil sensory quality (Morales et al., 1995, Angerosa et al., 2000z, Morales et al., 1997). Post-harvest olive fruit handling has been shown to affect the sensory quality of olive oil (Gutierrez et al., 2000, Garcia et al., 1996b). In this study, low temperature post-harvest fruit storage showed a decrease in levels of E-2-hexenal and hexanal with respect to the mean concentrations (expressed as statistical z-values) at weeks 1 and 3 (**Figure 4.1**), which coincided with oil of poor sensory quality (**Table 4.2**), and can be associated with a decrease in enzyme activity. Both E-2-hexenal and hexanal are reported to be generated enzymatically (Sanchez & Salas, 2000, Olias et al., 1993) with the later also formed through chemical oxidation (Morales et al., 1997, Vichi et al., 2003b). While there is a decrease in levels of E-2-hexenal and hexanal at weeks 1 and 3, a concurrent increase in E-2-hexenol (**Figure 4.1**) is noted, which might indicate a possible enzymatic reduction of E-2-hexenal to E-2-hexenol with the aid of alcohol dehydrogenase (Olias et al., 1993). The probable activation of alcohol dehydrogenase was earlier observed (Koprivnjak et al., 2000) where hexanal was reduced to hexanol during air storage of olive fruits.

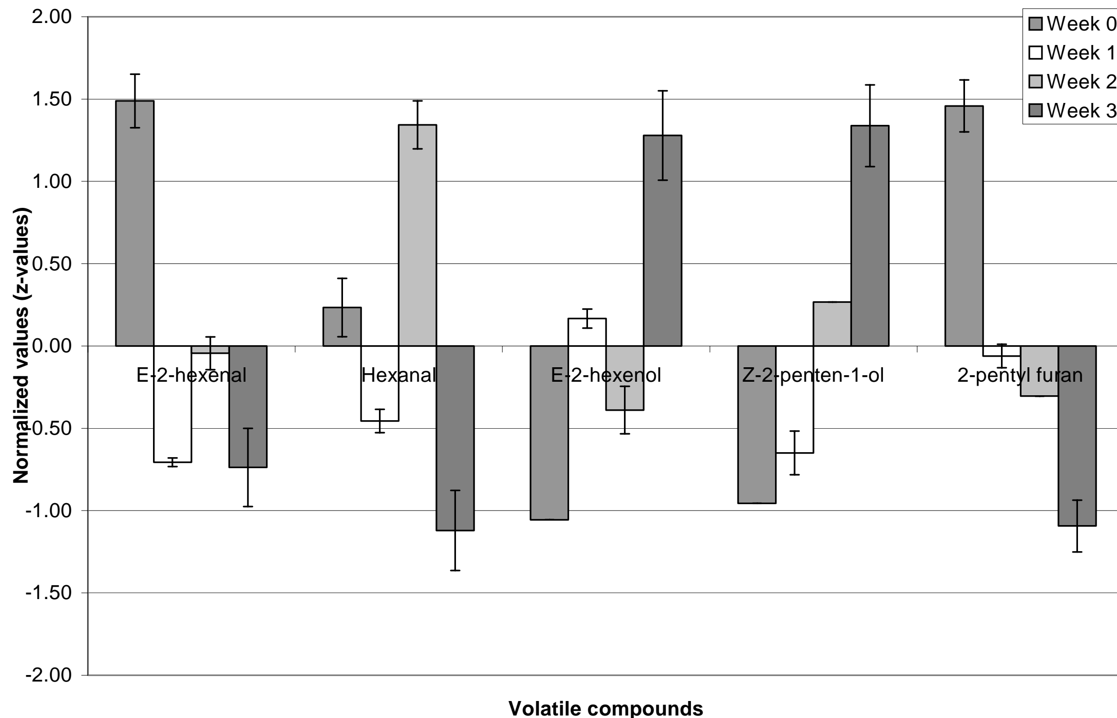

Figure 4.1 Volatile compounds trends during low temperature fruit storage.

The increase in E-2-hexenal with fruit storage has been rarely reported. An exception is Koprivnjak et al. (Koprivnjak et al., 2000) who reported increase in concentration of E-2-hexenal with olive fruit storage for 10 days in cool dry air. Our results (**Table 4.2**) show significantly ($p < 0.05$) higher concentrations for E-2-hexenal in fresh oil (week 0) than in oil extracted from low temperature stored fruit at weeks 1, 2 and 3 with a slight increase during fruit storage at week 2 (**Table 4.2**), consistent with the observations of Koprivnjak et al. (2000).

Hexanal had significantly higher concentrations at 2 weeks of low temperature fruit storage than at weeks 0, 1 and 3 (**Table 4.2**). The increase in concentration for hexanal in this study is not consistent with earlier observations (Koprivnjak et al., 2000) based on *Bjelica* olive cultivar where 90% of hexanal was lost after storing olive fruits in the open air for 10 days. However, the high levels of E-2-hexenal and hexanal, which coincided with positive sensory characteristics (**Table 4.2**), is consistent with earlier reports (Reiners & Grosch, 1998, Morales et al., 1997) that associate high levels of E-2-hexenal and hexanal with positive sensory characteristics reminiscent of premium olive oil quality. Both E-2-hexenal and hexanal are enzymatically formed through the cleavage of unsaturated fatty acid hydroperoxides catalysed by hydroperoxide lyase (Olias et al., 1993), which suggests an activation of the enzyme during low temperature fruit storage.

Apart from E-2-hexenal, hexanal and E-2-hexenol, that changed with the sensory quality of olive oil, Z-2-penten-1-ol and 2-pentyl furan significantly ($p < 0.05$) changed with duration of low temperature fruit storage (**Table 4.2**). Levels of Z-2-penten-1-ol increased with weeks of low temperature fruit storage whereas 2-pentyl furan decreased (**Figure 4.1**). Volatile alcohols with five carbon atoms, such as Z-2-penten-1-ol, have been reported (Angerosa et al., 1998) to increase with time during olive oil extraction while an increase in 2-pentyl furan was observed (Vichi et al., 2003b) with olive oil storage time. The increase in Z-2-penten-1-ol during fruit storage is in agreement with its behaviour during oil extraction whereas a decrease in 2-pentyl furan is a reverse of what happens during olive oil storage, which illustrates the different effects on sensory quality between oil and fruit storage. In the fruit, storage increases interactions between enzymes and substrates as a result of cell wall degradation, which might promote enzymatic generation of volatile compounds (Koprivnjak et al., 2000) associated with positive sensory quality while suppressing the chemical formation of volatile compounds linked to oxidative rancidity, such as 2-pentyl furan (Vichi et al., 2003b).

4.3.3 Trends in levels of phenolic compounds of olive oil during low temperature fruit storage

Olive oil phenolic compounds are components of the fruit unlike volatile compounds that are predominantly generated during the oil extraction process (Sanchez & Salas, 2000, Olias et al., 1993). Among the phenolic compounds detected in olive oil, lignans were not detected in olive fruit (**Table 4.1**). A common lignan in olive oil is acetoxypinoresinol, which interestingly showed a similar but opposite trend (**Figure 4.2**) with hexanal and E-2-hexenal (**Figure 4.1**) during low temperature fruit storage.

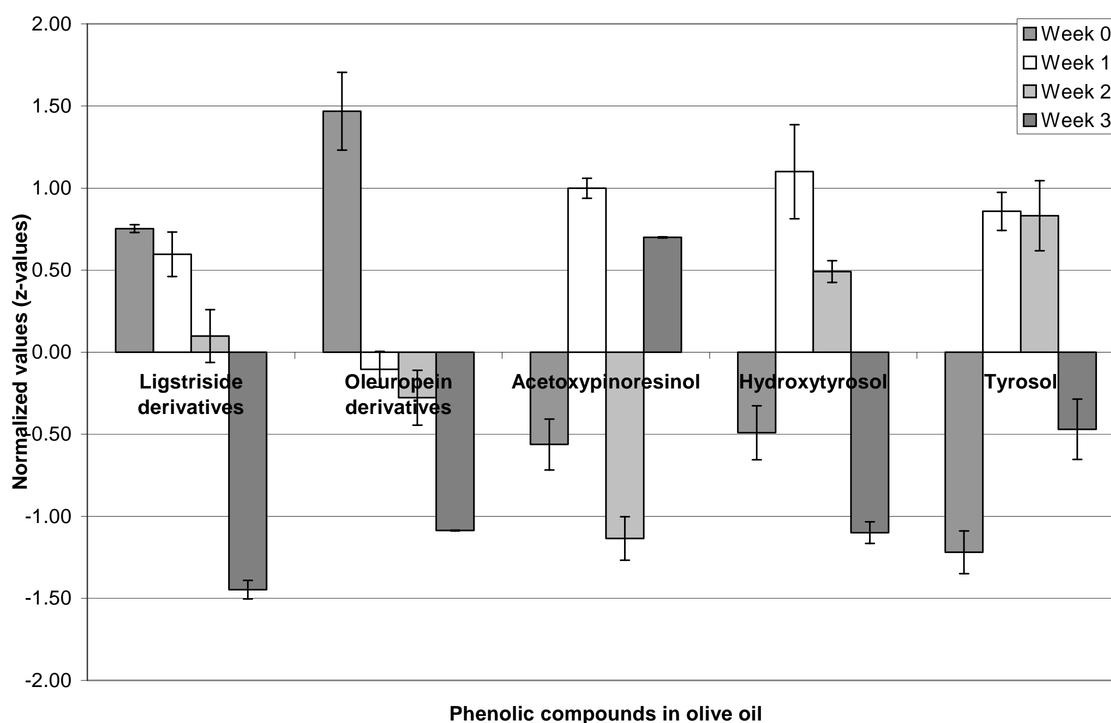

Figure 4.2. Olive oil phenolic compounds trends during low temperature fruit storage.

Acetoxypinoresinol increased at weeks 1 and 3 (**Figure 4.1**), which coincided with poor sensory quality (**Table 4.2**). This illustrates that the conditions conducive to the formation of acetoxypinoresinol are similar to the conditions for production of poor sensory quality olive oil. Results presented in **Section 6.3**, identified acetoxypinoresinol as a discriminating variable characterising low temperature olive oil storage indicating its importance as an indicator of further deterioration of olive oils at low temperatures.

Oleuropein and ligstroside derivatives continuously decreased during low temperature fruit storage (**Figure 4.2**). The statistical z-value for oleuropein derivatives was negative only after one week of fruit storage compared to two weeks for ligstroside derivatives (**Figure 4.2**) indicating faster conversion of oleuropein derivatives with subsequent partitioning into the lipid phase. The significantly ($p < 0.05$) higher values for oleuropein and ligstroside derivatives for fresh fruit (week 0) than stored fruit (week 3) relative to sensory quality (**Table 4.2**) is consistent with earlier reports (Andrewes et al., 2003, Beauchamp et al., 2005, Mateos et al., 2004) that associated these phenolic compounds with bitterness and pungency. A similar effect of low temperature on taste of olive oil was reported (Morello et al., 2003) earlier where oils extracted from frost damaged olives were less pungent and had no bitterness. The change in sensory properties was attributed to the decrease of oleuropein derivatives and slight rises in concentrations of simple phenolic compounds such as vanillic acid that gave rise to sweeter oils (Morello et al., 2003).

These simple phenolic compounds, for instance hydroxytyrosol and tyrosol, are formed from the hydrolysis of high molecular weight glycosylated phenolic compounds, such as oleuropein and ligstroside compounds (Brenes et al., 2001, Amiot et al., 1989, Ryan et al., 1999). A shift from high molecular weight compounds to low molecular weight compounds during olive fruit aging was earlier reported (Jimenez et al., 2001) and attributed to hydrolysis of glycosylated compounds. In our study, an increasing trend for hydroxytyrosol and tyrosol in weeks 1 and 2, indicated through positive z-values (**Figure 4.2**), suggests a possible increase in the hydrolytic activity in the fruit. This is further supported by an increase in FFA, a hydrolytic product of major lipid component – triglycerides, which coincided with a significant ($p < 0.05$) increase in hydroxytyrosol and tyrosol (**Table 4.2**).

The negative z-values for hydroxytyrosol and tyrosol in the oil at three weeks of low temperature fruit storage (**Figure 4.2**), indicates the advanced stages of oil quality deterioration where the simple phenolic compounds are consumed as the oil oxidises and the remaining compounds preferentially partition into the hydrophilic waste stream. The evidence of enhanced hydrolytic activity in the third week can be observed from the low oil yield (**Table 4.2**).

4.3.4 Trends in phenolic compounds of olive fruit during low temperature storage

Changes in the phenolic compounds of olive fruit during low temperature may have subsequent effects on virgin olive oil composition and quality. Changes in olive fruit components, such as phenolic compounds, may indicate interactions between intra- and extra-cellular components culminating in additional oil components, which can assist in understanding virgin olive oil quality. For instance, low temperature fruit storage showed an increase in levels of fruit ligstroside derivatives and tyrosol at week 1 and 3 (**Figure 4.3**), which coincided with oil of poor sensory quality (**Table 4.2**).

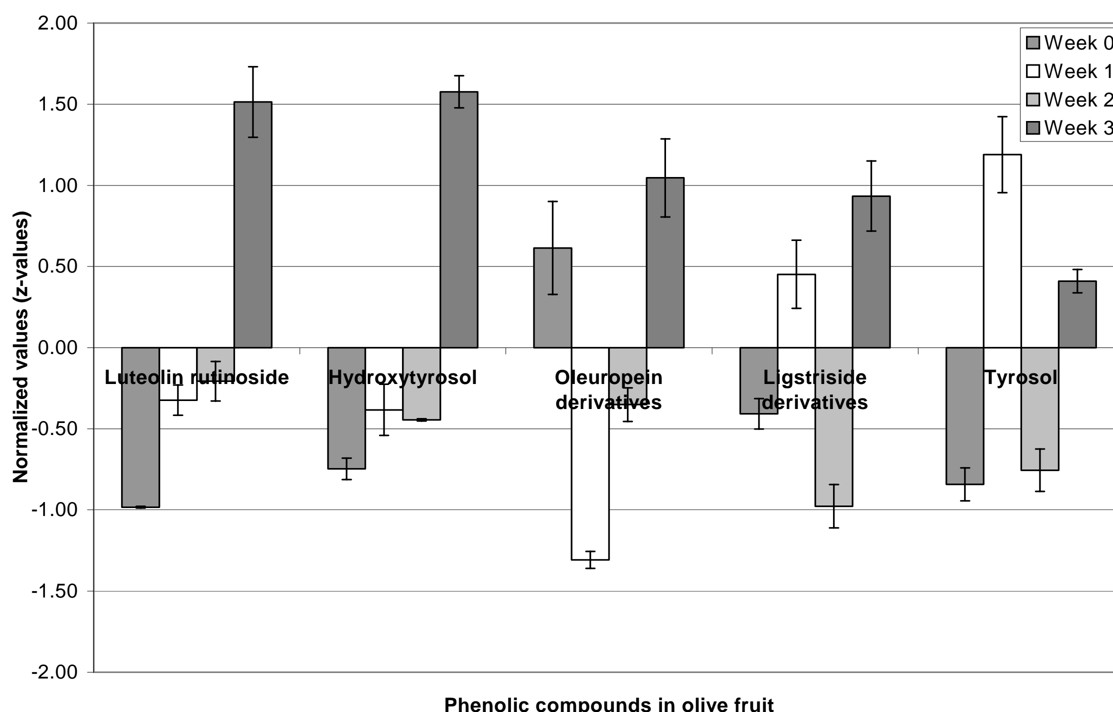

Figure 4.3. Olive fruit phenolic compounds trends during low temperature fruit storage.

Increase in concentrations of ligstroside derivatives and tyrosol in the fruit at weeks 1 and 3 (**Table 4.2**) can be associated with their formation before and after cell wall degradation at weeks 1 and 3 respectively. The increasing concentration during the first week of storage might indicate a shift in equilibrium where the fruit components try to oppose any change to the initial status. The re-establishment of equilibrium can be observed with fruit hydroxytyrosol (a hydrolytic product of oleuropein) and oleuropein derivatives (**Figure 4.3**) where a change from positive to negative z-values from weeks 0 to 1 for oleuropein derivatives is accompanied by an increase towards more positive z-values for hydroxytyrosol (**Figure 4.3**).

The general trend of increase in z-values with duration of low temperature fruit storage was observed for hydroxytyrosol and luteolin rutinoside in olive fruit (**Figure 4.3**). Luteolin rutinoside was earlier (Kalua et al., 2005) observed to increase with fruit maturity, which corresponds to increase in the porosity of cell wall (Jimenez et al., 2001).

Quantitative data on all fruit phenolic compounds that were significantly ($p < 0.05$) affected during low temperature storage (**Table 4.2**), shows that at week 3 of low temperature storage, fruit phenolic compounds (**Figure 4.3**) increased. The increase in fruit phenolic compounds during low temperature fruit storage provides further evidence of cell structure destruction; where bound phenolic compounds are released and they are free interact. The interactions between phenolic compounds showed different effects in olive oil and fruit. Phenolic compounds in the oil (oleuropein and ligstroside derivatives) continuously decreased with fruit storage (**Figure 4.2**) whereas phenolic compounds in the fruit (hydroxytyrosol and luteolin rutinoside) continuously increased (**Figure 4.3**). This main difference of an increase in fruit phenolic compounds and a decrease in oil phenolic compounds might indicate an interaction between reactive phenolic compounds (oleuropein and ligstroside) with other substrates that are released with increase in the porosity of the cell wall. Accelerated cell wall degradation using enzymes during mechanical extraction process of virgin olive oil has been found to increase the concentration of phenolic compounds in olive paste and oil (Vierhuis et al., 2001). In our case of slow cell wall degradation, the time for the interactions between intra- and extra-cellular components is extended, and does not always lead to an increase in phenolic compounds (**Figure 4.3**), but does result in an increased association between minor components affecting virgin olive oil quality.

4.3.5 Associations of olive minor components with olive oil quality during fruit storage

The interactions of the major and minor constituents under different environmental conditions, such as low temperature, change the composition of virgin olive oil that subsequently affects the oxidative stability of oil (Velasco & Dobarganes, 2002). It is generally accepted that oxidative status of virgin olive oil is strongly related to sensory defects and that sensory characteristics determine the quality of virgin olive oil (Psomiadou et al., 2003). To maintain the quality of virgin olive oil, it is therefore paramount to control the oxidation status that can be achieved by having control over some minor constituents, which act as antioxidants. Antioxidants deter the generation of volatile compounds from chemical oxidation responsible for sensory defects as well as volatile compounds from enzymatic oxidation that impart positive sensory characteristics (Velasco & Dobarganes, 2002, Psomiadou et al., 2003). Phenolic compounds are among the minor components in olive oil that are known for their antioxidant activity (Baldioli et al., 1996). An association between phenolic compounds and measures of olive oil quality during low temperature fruit storage may provide an early indication of quality changes.

In this study, trends in both volatile and phenolic compounds have been presented (**Figures 4.1 – 4.3**) with little association to the overall quality of virgin olive oil. Associations between the measured parameters (**Table 4.1**) were explored using multiple linear regression and results with reference to R^2 (**Table 4.3**), explained most of the variations (70.5 to 100.0 %) in quality indices, volatile, and phenolic compounds during low temperature fruit storage.

The associations between minor components and quality indices (**Table 4.3**) illustrate the correlations between compounds in the fruit and oil and eventually how that correlation affects the overall quality of virgin olive oil produced after low temperature fruit storage.

Table 4.3. Associations of quality indices, volatile and phenolic compounds during fruit storage.

Dependent Var (Y)	R^2	Independent Var (X) – Negative[C]	β (-)	Independent Var (X) – Positive[D]	β (+)
Fruit Phenolic Compounds					
Hydroxytyrosol	1.000	Hydroxytyrosol (Oil)	- 0.301		
		Z-2-penten-1-ol	- 0.250		
		1-penten-3-ol	-1.053		
Luteolin glucoside	0.982			Hydroxytyrosol (Fruit)	0.776
				Acetoxypinoresinol	0.359
Luteolin rutinoside	0.968	1-penten-3-ol	- 0.984		
Ligstroside derivatives	0.858			Acetoxypinoresinol	0.926
Tyrosol	0.809			Ligstroside derivatives (Fruit)	0.899
Oleuropein derivatives	0.756	Hydroxytyrosol	- 0.870		
Oil Phenolic Compounds					
Vanillic acid	0.975	FFA [B]	-0.318	2-pentyl furan	0.813
Oleuropein derivatives	0.926			2-pentyl furan	0.962
Oleuropein aglycon	0.884			Oleuropein derivatives (Oil)	0.940
Ligstroside derivatives	0.855	Z-2-penten-1-ol	- 0.925		
Pinoresinol	0.765			Vanillic acid	0.875
Hydroxytyrosol	0.756	Oleuropein derivatives (Fruit)	- 0.870		
Acetoxypinoresinol	0.727			ΔK	0.852
Tyrosol	0.705			PV [A]	0.840
Oil Volatile Compounds					
E-2-hexenal	1.000			Total Volatiles	0.716
				Pinoresinol	0.199
				2-pentyl furan	0.216
				Tyrosol (Fruit)	0.042
Total Volatiles	1.000	Pinoresinol	- 0.278	E-2-hexenal	1.397
		2-pentyl furan	- 0.302		
		Tyrosol (Fruit)	- 0.059		
2-pentyl furan	0.926			Oleuropein derivatives (Oil)	0.962
Z-2-penten-1-ol	0.855	Ligstroside derivatives	- 0.925		
E-2-hexen-1-ol	0.786	1-penten-3-ol	- 0.886		
Hexanal	0.710	Luteolin glucoside (Fruit)	- 0.843		
Oil Quality					
K270	0.944	Total Volatiles	- 0.511	6-methyl-5-hepten-2-one	0.703
PV [A]	0.803			K232	0.896
K232	0.803			PV [A]	0.896

[A] Peroxide Value expressed as milli-equivalents oxygen per kg oil

[B] Free Fatty Acid as % oleic acid

[C] Variables in the multiple linear regression equation with a negative coefficient, β (-)

[D] Variables in the multiple linear regression equation with a positive coefficient, β (+)

4.3.6 Associations of phenolic compounds in the fruit

Not surprisingly, fruit phenolic compounds are shown to have a dependent association with both volatile and phenolic compounds in the oil produced. A negative dependent association was observed between fruit phenolic compounds and volatile compounds that were predominantly C5 compounds (**Table 4.3**). Both fruit hydroxytyrosol and luteolin rutinoside showed a negative dependent association with 1-penten-3-ol shown though negative standardized β-values (**Table 4.3**). Fruit hydroxytyrosol also showed a negative dependent association with Z-2-penten-1-ol (**Table 4.3**). The increase in fruit hydroxytyrosol and luteolin rutinoside (**Figure 4.3**) with a negative association with C5 volatile compounds suggests that low temperature fruit storage had a suppression effect on the formation of C5 volatile compounds.

The positive dependent associations were observed for the fruit phenolic compounds: luteolin glucoside, ligstroside derivatives and tyrosol with fruit hydroxytyrosol, acetoxypinoresinol and fruit ligstroside respectively (**Table 4.3**) and this suggests a simultaneous formation between the phenolic compound pairs during low temperature fruit storage. While there was a clear positive dependent association between phenolic compounds, hydroxytyrosol (**Table 4.3**) was an exception. Fruit hydroxytyrosol showed a negative dependent association with oil hydroxytyrosol (**Table 4.3**) suggesting a decrease in the partitioning of hydroxytyrosol from fruit to oil during low temperature fruit storage.

4.3.7 Associations of phenolic compounds in the oil

Phenolic compounds in olive oil were shown to have a dependent association with quality indices, volatile and phenolic compounds (**Table 4.3**), which can be explained with reference to formation of volatile and phenolic compounds during low temperature fruit storage. A negative dependent association was observed for oil hydroxytyrosol with fruit oleuropein derivatives (**Table 4.3**). This association is further illustrated with the trends where oil hydroxytyrosol has positive z-values in weeks 1 and 2 (**Figure 4.2**) with concomitant negative z-values in weeks 1 and 2 for fruit oleuropein derivatives (**Figure 4.3**) suggesting that oil hydroxytyrosol is formed from fruit oleuropein derivatives during low temperature fruit storage. The formation of hydroxytyrosol from fruit oleuropein derivatives is consistent with earlier reports (Brenes et al., 2001, Amiot et al., 1989, Ryan et al., 1999) where simple phenolic compounds, such as hydroxytyrosol and tyrosol, are formed from the hydrolysis of high molecular weight glycosylated phenolic compounds, such as oleuropein and ligstroside compounds.

Oil oleuropein agylcon was positively dependent on oil oleuropein derivatives (**Table 4.3**). Oil oleuropein derivatives decreased with low temperature fruit storage (**Figure 4.2**) which could have a similar effect on oleuropein aglycons, deducing from their positive association. The decrease of oleuropein compounds could be related to their antioxidant activity during storage (Baldioli et al., 1996) where the compounds are consumed to protect the oil from oxidative degradation. Tyrosol was an exceptional phenolic compound that showed a positive dependent association with PV during low temperature fruit storage (**Table 4.3**). Previous reports (Kiritsakis, 1998) based on olive oil storage associated high levels of tyrosol with low quality, which is synonymous to the above observation since high PV levels are also associated with low quality oil.

4.3.8 Associations of volatile compounds

Volatile compounds showed a dependent association on phenolic compounds with predominantly higher standardised β-values than the association among volatile compounds themselves (**Table 4.3**). The associations among volatile compounds indicated a positive association between E-2-hexenal and total volatiles and a negative association between 1-penten-3-ol and E-2-hexen-1-ol (**Table 4.3**). The oil phenolic compounds, oleuropein derivatives were positively associated with 2-pentyl furan (**Table 4.3**) and this observation is supported by trends of these minor components in virgin olive oil where both 2-pentyl furan (**Figure 4.1**) and oleuropein derivatives (**Figure 4.2**) decreased with low temperature olive fruit storage. Quantitative data (**Table 4.2**) further illustrates that low temperature fruit storage did not favour the formation of both 2-pentyl furan and oleuropein derivatives.

A negative dependent association was observed for Z-2-penten-1-ol and hexanal with oil ligstroside derivatives and fruit luteolin glucoside respectively (**Table 4.3**). The negative association between Z-2-penten-1-ol and ligstroside derivatives is supported by the trends for both compounds, Z-2-penten-1-ol (**Figure 4.1**) and ligstroside derivatives (**Figure 4.2**) where low temperature fruit storage favoured the formation of Z-2-penten-1-ol but did not favour the formation of ligstroside derivatives in virgin olive oil.

4.3.9 Associations of quality indices

During low temperature fruit storage, quality indices showed a dependent association with volatile compounds. Total volatiles were negatively associated to K_{270} as observed from the negative standardized β-value (**Table 4.3**). Total volatiles were also highly positively associated with E-2-hexenal, with a standardized β-value of 1.397 (**Table 4.3**). The negative association between E-2-hexenal and K_{270} is in agreement with reported observations where high E-2-hexenal levels are related to positive sensory quality (Kiritsakis, 1998, Cavalli et al., 2004) and an increase in K_{270} is synonymous with oxidized rancid olive oil (Gutierrez & Fernandez, 2002). The volatile compound, 6-methyl-5-hepten-2-one, had a positive standardised β-value in association with K_{270} (**Table 4.3**), which indicated that as the concentration of 6-methyl-5-hepten-2-one increased the value of K_{270} also increased. This is in agreement with reports (Gutierrez & Fernandez, 2002) where K_{270} gives a measure of secondary oxidation products, such as the ketone 6-methyl-5-hepten-2-one. The measure of primary oxidation products, K_{232}, had a positive standardised β-value (**Table 4.3**) in its association with PV, which is in agreement with literature (Gutierrez & Fernandez, 2002), where both K_{232} and PV gives an indication of primary oxidation products (hydroperoxides).

5. Changes in Volatile and Phenolic Compounds with Malaxation Time and Temperature during Virgin Olive Oil Production

5.1 Introduction

Mechanical oil extraction affects formation of volatile compounds and the release of phenolic antioxidants, which greatly influence the quality of virgin olive oil (De Stefano et al., 1999, Olias et al., 1993). During mechanical extraction of virgin olive oil, the olive paste, formed after crushing the fruit, is mixed in a process called malaxation (Di Giovacchino et al., 2002a, Angerosa et al., 2001). Malaxation induces the coalescence of minute oil droplets into large droplets and the subsequent formation of a continuous lipid phase, which is then separated from the paste (Ranalli et al., 2003b). To assist in the coalescence process, temperature of the paste is raised to decrease the viscosity of the mix. Higher oil yields are obtained by malaxing at higher temperatures (Amirante et al, 2002), but oil quality may deteriorate if the temperature is too high. Thus a balance between oil yield and quality must be achieved. Furthermore, higher malaxation temperatures and shorter malaxation times may be advantageous through increasing oil yield and daily production capacity, respectively, provided that such a combination retains the extra virgin status of the oil.

Several studies have shown malaxation time and temperature are important factors that strongly influence the quality and yield of virgin olive oil (Angerosa et al., 2001, Ranalli et al, 2003b, Morales & Aparicio, 1999, Koutsaftakis et al., 1999, Ranalli, et al, 2001). These studies have investigated the effect of malaxation time and temperature either as sole variables, or in combination with other variables e.g. cultivar. For instance, Ranalli's group have studied the effect of malaxation temperature (Ranalli, et al, 2001) and time (Ranalli, et al, 2001) on oil quality and yield based on *Caroleo*, *Leccino* and *Dritta* cultivars. Several studies (Angerosa et al., 2001, Ranalli et al, 2003b, Morales & Aparicio, 1999*,* Servili et al., 2003) have investigated the effect of both malaxation time and temperature on olive oil quality during mechanical extraction. Across all of these studies, a limited range of malaxation temperature (20 – 38°C) and time (15 - 90 min) have been reported (Angerosa et al., 2001, Ranalli et al, 2003b, Morales & Aparicio, 1999*,* Ranalli, et al, 2001, Servili et al., 2003, Salas and Sanchez, 1999).

Malaxing olive paste at 30 ºC for at least 45 min (Ranalli et al, 2003b) produced both pleasant green extra-virgin olive oil and satisfactory oil extraction outputs, but malaxing at 35 ºC introduced numerous defects in the oil without substantially increasing oil yield (Ranalli, et al, 2001). Conversely, it has been reported (Khfif et al, 2003) that malaxing at 35°C for 60 min produces best quality olive oil and yield. Anecdotal evidence suggests that some processors are malaxing at temperatures significantly lower than 25°C mainly due to low ambient temperatures early in the processing day, without guidance as to how this may impact oil yield and quality. Little is known about the changes extreme processing conditions can introduce into virgin olive oil. Morales and coworkers (1999) suggested an alternative way of obtaining pleasant green olive oils through processing at higher temperatures (> 35°C) with minimum malaxation times (< 30 min). A recent report by Garcia et al (Garcia et al., 2005) on the treatment of olives with hot water (60°C and 72°C) prior to oil extraction, raises the question of how high a temperature is possible, before oil quality deteriorates below the virgin classification. In the case of oil extraction from *Coratina* olive fruit (Angerosa et al., 2001), investigations of extreme high malaxation temperatures (35°C and 45°C) were not carried out due to technical troubles.

In evaluating the various malaxation time and temperature combinations for processing, different parameters have been used to describe the effect of these variables on virgin olive oil quality. For example, some studies have focused on volatile compounds only (Morales & Aparicio, 1999); some on volatile compounds and sensory analysis (Angerosa et al., 2001); and phenolic and volatile compounds combined with sensory analysis (Servili et al., 2003); whereas Ranalli's group (2001, 2003b) have considered a diverse array of measures including quality indices, oil yield, volatile and phenolic compounds as well as sensory analysis.

Because of wide variations in experimental designs, different ranges of malaxation times and temperatures, and the different parameters used to define the changes of virgin olive oil quality, it is difficult to ascertain the key oil quality parameters that are indicative of, or predictors for, quality changes in the oil due to a combination of malaxation time and temperature. For instance, studies that considered a wide spectrum of quality attributes (Ranalli et al., 2001, 2003b), did not consider the simultaneous effect of malaxation time and temperature. On the other hand, a study (Morales & Aparicio, 1999) with a sound statistical experimental design and data analysis that simultaneously investigated the effect of malaxation time and temperature considered only volatile compounds, and no other quality parameters.

The objective of this study was to systematically identify volatile and phenolic compounds that significantly ($p < 0.01$) change with simultaneous changes in malaxation time and temperature during virgin olive oil production. This study is unique as it applies complete four level factorial combinations of malaxation time and temperature over a wide range to explore changes in volatile and phenolic compounds, quality indices, and oil yield in a single study. The systematic approach of using response surfaces, contour plots and multivariate analysis applied in this study is rare in studies of olive oil processing conditions. The extremes of malaxation temperatures and times can reveal some of the changes in virgin olive oil quality that can be explored for the benefit of possible future advancement in olive processing technology.

5.2 Methodology

5.2.1 Materials

Reagents, phenolic and volatile standards from the indicated sources were used without further purification: acetic acid (Biolab, Sydney, Australia); hexane and methanol (Mallinckrodt Chemicals, Paris, France); acetonitrile (J.T. Baker, Phillipsburg, USA); formic acid (Sigma, St. Louis, USA); caffeic acid, *p*-coumaric acid and gallic acid (Sigma, St. Louis, USA); tyrosol (Aldrich, Milwaukee, USA); hydroxytyrosol (Sapphire Bioscience, Sydney, Australia); oleuropein (Extrasynthese, Genay, France). Verbascoside was kindly donated by Prof. Okuyama of Chiba University, Japan. Standards were prepared in methanol + water (50 + 50 v/v) and filtered through 0.45 µm plastic non-sterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for chromatographic preparations.

The volatile standards used were as follows: pentanal, *E*-2-hexenal and nonanol (Merck, Hohenbrunn, Germany); hexanal, heptanal, *E*-2-octenal, *E*-2-nonenal, 1-penten-3-ol, 2-penten-1-ol, heptanol, octanol, hexyl acetate, methyl isobutyl ketone (MIBK) and 2-nonanone (Aldrich, Milwaukee, USA); octanal, octane, nonane, decane, undecane and dodecane (Sigma, St. Louis, USA); benzaldehyde (Ajax chemicals, Auburn, Australia), ethanol (Biolab, Sydney, Australia); ethyl acetate (Mallinckrodt Chemicals, Paris, France), and hexanol (Riedel de Haen, Seelze, Germany).

Reagents were used in the determination of peroxide values (PV), UV absorbances (K_{232}, K_{270} and ΔK) and free fatty acid (FFA) were as follows: chloroform, acetic acid, and potassium iodide (Biolab, Sydney, Australia), sodium thiosulphate (Asia Pacific Speciality Chemicals Ltd., Seven Hills, Australia), and starch (Scharlau Chemie S. A., Barcelona, Spain) for PV; cyclohexane spectrophotometric grade (Sigma, St. Louis, USA) for UV absorbances; and propan-2-ol

(Mallinckrodt Chemicals, Paris, France), sodium hydroxide (Ajax chemicals, Auburn, Australia), and phenolphthalein indicator (Sigma, St. Louis, USA) for FFA determination.

Frantoio olive fruit (50 kg) was hand-picked from Cookathama farm, near Darlington Point in southwestern New South Wales, Australia during the 2004-harvest season. The fruit was harvested at thirty-four weeks after flowering when the skin color was red to black (maturity index = 3.7 ± 0.1). The oil extracted from the olive fruit was stored (< 1 week) in the dark at room temperature prior to analysis of quality indices (PV, FFA, K-values), volatile and phenolic compounds.

5.2.2 Olive oil extraction

Forty-eight samples (16 treatments × 3 replicates × 1 kg olive fruit/extraction) were extracted using a cold press Abencor extraction unit (Abencor, Spain). Olive oil was extracted according to the time – temperature processing conditions based on a four-level (4^2) complete factorial experimental design with malaxation time (30, 60, 90 and 120 min) and malaxation temperature (15, 30, 45 and 60°C) as factors. Water (100 mL/kg fruit) was added at processing temperature to improve the rheology of the paste. The oil and paste mixture was separated into two phases after centrifugation and the top oil layer was decanted into foil-covered pharmaceutical bottles (200 mL) prior to analysis.

5.2.3 Determination of quality parameters

Determination of FFA, PV, and UV absorbances (K-values) were performed according to Section 2.4, 2.5 and 2.6, respectively, and were used as independent variables in the characterization of malaxation time and temperature (**Table 5.1**).

5.2.4 Qualitative and quantitative analysis of volatile compounds

See Section 2.2 for details of the determination of volatile compounds in oil as listed in **Table 5.1**.

5.2.5 Qualitative and quantitative analysis of phenolic compounds

See Section 2.3 for details of the determination of phenolic compounds in oil as listed in **Table 5.1**.

Table 5.1. Variables for the characterization of malaxation time and temperature in virgin olive oil from *Frantoio* fruit.

Volatile compounds	Phenolic compounds	Other variables
Acetic acid	Tyrosol	Free Fatty Acid (FFA)
1-penten-3-one	Vanillic acid	Peroxide Value (PV)
1-penten-3-ol	3,4-DHPEA-DEDA[A]	K_{232}
Z-2-penten-1-ol	(+) – acetoxypinoresinol	K_{270}
Octane	Oleuropein aglycone	ΔK
Hexanal	Hemiacetal of oleuropein	Oil Yield
E-2-hexenal		
E-2-hexen-1-ol		
Hexanol		
6-methyl-5-hepten-2-one		
2-pentyl furan		
Octanal		
Hexyl acetate		
Octanol		

[A] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde

5.2.6 Statistical data analysis

See Section 2.9.2 for details of sample characterisation with stepwise linear discriminant analysis (SLDA)

5.3 Results and Discussion

Virgin olive oils extracted from *Frantoio* fruit were used to identify volatile and phenolic compounds that significantly ($p < 0.01$) change with malaxation time and temperature. In order to identify significant ($p < 0.01$) changes in virgin olive oil, multivariate statistical methods were applied. The global indicators of changes in virgin olive oil quality (FFA, PV, K_{232}, K_{270} and ΔK); olive oil phenolic compounds; volatile compounds; and oil yield (**Table 5.1**) with processing have been identified and the changes for selected individual phenolic and volatile compounds with malaxation time and temperature have also been explored.

5.3.1 Malaxation time and temperature discrimination

Discrimination of malaxation time and temperature with SLDA was undertaken to recognize patterns and identify discriminating variables. The highest cumulative % variance explained (99.7%) was observed for malaxation time discrimination (**Table 5.2**), indicating an overall success in the discrimination. However, the strong influence of discriminant Function 1, explaining 98.5% of the variance (**Table 5.2)** limited malaxation time discrimination along the y-axis (**Figure 5.1**). The Wilks' Lambda statistic for discriminant Function 2 was close to one and the means of the scores were not significantly different ($p > 0.05$), consistent with earlier reports (Ranalli et al., 2003b) on the limited influence of malaxation time during virgin olive oil production. Malaxing for 30 min produced oil that was separated from the other malaxation times (60, 90 and 120 min) whereas malaxing for 90 and 120 min formed a cluster that was not mutually exclusive (**Figure 5.1**). The formation of a cluster indicates that there were no significant ($p < 0.01$) differences in the oils produced at 90 and 120 min, while malaxing for 30 min produced a significantly ($p < 0.01$) different virgin olive oil.

Discrimination based on malaxation temperature separated the group centroids, apart from 30 and 45°C, which formed a cluster (**Figure 5.2**). Malaxing at 15°C and 60°C displayed a distinct difference on the x-axis (**Figure 5.2**) indicating differences in quality and composition of minor components with processing at the respective malaxation temperatures. The extreme temperatures (15 and 60°C) were also separated from the intermediate malaxation temperatures (30 and 45°C) on the y-axis (**Figure 5.2**).

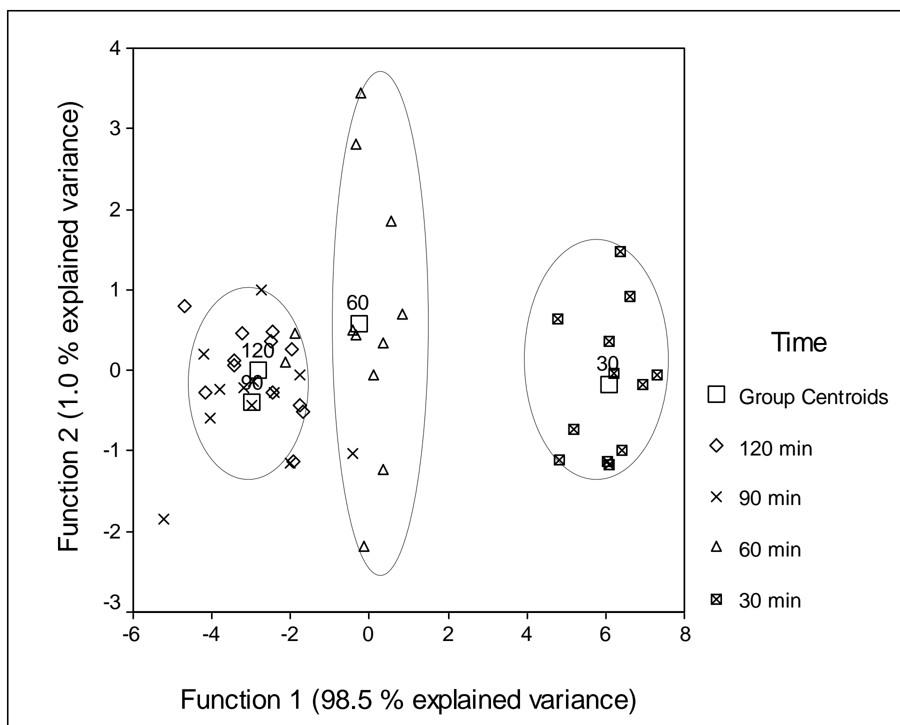

Figure 5.1. Scatter plot for the first two canonical discriminant function separating malaxation time.

Malaxation temperature explained a lower cumulative variance (90.8%) than malaxation time (99.5%) but had a higher % variance explained for Function 2 (**Table 5.2**). Malaxation temperature separated the group centroids better than malaxation time (**Figures 5.1** and **5.2**). Other reports (Angerosa et al., 2001, Ranalli et al, 2001, Servili et al., 2003, Salas & Sanchez, 1999) have shown that malaxation temperature is important in the production of premium quality virgin olive oil.

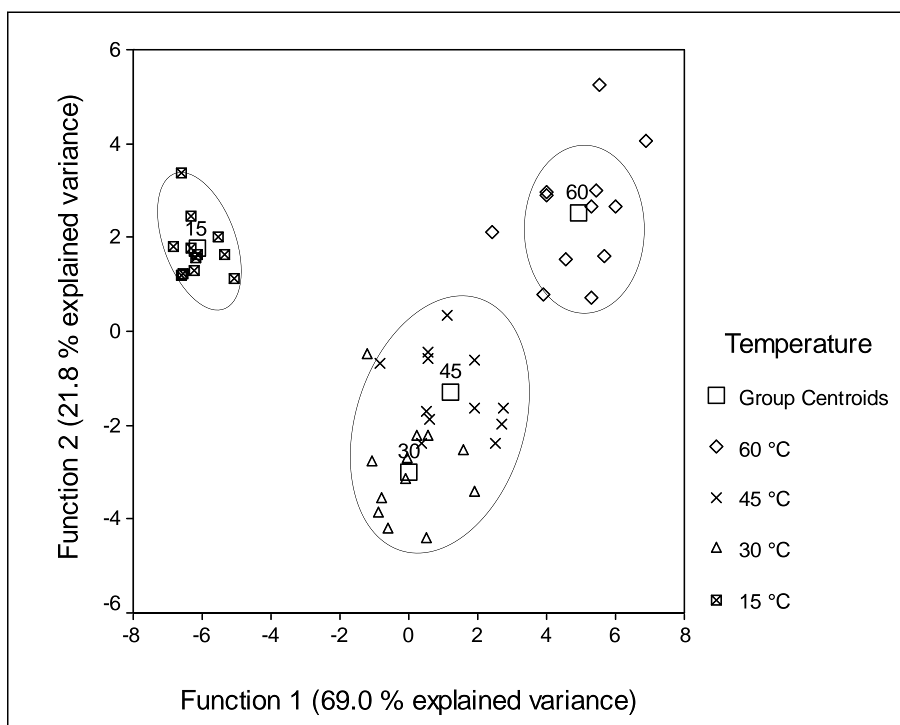

Figure 5.2. Scatter plot for the first two canonical discriminant function separating malaxation temperature.

Table 5.2. Discriminating variables for malaxation times and temperatures during virgin olive oil production.

Discriminated Groups	% Variance explained (Function 1, V_1)	% Variance explained (Function 2, V_2)	% Variance explained (Cumulative)	Discriminating Variables
Time	**98.5***	**1.0**	**99.5**	*Z*-2-penten-1-ol
				Hexanal
				3,4-DHPEA-DEDA[A]
				Acetoxypinoresinol
				FFA[B]
				Yield
Temperature	**69.0***	**21.8***	**90.8**	1-penten-3-ol
				Hexanal
				E-2-hexenal
				Octane
				Tyrosol
				Vanillic acid
				3,4-DHPEA-DEDA[A]
				FFA[B]

* Wilks' Lambda statistic significantly ($p < 0.05$) different.

[A] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde.

[B] Free Fatty Acid as % oleic acid

5.3.2 Parameters that discriminate malaxation times

Discrimination of malaxation times along the x-axis of **Figure 5.1** is given in the linear discriminant equation (V_1, **Equation i**) below.

$V_1 =$	1.29[hexanal] + 1.44[DHPEA-DEDA] + 0.83 × FFA	**(i)**
	– 1.12[*Z*-2-penten-1-ol] – 0.73[acetoxypinoresinol] – 1.65 × yield	

A malaxation time of 30 min, which is on the positive side of the scatter plot (**Figure 5.1**), was discriminated by hexanal, 3,4-DHPEA-DEDA and FFA. Long malaxation times (90 and 120 min), which lie on the negative side of the scatter plot, were discriminated by *Z*-2-penten-1-ol, acetoxypinoresinol and oil yield. Discrimination on the y-axis was not distinct as almost all the malaxation times lie along the axis at Function 2 equal to zero (**Figure 5.1**). Nevertheless, there was a slight separation between the group centroids for malaxing at 30 and 60 min, which lie on the negative and positive side respectively of Function 2. Discrimination of malaxation times along Function 2 of **Figure 5.1** is given in the linear discriminant equation (V_2, **Equation ii**) below.

$V_2 =$	0.58[hexanal] + 0.52[*Z*-2-penten-1-ol] + 0.10 × FFA	**(ii)**
	+ 0.16[acetoxypinoresinol] – 0.34 × yield – 0.44[DHPEA-DEDA]	

With the separation of the group centroids for malaxing at 30 and 60 min alluded above, oil yield and 3,4-DHPEA-DEDA, variables with negative coefficients in **Equation ii**, discriminated virgin olive oil with 30 min malaxation time whereas hexanal, *Z*-2-penten-1-ol, FFA and acetoxypinoresinol discriminated virgin olive oil with 60 min malaxation time, which lies on the positive side of Function 2 (**Figure 5.1**).

The validity of discrimination was checked by examining the variance explained and the significance of separating the malaxation time group centroids. The discrimination by Function 1, V_1 explained more variance (98.5%) than Function 2, V_2 (1.0%) with a non-significant ($p > 0.05$) Wilks' Lambda statistic (**Table 5.2**). The non-significant Wilks' Lambda statistic confirmed the poor separation of malaxation times on the y-axis (**Figure 5.1**), which is explained by discriminant Function 2, V_2 (**Table 5.2**). Compilation of discriminating variables that separated individual malaxation times (**Table 5.3**) was accomplished by considering the significance of the discriminant Function and the percentage variance explained by the Function in the scatter plot (**Figure 5.1**).

Table 5.3. Variables separating individual malaxation times and temperatures.

Time (min)	Discriminating variables
30	Hexanal, 3,4-DHPEA-DEDA[A] and FFA[B]
60	*Z*-2-penten-1-ol, hexanal, acetoxypinoresinol and FFA[B]
90	*Z*-2-penten-1-ol, acetoxypinoresinol and yield
120	*Z*-2-penten-1-ol, acetoxypinoresinol and yield
Temperature (°C)	
15	1-penten-3-ol, E-2-hexenal and vanillic acid
30	E-2-hexenal
45	Tyrosol and FFA[B]
60	Hexanal, octane and 3,4-DHPEA-DEDA[A]

[A] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde.

[B] Free Fatty Acid as % oleic acid

A malaxation time of 30 min produced significantly different virgin olive oils to those malaxed for 60, 90 and 120 min (**Figure 5.1**). Quantitative data in **Table 5.4** is consistent with this observation for 3,4-DHPEA-DEDA, which discriminated 30 min malaxation (**Table 5.3**) where the concentrations at 30 min were significantly ($p < 0.01$) higher than the other malaxation times. Apart from FFA, malaxation time discriminating variables (**Table 5.2**) are not directly associated with virgin olive oil quality. Results of this study are consistent with earlier reports (Ranalli et al., 2003, Servili et al. 2003) that observed minimal influence of malaxation time on the quality of virgin olive oil.

It can be observed in **Table 5.2** that concentrations of hexanal, 3,4-DHPEA-DEDA and FFA, changed with both malaxation time and temperature, whereas concentrations of *Z*-2-penten-1-ol, (+)-acetoxypinoresinol and oil yield significantly ($p < 0.01$) changed with time only; and 1-penten-3-ol, *E*-2-hexenal, octane, tyrosol and vanillic acid concentration changed with temperature only. The different discriminating variables of processing conditions illustrate the dependence of virgin olive oil quality on malaxation time and temperature.

Table 5.4. Quantitative data for different malaxation time-temperature combinations in the production of virgin olive oil.

Processing conditions	Hexanal (μg/g)	Octane (μg/g)	3,4-DHPEA-DEDA [A]	Tyrosol (μg/g)	FFA [B]	PV [C]	Oil Yield (% m/m)
30 min – 15°C	$27 \pm 3^{c, d}$	$0.30 \pm 0.01^{a, b}$	< 0.1	2.6 ± 0.6^{a}	$0.35 \pm 0.02^{a, b}$	$15.3 \pm 0.8^{a, b}$	34.6 ± 0.6^{a}
60 min – 15°C	29 ± 8^{d}	$0.37 \pm 0.04^{a, b}$	< 0.1	3.4 ± 0.4^{a}	$0.37 \pm 0.01^{a, b, c}$	$14 \pm 2^{a, b}$	$40.0 \pm 0.1^{e, f}$
90 min – 15°C	$25 \pm 2^{a, b, c, d}$	$0.31 \pm 0.02^{a, b}$	< 0.1	3.2 ± 0.4^{a}	$0.37 \pm 0.01^{a,b,c}$	$14 \pm 2^{a, b}$	$42.0 \pm 0.1^{g, h}$
120 min – 15°C	$26 \pm 4^{b, c, d}$	$0.36 \pm 0.04^{a, b}$	< 0.1	3.5 ± 0.2^{a}	$0.37 \pm 0.02^{a,b,c}$	$14 \pm 1^{a, b}$	$43.0 \pm 0.1^{h, I}$
30 min – 30°C	$21.8 \pm 0.3^{a,b,c,d}$	0.16 ± 0.01^{a}	2.3 ± 0.6^{b}	5.5 ± 0.5^{b}	$0.38 \pm 0.01^{b, c}$	$13.7 \pm 0.4^{a, b}$	$38.0 \pm 0.1^{c, d}$
60 min – 30°C	$19 \pm 2^{a, b, c}$	0.16 ± 0.01^{a}	0.5 ± 0.9^{a}	$6.1 \pm 1.2^{b, c}$	$0.37 \pm 0.02^{b, c}$	16 ± 2^{b}	$40.3 \pm 0.6^{e, f}$
90 min – 30°C	$20 \pm 3^{a, b, c}$	0.25 ± 0.04^{a}	0.6 ± 0.9^{a}	$6.7 \pm 0.6^{b, c}$	$0.42 \pm 0.02^{d, e}$	$16 \pm 2^{a, b}$	44.3 ± 0.6^{j}
120 min – 30°C	$24 \pm 1^{a, b, c, d}$	$0.30 \pm 0.03^{a, b}$	< 0.1	$5.8 \pm 1.0^{b, c}$	0.43 ± 0.01^{e}	$15 \pm 1^{a, b}$	$44.0 \pm 0.1^{I, j}$
30 min – 45°C	$18 \pm 2^{a, b, c}$	$0.26 \pm 0.03^{a, b}$	2.8 ± 0.6^{b}	5.2 ± 0.4^{b}	0.34 ± 0.01^{a}	12.4 ± 0.9^{a}	33.8 ± 0.2^{a}
60 min – 45°C	$21 \pm 4^{a, b, c, d}$	$0.51 \pm 0.08^{a, c}$	0.5 ± 0.9^{a}	$6.1 \pm 0.4^{b, c}$	$0.38 \pm 0.01^{b, c}$	$14.5 \pm 0.9^{a, b}$	$37.0 \pm 1.0^{b, c}$
90 min – 45°C	16 ± 2^{a}	$0.52 \pm 0.07^{a, b, c}$	0.5 ± 0.9^{a}	5.5 ± 0.8^{b}	$0.37 \pm 0.01^{a,b,c}$	$13 \pm 1^{a, b}$	38.4 ± 0.5^{d}
120 min – 45°C	$18 \pm 3^{a, b}$	$0.8 \pm 0.2^{c, d}$	< 0.1	5.7 ± 0.4^{b}	$0.39 \pm 0.01^{c, d}$	$13.5 \pm 0.4^{a, b}$	39.6 ± 0.6^{e}
30 min – 60°C	$23.6 \pm 1.0^{a,b,c,d}$	$0.6 \pm 0.1^{b, c}$	2.8 ± 0.3^{b}	$6.0 \pm 1.6^{b, c}$	$0.39 \pm 0.01^{b, c}$	$13.0 \pm 0.8^{a, b}$	36.3 ± 0.6^{b}
60 min – 60°C	$25 \pm 5^{b, c, d}$	$1.0 \pm 0.2^{c, d}$	0.6 ± 1.1^{a}	7.7 ± 0.6^{c}	0.43 ± 0.02^{e}	$12.7 \pm 0.7^{a, b}$	$40.3 \pm 0.6^{e, f}$
90 min – 60°C	$18 \pm 2^{a, b}$	1.4 ± 0.2^{e}	< 0.1	$6.7 \pm 0.4^{b, c}$	0.43 ± 0.01^{e}	$13 \pm 2^{a, b}$	$42.0 \pm 0.1^{g, h}$
120 min – 60°C	$18 \pm 2^{a, b}$	1.7 ± 0.4^{e}	< 0.1	$6.9 \pm 0.6^{b, c}$	0.45 ± 0.01^{e}	$14 \pm 1^{a, b}$	$41.0 \pm 0.1^{f, g}$

Different superscripts in a column indicate significantly ($p < 0.01$) different mean ± standard deviation of triplicate determinations.

[A] concentration of 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde in μg/g

[B] Free Fatty Acid as % oleic acid

[C] Peroxide Value expressed as milli-equivalents oxygen per kg oil

5.3.3 Parameters that discriminate malaxation temperatures

Virgin olive oil produced at different malaxation temperatures was separated by selected variables (**Table 5.2**) and not all parameters measured in olive oil from *Frantoio* fruit (**Table 5.1**). Discrimination of malaxation temperatures along the x-axis of **Figure 5.2** is given in the linear discriminant equation (V_1, **Equation iii**) below.

$V_1 =$	0.32[octane] + 0.14[hexanal] + 0.49 × FFA + 0.68[tyrosol] + 1.26[DHPEA-DEDA]	**(iii)**
	– 0.37[1-penten-3-ol] – 0.13[*E*-2-hexenal] – 0.96[vanillic acid]	

Discriminating variables with positive coefficients (octane, hexanal, FFA, tyrosol and 3,4-DHPEA-DEDA) discriminated high malaxation temperatures (45 and 60°C), which are on the positive side of **Figure 5.2**. Low temperature (15°C) malaxation, which lies on the negative side of **Figure 5.2**, is discriminated by compounds with negative coefficients (1-penten-3-ol, *E*-2-hexenal and vanillic acid). Discrimination by V_1 explained more variance (69.0%) than V_2 (21.8%). Discriminant analysis on the y-axis (V_2, **Equation iv**) separates the extreme of malaxation temperatures (15 and 60°C), which lie on the positive side of the y-axis (**Figure 5.2**), from the intermediate temperatures (30 and 45°C).

$V_2 =$	1.08[octane] + 0.19[1-penten-3-ol] + 1.91[hexanal] + 0.09[vanillic acid]	(**iv**)
	+ 0.50[DHPEA-DEDA] – 1.87[*E*-2-hexenal] – 0.44 × FFA – 0.38[tyrosol]	

Extreme malaxation temperatures (15 and 60°C) on the positive side of the y-axis (**Figure 5.2**), are discriminated by (octane, 1-penten-3-ol, hexanal, vanillic acid and 3,4-DHPEA-DEDA). *E*-2-hexenal, FFA and tyrosol discriminate intermediate malaxation temperatures (30 and 45°C) on the negative side of the y-axis (**Figure 5.2**). Discriminating variables that significantly ($p < 0.01$) discriminated individual malaxation temperatures were deduced (**Table 5.3**) from discriminant functions V_1 and V_2, above.

As shown in **Table 5.3,** virgin olive oils produced by malaxing at lower temperatures (15 and 30°C) were discriminated by compounds (1-penten-3-ol, *E*-2-hexenal and vanillic acid) associated with freshness of olive oil (Angerosa et al., 2001, Morales &; Aparicio, 1999, Ranalli et al., 2001, Cavalli et al., 2004). Similarly, high malaxation temperatures (45 and 60°C) produced oils that were discriminated by variables (hexanal, octane, tyrosol and FFA), which are often associated with low quality olive oil (Kiritsakis, 1998, Angerosa et al., 2000z). Interestingly, 3,4-DHPEA-DEDA is also among the high temperature discriminating variables. Increasing levels of this phenolic compound may protect the oil from oxidation, consistent with the non-significant changes in PV and K-values at higher temperatures. The identification of high malaxation temperature discriminating variables is consistent with quantitative data (**Table 5.4**) where some of these variables (octane, 3,4-DHPEA-DEDA, tyrosol and FFA) have significantly ($p < 0.01$) higher values at high temperatures (45 and 60°C) than at low temperatures (15 and 30°C).

5.3.4 Effect of malaxation time-temperature combination on virgin olive oil quality and yield

Malaxation time and temperature significantly changed the volatile and phenolic profile of virgin olive oil, which are important in the sensory quality of virgin olive oil (Olias et al., 1993, Angerosa et al., 2000z, Psomiadou et al., 2003) in addition to changing FFA content, which is used to grade olive oil into different commercial classes (IOOC, 2003). Hence, it is not surprising that FFA, volatile and phenolic compounds appear as discriminating variables in **Table 5.2**. Common oxidation indicators of olive oil, PV, K_{232} and K_{270}, were not among the variables that significantly ($p < 0.01$) discriminated malaxation times and temperatures. This observation is supported by quantitative data (**Table 5.4**)

where PV shows minimum significant ($p < 0.01$) changes with both malaxation time and temperature. Previous studies (Ranalli et al., 2001) have shown an acceleration of lipolysis and oxidation processes with an increase in malaxation temperatures. The non-significant ($p > 0.01$) influence of oxidation indicators compared to the significant ($p < 0.01$) influence of FFA indicates that lipolysis is more important than oxidation during virgin olive oil production.

The processing parameters also affected oil yield. Malaxation time showed a highly significant effect on oil yield (**Table 5.2**) with a 30 min malaxation time producing significantly ($p < 0.01$) less oil (**Table 5.4**) than the other malaxation times (60, 90 and 120 min) at all malaxation temperatures (15, 30, 45 and 60°C). Malaxing at 45°C had significantly ($p < 0.01$) lower yields compared to other temperatures (15, 30 and 60°C), which might be due to change in the rheology of the paste and increased interactions between lipids, proteins and carbohydrates culminating in the entrapment of oil in the olive paste. Amirante et al. (2002) observed that raising the temperature of the olive paste reduces the viscosity leading to better separation and higher oil yields; this was not the case in our study as malaxation time had a more significant ($p < 0.01$) effect on oil yield. Earlier studies (Ranalli et al., 2003b) reported a small decrease in oil yields with time from 60 – 75 min, which was attributed to a re-formation of oil-water or oil-solid emulsions. In our study, decrease in yield with time was not observed (**Table 5.4**). These variations may possibly be attributed to the different paste rheologies probably arising from different cultivars and maturity stages.

5.3.5 Changes in phenolic compounds with processing

In addition to affecting the volatile and phenolic compounds of virgin olive oil in relation to other virgin olive oil components, malaxation time and temperature affected concentrations of individual compounds in different ways. In the case of tyrosol, it is observed from the response surface (**Figure 5.3a**) that concentration predominantly increases with malaxation temperature.

A closer look at the contour plot reveals that the concentration predominantly increases along the temperature axis (**Figure 5.3b**). The low density of contour lines at temperatures above 30°C (**Figure 5.3b**), indicates less sensitivity of tyrosol formation towards high malaxation temperatures. The formation of tyrosol is sensitive at low temperatures and quantitative data (**Table 5.4**) shows a significant ($p < 0.01$) difference in tyrosol concentration at 15°C with minimal significant differences at higher malaxation temperatures. No significant ($p > 0.01$) differences of tyrosol concentration with malaxation time (**Table 5.4**) were found and consistent with an earlier observation that tyrosol is significantly affected by temperature only (**Table 5.2**).

Looking at an example of a discriminating variable that predominantly changed with malaxation time, 3,4-DHPEA-DEDA, the response surface (**Figure 5.4a)** clearly shows high concentrations at short malaxation times in agreement with earlier observations in this study (**Table 5.3**). Complimentary observations are made on the contour plot (**Figure 5.4b**) where contour lines span along the time axis and concentration increases towards shorter times with a high density below 60 min indicating a high sensitivity in 3,4-DHPEA-DEDA formation at short malaxation times. Quantitative data (**Table 5.4**) show low concentrations of 3,4-DHPEA-DEDA at 15°C and a significant ($p < 0.01$) difference was found at 30 min malaxation time for higher temperatures (30, 45 and 60°C).

The observed low concentrations of the phenolic compounds (tyrosol and 3,4-DHPEA-DEDA) at low temperatures (15°C) suggest their levels in virgin olive oil are strongly influenced by processing temperature. Malaxation temperature plays a crucial role in the formation and degradation of such phenolic compounds. Degradation may be accelerated at elevated temperatures, while formation may involve bond cleavages to release phenolic compounds that are bound to other molecules in the olive fruit. This is in line with earlier observations (Montedoro et al., 1992b, Ryan et al., 1999, Amiot et al., 1989) on the formation of phenolic compounds, such as 3,4-DHPEA-DEDA and tyrosol from high molecular weight phenolic compounds. Reaction kinetics will determine the concentration of all compounds in the oil. In the cases where malaxation time emerges as a critical variable, the

importance of reaction kinetics also emerges. For instance, in the case of 3,4-DHPEA-DEDA, it is apparent from the density of contour lines (**Figure 5.4b**) that short processing times (< 60 min) favor higher rates of 3,4-DHPEA-DEDA formation relative to degradation whereas at higher processing temperatures/longer times the degradation rate of 3,4-DHPEA-DEDA increases.

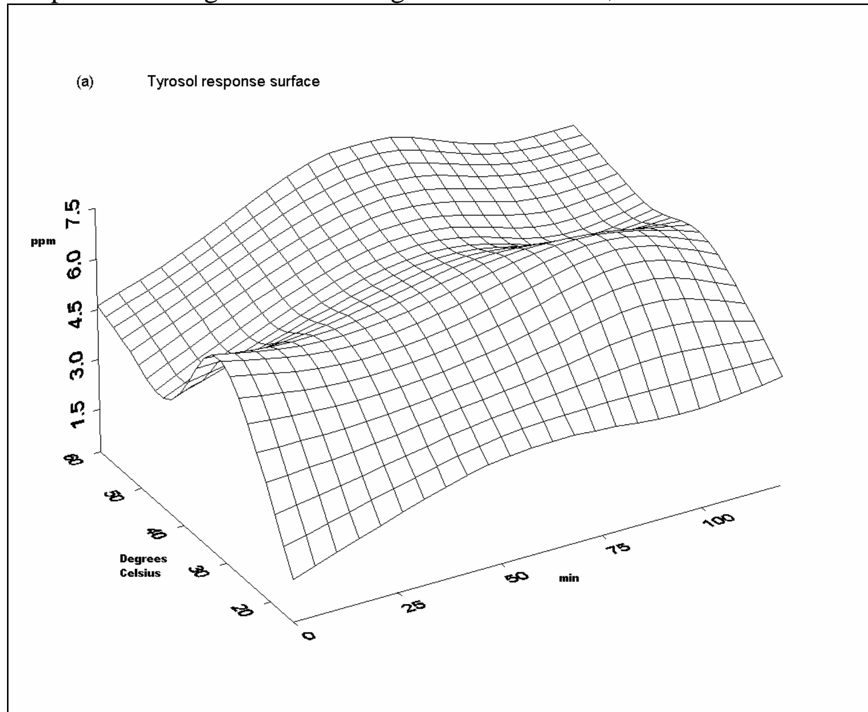

Figure 5.3a. Malaxation time – Temperature response surface for tyrosol.

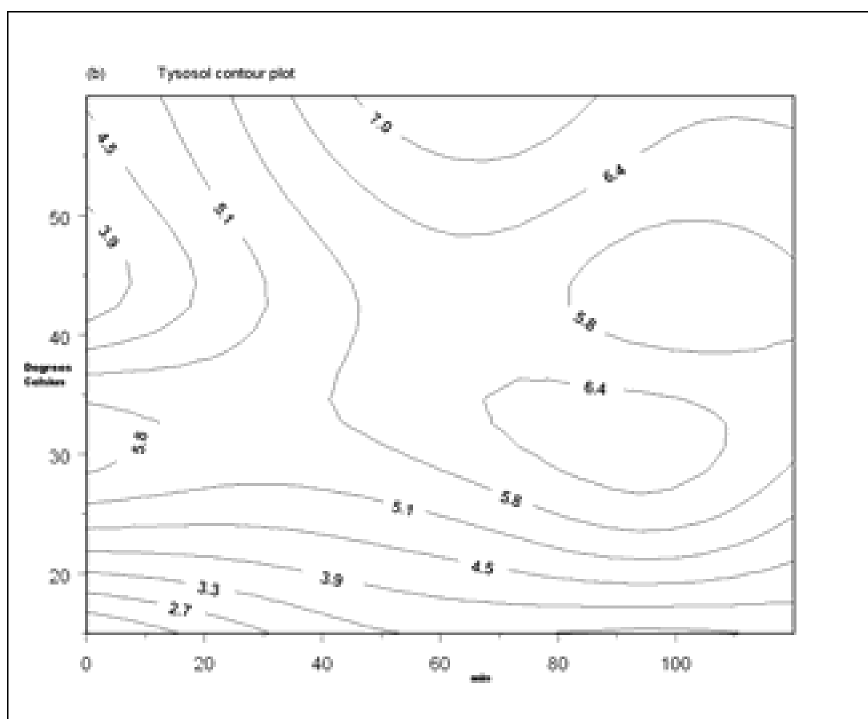

Figure 5.3b. Malaxation time – Temperature contour plot for tyrosol with numbers in the plot representing concentration in µg/g oil.

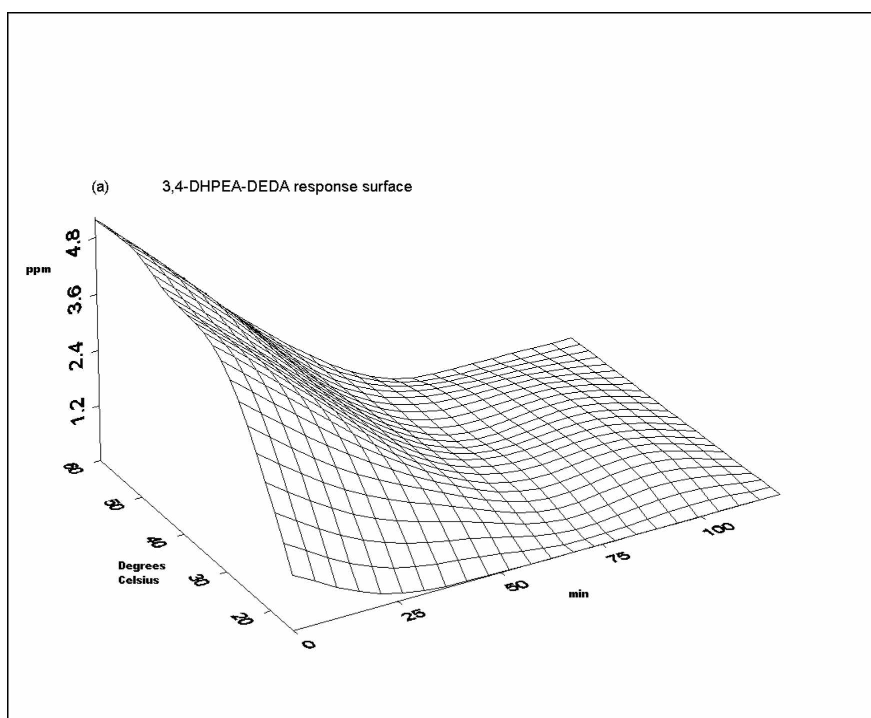

Figure 5.4a. Malaxation time – Temperature response surface for 3,4-DHPEA-DEDA formation.

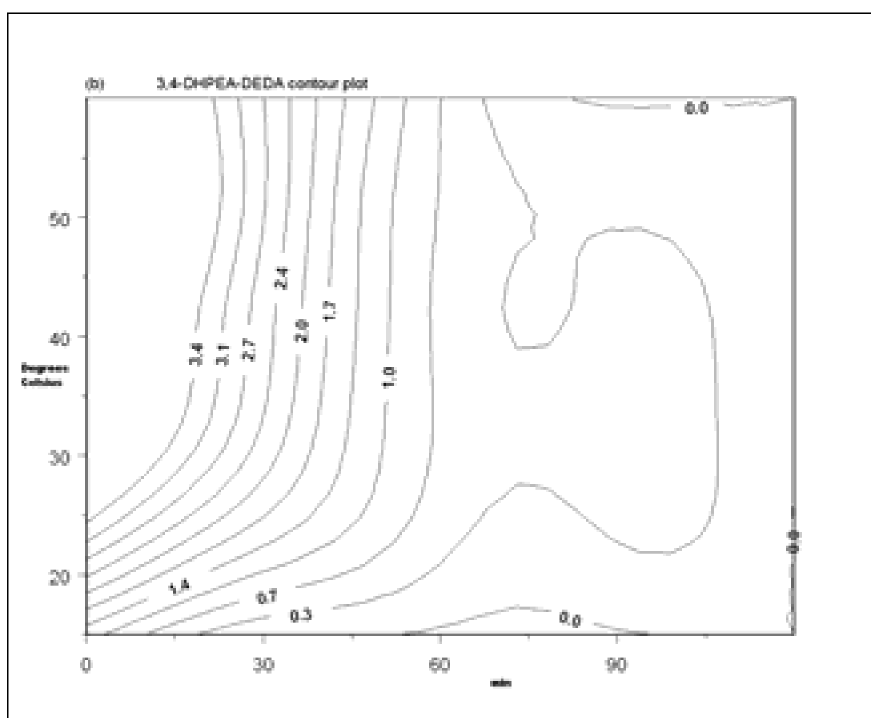

Figure 5.4b. Malaxation time – Temperature contour plot for 3,4-DHPEA-DEDA with numbers in the plot representing concentration in μg/g oil.

5.3.6 Changes in volatile compounds with processing

Volatile compounds were the most common discriminating variables (**Table 5.2**) of malaxation times and temperatures. The discriminating volatile compounds were found to increase in chain length with malaxation temperature where 1-penten-3-ol, *E*-2-hexenal and octane were discriminating variables at 15, 30 and 60°C respectively (**Table 5.3**). The increase towards long chain volatile compounds with processing temperature is consistent with earlier observations (Khfif et al., 2003) where virgin olive oil C6 volatile compounds decreased with high malaxation temperature. Unlike malaxation temperature, malaxation time showed a decrease with chain length where the C6 volatile compound (hexanal) significantly ($p < 0.01$) discriminated shorter times (30 and 60 min) whereas longer times (90 and 120 min) were discriminated by the C5 volatile compound, *Z*-2-penten-1-ol (**Table 5.3**).

It should be recognized that volatile compounds in virgin olive oil do not originate from the fruit, per se, they are formed during processing (Olias et al., 2003, Khfif et al., 2003) which points us towards the importance of thermodynamic conditions. This idea is supported by data in **Table 5.2**, where four volatile compounds (1-penten-3-ol, octane, hexanal, *E*-2-hexenal) significantly ($p < 0.01$) discriminated malaxation temperatures compared to only *Z*-2-penten-1-ol that discriminated malaxation time. Response surfaces and contour plots for octane and hexanal (**Figures 5** and **6**) illustrate the dominant influence of temperature in volatile formation.

Octane is produced from decomposition of 10-hydroxyperoxide of oleic acid and correlated with “fusty” defect in olive oil (Di Giovacchino, 2000). High malaxation temperatures favored the formation of octane as illustrated by the response surface (**Figure 5.5a**). The increase of octane concentration with malaxation temperature is also apparent on the contour plots (**Figure 5.5b**). The formation of octane becomes more sensitive at elevated malaxation temperatures (> 40°C) indicated by a high density of contour lines (**Figure 5.5b**). Octane concentrations (**Table 5.4**) were significantly ($p < 0.01$) different at elevated malaxation temperatures (45 and 60°C) but were not significantly ($p > 0.01$) different at low temperatures (15 and 30°C).

The hexanal time-temperature response surface (**Figure 5.6a**) shows that as processing temperature is increased, the concentration of hexanal initially decreases, reaches a minimum at about 50°C, then increases as temperature is further increased. The 50°C minimum forms a valley that runs virtually parallel with the time axis. An earlier study (Morales & Aparicio, 1999) reported that hexanal levels were more influenced by malaxation time than temperature. This is not consistent with our observations probably due to the inclusion of higher malaxation temperatures (45°C and 60°C) in our study compared to the malaxation temperature range in the work by Morales & Aparicio (1999). The contour lines in **Figure 5.6b** span diagonally along the time and temperature axis showing that neither malaxation time nor temperature exerts a dominant influence on the formation of hexanal.

It is intriguing that hexanal concentration increases along opposite directions of the temperature axis (**Figure 5.6b**). This behavior suggests that there may be two different modes of hexanal formation during virgin olive oil extraction. Previous studies have reported that hexanal may be formed through both enzymatic and non-enzymatic pathways (Vichi et al., 2003b, Morales et al., 1997). The contour plot (**Figure 5.6b**) can be interpreted as displaying the two modes of hexanal formation; non-enzymatic mode (hexanal concentration increasing towards high temperature/long time) and enzymatic mode (low temperature/short time) illustrated by the direction of the arrows pointing towards increasing hexanal concentration (**Figure 5.6b**). The enzymes responsible for hexanal formation have a high activity at low temperatures (Salas & Sanchez, 1999) and lose their activity at high temperatures, consistent with the increase in the enzymatic hexanal formation towards low temperatures in this work. At high temperatures (> 50°C) and shorter malaxation times (< 75 min), an increase in hexanal concentration is observed (**Figure 5.6b**). Quantitative data (**Table 5.4**) show the lowest concentration at 90 min – 45°C, which is along the 50°C valley of the response surface (**Figure 5.6a**). Significantly ($p < 0.01$) higher hexanal concentrations (**Table 5.4**) were observed at short

malaxation times (30 and 60 min) and low temperature (15°C) consistent with an earlier report (Salas & Sanchez, 1999) where maximum hexanal production was observed at 15°C.

It can be hypothesized that the generation of hexanal at high temperatures is non-enzymatic since at such high temperatures enzyme activity is lost. The non-enzymatic nature of hexanal formation during oil extraction has been rarely reported probably because most of the studies (Angerosa et al., 2001, Morales & Aparicio, 1999, Ranalli et al., 2001, Servili et al., 2003) on effects of malaxation temperature were conducted at low temperatures (below 40°C). The non-enzymatic formation of hexanal at elevated temperatures is in agreement with the formation of other volatile compounds, such as octane shown above and other C8 and C9 volatile compounds known to be formed through non-enzymatic oxidation during olive oil storage (Kiritsakis, 1998, Reiners & Grosch, 1998).

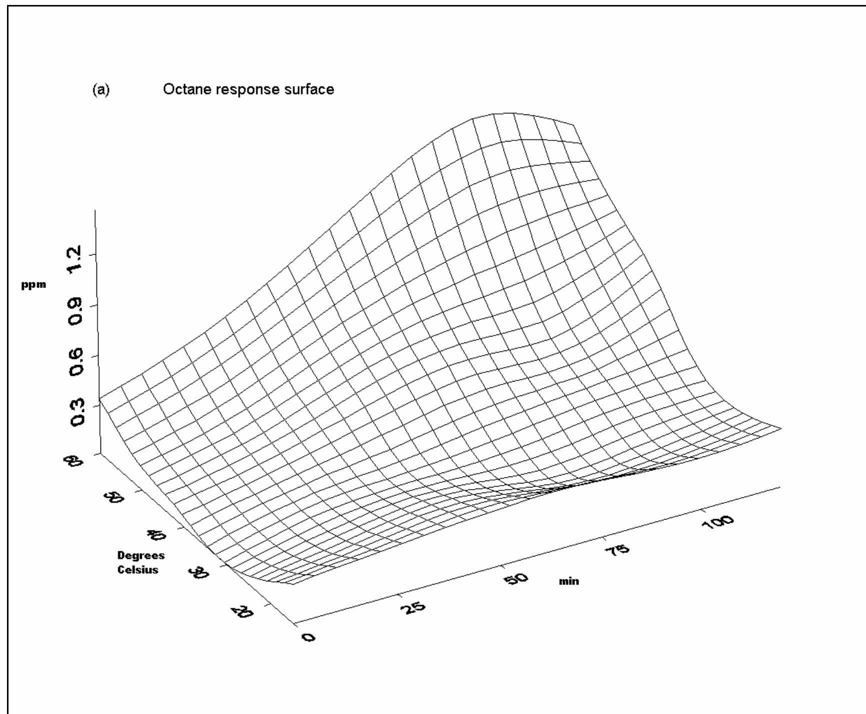

Figure 5.5a. Malaxation time – Temperature response surface for octane formation.

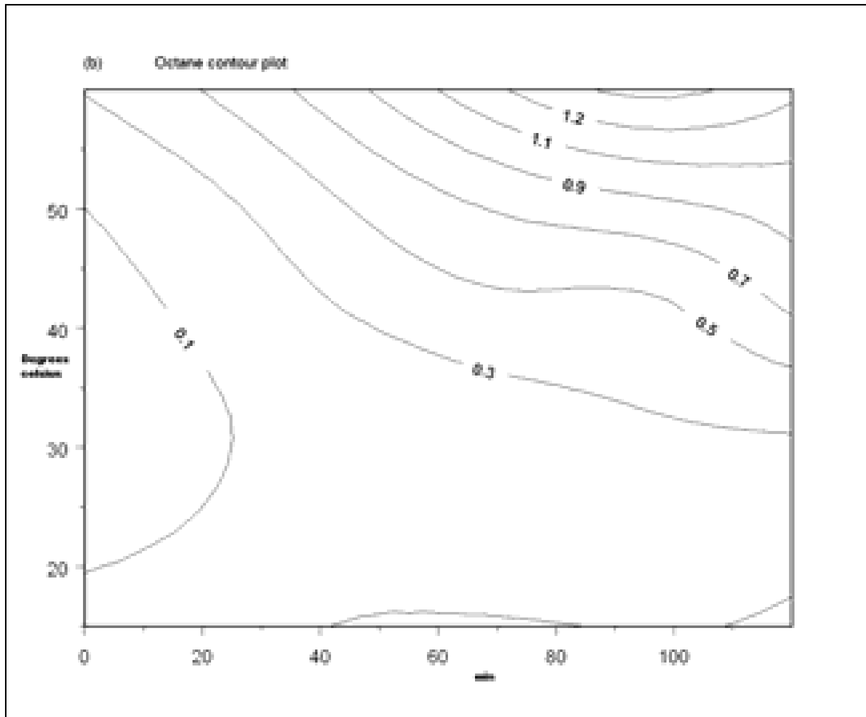

Figure 5.5b. Malaxation time – Temperature contour plot for octane with numbers in the plot representing concentration in µg/g oil.

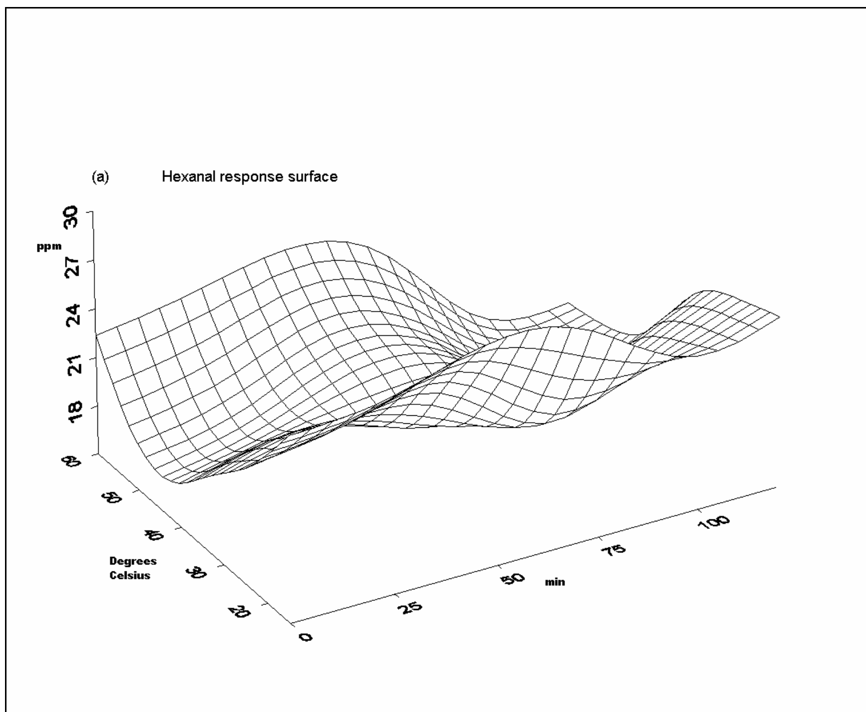

Figure 5.6a. Malaxation time – Temperature response surface for hexanal formation.

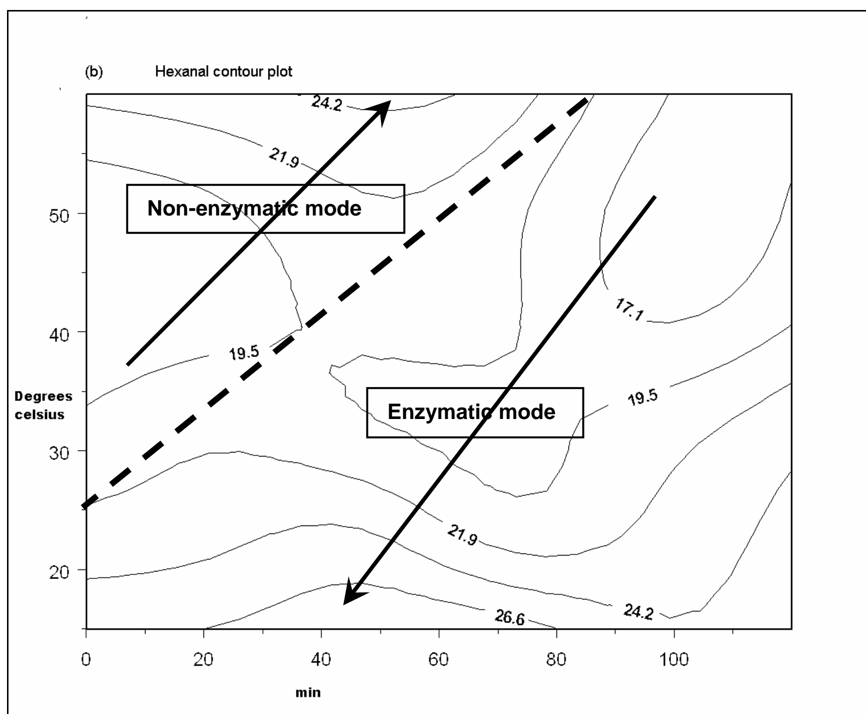

Figure 5.6b. Malaxation time – Temperature contour plot for hexanal with numbers in the plot representing concentration in μg/g oil.

5.3.7 Industrial and Bench Scale Oil Extraction Comparison

Clear differences in characteristics between virgin olive oils processed at industrial and bench scale have been reported (Angerosa, Mostallino, et al. 2000) at the same malaxation temperature regardless of the different malaxation times. What is still unclear is whether industrial or bench scale oil extraction show similar trends and optimal extraction conditions. Investigations on virgin olive oil quality; composition trends; and optimal extraction conditions cannot be fully performed at industrial scale since large amounts of olive fruit are required (Angerosa, Mostallino, et al. 2000). These large olive fruit quantities are not always available and investigations on optimal oil extraction conditions are more feasible with bench scale studies based on small homogeneous quantities of olive fruit.

Not surprising, most of the studies on factors affecting virgin olive oil quality and composition have been carried on bench scale. A study (Cerretani, Bendini, et al. 2005) that was carried out to compare low scale and industrial scale virgin olive oil extraction used different malaxation temperatures, 18 °C for low scale and 27 °C for industrial scale extraction, which makes it difficult to compare the extracted virgin olive oils. As results from this study show (**Table 5.4**), malaxation temperature has a significant influence on the quality and composition of virgin olive oil that confounds comparisons at different malaxation temperatures. In general, most of the studies that compare bench and industrial scale oil extraction were carried out on different non-comparable conditions (Garcia, Brenes, et al. 2001) (Cerretani, Bendini, et al. 2005) with the exception of one study (Angerosa, Mostallino, et al. 2000) where virgin olive oils were discriminated based on both bench and industrial scale oil extraction with minimal confounding factors. The study in this section compliments an earlier study (Angerosa, Mostallino, et al. 2000) carried out at a single malaxation temperature and compares trends in oil characteristics and extraction conditions at several malaxation temperatures between industrial and bench scale oil extractions.

Comparisons of overall optimal conditions obtained for bench scale oil extraction (**Table 5.5**) were similar to industrial scale conditions (**Table 5.6**). In terms of volatile compounds, *trans*-2-hexenal and hexanal showed highest levels with malaxing at 15 °C for 30 min (**Table 5.5**), close to their optimum malaxation temperatures (**Table 5.6**) and 1-penten-3-ol showed highest levels with malaxing at 30 °C for 60 min (**Table 5.5**) also close to its optimum malaxation temperature (**Table 5.5**). Similarly, octane that had optimum malaxation temperature at high temperature (**Table 5.5**) showed the highest and significantly different concentration with malaxing at 45 °C for 120 min and not detected at all with malaxing at 15 °C for 30 min with industrial scale oil extraction (**Table 5.6**). Similar agreement between bench (**Table 5.5**) and industrial scale oil extractions (**Table 5.6**) were observed for the phenolic compound, oleuropein aglycon and quality indices, PV and FFA.

However, some observations were not immediately transferable from bench to industrially extracted oils. For instance, highest concentrations of acetoxypinoresinol were favoured at low temperatures with industrial scale processing (**Table 5.6**) whereas high optimum malaxation temperatures during bench scale oil extractions were observed for acetoxypinoresinol (**Table 5.6**). Similarly, oil yields significantly and continuously increased with increasing malaxation temperature at industrial scale (**Table 5.6**) whereas bench scale oil extractions showed minimum significant differences with oil yields at 30 °C higher than 45 °C (**Table 5.7**).

Elevation of malaxation temperatures may also affect the chemical characteristics of the extracted oil in addition to the olive paste, which consequently affects oil yield. The oil characteristics differences might be attributed directly to the differences in contact surface area to mass ratio. In bench scale oil extractions, the ratio is large resulting in more exposure of air to the olive paste and a faster heat penetration that might favour oxidative and hydrolytic changes in the olive paste and hence higher volatile and phenolic compounds imparted from the olive fruit to oil. On the other hand, the small contact surface area to mass ratio for industrial scale oil extraction may imply that less oxygen is exposed to the paste and also less heat contact with the olive paste resulting in lower tendencies to oxidative processes during oil extraction (Cerretani, Bendini, et al. 2005). In summary, elevated

malaxation temperatures might favour chemical changes in both bench and industrially extracted virgin olive oils while changes in the olive paste rheology due to elevated temperatures might not favour higher oil yields during bench scale oil extraction.

The contrast in observations between bench and industrial scale oil extraction may be attributed to the differences in contact surface area to mass ratio that influences heat transfer and olive paste air exposure. Unsteady state heat transfer, where olive paste temperature is a function of both location and time, dominate industrial scale oil extraction and may cause differences in oil yield. The unsteady state regime allows oil droplets to slowly form a continuous lipid phase before denaturation of proteins and interaction of olive components, such as proteins and carbohydrates, which might slowly change the paste rheology. The slow heat transfer during industrial scale oil extraction may imply a slow change in paste rheology and a slow coalescence of small oil droplets forming a continuous lipid phase with increasing malaxation temperature giving enough time for the continuous lipid phase to separate from the paste. In contrast during bench scale oil extraction, steady state heat transfer conditions, where olive paste temperature varies with location only, were attained faster than in industrial scale extraction and may have resulted in faster changes in paste rheology and rapid coalescence of small oil droplets forming a continuous lipid phase, which possibly was rapidly and consistently trapped in the changed olive paste matrix. The rapid and consistent retention of the oil in the olive paste with elevation of malaxation temperature during bench scale oil extraction entailed that significant changes in oil yield were minimal as observed above.

Table 5.5 Bench scale Production time and temperature conditions maximising levels of variables generated from Gaussian curve fit.

Low Temperature Variables	Temperature (T_0)/ °C	Time (t_0)/minutes	R^2	MSR
Volatile Compounds (μg/g)				
1-penten-3-ol	22 ± 5 [a, b]	46 ± 9 [a]	0.5 ± 0.2 [a, b, c, d, e, f, g]	0.06 ± 0.04 [a]
Hexanal	19 ± 2 [a, b]	72 ± 25 [a]	0.3 ± 0.3 [a, b, c, d, e]	0.06 ± 0.04 [a]
Hexanal *	15.4 ± 0.9 [a, b]	66 ± 34 [a]	0.83 ± 0.05 [g]	0.03 ± 0.03 [a]
Trans-2-hexenal	20 ± 2 [a, b]	64 ± 20 [a]	0.5 ± 0.2 [a, b, c, d, e, f, g]	0.08 ± 0.06 [a]
Hexanol	26 ± 9 [b]	107 ± 2 [a]	0.07 ± 0.02 [a, b]	0.01 ± 0.01 [a]
Hexanol *	17 ± 2 [a, b]	90 ± 20 [a]	0.3 ± 0.3 [a, b, c, d]	0.12 ± 0.09 [a,, b]
Phenolic Compounds (μg/g)				
Oleuropein aglycon	18 ± 1 [a, b]	63 ± 13 [a]	0.2 ± 0.3 [a, b, c, d]	0.09 ± 0.02 [a]
Oleuropein aglycon *	14 ± 2 [a]	59 ± 21 [a]	0.7944 ± 0.0010 [f, g]	0.03 ± 0.04 [a]
Quality Indices and Oil Yield				
PV [A]	20 ± 2 [a, b]	86 ± 6 [a]	0.5 ± 0.1 [a, b, c, d, e, f, g]	0.05 ± 0.05 [a]
Oil Yield (% m/m)	23 ± 7 [a, b]	107 ± 2 [a]	0.50 ± 0.09 [a, b, c, d, e, f, g]	0.09 ± 0.05 [a]
High Temperature Variables	**Temperature (T_0)/ °C**	**Time (t_0)/minutes**	**R^2**	**MSR**
Volatile Compounds (μg/g)				
Acetic acid	55 ± 2 [c, d]	53 ±10 [a]	0.47 ± 0.08 [a, b, c, d, e, f,, g]	0.035 ± 0.004 [a]
Cis-2-penten-1-ol	57 ± 2 [c, d]	78 ± 48 [a]	0.56 ± 0.07 [b, c, d, e, f, g]	0.03 ± 0.02 [a]
Hexanol **	50 ± 6 [c]	45 ± 12 [a]	0.70 ± 0.02 [d, e, f, g]	0.006 ± 0.002 [a]
Octane	54.9 ± 0.9 [c, d]	101.8 ± 0.6 [a]	0.4 ± 0.2 [a, b, c, d, e, f, g]	0.10 ± 0.04 [a]
Phenolic Compounds (μg/g)				
Hydroxytyrosol	48 ± 10 [c]	47 ± 6 [a]	0.4 ± 0.1 [a, b, c, d, e]	0.01 ± 0.01 [a]
3,4-DHPEA-DEDA [B]	52 ± 2 [c, d]	62 ± 34 [a]	0.48 ± 0.03 [a, b, c, d, e, f, g]	0.11 ± 0.03 [a]
(+)-acetoxypinoresinol	59 ± 5 [c, d]	67 ± 20 [a]	0.48 ± 0.04 [a, b, c, d, e, f, g]	0.03 ± 0.03 [a]
Tyrosol	56 ± 3 [c, d]	90 ± 15 [a]	0.4 ± 0.3 [a, b, c, d, e]	0.046 ± 0.005 [a]
Tyrosol **	61 ± 5 [d]	73 ± 49 [a]	0.83 ± 0.05 [g]	0.009 ± 0.004 [a]
Quality Indices				
K_{232}	53.4 ± 0.5 [c, d]	70 ± 20 [a]	0.611 ± 0.006 [c, d, e, f, g]	0.04 ± 0.03 [a]
K_{270}	55 ± 2 [c, d]	104 ± 3 [a]	0.27 ± 0.01 [a, b, c, d, e]	0.05 ± 0.01 [a]
FFA [C]	54 ± 1 [c, d]	101 ± 13 [a]	0.6 ± 0.1 [b, c, d, e, f, g]	0.06 ± 0.02 [a]

Different superscripts indicate significantly ($p < 0.05$) different mean ± standard deviation of triplicate values.
* and ** Optimum conditions predicted from malaxation temperatures < 45 °C and ≥ 45 °C respectively.
[A] Peroxide Value expressed as milli-equivalents oxygen per kg oil
[B] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde (**Appendix 1**)
[C] Free Fatty Acid as % oleic acid

Table 5.6 Quantitative data from industrial scale production of virgin olive oil at different malaxation time-temperature combinations.

Industrial Scale Conditions	30 min – 15°C	60 min – 30°C	120 min – 45°C
Volatile Compounds (μg/g)			
Acetic acid	< 0.05	0.10 ± 0.01 [a]	0.10 ± 0.01 [a]
1-penten-3-ol	0.20 ± 0.01 [a]	0.30 ± 0.01 [a]	0.20 ± 0.01 [a]
Trans-2-hexenal	6.50 ± 0.01 [a]	5.9 ± 0.3 [a]	1.5 ± 0.3 [b]
Hexanal	1.20 ± 0.01 [a]	0.95 ± 0.07 [a]	0.6 ± 0.1 [a]
Hexanol	0.20 ± 0.01 [a]	0.10 ± 0.01 [a]	0.10 ± 0.01 [a]
Octane	< 0.01	0.10 ± 0.01 [a]	0.35 ± 0.07 [b]
Phenolic Compounds (μg/g)			
Tyrosol	< 3.0	< 3.0	< 3.0
Acetoxypinoresinol	18 ± 2 [a]	17.5 ± 0.7 [a]	16.5 ± 0.1 [a]
Oleuropein aglycon	21.5 ± 0.7 [a]	23 ± 1 [a]	19 ± 1 [a]
Quality Indices and Oil Yield			
FFA [A]	0.22 ± 0.01 [a]	0.22 ± 0.01 [a]	0.23 ± 0.01 [a]
PV [B]	12 ± 2 [a]	10.3 ± 0.9 [a]	10.5 ± 0.9 [a]
K_{232}	1.62 ± 0.01 [a]	1.66 ± 0.01 [a]	1.64 ± 0.01 [a]
K_{270}	0.10 ± 0.01 [a]	0.10 ± 0.01 [a]	0.10 ± 0.01 [a]
Oil Yield (% m/m)	17.78 ± 0.03 [a]	22.22 ± 0.03 [b]	30.12 ± 0.06 [c]

Different superscripts in a row indicate significantly ($p < 0.05$) different mean ± standard deviation of duplicate determinations.
[A] Free Fatty Acid as % oleic acid
[B] Peroxide Value expressed as milli-equivalents oxygen per kg oil

Regardless of the contrasting influences of processing conditions on bench and industrial scale processing, this study does not show clear benefits of processing at elevated temperatures. In both bench and industrial scale oil extraction, volatile and phenolic compounds that are synonymous with premium quality virgin olive oil were promoted at low malaxation temperatures. At such low malaxation temperature, oil yield was not favoured with industrial scale extraction entailing a possible trade off between quality and quantity during virgin olive oil production. Depending on paste rheology, which might change with cultivar and weather conditions during fruit ripening, elevated malaxation temperatures might not always favour enhanced oil yields (IOOC, 1990)(Amirante, Dugo, et al., 2002)

Table 5.7 Quantitative bench scale production data for different malaxation time-temperature combinations in the production of virgin olive oil from *Frantoio* olive fruit.

Processing conditions	Hexanal (μg/g)	Octane (μg/g)	3,4-DHPEA-DEDA [A]	Tyrosol (μg/g)	FFA [B]	PV [C]	Oil Yield (% m/m)
30 minutes – 15°C	$27 \pm 3^{c,d}$	$0.30 \pm 0.01^{a,b}$	< 0.1	2.6 ± 0.6^{a}	$0.35 \pm 0.02^{a,b}$	$15.3 \pm 0.8^{a,b}$	34.6 ± 0.6^{a}
60 minutes – 15°C	29 ± 8^{d}	$0.37 \pm 0.04^{a,b}$	< 0.1	3.4 ± 0.4^{a}	$0.37 \pm 0.01^{a,b,c}$	$14 \pm 2^{a,b}$	$40.0 \pm 0.1^{e,f}$
90 minutes – 15°C	$25 \pm 2^{a,b,c,d}$	$0.31 \pm 0.02^{a,b}$	< 0.1	3.2 ± 0.4^{a}	$0.37 \pm 0.01^{a,b,c}$	$14 \pm 2^{a,b}$	$42.0 \pm 0.1^{g,h}$
120 minutes – 15°C	$26 \pm 4^{b,c,d}$	$0.36 \pm 0.04^{a,b}$	< 0.1	3.5 ± 0.2^{a}	$0.37 \pm 0.02^{a,b,c}$	$14 \pm 1^{a,b}$	$43.0 \pm 0.1^{h,I}$
30 minutes – 30°C	$21.8 \pm 0.3^{a,b,c,d}$	0.16 ± 0.01^{a}	2.3 ± 0.6^{b}	5.5 ± 0.5^{b}	$0.38 \pm 0.01^{b,c}$	$13.7 \pm 0.4^{a,b}$	$38.0 \pm 0.1^{c,d}$
60 minutes – 30°C	$19 \pm 2^{a,b,c}$	0.16 ± 0.01^{a}	0.5 ± 0.9^{a}	$6.1 \pm 1.2^{b,c}$	$0.37 \pm 0.02^{b,c}$	16 ± 2^{b}	$40.3 \pm 0.6^{e,f}$
90 minutes – 30°C	$20 \pm 3^{a,b,c}$	0.25 ± 0.04^{a}	0.6 ± 0.9^{a}	$6.7 \pm 0.6^{b,c}$	$0.42 \pm 0.02^{d,e}$	$16 \pm 2^{a,b}$	44.3 ± 0.6^{j}
120 minutes – 30°C	$24 \pm 1^{a,b,c,d}$	$0.30 \pm 0.03^{a,b}$	< 0.1	$5.8 \pm 1.0^{b,c}$	0.43 ± 0.01^{e}	$15 \pm 1^{a,b}$	$44.0 \pm 0.1^{I,j}$
30 minutes – 45°C	$18 \pm 2^{a,b,c}$	$0.26 \pm 0.03^{a,b}$	2.8 ± 0.6^{b}	5.2 ± 0.4^{b}	0.34 ± 0.01^{a}	12.4 ± 0.9^{a}	33.8 ± 0.2^{a}
60 minutes – 45°C	$21 \pm 4^{a,b,c,d}$	$0.51 \pm 0.08^{a,b,c}$	0.5 ± 0.9^{a}	$6.1 \pm 0.4^{b,c}$	$0.38 \pm 0.01^{b,c}$	$14.5 \pm 0.9^{a,b}$	$37.0 \pm 1.0^{b,c}$
90 minutes – 45°C	16 ± 2^{a}	$0.52 \pm 0.07^{a,b,c}$	0.5 ± 0.9^{a}	5.5 ± 0.8^{b}	$0.37 \pm 0.01^{a,b,c}$	$13 \pm 1^{a,b}$	38.4 ± 0.5^{d}
120 minutes – 45°C	$18 \pm 3^{a,b}$	$0.8 \pm 0.2^{c,d}$	< 0.1	5.7 ± 0.4^{b}	$0.39 \pm 0.01^{c,d}$	$13.5 \pm 0.4^{a,b}$	39.6 ± 0.6^{e}
30 minutes – 60°C	$23.6 \pm 1.0^{a,b,c,d}$	$0.6 \pm 0.1^{b,c}$	2.8 ± 0.3^{b}	$6.0 \pm 1.6^{b,c}$	$0.39 \pm 0.01^{b,c}$	$13.0 \pm 0.8^{a,b}$	36.3 ± 0.6^{b}
60 minutes – 60°C	$25 \pm 5^{b,c,d}$	$1.0 \pm 0.2^{c,d}$	0.6 ± 1.1^{a}	7.7 ± 0.6^{c}	0.43 ± 0.02^{e}	$12.7 \pm 0.7^{a,b}$	$40.3 \pm 0.6^{e,f}$
90 minutes – 60°C	$18 \pm 2^{a,b}$	1.4 ± 0.2^{e}	< 0.1	$6.7 \pm 0.4^{b,c}$	0.43 ± 0.01^{e}	$13 \pm 2^{a,b}$	$42.0 \pm 0.1^{g,h}$
120 minutes – 60°C	$18 \pm 2^{a,b}$	1.7 ± 0.4^{e}	< 0.1	$6.9 \pm 0.6^{b,c}$	0.45 ± 0.01^{e}	$14 \pm 1^{a,b}$	$41.0 \pm 0.1^{f,g}$

Different superscripts in a column indicate significantly ($p < 0.01$) different mean ± standard deviation of triplicate determinations;
[A] concentration (μg/g) of 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde (**Appendix 1**);
[B] Free Fatty Acid as % oleic acid;
[C] Peroxide Value expressed as milli-equivalents oxygen per kg oil.

5.4 Conclusions

The above results suggest that alternative processing time-temperature combinations may be suitable for the production of virgin olive oil with pleasant sensory characteristics. It has been proposed (Morales & Aparicio, 1999) that elevated temperatures (> 35°C) and short malaxation times (< 30 min) can produce pleasant olive oils with green aroma characteristics. These conditions represent a departure from the typically recommended processing temperature of 30°C and malaxing for at least 45 min, according to olive paste rheology (Ranalli et al., 2003b). The higher malaxation temperatures (> 35°C) and shorter times (< 30 min) proposed by Morales & Aparicio (1999) fall in the region of non-enzymatic hexanal formation (**Figure 5.6b**), where hexanal concentration increases with malaxation temperature. In a similar high temperatures (> 35°C) and short times (< 30 min) region (**Figure 5.5b**), octane formation is minimized. This result of maximizing hexanal, associated with green aroma characteristic olive oils (Reiners & Grosch, 1998), and minimizing octane, associated with low quality olive oils (Di Giovacchino, 2000), supports the alternative approach of shorter time and higher malaxation temperature for the production of premium virgin olive oil.

This study does not show clear benefits of processing at elevated temperatures as oil quality was minimised for both bench and industrial scale oil extraction while oil yield was maximised during industrial scale oil extraction.

6.0 Discrimination of Storage Conditions and Freshness in Virgin Olive Oil

6.1 Introduction

As soon as virgin olive oil is extracted from the olive fruit there is potential for quality to deteriorate. Commercially, olive oil is stored with minimal oxygen exposure to protect quality, however, during domestic consumption, oxygen ingress is inevitable and may hasten oxidation and loss of freshness. To maintain the quality of virgin olive oil, it is paramount to control the oxidation status so that oil composition is consistent from production to consumption. This can be achieved through an understanding and control of both external factors i.e. oxygen concentration, temperature and light (Rahmani & Csallany. 1998, Velasco & Dobarganes, 2002), and internal factors i.e. major and minor constituents of virgin olive oil that influence oxidation (Rahmani & Csallany. 1998, Velasco & Dobarganes, 2002, Gutierrez & Fernandez 2002, Aparicio et al., 1999, Salvador et al., 1999).

Light exposure, temperature and oxygen concentration influence virgin olive oil quality and freshness during transportation, storage and consumption (Velasco & Dobarganes, 2002). Commercially, virgin olive oil is usually stored and transported in the dark but often packaged in transparent bottles in response to consumer preferences (Velasco & Dobarganes, 2002, Gutierrez & Fernandez 2002) thereby exposing the oil to light before and after purchase. Temperature variation during virgin olive oil storage and transportation is common and may be attributed to natural climatic changes and in some cases to intentional temperature control. Virgin olive oil is rarely stored at low temperature commercially, although low temperature storage before laboratory analysis has been widely reported to preserve the freshness of olive oil (Tovar et al., 2001, Beltran et al., 2005, Salvador et al., 2001, Mousa et al., 1996, Ranalli et al., 2000, Psomiadou et al., 2003). An understanding of olive oil oxidation at low temperature may find wide industrial applications in numerous areas, including oil-rich frozen food products.

Monitoring of oxidation in virgin olive oil during storage has been based on the change in major and minor constituents of virgin olive oil usually investigated through univariate statistical approaches using a variety of oxidation indicators. Phenolic and volatile compounds are the common minor constituents that are measured and shown to change during virgin olive oil storage (Psomiadou et al., 2003, Pagliarini et al., 2000, Tsimidou et al., 1992, Cavalli et al., 2004, Vichi et al., 2003). Understanding and control over phenolic compounds, which act as antioxidants (Psomiadou et al., 2003, Baldioli et al., 1996), can prevent oxidative deterioration of virgin olive oil. Antioxidants maintain levels of volatiles that impart positive sensory characteristics (Angerosa, 2002), and deter the generation of C7 to C12 volatile compounds responsible for sensory defects (Angerosa, 2002). To date, the focus in determining the extent of oxidation has been on the volatile compounds that are formed, and not necessarily on the compounds that are lost, as virgin olive oil loses its freshness.

Many studies on oxidation of virgin olive oils (Aparicio et al., 1999, Tsimidou, et al., 1992, Baldioli et al., 1996, Tsimidou, 1998, Litridou et al., 1997, Gutierrez et al., 2002) have reported good correlations between changes in compounds and stability, as measured by accelerated tests, and consequently such compounds have been identified as markers of oxidation. However, the application of accelerated studies to real-time (non-accelerated) shelf life studies remains questionable (Okogeri, & Tasioula-Margari, 2002). The extreme conditions in accelerated tests - high temperatures and with air bubbled into the oil - do not simulate actual storage conditions and may lead to qualitative and quantitative changes to the oil that are not related to real-time storage. This may lead to difficulties in choosing markers of oxidation that could be used to indicate deterioration of quality under different storage conditions. Here we define a marker as a parameter (compound or physical measurement) that is uniquely and significantly correlated with a particular treatment of an oil.

Regardless of the drawbacks of accelerated tests in shelf life investigations, studies based on real-time shelf life conditions are rare, and where they exist they are usually extrapolated to apply to storage conditions not used in the original shelf life study. Studies based on single storage conditions such as light (Psomiadou & Tsimidou, 2002a) and dark (Psomiadou & Tsimidou, 2002b) have been reported. Some studies have combined different storage conditions, for instance, dark and low temperatures (Cinquanta et al., 2001) and dark storage, uncontrolled light and uncontrolled temperature (Pagliarini, et al. 2000). Univariate statistical approaches were applied in most of these shelf-life studies except for Pagliarini et al. (2000) where a multivariate statistical approach was used.

Univariate statistical analyses limit consideration of the interactions that may occur between several external and internal factors. Multivariate statistical analysis can be applied to simultaneously explore factors influencing oxidation of virgin olive oil when stored under different conditions. The use of exploratory and classification statistical approaches such as stepwise linear discriminant analysis (SLDA) and principal component analysis (PCA) can identify patterns in samples and variables contributing to the clustering of samples (Miller & Miller, 2005).

The objective of this study was to investigate how different storage conditions affect oil quality relative to that of fresh oil. A multivariate statistical approach with SLDA was applied to simultaneously compare the effect of light, dark and low temperature storage, in the presence and absence of oxygen, on virgin olive oil. To the best of our knowledge, this is the first study of its kind that has identified parameters that significantly ($p < 0.01$) discriminate oil storage conditions in a real-time shelf-life study lasting one year. From this study, parameters that were uniquely associated with storage conditions were identified, and these may be used as markers for particular storage conditions.

6.2 Methodology

6.2.1 Materials

Standards and reagents from the indicated sources were used without further purification. Phenolic standards: caffeic acid, *p*-coumaric acid and gallic acid (Sigma, St. Louis, USA), tyrosol (Aldrich, Milwaukee, USA), hydroxytyrosol (Sapphire Bioscience, Sydney, Australia), oleuropein (Extrasynthese, Genay, France). Standards were prepared in methanol + water (50 + 50 v/v) and filtered through 0.45 µm plastic non-sterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for chromatographic preparations.

Volatile standards: pentanal, *E*-2-hexenal and nonanol (Merck, Hohenbrunn, Germany); hexanal, heptanal, *E*-2-octenal, *E*-2-nonenal, 1-penten-3-ol, 2-penten-1-ol, heptanol, octanol, hexyl acetate, methyl isobutyl ketone (MIBK) and 2-nonanone (Aldrich, Milwaukee, USA); octanal, octane, nonane, decane, undecane and dodecane (Sigma, St. Louis, USA); benzaldehyde (Ajax chemicals, Auburn, Australia), ethanol and acetic acid (Biolab, Sydney, Australia); ethyl acetate (Mallinckrodt Chemicals, Paris, France), and hexanol (Riedel de Haen, Seelze, Germany).

Reagents were as follows: chloroform, acetic acid, and potassium iodide (Biolab, Sydney, Australia), sodium thiosulphate (Asia Pacific Speciality Chemicals Ltd., Seven Hills, Australia), and starch (Scharlau Chemie S. A., Barcelona, Spain) for peroxide values (PV); cyclohexane spectrophotometric grade (Sigma, St. Louis, USA) for UV absorbances (K_{232}, K_{270} and ΔK); and propan-2-ol (Mallinckrodt Chemicals, Paris, France), sodium hydroxide (Ajax chemicals, Auburn, Australia), and phenolphthalein indicator (Sigma, St. Louis, USA) for free fatty acid (FFA) determination. Acetic acid (Biolab, Sydney, Australia), hexane and methanol (Mallinckrodt Chemicals, Paris, France), acetonitrile (J.T. Baker, Phillipsburg, USA), formic acid (Sigma, St. Louis, USA) were used in phenolic compounds analysis.

Olive oil samples. Fresh extra-virgin olive oil samples (3 x 5 litres), commercially extracted from *Paragon* olive fruit during the 2003 harvest season, were supplied by Riverina Olive Grove. The sensory description of the fresh extra-virgin olive oil, as provided by the supplier, was pronounced banana fruit, mild pepper and pungency. Aggregated quantitative data monitored over a 12-month period for common olive quality indices and major volatile and phenolic compounds are provided in **Table 6.1**.

Table 6.1. Quantitative data for different storage conditions and fresh virgin olive oil.

Variables	Fresh virgin olive oil	Without headspace			With headspace		
		Cold stored oil	Dark stored oil	Light stored oil	Cold stored oil	Dark stored oil	Light stored oil
Volatile compounds							
Acetic acid	1.8 ± 0.3 ^{c}	1.2 ± 0.3 ^{b}	1.1 ± 0.3 ^{b}	1.3 ± 0.3 ^{b}	0.13 ± 0.02 ^{a}	0.12 ± 0.01 ^{a}	0.15 ± 0.01 ^{a}
1-penten-3-ol	0.49 ± 0.07 ^{c}	0.23 ± 0.09 ^{b}	0.21 ± 0.09 ^{b}	0.22 ± 0.07 ^{b}	0.02 ± 0.01 ^{a}	0.049 ± 0.008 ^{a}	0.052 ± 0.006 ^{a}
Pentanal	2.1 ± 0.3 ^{d}	0.9 ± 0.5 ^{b}	1.0 ± 0.4 ^{b}	1.5 ± 0.3 ^{c}	0.08 ± 0.01 ^{a}	0.08 ± 0.01 ^{a}	0.05 ± 0.04 ^{a}
Hexanal	4.9 ± 0.6 ^{c}	2.7 ± 0.8 ^{b}	3.1 ± 0.9 ^{b}	6 ± 1 ^{d}	0.08 ± 0.01 ^{a}	0.09 ± 0.01 ^{a}	0.37 ± 0.05 ^{a}
E-2-hexenal	7.2 ± 0.7 ^{c}	4 ± 2 ^{b}	4 ± 2 ^{b}	4 ± 2 ^{b}	1.4 ± 0.2 ^{a}	2.1 ± 0.3 ^{a}	3.5 ± 0.4 ^{b}
E-2-hexen-1-ol	9 ± 1 ^{c}	5 ± 2 ^{b}	5 ± 2 ^{b}	5 ± 2 ^{b}	1.9 ± 0.2 ^{a}	2.2 ± 0.3 ^{a}	1.8 ± 0.1 ^{a}
Hexanol	4.5 ± 0.4 ^{b}	2.7 ± 0.9 ^{a}	3 ± 1 ^{a}	2.6 ± 0.9 ^{a}	2.7 ± 0.3 ^{a}	2.7 ± 0.4 ^{a}	2.7 ± 0.2 ^{a}
Octane	0.38 ± 0.04 ^{c}	0.19 ± 0.08 ^{b}	0.19 ± 0.08 ^{b}	0.7 ± 0.2 ^{d}	0.049 ± 0.009 ^{a}	0.04 ± 0.02 ^{a}	0.06 ± 0.02 ^{a}
Octanal	< 0.16	< 0.16	< 0.16	< 0.16	0.31 ± 0.02 ^{a}	0.36 ± 0.06 ^{b}	0.28 ± 0.09 ^{a}
E-2-nonen-1-ol	< 0.07	< 0.07	< 0.07	< 0.07	0.2 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	0.13 ± 0.03 ^{a}
2-pentyl furan	0.80 ± 0.07 ^{c}	0.5 ± 0.2 ^{b}	0.5 ± 0.2 ^{b}	0.5 ± 0.2 ^{b}	0.11 ± 0.01 ^{a}	0.14 ± 0.03 ^{a}	0.11 ± 0.04 ^{a}
Phenolic compounds							
Hydroxytyrosol	25 ± 2 ^{d}	14 ± 9 ^{a}	21.2 ± 0.9 ^{c}	21 ± 1 ^{b,c}	14.2 ± 0.6 ^{a}	17 ± 2 ^{a,b}	19 ± 1 ^{b,c}
Tyrosol	35 ± 3 ^{d}	27 ± 1 ^{b}	31 ± 2 ^{c}	29 ± 2 ^{c}	20.7 ± 0.9 ^{a}	25 ± 2 ^{b}	30 ± 2 ^{c}
Ligstroside dialdehyde	98 ± 8 ^{c}	36 ± 14 ^{a}	31 ± 19 ^{a}	38 ± 7 ^{a}	54 ± 10 ^{b}	31 ± 7 ^{a}	31 ± 4 ^{a}
(+)-acetoxy-pinoresinol	185 ± 17 ^{c}	97 ± 20 ^{b}	99 ± 32 ^{b}	102 ± 30 ^{b}	74 ± 11 ^{a}	53 ± 8 ^{a}	62 ± 6 ^{a}
Oleuropein aglycon	82 ± 16 ^{d}	43 ± 21 ^{b,c}	34 ± 20 ^{a,b,c}	52 ± 26 ^{c}	22 ± 7 ^{a}	22 ± 4 ^{a}	32 ± 7 ^{a,b}
Quality indices							
FFA [A]	0.302 ± 0.007 ^{a,b}	0.37 ± 0.07 ^{c}	0.39 ± 0.08 ^{c}	0.37 ± 0.09 ^{c}	0.30 ± 0.01 ^{a,b}	0.35 ± 0.02 ^{b,c}	0.27 ± 0.03 ^{a}
K_{232}	1.72 ± 0.01 ^{a}	1.77 ± 0.05 ^{a,b}	1.9 ± 0.1 ^{c}	1.85 ± 0.04 ^{b,c}	1.78 ± 0.03 ^{a,b}	2.4 ± 0.2 ^{e}	2.13 ± 0.04 ^{d}
K_{270}	0.15 ± 0.01 ^{b}	0.142 ± 0.009 ^{b}	0.144 ± 0.009 ^{b}	0.20 ± 0.01 ^{d}	0.13 ± 0.01 ^{a}	0.168 ± 0.008 ^{c}	0.237 ± 0.005 ^{e}
PV [B]	12.0 ± 0.4 ^{a}	17 ± 1 ^{b}	18 ± 2 ^{b,c}	18 ± 1 ^{b,c}	20 ± 2 ^{c}	26 ± 3 ^{d}	36 ± 2 ^{e}

Different superscripts in a row indicate significantly different ($p < 0.01$) mean ± standard deviation in µg/g of twelve analyses over a year.

[A] Free Fatty Acid as % oleic acid

[B] Peroxide Value expressed as milli-equivalents oxygen per kg oil

6.2.2 Virgin olive oil storage conditions.

Virgin olive oil (approx. 100 mL) was transferred into clear pharmaceutical bottles (6 x 100 mL/storage condition) and stored in the light at ambient temperature, dark at ambient temperature and at low temperature in the dark. Virgin olive oil bottles for dark and low temperature storage were wrapped in aluminium foil to exclude light. Virgin olive oil samples for light storage were placed on a laboratory shelf out of exposure to direct sunlight, and low temperature samples were stored in a refrigerator (1.0 ± 1.0°C). Virgin olive oil was analysed at bottling to provide data on fresh oil. Thereafter, one bottle per storage condition was analysed every two months for 12 months. Virgin olive oil was stored both without headspace and with a fifty percent headspace.

6.2.3 Qualitative and quantitative analysis of phenolic compounds

See Section 2.3 for details of the determination of phenolic compounds in as listed in **Table 6.2**.

6.2.4 Qualitative and quantitative analysis of volatile compounds

See Section 2.2 for details of the determination of volatile compounds as listed in **Table 6.2**.

Table 6.2. Variables for the characterization of freshness and storage conditions.

Volatile compounds	Phenolic compounds	Other variables
Acetic acid	Hydroxytyrosol	Free Fatty Acid (FFA)
1-penten-3-one	Tyrosol	Peroxide Value (PV)
1-penten-3-ol	Caffeic acid	K_{232}
Pentanal	3,4-DHPEA-DEDA[A]	K_{270}
Pentan-1-ol	Ligstroside dialdehyde	ΔK
Z-2-penten-1-ol	Ligstroside acetals	
Octane	Oleuropein derivatives	
Hexanal	(+) – pinoresinol	
E-2-hexenal	(+) – acetoxypinoresinol	
E-2-hexen-1-ol	Oleuropein aglycone	
Hexanol	Hemiacetal of oleuropein	
6-methyl-5-hepten-2-one		
2-pentyl furan		
Octanal		
E-2-nonen-1-ol		

[A] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde

6.2.5 Determination of quality parameters

Determination of FFA, PV and UV absorbances (K-values) was performed according to the standard EC and IOOC methods (EC, 1991, IOOC, 2003) as detailed in Sections 2.4, 2.5 and 2.6, respectively.

6.2.6 Statistical data analysis

Patterns that best separated storage conditions were identified by Stepwise Linear Discriminant Analysis (SLDA, Section 2.9.2) using quality indices and concentrations of volatile and phenolic compounds (**Table 6.2**) as independent variables with SPSS 12.0 (SPSS Inc., Chicago, USA). Linear discriminant analysis is a standard statistical technique for projecting data from a high dimensional space onto a perceivable reduced subspace such that the data can be separated by visual inspection (Li et al., 1999). For instance in our case, thirty-one variables with over 4,500 data points were significantly reduced to fifteen representative variables depicting only data points that identify trends and patterns in the original 4,500 points, which may not be evident from the use of univariate statistics. The outcome of discriminant analysis in recognizing storage conditions patterns was visualized in two dimensions by combined-group scatter plots (**Figures 6.1 – 6.3**), where the x-axis plots the values of discriminant Function 1 and the y-axis plots the values of discriminant Function 2.

Significant ($p < 0.01$) differences for parameters measured under different storage conditions (**Table 6.1**) were examined using one-way ANOVA post hoc multiple comparison tests using Duncan's test with SPSS 12.0 (SPSS Inc., Chicago, USA) as detailed in Section 2.9.1.

6.3 Results and Discussion

Virgin olive oil is best when consumed fresh. Storage has the potential to lower the quality of virgin olive oil. In order to more fully understand the impact of different storage conditions, multivariate analysis with SLDA was used to recognize storage patterns with scatter plots (**Figures 6.1 – 6.3**) and discriminating variables were identified. A chemometric multivariate approach was used to identify potential markers of freshness and storage under light, dark and low temperature conditions both in the presence and absence of oxygen. This approach gave insights into the chemical changes occurring in the oil during storage.

6.3.1 Discrimination of storage conditions relative to freshness

Fresh virgin olive oil and olive oil stored in the light; dark; and at low temperatures were significantly ($p < 0.01$) separated (**Figures 6.1 – 6.3**) showing distinct differences in the quality of olive oil under different storage conditions. The separation based on the first two discriminant functions had a significant ($p < 0.05$) Wilks' Lambda statistic on both functions (**Table 6.3**) indicating the suitability of SLDA in discriminating the different storage conditions. The separation of different storage conditions in this study differs markedly from an earlier report (Pagliarini, et al., 2000) in which storage conditions (uncontrolled light, temperature, and dark storage) for up to 14 months did not show a statistically significant influence.

The successful separation of the different storage conditions is illustrated in **Table 6.3** where the cumulative variance explained of 100% was achieved for the first two discriminant functions with storage conditions alone and 73% for fresh oil relative to different storage conditions. The lower cumulative variance explained (73%) for fresh oil relative to different storage conditions compared to 100% for the storage conditions alone indicate the closeness of some storage conditions to fresh oil. The presence of oxygen causes the most significant departure from fresh oil for all storage conditions (**Figure 6.1**). Furthermore, the presence of oxygen enhances the separation of the different storage conditions from each other.

Table 6.3. Discrimination of storage conditions showing % variance explained and significance of discriminant functions.

Discriminated Groups	% Variance explained (Function 1, V_1)	% Variance explained (Function 2, V_2)	% Variance explained (Cumulative)
Fresh oil relative to storage condition	**46.7***	**26.2***	**73.0**
Storage without headspace	**91.8***	**8.2***	**100.0**
Storage with headspace	**80.0***	**20.0***	**100.0**

* Wilks Lambda statistic significantly ($p < 0.05$) different.

The observations above indicate how differences in storage conditions can cause variations in olive oil composition and quality. A wider departure from fresh oil was observed with oil exposed to oxygen (with headspace) showing higher significant ($p < 0.01$) differences than oil stored in the absence of oxygen (without headspace). It was also observed that oil stored at low temperature was comparable to fresh oil (**Figure 6.1**). In addition, olive oil stored at low temperature; and in the dark formed a cluster in the absence of oxygen but the oils stored under the same conditions in the presence of oxygen were separated and significantly ($p < 0.01$) different from each other. The formation of a cluster for virgin olive oil stored in the dark and at low temperature in the absence of oxygen (**Figure 6.1**) indicates minimal differences in composition and quality, which later emerge when the bottles are opened and exposed to oxygen.

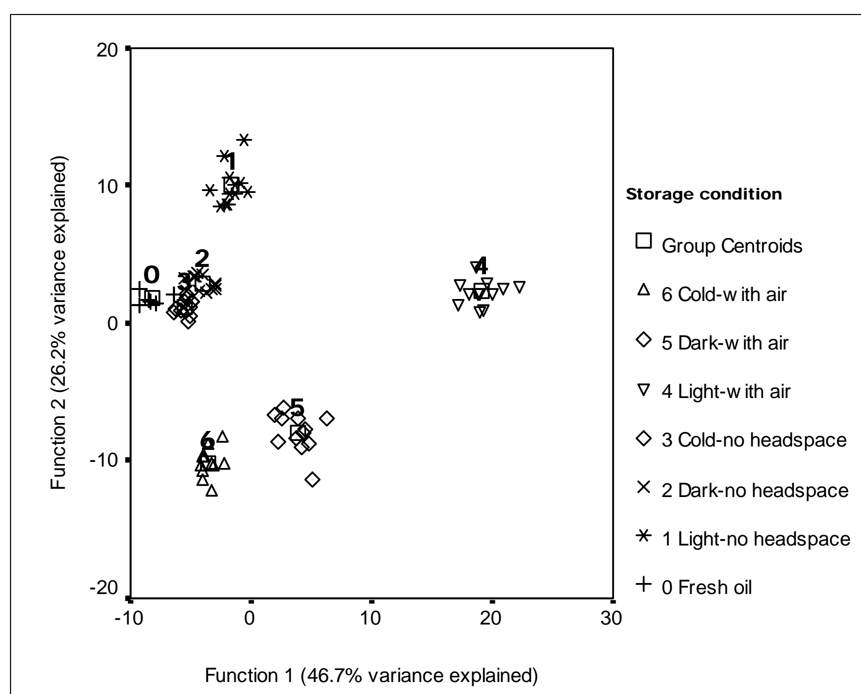

Figure 6.1. Scatter plot for the first two canonical discriminant function separating fresh and stored olive oil.

6.3.2 Discrimination of storage conditions in absence of oxygen

Storage of olive oil in the absence of oxygen simulates the conditions during transportation and storage at commercial level, before consumption at household level. Even though virgin olive oil is not exposed to oxygen, which would promote oxidative rancidity (Velasco & Dobarganes, 2002,

Hamilton et al., 1997), the discrimination in **Figure 6.1** establishes significant ($p < 0.01$) differences when the oil is exposed to light and when it is subjected to different storage temperatures. The differences are further explored below (**Figure 6.2**) to identify the discriminating variables that characterise storage conditions of virgin olive oil in the absence of oxygen.

It should be noted that not all parameters measured in virgin olive oil (**Table 6.2**) discriminated storage conditions. Only those parameters that had a significant ($p < 0.01$) contribution in separating the storage conditions were entered in the Functions of the scatter plot (e.g. **Figure 6.2**). The discrimination of storage conditions along Function 1 of **Figure 6.2** is given in the linear discriminant equation (V_1, **i**) below.

$V_1 =$	2.56[hexanol] + 0.83 x K_{232} + 0.22[tyrosol] - 1.76[octane]	(**i**)
	– 1.05 x K_{270} - 0.52[acetoxypinoresinol]	

Octane, acetoxypinoresinol and K_{270} (parameters with negative coefficients) discriminated olive oil stored in the light, which is on the negative side of Function 1 in **Figure 6.2**. Olive oil stored in the dark and at low temperature, which lie on the positive side of Function 1 in **Figure 6.2**, were discriminated with hexanol, tyrosol and K_{232} (positive coefficients). Olive oil samples stored in the dark and at low temperature, which were not discriminated on Function 1, were further separated from each other on Function 2 in **Figure 6.2**, defined by the linear discriminant equation (V_2, **ii**) below.

$V_2 =$	1.11[hexanol] + 1.16 x K_{232} + 1.22[tyrosol] + 0.52[octane]	(**ii**)
	– 0.25 x K_{270} - 1.45[acetoxypinoresinol]	

Olive oil stored at low temperature falls on the negative side of Function 2 in **Figure 6.2**, which was discriminated with acetoxypinoresinol and K_{270} (parameters with negative coefficients) in (**ii**). Parameters with positive coefficients (octane, hexanol, tyrosol and K_{232}) in (**ii**) discriminated light and dark stored olive oil that appears on the positive side of Function 2 in **Figure 6.2**.

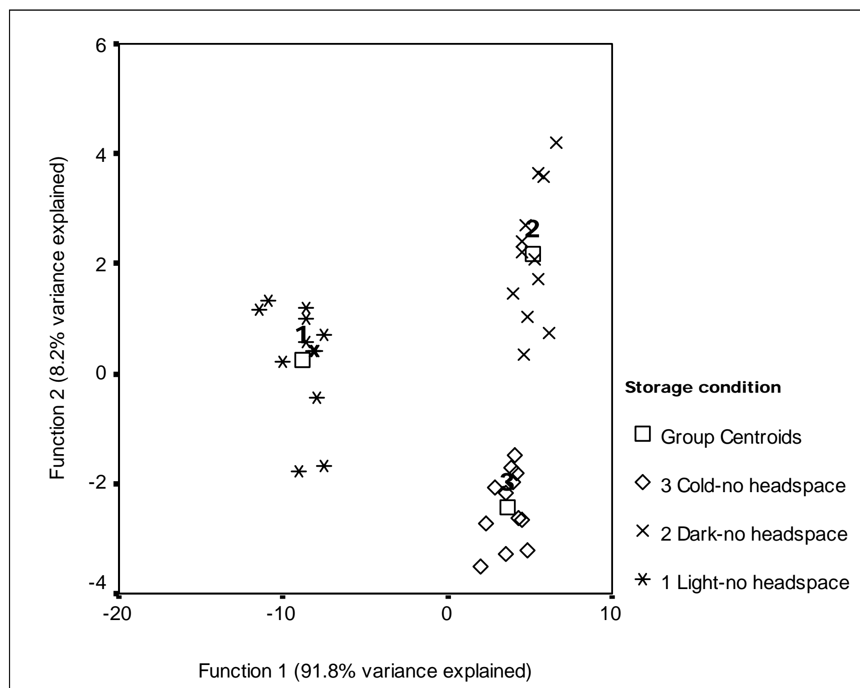

Figure 6.2. Scatter plot for the first two canonical discriminant function separating storage conditions in the absence of oxygen.

6.3.3 Discrimination of storage conditions in presence of oxygen

The introduction of headspace during storage, which simulates the conditions during consumption at household level, produced virgin olive oil clusters (**4, 5** and **6)** that were significantly ($p < 0.01$) different from those stored in the absence of oxygen (**1, 2** and **3**) as illustrated above in **Figure 6.1**. This implies that given the same storage conditions, the composition of virgin olive oil differs at commercial distribution and storage level (oxygen exposure minimized) and at household consumption level (oil exposed to oxygen). The discrimination of samples stored in the presence of oxygen was better on the y-axis as illustrated by a higher % variance explained (20.0%) in Function 2 compared to 8.2% for virgin olive oil stored without headspace (**Table 6.3**). The higher % variance explained for virgin olive oil stored in the presence of oxygen (**Figure 6.3**) indicates that the differences in composition and quality with storage conditions are more pronounced at household consumption level than during transportation and storage at commercial level.

The presence of oxygen had a pronounced effect on the variables that significantly ($p < 0.01$) discriminated storage conditions as revealed by comparing equation **i** with equation **iii**, the linear discriminant equation for Function 1 of **Figure 6.3**.

$V_1 =$	1.63[hexanal] + 0.42 x K_{232} + 0.62 x K_{270} + 0.54[hydroxytyrosol]	(**iii**)
	- 0.44[acetic acid] - 0.66[*E*-2-hexenal] – 0.50 x FFA	

Equation (**iii**) indicates that hexanal, hydroxytyrosol, K_{232} and K_{270} (parameters with positive coefficients) discriminated virgin olive oil stored in the light (**Figure 6.3**). These parameters contrast with those found for light storage in the absence of oxygen (equation **i**) – octane, acetoxypinoresinol and K_{270}. Virgin olive oil stored in the dark and at low temperature (**Figure 6.3**) was discriminated by acetic acid, *E*-2-hexenal and FFA (parameters with negative coefficients). There was poor separation of dark and low temperature storage conditions with respect to Function 1 in **Figure 6.3** but Function 2 clearly discriminated these storage conditions through linear discriminant equation (V_2, **iv**) below.

$V_2 =$	1.53[*E*-2-hexenal] + 0.72 x K_{232} + 0.21 x K_{270} + 0.75 x FFA 0[hydroxytyrosol]	(**iv**)
	+ 0.70[hydroxytyrosol] - 0.65[acetic acid] - 1.22[hexanal]	

From equation (**iv**), *E*-2-hexenal, hydroxytyrosol, K_{232}, K_{270} and FFA characterise virgin olive oil stored in the dark (**Figure 6.3**). Parameters with negative coefficients (acetic acid and hexanal) discriminated light and low temperature olive oil storage (**Figure 6.3**).

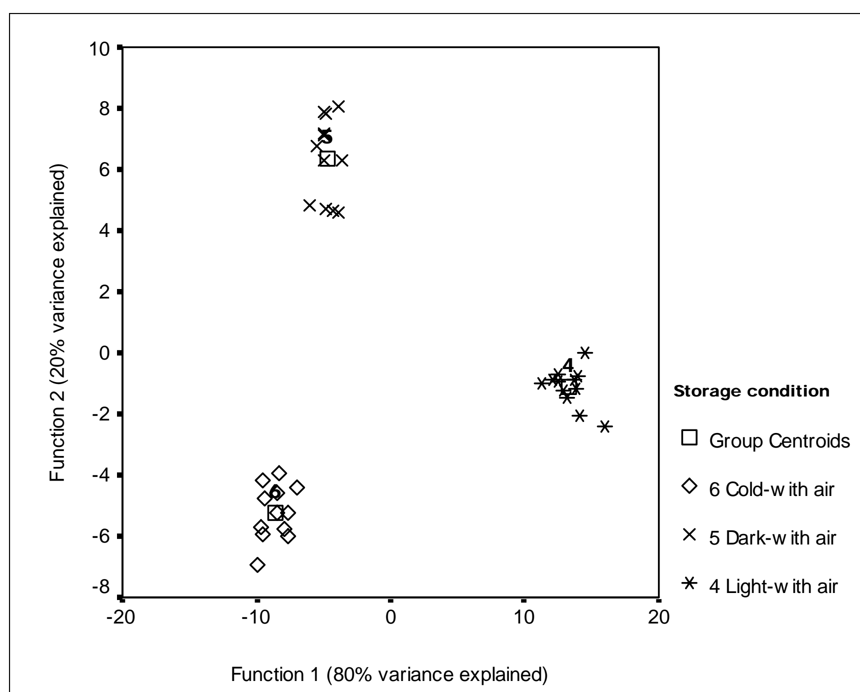

Figure 6.3. Scatter plot for the first two canonical discriminant function separating storage conditions in the presence of oxygen.

Parameters that significantly ($p < 0.01$) discriminated storage conditions as presented above in discriminant equations **i** – **iv** are compiled in **Table 6.4**. These parameters are investigated further, below, to determine which are unique to a particular set of storage conditions and hence can be considered to be a marker of those conditions. Furthermore, quantitative changes in these parameters may reveal insights into oil chemistry relative to storage conditions.

Table 6.4. Variables separating the different storage conditions of virgin olive oil.

Storage condition	Discriminating variables (without headspace)	Discriminating variables (with headspace)	Headspace independent discriminating variables
Low temperature	K_{270} *E*-2-hexen-1-ol Ligstroside dialdehyde (+)-acetoxypinoresinol	Acetic acid Pentanal PV^A	-
Dark	*E*-2-hexenal K_{232} PV^A Tyrosol Hexanol	*E*-2-hexenal K_{232} K_{270} FFA^B Hydroxytyrosol	*E*-2-hexenal K_{232}
Light	Octane *E*-2-hexen-1-ol K_{270} Ligstroside dialdehyde (+)-acetoxypinoresinol	Hexanal K_{232} K_{270} PV^A Hydroxytyrosol	K_{270}

[A] Peroxide Value expressed as milli-equivalents oxygen per kg oil

[B] Free Fatty Acid as % oleic acid

6.3.4 Parameters that characterize low temperature storage

Without headspace. Low temperature storage maintains the quality of olive oil close to that of fresh oil as observed by their proximity in **Figure 6.1**. These conditions resulted in the least significant ($p < 0.01$) increase in PV, and lower values of K_{270} than fresh virgin olive oil (**Table 6.1**). This suggests that hydroperoxides (as measured by PV) increase slowly over a twelve month storage period

(presumably due to oxygen present at bottling, or adventitious ingress through incomplete seals) and that their decomposition to secondary oxidation products is inhibited (low K_{270}). The slow oxidation rate of olive oil at low temperature is consistent with the report of Velasco and Dobarganes (2002), who state that at low or moderate temperatures, hydroperoxides are the major compounds formed.

All phenolic compounds significantly ($p < 0.01$) decreased in concentration during low temperature storage. While conditions that lead to oxidative damage to oils (light, heat, O_2) were kept to a minimum, it is apparent that oxidative chemistry was still occurring, leading to a decrease in levels of the antioxidant compounds. SLDA identified ligstroside dialdehyde and acetoxypinoresinol as the phenolic compounds whose change in concentration was most characteristic of low temperature storage in the absence of oxygen. However, since these compounds also discriminate light storage, without headspace (**Table 6.4**), they are not unique to one set of storage conditions, and therefore cannot be classified as markers.

E-2-hexen-1-ol was the only volatile compound whose concentration was found to be discriminating by SLDA (**Table 6.4**) for low temperature storage without headspace. The concentration of *E*-2-hexen-1-ol decreased during the storage period. This C6 compound is associated with the lipoxygenase pathway – a series of enzyme catalysed transformations leading to volatile compounds with favourable "green" aromas (Olias et al., 1993, Ridolfi et al., 2002). Since this particular compound is reported to have a "green" odour, loss of this compound could lead to a "flattening" of the aroma of olive oil. *E*-2-hexen-1-ol was not uniquely associated with low temperature storage (**Table 6.4**) and therefore cannot be classified as a marker of this storage condition. In fact, low temperature storage in the absence of oxygen, showed no marker (**Table 6.5**) supporting the observation (**Figure 6.1**) on the similarity of fresh oil to that stored at low temperature.

With headspace. Low temperature storage brought about the least change in the oil (**Figure 6.1**) compared to the other storage conditions in the presence of oxygen. Not surprisingly, the presence of headspace O_2, resulted in PV being identified as a discriminating variable by SLDA (**Table 6.4**). However, it is not uniquely associated with low temperature storage and is hence not a marker (**Table 6.5**). As above (*Without headspace* discussion), low temperature appeared to slow the rate of conversion of hydroperoxides to secondary oxidation products as indicated by the low value of K_{270}.

Levels of phenolic compounds decreased during storage, similar to that observed in the absence of headspace (see above). SLDA did not identify any phenolic compounds as discriminating variables for the storage of oil at low temperature and with headspace (**Table 6.4**).

The volatile compounds acetic acid and pentanal exclusively discriminated low temperature storage (**Table 6.4**) and hence can be classified as markers of this storage condition (**Table 6.5**). As with *E*-2-hexen-1-ol (above), these compounds decrease in concentration during storage. It is not yet known whether they are lost chemically during the storage period, or whether they are lost during opening of the containers for sampling. Sensory evaluation would be required to investigate what impact, if any, loss of these compounds would have on stored oil.

Table 6.5. Potential oxidation markers of virgin olive oil stored in the light, dark and at low temperature

Storage condition	Markers (without headspace)	Markers (with headspace)	Common markers
Low temperature	-	Acetic acid Pentanal	-
Dark	Tyrosol Hexanol	FFA[A]	*E*-2-hexenal K_{232}
Light	Octane	Hexanal	K_{270}

[A] Free Fatty Acid as % oleic acid

6.3.5 Parameters that characterize dark storage.

Without headspace. As with low temperature storage, virgin olive oil kept in the dark in the absence of oxygen, showed a significant ($p < 0.01$) increase in PV (**Table 6.1**) compared to fresh oil. Under these conditions, a significant ($p < 0.01$) increase in K_{232} was also observed. These results are consistent with an earlier study (*24*), where slight increases in PV and K_{232} were observed for virgin olive oil stored under similar conditions. Storage in the dark leads to maximum values of K_{232} (**Table 6.1**), independent of the presence or absence of headspace. This suggests that non-photo-assisted, auto-oxidation reactions, leading to primary oxidation products, are prominent at ambient temperatures. This is further reflected in the fact that K_{232} was a discriminating variable for oils stored in the dark both in the presence and absence of headspace (**Table 6.4**). As such, it constitutes a marker for dark storage for both conditions (**Table 6.5**).

Oils stored in the dark showed decreased levels of all phenolic compounds (**Table 6.1**), indicating ongoing oxidation reactions during the storage period. However, only tyrosol was found by SLDA to discriminate this storage condition (**Table 6.4**) and as it was uniquely associated with this storage condition, is classified as a marker compound for dark storage in the absence of oxygen (**Table 6.5**).

SLDA identified hexanol and *E*-2-hexenal as volatile compounds that discriminated oils stored in the dark. Loss of these C6 compounds (see above) during storage may lead to oil with less favourable aroma. Hexanol was uniquely associated with this storage condition and is therefore a marker (**Table 6.5**) compound. *E*-2-hexenal was also associated with dark storage with headspace (**Table 6.4**), and is a marker for dark storage regardless of the presence or absence of oxygen (**Table 6.5**).

Cavalli et al (2004) reported a reduction in *E*-2-hexenal content and an increase in C6 alcohols and C5 ketones in olive oil stored in the dark at ambient temperature, and these compounds have been proposed as markers of virgin olive oil freshness. In this study, a decrease in *E*-2-hexenal was observed, but no increase in C6 alcohols nor C5 ketones was detected. In fact, in our study, the C6 alcohol hexanol decreased in concentration during storage.

Another study (Vichi et al., 2003b) on dark storage of virgin olive oil, but this time under accelerated conditions (60°C), reported an increase in a number of volatile compounds. Nonanal was proposed as the most sensitive marker to oxidative deterioration. Under the non-accelerated conditions used in this study, nonanal was not detected during storage. This highlights the need to carefully interpret oxidation markers evaluated under different conditions.

With headspace. In the current study, K_{232} was a common discriminating variable for virgin olive oils stored both in the presence and absence of headspace (**Table 6.4**). Quantitatively, K_{232} values were significantly ($p < 0.01$) higher in the presence of headspace than in the absence of headspace (**Table 6.1**) indicating increased oxidation, consistent with this storage condition.

FFA was identified as a discriminating variable that was significantly ($p < 0.01$) greater in virgin olive oil stored in the dark with headspace (**Table 6.1**) than fresh oil, suggesting possible hydrolytic reactions. Oxidative reactions leading to a rise in FFA have been attributed to the production of volatile acids from the decomposition of hydroperoxides and oxidation of aldehydes (*20*). This was not consistent with our findings (**Table 6.1**) where no concomitant increase in volatile acids with FFA was observed.

E-2-hexenal was the only volatile compound to be found to be discriminating for dark storage in the presence of headspace (**Table 6.4**). As discussed above, it is a general marker for dark storage (**Table 6.5**) since it was also discriminating for dark storage in the absence of headspace. *E*-2-hexenal is reported to be one of the most important volatile compounds contributing to the pleasant aroma of extra virgin olive oil (Cavalli et al., 2004). Loss of this C6 aldehyde during storage will lead to oil that has less desirable sensory properties compared to the fresh oil.

The phenolic compound hydroxytyrosol was selected by SLDA as a discriminating variable for dark storage in the presence of headspace (**Table 6.4**). However, it is not unique to this storage condition and is hence not able to act as a marker (**Table 6.5**). It is interesting that this ortho-diphenol is a discriminating variable in the presence of headspace (under both dark and light storage conditions) and may reflect its reactivity as an antioxidant in the presence of oxygen (Gutierrez et al., 1999, Gutfinger, 1981). Earlier studies (Pagliarini et al., 2000, Cinquanta et al., 2001) have reported a significant influence of hydroxytyrosol in maintaining virgin olive oil quality.

6.3.6 Parameters that characterize light storage

Without headspace. Olive oil stored in the light showed the most significant departure from fresh oil (**Figure 6.1**). Photo-assisted oxidation is a well known cause of defective oil (Velasco & Dobarganes, 2002), yet colourless, glass containers are common, even though they may be exposed to light 24 hours per day on a supermarket shelf. Interestingly, the only quality index that discriminated this storage condition was K_{270}. Its value significantly increased (**Table 6.1**) during storage in the light in the absence of oxygen (**Table 6.4**). K_{270} is associated with secondary oxidation (Gutierrez & Fernandez, 2002, EC, 1991), and the fact that it was the discriminatory quality index would suggest that photo-assisted secondary oxidation, rather than primary oxidation (as indicated by K_{232}, PV values), is the dominant mechanism for oil deterioration under these storage conditions.

That secondary oxidation is an important process under these storage conditions is reinforced by considering the increase in octane levels (**Table 6.1**). Octane has been linked to the breakdown of 10-hydroxyperoxide of oleic acid and correlated with sensory defects in olive oil (Di Giovacchino, 2000). Light storage in the absence of oxygen was the only condition that led to the increased concentrations of octane in the oil (**Table 6.1**). As octane exclusively discriminated this storage condition, it qualifies as a marker compound (**Table 6.5**).

E-2-hexenol was another volatile compound that was identified as a discriminating variable by SLDA (**Table 6.4**). Its concentration decreased during storage in the light in the absence of oxygen (**Table 6.1**). In general, the C6 compounds all decreased under this storage condition, pointing to a loss of freshness in the oil as discussed above. The exception was hexanal, which increased in concentration (**Table 6.4**). Hexanal is known to be associated with the oxidation of oil (Morales et al., 1997), however, the increase observed under the storage conditions employed here did not result in it being identified as a discriminating variable.

Levels of all phenolic compounds significantly decreased (**Table 6.1**) during light storage, as was the case for the other storage conditions. SLDA identified acetoxypinoresinol and ligstroside dialdehyde as discriminating variables for this storage condition in the absence of oxygen (**Table 6.4**), however, as they were not uniquely discriminating, they are not marker compounds. Loss of both phenolic and volatile compounds suggests that both aroma and taste were affected by storage.

With headspace. All quality indices associated with oxidation, i.e. PV, K_{232} and K_{270}, significantly ($p < 0.01$) increased (**Table 6.1**) when virgin olive oil was stored in the light with a headspace. The presence of oxygen therefore resulted in a rate of formation of hydroperoxides that was faster than the decomposition rate as signified through increased concentrations of primary oxidation products (i.e. PV and K_{232} values). The data in **Table 6.1**, suggest that secondary oxidation products (i.e. K_{270} values) are linked to light exposure regardless of whether oxygen is present or not. Thus, K_{270} is a common marker for light storage (**Table 6.5**).

All volatile compounds found in fresh oil decreased during storage in the light in the presence of oxygen (**Table 6.1**). Hexanal was found to be a discriminating variable (**Table 6.4**) and since it was uniquely linked to this storage condition, it may be classified as a marker (**Table 6.5**). The low level of hexanal found in oil stored in the light and with a headspace is indicative of oil that has lost its original

freshness. Such oils would rate low on sensory scores where flavour intensity is rated (Angerosa, 2002).

As discussed above (dark storage with headspace), hydroxytyrosol is a discriminating variable (**Table 6.4**) for oils stored with a headspace. The amount of light exposure did not affect the levels of hydroxytyrosol found during storage (**Table 6.1**). This suggests that hydroxytyrosol is not directly photo-degraded, but rather reacts with other species that are generated in the presence of oxygen.

6.3.7 Effect of oxygen exposure during virgin olive oil storage

Oxygen is usually introduced in accelerated methods in an effort to enhance lipid oxidation and, for example, attempt to correlate an oil's resistance to oxidation with levels of endogenous antioxidants (Baldioli et al., 1996). We are not aware of studies where oxygen is deliberately introduced as a variable in a real-time storage trial. Yet oxygen exposure is an inevitable consequence of consumer use and storage, and chemical changes occurring during this period are an important consideration in a product's quality and reliability. This aspect of the "supply chain" has received little attention. Here, the inclusion of oxygen coupled with storage at ambient temperatures, i.e. non-accelerated conditions, allows some insights into this the final stage of the supply chain.

The major difference between oils stored with or without headspace is the appearance of longer chain volatile compounds, *vis* octanal and *E*-2-nonen-1-ol, which were only detected in oils exposed to oxygen (**Table 6.1**). The formation of octanal is linked to the breakdown of 13-hydroxyperoxy oleic acid (Vichi et al., 2003) and *E*-2-nonen-1-ol is formed from 9-hydroxyperoxy linoleic acid (Fisher & Scott, 1997, Frankel, 1998). The higher concentration of oxygen is expected to increase the formation of peroxides, and this is generally supported by an increase in PV, but the appearance of octanal and *E*-2-nonen-1-ol suggest that the breakdown of hydroperoxides is also linked to levels of oxygen. Longer chain volatile compounds are typically reported in accelerated studies e.g. Gutierrez et al (2002), however, such studies also lead to high levels of hexanal and acetic acid. Under the conditions employed in this study, concentrations of hexanal and acetic acid decreased with storage in the presence of oxygen. This reinforces our earlier observation that results from accelerated oxidation of oil must be extrapolated with caution to real time shelf life studies.

As noted earlier, loss of particular compounds may be just as important an indicator of loss of freshness as the generation of new compounds during storage. The presence of oxygen during storage significantly ($p < 0.01$) lowered concentrations of acetic acid, 1-penten-3-ol, *E*-2-hexen-1-ol, and acetoxypinoresinol relative to storage in the absence of oxygen (**Table 6.1**). In addition to monitoring the generation of new compounds, monitoring the loss of these compounds may be important when investigating the effect of oxygen exposure during real-time virgin olive oil storage.

6.3.8 Potential oxidation and freshness markers of virgin olive oil

The change of oxidation markers with storage conditions (**Table 6.5**) may explain why diverse oxidation markers have been previously reported for virgin olive oil. Some proposed markers include nonanal (Vichi et al., 2003b) and the ratio of hexanal/nonanal (Morales et al., 1997, Kiritsakis, 1998). While most studies have used nonanal as a primary indicator of rancidity, Solinas et al. (1987) observed that 2-pentenal and 2-heptenal were the main rancidity indicators. Neither nonanal, nor 2-pentenal or 2-heptenal were identified as oxidation markers in this study. Although hexanal levels change with olive oil storage and it was identified statistically (**Table 6.5**) as a marker of storage in the light with headspace, hexanal is not favoured as a marker compound. This is because the amount of hexanal does not distinguish oxidised oils from virgin oils, since hexanal originates from both enzymatic and chemical oxidation (Vichi et al., 2003b, Angerosa, 2002, Morales et al., 1997).

Gutierrez et al (2002), proposed the use of phenolic compounds to establish the average life of olive oils subjected to oxidation with the Rancimat method. In this study, all phenolic compounds decreased in concentration regardless of storage conditions. This suggests that oxidative processes are occurring even under mild conditions. It is interesting to note that in an oxygen limited environment, SLDA identified mono-hydroxy compounds as discriminating variables (ligstroside dialdehyde and (+)-acetoxypinoresinol, (**Table 6.4**), whereas in the presence of oxygen, the ortho-diphenol hydroxytyrosol, was a discriminating variable (**Table 6.4**). Thus phenolic compounds are not all equally affected by storage conditions and tyrosol was the only phenolic compound attributed as a marker compound (**Table 6.5**).

Parameters – *E*-2-hexenal, K_{232} and K_{270} – that significantly ($p < 0.01$) discriminated virgin olive oil stored both with and without headspace (**Table 6.4**) were identified as common oxidative markers (**Table 6.5**). These parameters were markers for oils stored at ambient temperature, but not for oils stored at low temperature (**Table 6.5**). The absence of any common oxidative markers for low temperature stored oil, which was shown earlier (**Figure 6.1**) to be closest to fresh oil, indicates that departure from freshness may be detected by changes in levels of *E*-2-hexenal, K_{232} and K_{270}. Among these parameters, K_{232} and K_{270} are included in the classification of virgin olive oil quality (EC, 1991, IOOC, 2003) and *E*-2-hexenal was previously reported (Cavalli et al., 2004) as a marker of olive oil quality and freshness. Currently, *E*-2-hexenal is not included in the classification of virgin olive oil quality, with variations in concentrations in fresh oil attributed to cultivar and maturity effects (Kalua et al., 2005). However, *E*-2-hexenal may be included as a parameter for classification of virgin olive oil quality and freshness with reference to its odour activity value to set a minimum value for its sensory impact.

7. Changes from Olive Fruit to Oil during Virgin Olive Oil Production.

Changes in virgin olive oil during production have been investigated in **Sections 3 – 6** for individual production steps, without consideration of possible inter-relationships between the steps. This approach, of investigating changes for single production steps in isolation, is common and does not consider the realistic relative effects of other processes down along the production line from olive fruit to oil. This section now investigates these inter-relationships and statistically identifies critical steps for quality indices; volatile compounds; and phenolic compounds from olive fruit to oil (including domestic storage). **Section 7** is organised around three major areas: research strategy; discrimination of virgin olive oil production steps relative to each other; and relative changes during virgin olive oil production from fruit to oil.

7.1 Introduction

The production of virgin olive oil commences from the olive grove where characteristics of the raw material, the olive fruit, determine the quality of virgin olive oil (Rontondi et al., 2004, Garcia et al., 1996a, Agar et al., 1998, Mousa et al., 1996). During virgin olive oil extraction, under good manufacturing practices, any oil can be classified as extra-virgin (**Table 1.1**) since it is likely to conform to legal limits of quality indices, such as FFA and PV. However, it is less likely to remain extra-virgin during consumption due to loss of stability associated with oxygen ingress under domestic storage conditions (Monteleone et al., 1998). Stability studies of virgin olive oil usually focus on the oil (Velasco & Dobarganes, 2002) with minimal regard to fruit characteristics from which the oil was extracted.

Evaluation of quality at extraction and assurance of adequate product life have been the primary concern for monitoring virgin olive oil production (Kiritsakis et al., 2002). Recently, Pardo et al., (2006) have proposed a new approach to issues of quality. They have proposed the terms "potential quality" and "real quality" as a means of incorporating fruit characteristics as parameters in the overall quality of the oil. Potential quality of olive oils is reached when healthy and clean olive fruits have been selected at optimum maturity; processed at optimum conditions with quick separation of residues and by-products. Real quality is that found in olive oils sampled randomly from storage tanks (Pardo et al., 2006). Once the potential and real quality are evaluated, stability is assessed to determine the commercial quality of the oils at the end of the maximum possible time of storage, when it is bottled and distributed in supermarkets or retailers (Pardo et al., 2006). Experience from this project has shown that virgin olive oil quality rapidly deteriorates once the oil is out of supermarkets or retailers and into our kitchens. We believe that it is important, particularly for the Australian industry, to educate consumers about the quality changes that occur during domestic consumption to ensure that oil is used before quality defects (especially sensory quality) are noticed.

The assessment of potential quality can pose several challenges: such as deciding upon the optimum fruit characteristics to obtain premium quality virgin olive oil; or to match these with optimum processing conditions. Once the oil is extracted another challenge arises as to when is the optimum time to assess the real quality – is it when the oil is just transferred to the storage tanks or just before bottling?. The emphasis on quality after bottling has often been on how a product will survive the distribution chain with little emphasis on how the oil will perform during consumption. With multiple steps along the virgin olive oil production line, which steps are critical to the production of quality virgin olive oil; and maintenance or enhancement of quality? Many factors have been attributed to the virgin olive oil quality differences along virgin olive oil production line (**Figure 1.1**) and it has been

difficult to identify the critical factors that promote quality. It has been observed that agronomic and climatic aspects such as cultivar; fruit ripeness; climatic conditions; and area of origin and technological aspects such as fruit storage; malaxation; and oil storage affect the quality of virgin olive oil and the composition of volatile and phenolic compounds (Angerosa et al., 2004z, Servili et al., 2004). However, almost invariably, these factors are identified by looking at quality changes at single l production steps.

The diversity and inter-relationships of factors affecting virgin olive oil quality and composition makes it tremendously difficult to carry out a complete quality characterisation of virgin olive oils (Aparicio & Luna, 2002). Consequently, quality characterisation of virgin olive oils should be carried out with samples identified by a large number of variables and data should be analysed with statistical techniques or artificial intelligence systems (Aparicio & Luna, 2002). Among the variables that have been widely attributed to the quality characterisation of virgin olive oil are volatile and phenolic compounds (Angerosa et al., 2004z, Servili et al., 2004, Angerosa, 2002). The objective of this section was to statistically identify, through changes in volatile and phenolic compounds, the critical steps during oil production that either enhance the quality of the oil or promote certain quality attributes,.

7.2 Methodology

Identification of critical control steps during virgin olive oil production is accomplished through simultaneous consideration of discriminating variables in virgin olive oil from olive fruit to oil storage with headspace (i.e. domestic use). Discriminating variables for fruit maturity; post-harvest fruit storage; virgin olive oil extraction; and oil storage are compiled to identify volatile compounds, quality indices, and phenolic compounds that characterise single or multiple virgin olive oil production steps (**Section 7.3**). The changes from fruit to oil during virgin olive oil production are determined through comparisons of statistical z-scores and steps that were critical in either maximizing or minimizing quality attributes from volatile and phenolic compounds are identified (**Section 7.4**).

7.2.1 Virgin oive oil production steps and sample selection

The optimum conditions for the production and maintenance of quality virgin olive oil, identified in **Sections 3 – 6**, have been used to compare the relative changes from fruit to oil at consumer level during virgin olive oil production. Virgin olive oil from spotted *Corregiola* olive fruits was compared with virgin olive oil extracted from *Corregiola* olive fruits with malaxation temperature of 30°C for 60 minutes. Changes in virgin olive oil arising from the fruit maturity step were compared with oil storage in the dark (in absence of oxygen for 4 months and in the presence of oxygen for 2 months). Dark oil storage conditions were chosen since such conditions are recommended for the preservation of virgin olive oil quality (IOOC, 1990). In order to identify the relative changes post-harvest fruit storage might have on the normal virgin olive oil production process, fruit storage for two weeks, which produced the best storage results (**Section 5**), was compared with normal virgin olive oil production steps from fruit to oil at consumer level.

The choice of fruit maturity and fruit storage represents changes in potential quality (Pardo et al., 2006) of virgin olive oil. Real quality (Pardo et al., 2006) was represented through changes in quality just after oil extraction whereas changes during the distribution and supply chain were represented with oil storage in the absence of oxygen. Storage of oil in presence of oxygen simulates virgin olive oil changes that might occur during consumer use. This consideration therefore covers the entire virgin olive oil production process from olive fruit to oil at consumer level.

Oils used to investigate the changes during virgin olive oil production in this chapter were from fruits of similar maturation (Maturity Index = 2.42 ± 0.09), which represents a coefficient of variation (CV) of 3.65 %, a CV small enough to rule out any effect from maturity. The olive fruits used were also from the same *Frantoio* family; *Corregiola* and *Paragon* (Kailis & Considine, 2002), which have been reported (Kalua et al., 2005) to have similar volatile and phenolic profiles.

7.2.2 Data treatment and analysis.

Volatile compounds, quality indices and phenolic compounds from different virgin olive oil production steps were of different magnitudes and to enable comparison of trends from olive fruit to oil (stored with headspace) on a similar reference scale, standardized normal variables (statistical z-scores) were used. Changes in virgin olive oil parameters associated with particular production steps are shown in **Figures 7.1 – 7.3** and were achieved through normalisation of statistical z-scores from single optimal steps as stipulated above (**Section 7.2.1**). Statistical z-scores from the optimal single steps during virgin olive oil production were normalized through subtraction from the mean of z-scores for the entire production process (including simulated domestic storage) and dividing by the standard deviation. Normalization of z-scores from single steps during virgin olive oil production ensured that the single processes are compared to the entire production process.

Identification of critical control steps from olive fruit to oil during consumption was achieved through a combined consideration of normalized z-score changes and discriminating variables from Stepwise Linear Discriminant Analysis (SLDA) that were compiled to identify changes in virgin olive oil based on volatile compounds; quality indices; and phenolic compounds (**Table 7.1**). Virgin olive oil production steps that were discriminated once were critical in transferring quality attributes associated with particular variables. In cases where multiple production steps were discriminated, critical control steps were based on steps that simultaneously discriminated a particular production step and caused the maximum or minimum change.

7.3 Discrimination of Virgin Olive Oil Production Steps

Variables that characterize steps along the virgin olive oil production line (**Figure 1.1**) may discriminate single or multiple production steps (**Table 7.1**). Discrimination of a single production step simplifies the process of optimising the levels of certain quality attributes in virgin olive oil as changing the variable at a particular step, where it is discriminated, has a non-significant effect on the other production steps. Changing variables that discriminate multiple processes along the production line does not necessarily mean that the change will be maintained further down the production line. In cases where a variable is discriminated by multiple steps along the production line, the impact of the other steps should be assessed. For instance, the concentration of a volatile or phenolic compound might be enhanced at a point along the production line, but then reduced at a subsequent step.

This section explores the variables that characterise changes along virgin olive oil production line for single steps, which might be used to optimise quality attributes in virgin olive oil (**Section 7.3.1**). On the other hand, variables that discriminate multiple production steps are also explored in **Section 7.3.2** and considered relative to the other unit processes during virgin olive oil production for the identification of critical steps.

Table 7. 1 Discriminating variables for virgin olive oil production steps.

Time (weeks)	Fruit Maturity	Fruit Storage	Oil Extraction	Oil Storage (-O_2)	Oil Storage (+O_2)	Frequency [A]
Oil Phenolic Compounds						
Hydroxytyrosol	✗	✓	✗	✗	✓	2
Tyrosol	✓	✓	✓	✓	✗	4
Oleuropein aglycon	✓	✓	✓	✗	✗	3
Oleuropein derivatives	✓	✓	✓	✗	✗	3
Oleuropein hemiacetal	✓	✗	✗	✗	✗	1
3,4-DHPEA-DEDA[B]	✗	✗	✓	✗	✗	1
Ligstroside dialdehyde	✗	✓	✗	✓	✗	2
Ligstroside derivatives	✗	✓	✗	✓	✗	2
Vanillic acid	✗	✓	✗	✗	✗	1
Pinoresinol	✗	✓	✗	✗	✗	1
Acetoxypinoresinol	✗	✓	✗	✓	✗	2
Luteolin	✓	✗	✗	✗	✗	1
Oil Volatile Compounds						
Pentanal	✗	✗	✗	✗	✓	1
Hexanal	✗	✓	✗	✗	✓	2
Trans-2-hexenal	✓	✓	✓	✓	✓	5
Cis-2-penten-1-ol	✓	✓	✗	✗	✗	2
1-penten-3-ol	✓	✗	✓	✗	✗	2
Trans-2-hexen-1-ol	✗	✓	✗	✓	✗	2
Hexanol	✓	✗	✓	✓	✗	3
Acetic acid	✗	✗	✓	✗	✓	2
Octane	✗	✗	✓	✓	✗	2
2-pentyl furan	✗	✓	✗	✗	✗	1
Quality Indices						
FFA	NA[C]	✓	✓	✗	✓	3
PV	NA	✓	✗	✓	✓	3
K_{232}	NA	✓	✗	✓	✓	3
K_{270}	NA	✓	✗	✓	✓	3

[A] Number of times a variable discriminates a process along virgin olive oil production line.

[B] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde (**Appendix 1**).

[C] Not analysed in the maturity study

7.3.1 Variables characterizing single processes

Only seven of the 22 volatile and phenolic compounds discriminated single steps along the virgin olive oil production line(**Table 7.1**). Five out of these seven discriminating variables were in steps that involved the olive fruit. For instance, the phenolic compounds oleuropein hemiacetal and luteolin discriminated fruit maturity only (**Table 7.1**). Thus targeting these compounds by harvesting olive fruit at certain maturation may lead to their incorporation in oil without being influenced by subsequent processes along the production line. Vanillic acid, pinoresinol and 2-pentyl furan were another set of discriminating variables that were affected by olive fruit post-harvest storage only.

Oil extraction is a critical control step for levels of 3,4-DHPEA-DEDA (**Table 7.1**) and oil storage a critical control step for levels pentanal (**Table 7.1**). It was previously observed (**Section 5.3.5)** that

formation of 3,4-DHPEA-DEDA was favoured with malaxation at high temperatures and for short periods of time, which might be critical processing conditions in transferring this phenolic compound from olive fruit to oil. Similarly, low temperature oil storage in the presence of oxygen (**Section 6.3.3**) results in the enhancement of pentanal in virgin olive oil (Morales et al., 2005). These two processes – vis. high temperature short time malaxation and low temperature storage in O_2 are not usually associated with optimum production conditions. Indeed these steps can be counter-productive on other variables. However, the example illustrates the fact that some productions steps can lead to elevated levels of desirable compounds that otherwise remain independent of prior or subsequent steps.

The emergence of volatile and phenolic compounds that discriminate single steps along the production line suggests that these compounds can be promoted or diminished in virgin olive oil through control of such steps, for instance fruit maturation and post-harvest fruit storage above. Solely discriminated production steps can be envisaged as critical control steps in the transferring of desirable or undesirable attributes, imparted through the volatile and phenolic compounds, from the olive fruit to oil.

7.3.2 Variables Characterizing Multiple Processes

In keeping with **Figure 1.1**, it is useful to consider variables associated with multiple production steps as those associated with those under agronomic control, technological control, chemical control or some combination of these factors. For example, oil extraction and storage were discriminated with acetic acid and octane (**Table 7.1**), but these variables were not discriminating for fruit characteristics during maturity nor post-harvest storage. This observation implies that acetic acid and octane are significantly formed during oil extraction and storage, independent of any influence from fruit characteristics under optimal conditions (**Section 7.2.1**). This is consistent with earlier reports (Vichi et al., 2003b) on the generation of these volatile compounds from oxidative quality deterioration during virgin olive oil storage. It can therefore be suggested that with proper control of oil extraction and oil storage conditions, octane and acetic acid may be minimised to enhance the quality of virgin olive oil. This consideration appears to be quite general regardless of the origin of the fruit.

In contrast, some compounds discriminate production steps before oil extraction and do not significantly discriminate unit processes during and after oil extraction. For instance, *cis*-2-penten-1-ol (**Table 7.1**) discriminated fruit characteristics only, i.e. fruit maturity and fruit storage, but was not a discriminating variable for processes when the oil was separated from the plant material, i.e. oil extraction and oil storage. This suggests that *cis*-2-penten-1-ol levels may be significantly altered with changes in fruit characteristics, with minimal effect from production steps during and after oil extraction. Thus, in the case of *cis*-2-penten-1-ol manipulating processing or storage conditions is not expected to influence levels of this compound in the oil and agronomic practises would need to be employed if enhanced levels of this compound were desired.

By far the majority of compounds investigated in this study discriminated production steps associated with both fruit characteristics and oil properties. For instance, 1-penten-3-ol was discriminated at fruit maturity and during oil extraction (**Table 7.1**) and hexanol was discriminated from fruit maturity, during oil extraction and oil storage. Phenolic compounds, such as oleuropein aglycon and oleuropein derivatives, have shown a similar tendency. This implies that even when concentrations of these compounds are maximised (or minimised, in the case of "undesirable" compounds) in the olive fruit, care should still be taken during and after oil extraction to maintain quality of virgin olive oil from production to consumption. Most of the compounds that discriminated multiple production steps were C5 and C6 volatile compounds and oleuropein related compounds, which are associated with premium virgin olive oil (Angerosa et al., 2000z, Kiritsakis, 1998). Most previous studies on virgin olive oil quality enhancement focus on the transfer from olive fruit to oil of positive quality attributes (often from C5 and C6 volatile compounds and oleuropein related compounds) without considering possible effects on quality from different oil storage conditions and post-harvest olive fruit handling. This study demonstrates that much could be learned by taking a more holistic approach to quality by considering

how compounds attributed to positive attributes are affected by the multiple steps involved in oil production. This is especially true when discrimination of all production steps is observed. This is the case for *trans*-2-hexenal and tyrosol (**Table 7.1**); compounds that are closely associated with virgin olive oil quality (Kiritsakis, 1998, Angerosa et al., 2000z). In cases where all virgin olive oil production steps were discriminated with particular volatile compounds; quality indices or phenolic compounds, a comparison of changes from olive fruit to oil storage with headspace will be investigated (**Section 7.4**) to elucidate the critical steps that either maximise or minimise these parameters in virgin olive oil.

The quality indices (FFA, PV, K_{232}, K_{270}) are usually measured at extraction or at bottling following bulk storage. However, it is interesting to note that only FFA discriminates the extraction step (**Table 7.1**). This suggests that just after extraction may not be an appropriate point in production to measure the oxidative indicators. On the other hand, all quality indices discriminated fruit storage and oil storage with headspace. It appears that storage in general, either fruit or oil, is a process where quality indices are susceptible to change. Even in oil storage without oxygen, the oxidative indices discriminated this process. Thus correct storage is critical in the production of high quality virgin oils. In this study, quality indices were not measured in the study on maturity stage. Previous work in this area has pointed to fruit maturity as influencing the quality indices. For instance, FFA is reported (Salvador et al., 2001) to increase during fruit maturity and fruit maturity has been reported (Garcia et al., 1996a, Salvador et al., 2001) to influence PV, K_{232} and K_{270}.

7.4 Identification of Critical Steps in Virgin Olive Oil Production.

Through the use of SLDA variables, i.e. quality indices, volatile and phenolic compounds, have been found that discriminate the different steps in virgin olive oil production (**Table 7.1**). However, identifying a discriminating variable does not give us any measure of the magnitude of change in that variable at a particular step. In order to ascertain whether a particular production step has a large effect on a discriminating variable, statistical z-scores (**Section 2.9**) must be used. . The different signs and magnitudes of z-scores for quality indices; volatile compounds; and phenolic compounds shown in **Figures 7.1 – 7.3** respectively, indicate that different processes along the virgin olive oil production line (**Figure 1.1**) influence virgin olive oil quality differently. Positive z-scores imply that optimising such steps maximise the variable, whereas negative z-scores indicate minimization of the variable. Minimal changes in z-scores (**Figure 7.1 – 7.3**) indicate controlled processes with minimal impact on virgin olive oil quality. A production step that showed a maximum or minimum z-score for a discriminating variable (**Figure 7.1 – 7.3**) (**Table 7.1**) may be identified as a critical step in controlling virgin olive oil quality, or transferring quality attributes from the olive fruit to oil.

7.4.1 Quality indices – trends from olive fruit to oil

Quality indices were earlier (**Section 7.3.2**) observed to characterise multiple processes during virgin olive oil production, which necessitates a look at trends to identify critical control steps. FFA levels were controlled during oil extraction and oil storage in absence of oxygen as shown by low z-scores, but showed an increase during fruit storage and oil storage in the presence of oxygen (**Figure 7.1**). FFA levels should be minimised to attain a premium virgin olive oil standard (IOOC, 2003), therefore, post-harvest fruit storage and oil storage in the presence of oxygen were critical control steps for this parameter. The positive FFA change with fruit storage (**Figure 7.1**) is consistent with earlier reports (Jimenez et al., 2001) on the increased hydrolytic activity with cell wall degradation, whereas the positive change during oil storage in the presence of oxygen (**Figure 7.1**) suggests that hydrolysis of triglycerides may proceed even after oil extraction.

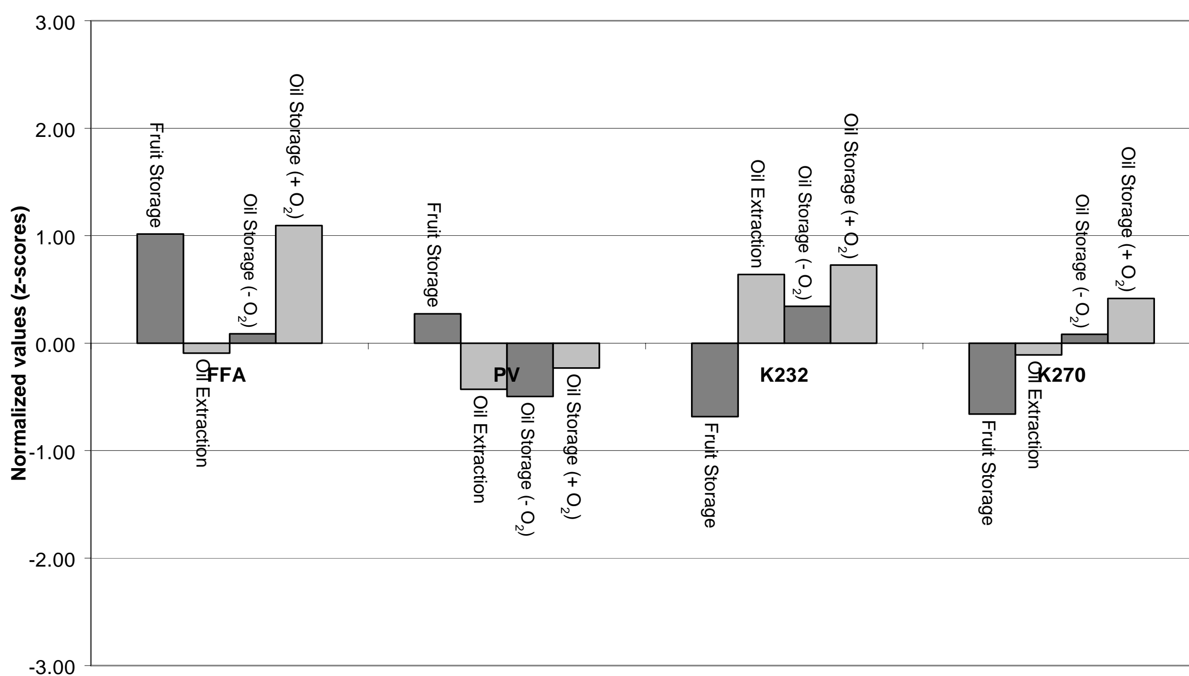

Figure 7.1 Quality Indices changes from olive fruit to oil.

Changes in PV were smaller than changes in FFA and K_{232} (**Figure 7.1**), which might indicate that PV is less suitable for monitoring changes in quality along the production line from olive fruit to oil stored with headspace. A comparison of PV and K_{232} changes (**Figure 7.1**) showed opposite sign changes, which is consistent with an earlier observation (**Table 5.7**) of oil extraction where PV was maximised at low temperatures and K_{232} at high temperatures. K_{232} has been reported (Di Giovacchino et al., 2002b) to mainly depend on primary oxidation during storage and correlated with PV, which was not the case for changes in K_{232} and PV in this study (**Figure 7.1**) where the two quality indices had z-scores with opposite signs. Furthermore, we found no correlation between K_{232} and PV values in our oil storage trial. This suggests that more investigation is needed to ascertain why these measurements are not more closely correlated.

K_{270}, a quality index related to secondary oxidation compounds especially those with a carbonyl functional group (Di Giovacchino et al., 2002b), showed an increase along virgin olive oil production line (**Figure 7.1**). The maximum K_{270} changes during oil storage in the presence of oxygen (**Figure 7.1**) is consistent with earlier observations (Gutierrez & Fernandez, 2002, Gomez-Alonso et al., 2006, Di Giovacchino et al., 2002b) where increases in K_{270} values were noted during oil storage. An increase in K_{270} has been reported (Gutierrez & Fernandez, 2002) to be an important indicator of the loss of extra-virgin olive oil quality and observations from this study suggests that control of oxygen exposure during virgin olive oil storage will minimise K_{270} (**Figure 7.1**). The low value of the K_{270} z-score for oil extraction (**Figure 7.1**) indicates minimal influence of this production step on the changes in secondary oxidation products. This is further shown through z-scores for volatile compounds in **Section 7.4.2**.

In general, oil extraction and oil storage in the absence of oxygen showed lower z-scores in comparison to those for fruit storage and oil storage in the presence of oxygen (**Figure 7.1**). This observation might imply that oil storage during consumption (where it is exposed to oxygen) and fruit storage production steps were critical in controlling the quality indices whereas oil extraction and oil storage in the absence of oxygen had a lesser impact on these parameters.

7.4.2 Volatile compounds – trends from olive fruit to oil

The z-scores for quality indices (**Figure 7.1**) were smaller than those for volatile compounds (**Figure 7.2**), which is consistent with an earlier observation (**Section 6.5.4**) on the potential of volatile compounds for detecting subtle changes in virgin olive oil quality and freshness before they are shown through quality indices. Almost all volatile compounds shown had maximum z-scores for fruit maturity indicating a dominant influence of fruit characteristics in determining the sensory properties of virgin olive oil. Fruit maturity z-scores were greater than those for oil extraction, which would tend to indicate that processing is secondary in importance in the production of high quality oil. Of course, controlling the extraction process is still important, however, it cannot create desirable traits in an oil that were not originally present in the fruit. Both oil extraction and oil storage resulted in negative z-scores for all volatile compounds – an exception being acetic acid during oil storage (**Figure 7.2**).

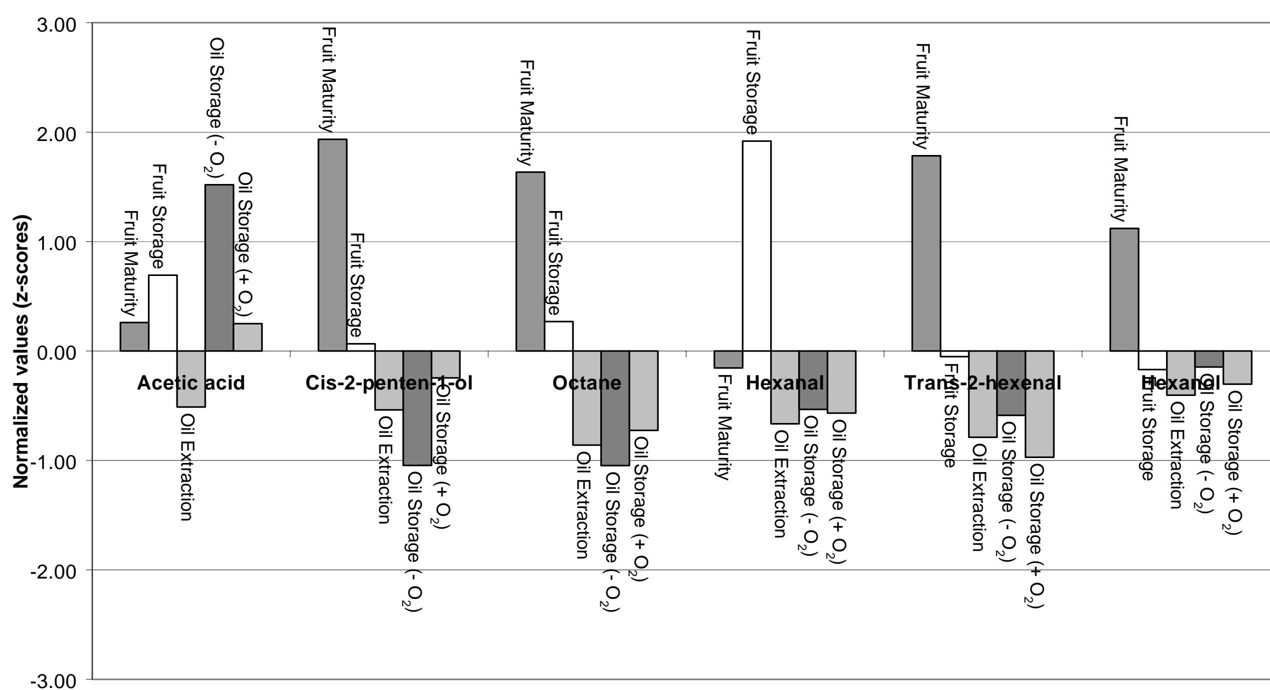

Figure 7.2 Volatile compounds changes from olive fruit to oil.

Fruit maturity, oil extraction, and oil storage in the absence of oxygen were discriminated with hexanol (**Table 7.1**) where fruit maturity maximised hexanol and the subsequent production steps minimised the volatile compound (**Figure 7.2**). This indicates the importance of fruit maturity in enhancing hexanol levels in virgin olive oil, which then later decline during oil extraction and oil storage. The larger magnitude for the hexanol z-score at fruit maturity compared with oil extraction and oil storage, suggests that fruit maturity is critical in controlling the levels of hexanol during virgin olive oil production.

Unlike hexanol, *trans*-2-hexenal discriminated all steps along virgin olive oil production line. Thus where the maximum z-score for *trans*-2-hexenal is observed (**Figure 7.2**), *vis*. fruit maturity, identifies critical control step. *Trans*-2-hexenal was earlier observed (**Table 3.6**) to be favoured in early maturity, whereas hexanol levels increased during late maturity, which indicates that different maturity stages can purposely be selected to impart certain sensory attributes from theses volatile compounds.

All post-harvest olive fruit handling steps showed negative z-scores for *trans*-2-hexenal and hexanol (**Figure 7.2**). The positive z-scores during fruit maturity and negative scores thereafter imply that *trans*-2-hexenal and hexanol might be maximised during fruit maturation but decline along the production line. This highlights the importance of good manufacturing practices (IOOC, 1990) in minimising the decline in these volatile compounds and hence maintaining the sensory quality of virgin olive oil.

Positive changes in z-scores for both fruit maturity and fruit storage were observed for acetic acid, octane and *cis*-2-penten-1-ol (**Figure 7.2**). Among these volatile compounds, *cis*-2-penten-1-ol discriminated both fruit maturity and fruit storage (**Table 7.1**) with a slight change during fruit storage and a maximum positive change during fruit maturity (**Figure 7.2**). Fruit maturity can therefore be suggested as a critical control step for *cis*-2-penten-1-ol, which is a desirable volatile compound due to its “green” aroma (Acree and Arn, 2004). On the other hand, acetic acid and octane did not discriminate the respective production steps, but these volatile compounds were discriminated during oil extraction and oil storage (**Table 7.1**). Oil extraction negatively changed the levels of acetic acid (**Figure 7.2**) probably due to volatilisation and degradation during malaxation, whereas oil storage positively changed acetic acid levels (**Figure 7.2**). Acetic acid is often linked to the vinegary defect in non-extra virgin oils (Morales et al., 2005), which are produced from fruit with microbial damage. As only sound fruit were used in this study, it would seem that the small amount of acetic acid present could be further reduced in the extraction step. Proper choice of fruit and oil storage conditions is also critical in minimising levels of acetic acid (**Figure 7.2**).

Oil extraction and oil storage in the absence of oxygen were discriminated with octane (**Table 7.1**) and the levels of octane negatively changed (**Figure 7.2**) relative to the other steps along virgin olive oil production line. This suggests that oil extraction and oil storage in the absence of oxygen are critical in controlling the levels of octane in virgin olive oil.Octane is also associated with sensory defects (Vichi et al., 2003b)(Morales et al., 2005), and therefore conditions that minimise levels are necessary. In **Section 5.3.6**, extraction temperatures of 30°C or lower were found to minimise octane levels as was storage in the absence of light (**Section 6.3.6**).

Hexanal was unique in that it showed a maximum positive z-score for fruit storage (**Figure 7.2**). Fruit and oil storage in presence of oxygen were discriminated with hexanal (**Table 7.1**) with the former showing maximum positive changes suggesting that fruit storage is critical for controlling the levels of hexanal in virgin olive oil. The switch from positive (during fruit storage) to negative (during and after oil extraction) for hexanal (**Figure 7.2**) may imply that olive fruit quality was critical in maximising the levels of this volatile compound in virgin olive oil. As fruit storage showed the largest z-score for hexanal, relative to other major volatile compounds, (**Figure 7.2**) this indicates that hexanal became the dominant volatile compound with olive fruit storage. It was shown in **Section 4.3.2** that the levels of hexanal found in two-week stored fruit, seemed to contribute to the return of the positive sensory characteristics of the oil derived from this fruit.

In conclusion, fruit maturity may be seen as the single most important influence on the levels of desirable volatile compounds in olive oil. Processing and storage reduce levels of these compounds, even under optimal conditions. Thus the aroma of virgin olive oil is predominantly linked to fruit characteristics. Flavour, being a combination of taste and aroma also depends on the phenolic compounds. We now investigate critical production steps involving phenolic compounds and how they may affect virgin olive oil sensory quality.

7.4.3 Phenolic compounds – trends from olive fruit to oil

The z-score changes for phenolic compounds (**Figure 7.3**) were smaller than changes for volatile compounds (**Figure 7.2**), which is consistent with an earlier observation (**Section 6.5.4**) on the potential for volatile compounds to detect subtle short-term changes in virgin olive oil quality and freshness. In contrast, phenolic compounds are associated with long-term oxidative quality changes. Comparison of the different processes along virgin olive oil production line show that oil storage in the presence of oxygen had a negative z-score for all phenolic compounds (**Figure 7.3**), which coincided with the positive changes in oxidative quality indices, K_{232} and K_{270} (**Figure 7.1**). The decrease in levels of phenolic compounds during oil storage is consistent with reports (Baldioli et al., 1996, Carrasco-Pancorbo et al., 2005) on the antioxidant activity of phenolic compounds. To preserve virgin olive oil quality, correct oil storage and minimal exposure to oxygen are critical. While exposure to oxygen is unavoidable during consumer use, this may not cause detectable loss of sensory quality if the oil is used rapidly (no more than 2 months).

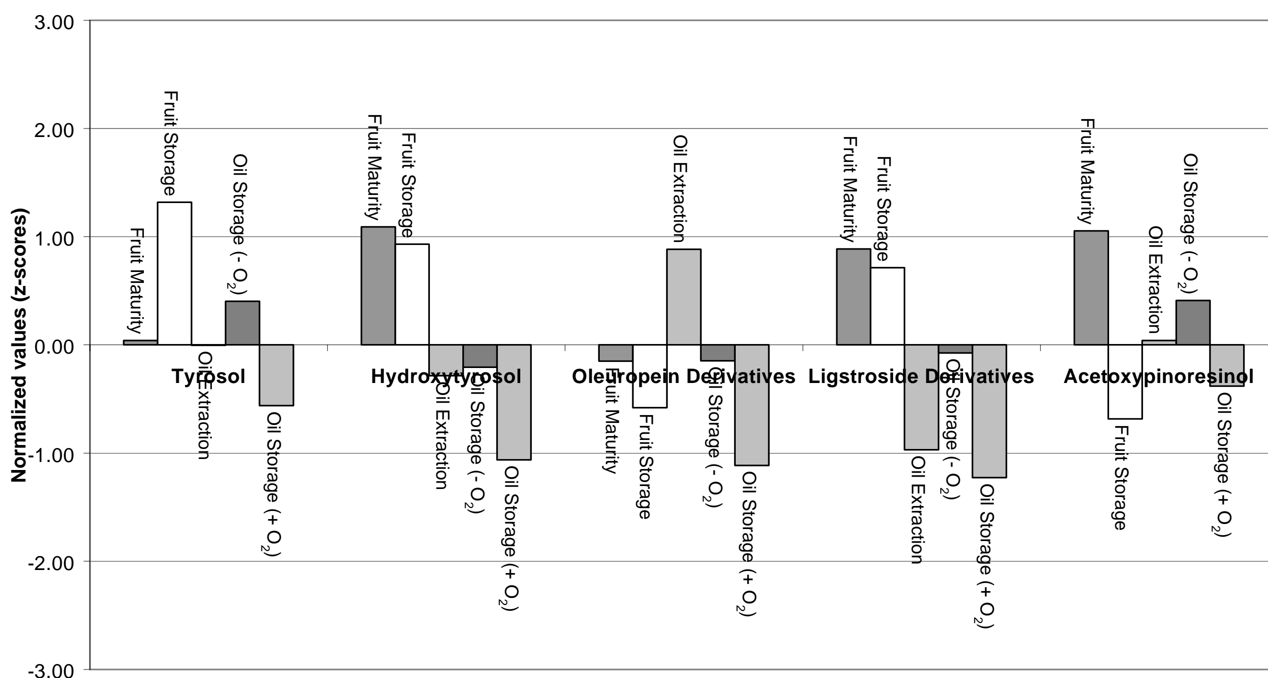

Figure 7.3 Phenolic compounds changes from olive fruit to oil.

Since concentrations of phenolic compounds decrease during consumer use, it may be desirable to attempt to maximise them through intervention at production steps with positive z-scores. For instance, in the case of hydroxytyrosol, ligstroside derivatives and (+)-acetoxypinoresinol (**Figure 7.3**), maximum positive changes were observed with fruit maturity, which suggests that the particular phenolic compounds can be maximised in virgin olive oil by harvesting olive fruits at a maturity stage when the levels of these compounds are at their peak.

Among the phenolic compounds, hydroxytyrosol and ligstroside derivatives (**Figure 7.3**) showed a positive change during fruit maturity and fruit storage indicating that changes in these phenolic compounds in olive oil are significantly influenced by fruit characteristics. These phenolic compounds can thus be maximised in the fruit, but subsequent steps show negative z-scores and thus must be carefully controlled to minimise loss of these compounds.

There are no clear trends in how phenolic compounds respond to fruit storage (**Figure 7.3**).Oleuropein derivatives and (+)-acetoxypinoresinol have negative z-scores, whereas tyrosol, hydroxytyrosol, and ligstroside derivatives had positive z-scores (**Figure 7.3**). The positive z-scores for hydroxytyrosol, tyrosol, and ligstroside derivatives during olive fruit storage (**Figure 7.3**), opposite to the z-score for K_{270} (**Figure 7.1**), might indicate the role of phenolic compounds in deterring secondary oxidation. The influence of phenolic compounds in deterring secondary oxidation was noted earlier (**Section 6.5.4**) in oil storage trials.

While fruit maturity and fruit storage are critical in determining the levels of phenolic compounds in virgin olive oils, oil extraction may also significantly change levels of phenolic compounds (**Figure 7.3**) with implications for oil taste. For instance, oleuropein derivatives had a positive z-score with oil extraction (**Figure 7.3**) indicating that bitter oils (Mateos et al., 2004, Gutierrez-Rosales et al., 2003) are favoured by oil extraction at the optimal time-Temperature combination (60 min, 30°C **Section 7.2.1**). On the other hand, ligstroside derivatives, which partly account for pungency (Andrewes et al., 2003) had a negative z-score under these conditions (**Figure 7.3**) indicating a loss of pungency. Achieving an oil that is balanced in terms of bitterness and pungency will require balancing of extraction conditions, perhaps to achieve a compromise in the levels of these compounds. Extraction conditions may need to be matched to fruit maturity, since this also has an effect on levels of phenolic compounds (Kalua et al., 2005).

7.5 Conclusions

Volatile compounds showed the greatest changes during production from olive fruit to oil (stored with headspace), followed by phenolic compounds and the least changes were observed with quality indices. This observation emphasises the importance of volatile and phenolic compounds in detecting virgin olive oil changes that could otherwise be missed when monitoring of oil based on quality indices alone.

Fruit characteristics were dominant in determining the sensory properties of virgin olive oil from volatile and phenolic compounds and hence were critical in controlling the sensory quality during virgin olive oil production. Extraction led to loss of volatile compounds, and opposite effects on bitterness and pungency (due to phenolic compounds), necessitating further work to find a compromise set of conditions. Subsequent steps in production – bulk storage, distribution, retail storage – and ultimately consumer use, inevitably lead to loss of sensory quality. To preserve virgin olive oil quality, minimising exposure to oxygen during oil storage is critical and should be avoided wherever possible. The rapid onset of deterioration upon exposure to oxygen has implications for how consumers use premium virgin olive oil and education campaigns may be necessary to ensure that consumers get the most from their oil.

8. Conclusions and Suggestions for Further Work.

8.1 Conclusions

This research has investigated various stages in the production of virgin olive oil, beginning with olive fruit right through to storage conditions that simulated domestic consumption. At each stage in the process, critical parameters have been identified, which may be controlled or optimised to enhance olive oil quality. We conclude this report by summarising the major findings and proposing several areas where future work may be targeted.

(i) Volatile and phenolic compounds that significantly ($p < 0.01$) discriminated cultivars and maturity stages were identified and changes in concentration during maturation were determined. Separation by stepwise linear discriminant analysis (SLDA) revealed that *Manzanilla* olive cultivar was separated from *Leccino*, *Barnea*, *Mission*, *Corregiola* and *Paragon* whereas *Corregiola* and *Paragon* formed a cluster. Volatile compounds discriminated both cultivars and all maturity stages – *vis*. green; spotted; red; and black. Phenolic compounds did not discriminate cultivars, but did discriminate oil derived from fruit in the early stages of maturity (green and spotted olives).

The trends and concentrations of the identified discriminating variables showed a cultivar dependency. For instance, the concentrations of *trans*-2-hexenal declined with maturity in *Corregiola* and *Paragon* while in *Barnea* they increased with maturity. Oleuropein derivatives showed no progression with maturity but increased in an interval during maturation that coincided with maximum levels of *trans*-2-hexenal and hexanal. This coincidence means both desirable aroma compounds (*trans*-2-hexenal and hexanal) and desirable taste compounds (oleuropein derivatives) may be optimised in oil by choosing fruit at the "spotted" maturity stage, to produce a well-balanced oil with green aromas and bitterness. Further work would need to be done to correlate these observations with yield data to determine if there needs to be a trade-off in yield to achieve best sensory results.

Statistical association of phenolic compounds further suggest that the presence of hesperidin in the fruit favours the formation of ligstroside dialdehydes to oleuropein derivatives, which might have implications on the taste of virgin olive oil with more pungent oils produced from fruits with hesperidin and more bitter oils produced from fruits where hesperidin is absent or at low levels. As yet, the underlying biochemical cause of this association is not known, and as far as we are aware, a link between the pathway responsible for hesperidin (a flavonoid) and oleuropein and ligstroside derivatives (secoiridoids) has not been proposed.

(ii) Studies on oil extraction have featured prominently in literature on olive oil production. This study was unique in that temperature extremes (15°C and ≥ 45°C) were investigated with a full-factorial designed experiment. Among the oil extraction conditions, malaxation temperature had a major influence on virgin olive oil quality whereas malaxation time affected oil yield. The optimum malaxation temperatures showed a clear split into variables that were favoured at high (> 45 °C) and low (< 30 °C) temperatures regardless of the olive fruit. Production of premium quality olive oil with high oil yields need a careful combination of processing parameters that do not favour the development of compounds associated with poor quality virgin olive oils. It was noted that optimum malaxation times that favour high oil yields were also conducive to the production of low quality oil. In contrast, compounds associated with premium quality olive oils resulted from shorter malaxation times (≤ 90 minutes), which favoured low oil yields. This study does not show clear benefits of processing at elevated temperatures as oil quality was minimised for both bench and industrial scale oil extraction while oil yield was maximised during industrial scale oil extraction. These changes

in virgin olive oil quality during oil extraction emphasizes the importance of processing conditions in the production of premium quality virgin olive oil as it can be observed that the positive potential sensory attributes from the raw material (the olive fruit) are lost during oil extraction.

Further changes in virgin olive oil occur when the olive fruit is subjected to oil extraction. Most of the volatile compounds that were potentially maximised during olive fruit maturity decline just after oil extraction. In addition to the detrimental changes in the aroma of virgin olive oil, oil extraction conditions changed the oil sensory attributes in different ways. For instance, bitterness was favoured during oil extraction whereas pungency was not. However, maximisation of sensory quality attributes from different olive fruits should be handled prudently since the levels and occurrence of phenolic compounds are maturity and cultivar dependent.

(iii) The distribution/supply chain is another step where correct handling of the oil is essential for slowing loss of quality as much as possible. Quality deterioration is worst when oxygen ingress occurs as is the case with domestic use. Virgin olive oil stored in the light showed the largest departure from freshness while oil stored at low temperature had characteristics closest to fresh oil. Different markers were observed for the different storage conditions, which illustrate the influence of storage conditions on changes in virgin olive oil quality. Under real-time shelf life conditions, volatile compounds marked the early subtle quality and freshness changes, whereas phenolic compounds were indicative of long-term oil oxidation. This study has highlighted the difficulties in using accelerated studies to represent quality loss during storage. Most of the oxidation indicators formed through accelerated studies were not among the markers for quality deterioration. On the other hand, loss of compounds already present in the oil (not formed during oxidation) were found to be indicative of quality and freshness changes in stored olive oil.

Exposure to oxygen separated virgin olive oils stored under different conditions illustrating that even though there might be minor differences in oil quality during transportation and storage at commercial level, quality differences between storage conditions may be significant at household level when virgin olive oil is exposed to oxygen. Additionally, storage time was reduced from 4 months to less than 2 months with oxygen exposure. The rapid loss in quality in the presence of oxygen calls for further consumer education and sensitization on the negative impact on virgin olive oil quality once the bottles are opened. In the distribution and retail chain, light exposure through packaging in transparent bottles should be avoided to maintain the quality of virgin olive oil through to domestic use. Furthermore, within the global market, the rationale for monitoring virgin olive oil quality either after oil extraction or at bottling should be revisited as results from this study show likely negative changes in quality if oils are stored for long periods or incorrectly. Consumers should have the confidence that oil labelled “Extra Virgin” is extra virgin at time of purchase.

(iv) During the entire virgin olive oil production process, volatile compounds showed the greatest changes followed by phenolic compounds and the least changes were observed with quality indices (FFA, PV, K_{232}, K_{270}). This observation emphasises the importance of volatile and phenolic compounds in detecting virgin olive oil changes that could otherwise be missed when monitoring oil quality is based on these indices alone.

When single virgin olive oil production steps were considered, olive fruit differences from cultivars and maturity were critical in determining the volatile and phenolic compounds. During oil extraction, the control of volatile compounds was critical. After the oil is extracted, volatile compounds were critical in the short term of oil storage but phenolic compounds and quality indices were significantly ($p < 0.01$) affected with prolonged storage of over two months. This identification of critical production steps may imply that both aroma and taste may be controlled through fruit properties whereas transfer of the best sensory attributes from

the fruit is critically controlled during oil extraction. Once olive oil is produced, maintenance of aroma and taste is critically controlled by the storage conditions.

However, virgin olive oil changes during production from olive fruit to oil are not affected in isolation with single production steps but multiple production steps are inter-related and define the ultimate quality of virgin olive oil during consumption. The dominant identification of critical changes in volatile compounds during fruit maturation and phenolic compounds during oil storage and fruit maturation illustrate the inter-relationships in virgin olive oil sensory changes from fruit to oil during consumption. In general, phenolic and volatile compounds quality attributes can be targeted for maximum transfer from olive fruit to oil while taking into account the impact on aroma, bitterness and pungency in fresh oils and the subsequent loss in quality during consumption. This study illustrates the importance of considering the entire virgin olive oil production process, from the olive fruit to oil during consumption, in the production and maintenance of premium quality virgin olive oil.

8.2 Suggestions for Further Work

8.2.1 Changes in olive oil quality and composition resulting from fruit storage.

Fruit storage is inevitable when harvesting capacity exceeds processing capacity. Ideally, storage conditions should preserve olive fruit quality and minimize fruit quality deterioration, which might introduce sensory defects in olive oil (IOOC, 1990, Di Giovacchino, 2000). Unfortunately, fruit storage has been reported to introduce defects in olive oil, such as “mustiness” and “fustiness”, which result from microbial damage during fruit storage (Morales et al., 2005).

However, in **Section 4**, a study with *Frantoio* olive fruit, indicated that virgin olive oil quality is lost within the first week of low temperature fruit storage and re-gained at two weeks. Changes in olive fruit components during low temperature fruit storage have shown similar trends with sensory notes (**Sections 4.3.2** and **4.3.3**). Phenolic compounds, such as oleuropein derivatives; ligstroside derivatives; and (+)-acetoxypinoresinol, showed similar trends to sensory notes related to taste (**Section 4.3.2**) whereas levels of *trans*-2-hexenal and hexanal corresponded to sensory notes associated with aroma (**Section 4.3.3**).

These observations suggest that low temperature olive fruit storage may be beneficial, with a possibility of moderating the sensory quality of virgin olive oils and increasing oil yield. These observations need further investigations to find out if the regaining of quality is cultivar and/or storage condition dependent, and finally determine the optimum storage period for the promotion of positive sensory attributes in olive oil. Variations in storage temperature and humidity might be explored for the benefit of oil yield and quality. Loss of water from the olive fruit skin surface through controlled humidity and storage temperature might be advantageous in difficult-to-extract olives, i.e. olive fruits with high moisture contents (IOOC, 1990), by reducing the moisture content thereby increasing the oil yield. Similarly, the reduced moisture content of the olive fruits realised through fruit storage might change the concentration of olive fruit components to levels where they are either maximally transferred from the olive fruit to oil or attain levels that are optimal for the generation of positive quality attributes.

8.2.2 Alternative processing conditions and oil extraction methods.

Alternative processing conditions of elevated malaxation temperatures (≥ 35°C) and short malaxation times (< 30 minutes) have been previously proposed (Morales & Aparicio, 1999) for the production of pleasant olive oils with green aroma characteristics. The alternative processing conditions proposed by Morales & Aparicio (1999) fall in the region of non-enzymatic hexanal formation (**Figure 5.6b**),

where hexanal concentration increases with malaxation temperature. In a similar high temperatures ($\geq$ 35°C) and short times (< 30 minutes) region (**Figure 5.5b**), octane formation is minimised. This result of maximising hexanal, associated with green aroma characteristic olive oils (Reiners & Grosch, 1998, Aparicio & Luna, 2002, Morales et al., 2005), and minimizing octane, related to poor quality virgin olive oils (Di Giovacchino, 2000), reinforces the alternative approach of shorter time and higher malaxation temperature for the production of premium virgin olive oil. However, this observation should be further investigated to determine the overall effect of alternative processing conditions on stability and quality attributes and establish if the effect is cultivar or maturity dependent.

Results from this study (**Section 5.4.3**) indicate that the general trend of oxidative degradation with malaxation time and temperature is cultivar dependent whereas hydrolytic degradation during virgin olive oil extraction is dependent on both maturity and cultivar. An option of malaxing at lower temperatures seems ideal for *Mission* during late maturity. However, for *Frantoio* and *Corregiola* oil yield is low with malaxing at lower temperatures unless at extended malaxation times where you gain oil yield at the expense of quality. These observations suggest that it might be imperative to identify the optimum conditions of virgin olive oil production for specific olive fruits to balance oil yield and quality.

Additionally, the validity of transferring bench scale results to industrial scale processing should be further tested. In this study (**Section 5.6**), the optimal processing conditions for virgin olive oil between bench and industrial scale processing were similar for C6 and C5 volatile compounds; secoiridoids (**Appendix 1**); PV; and FFA. However, some observations, such as optimal processing conditions for (+)-acetoxypinoresinol and oil yields (**Section 5.6**), were not immediately transferable from bench to industrially extracted oils. This calls for further investigations into factors that might affect the transfer of results from bench to industrial scale processing. Furthermore, the different industrial oil extraction systems, such as three-phase centrifugation; percolation; and pressing (Di Giovacchino, 2000), should be further investigated to identify critical production steps and establish commonalities between the different oil extraction systems.

8.2.3 Effect of Low Temperature Olive Oil Storage on Quality.

Once olive oil is extracted, it is stored under different storage conditions. Observations from **Section 6** indicate that storage conditions affect olive oil differently. Among the oil storage conditions investigated in **Section 6**, light; dark; and low temperature, olive oil stored at low temperature maintained its proximity to fresh oil as observed in **Figure 6.1**. Regardless of the presence or absence of headspace, low temperature appeared to slow the rate of conversion of hydroperoxides to secondary oxidation products as indicated by the low K_{270} value (**Section 6.4.1**), which is consistent with the report of Velasco and Dobarganes (2002). This slowing down in oxidative deterioration might be applied in the storage of bulk oils or oil-rich food products. Anecdotal evidence shows that currently low temperature oil storage is rare and further research is needed to determine what happens to other components of olive oil as freshness and quality of the oil are preserved.

On the other hand, observations from this research (**Section 6.5.2**) have shown that low temperatures storage might cause loss in compounds associated with bitterness, such as oleuropein derivatives, but with maintenance of pungency from ligstroside derivatives. This observation can be applied in debittering of olive oil, which has been achieved through heating the olive fruit (Garcia et al., 2005) producing olive oils with a decreased oxidative stability. Low temperature oil storage might be a cost effective way of debittering in terms of energy consumption since heat is only transferred with the product, olive oil, unlike in fruit debittering where some of the heat is spent on the plant material that eventually goes to the waste as pomace. Additionally, debittering based on oil maintains the freshness of olive oil (**Section 6.3.1**) with the maintenance of pungency (**Section 6.5.2**). Low temperature olive oil storage therefore calls for further investigations into the optimal storage temperatures and an exploration in the maximisation of the benefits of this type of oil storage in moderating and maintaining quality at consumer level.

9. References

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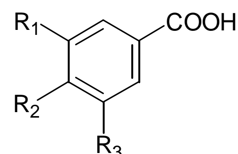
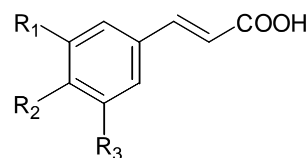
Appendix 1

Chemical Structures of Common Phenolic Compounds in Virgin Olive Oil and Olive Fruits.

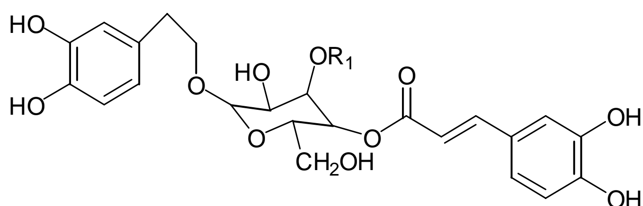
Phenolic acids - Benzoic acids	R_1	R_2	R_3
Gallic acid	OH	OH	OH
Protocatechuic acid	OH	OH	H
Vanillic acid	OCH_3	OH	H
Syringic acid	OCH_3	OH	OCH_3



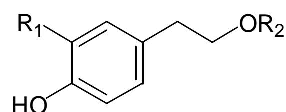
Phenolic acids - Cinnamic acids	R_1	R_2	R_3
Caffeic acid	OH	OH	H
p-Coumaric acid	H	OH	H
Ferulic acid	OCH_3	OH	H



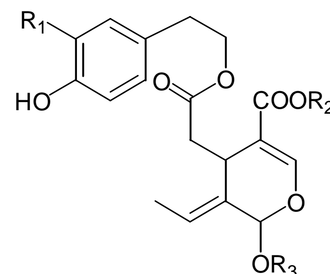
Phenolic acid derivative	R_1
Verbascoside	Rhamnose



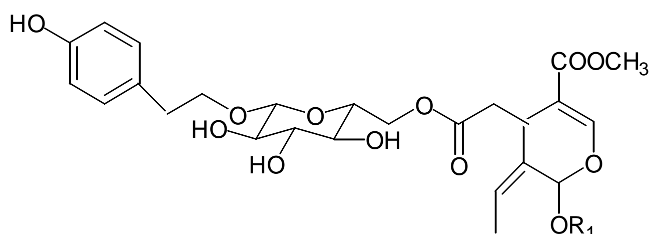
Phenolic alcohols	R_1	R_2
Hydroxytyrosol	OH	H
Hydroxytyrosol glucoside	OH	Glucose
Tyrosol	H	H
Tyrosol glucoside	H	Glucose



Secoiridoids	R_1	R_2	R_3
Oleuropein	OH	CH_3	Glucose
Oleuropein aglycon	OH	CH_3	H
Demethyloleuropein	OH	H	Glucose
Ligstroside	H	CH_3	Glucose

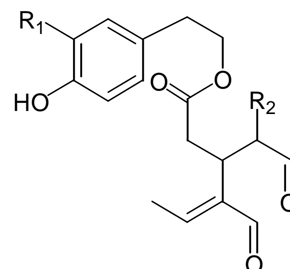


Secoiridoid	R_1
Nuzhenide	Glucose

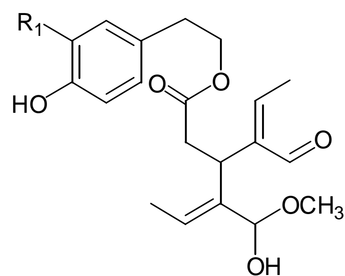


Secoiridoid dialdehydes	R_1	R_2
Ligstroside dialdehyde	H	H
3,4-DHPEA-DEDA [a]	OH	H

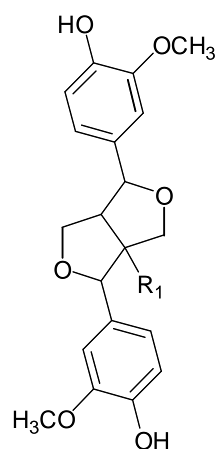
[a] 3, 4 – dihydroxy phenyl ethyl alcohol – decarboxymethyl elenolic acid dialdehyde



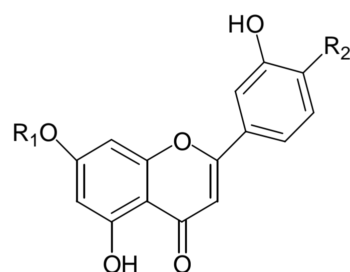
Secoiridoid hemiacetals	R_1
Oleuropein hemiacetal	OH
Ligstroside hemiacetal	H



Lignans	R_1
(+) - pinoresinol	H
(+) - acetoxypinoresinol	CH_3COO



Flavonoids	R_1	R_2
Luteolin	H	OH
Luteolin - 7- glucoside	Glucose	OH
Luteolin - 7- rutinoside	Rutinoside	OH
Hesperidin	Rutinoside	OCH_3



Appendix 2 Rapid assessment of oil defects with zNose® technology

Strictly speaking, the extra-virgin classification belongs to oils that have undergone sensory evaluation by a trained sensory panel. The aim is to detect oils that carry sensory defects – extra-virgin oils have no defects. Currently Australia has two IOOC accredited sensory panels. There is great demand on these panels through the shear volume of oil requiring sensory evaluation. In conjuction with Dr Andrea Bishop and Professor Graeme Batten of CSU (and funded by CSU), one of the authors investigated the use of zNose® technology to detect oils with defects with the aim of using the technology as an adjunct to sensory panel testing.

Rapid vapour analysis from oil headspace was undertaken with a zNose® - a new generation electronic nose. Electronic noses are very fast, portable gas chromatographs capable of rapid oil aroma analysis: analysis time is typically less than 2 minutes. Separated vapour phase components are detected and may be displayed on a "vapour print". **Figure 1** below illustrates vapour prints for oil defect standards and a range of commercial oils. The technique has considerable potential for the monitoring of volatile production, and hence in quality control applications.

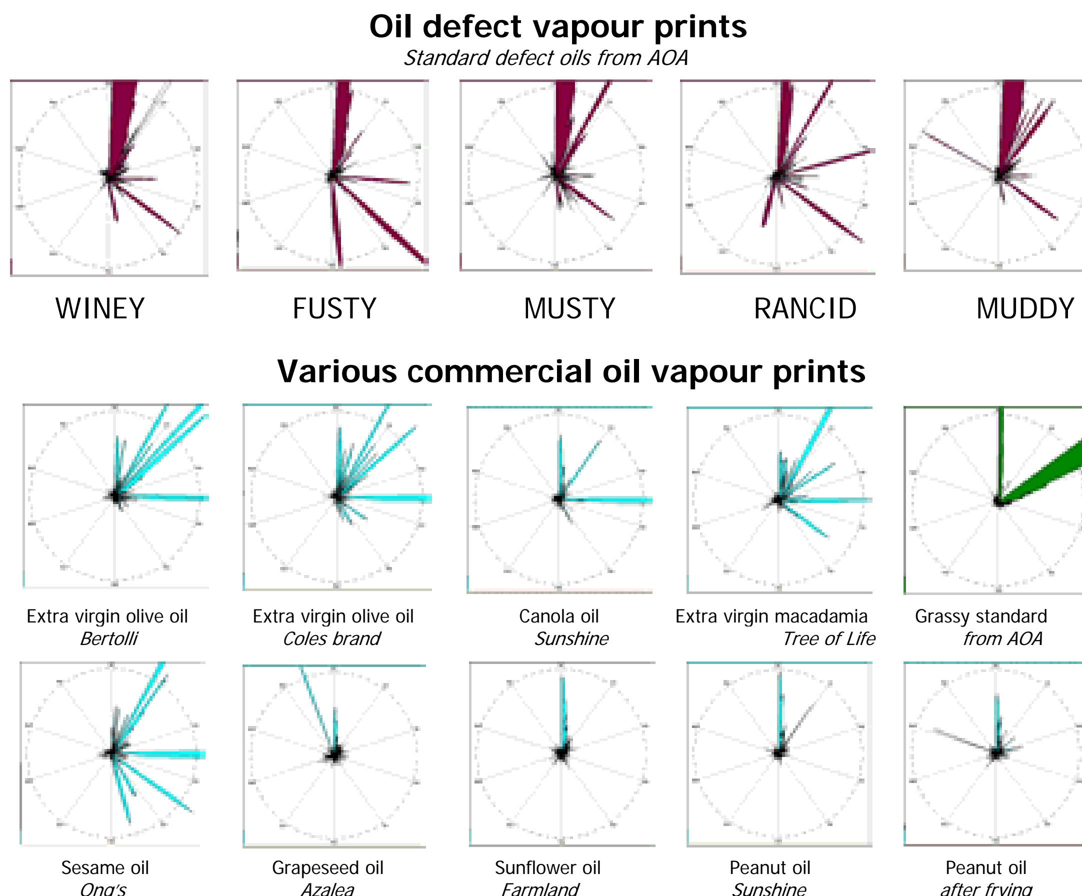


Figure 1. Vapour prints of standard defect olive oils and other commercial oils.

Very different vapour prints were generated from each of the standard defect olive oils. This work illustrates the enormous potential of the zNose® to detect oils with defects and hence relieve the strain on tasting panels. Objective, instrument based methods of analysis have obvious advantages over subjective, human-based testing – IF they prove to be as sensitive to particular aromas as the human nose. This aspect of the work needs further testing.