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Sterols in Australian Olive Oils

— The effects of technological and biological factors —

RIRDC Publication No. 10/173

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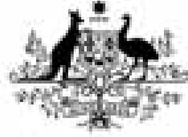

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Sterols in Australian Olive Oils

The effects of technological and biological factors

by Claudia Guillaume[1], Leandro Ravetti[1] and Joshua Johnson[2]

[1]Modern Olives

[2]Victoria University

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Researcher Contact Details

Leandro Ravetti
PO Box 92
LARA VIC 3212

03 5272 9550
03 5272 9599
l.ravetti@modernolives.com.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600

PO Box 4776
KINGSTON ACT 2604

Phone: 02 6271 4100
Fax: 02 6271 4199
Email: rirdc@rirdc.gov.au.
Web: http://www.rirdc.gov.au

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Foreword

Plant sterols have been found to be effective in lowering elevated cholesterol, and are now being added to a wide range of foods.

This report evaluates the horticultural and processing practices that impact on the sterol content and profile of the most important Australian olive varieties. The report also provides biochemical and genetic information which allow a better understanding of the dynamics of sterols in olives and olive oil. The research was conducted by Modern Olives and was supported by RIRDC, the Australian Olive Association and the Australian olive industry.

The outcome of this study reinforces that Australian oils have healthy and high quality characteristics, given their beneficial measured levels of total sterols and Campesterol/Stigmasterol ratio. This ratio provides additional information about processing conditions and their impact on oil quality. When utilised in combination with other traditional oil quality chemical parameters, it can allow an objective and cost effective comparison between the overall quality of different oils produced from the same variety. The results also indicate that Australian growers and processors should continue to focus on obtaining high quality olive oils by carefully monitoring malaxing temperature and times, as well as conducting prompt processing of the fruit immediately after harvest.

Where Campesterol levels for particular Australian oils are found to be above most common international standards, causes were found to be strongly related to a combination of genetics and environment. They bore no relationship with adulteration or oil quality. Consequently, it is proposed that Australian growers, processors, retailers and government work together towards a modern and consumer oriented olive oil trade standard that includes the natural chemistry of our oils. A new trade standard would also address a number of other issues in current international regulations.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Olives R&D program, which aims to manage investment in research and development by the olive industry and government to build a profitable industry through more efficient production methods; a strong reputation as a supplier of quality products; an expanded domestic and export market opportunities; and a sustainable use of land and water resources.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

About the Authors

Modern Olives is the leading advisory company of the Australian modern olive industry. A highly qualified team with solid horticultural knowledge, extensive experience in modern olive production combined with a vast national and international olive network strengthen its leadership position. Modern Olives Analytical Services and its state of the art olive oil laboratory is another important step in the company endeavour to offer the best possible technical advice and research support. The Modern Olives Technical Team is currently providing technical advice to companies and individuals who have planted more than two and a half million olive trees in various regions of Australia and that have produced approximately 60% of the Australian olive oil during the past two years. Modern Olives Analytical Services currently holds NATA ISO 17025, AOCS and IOC accreditations. The School of Molecular Sciences at Victoria University is highly recognised in the area of the biological research and has a long record of activity in this area.

Leandro Ravetti's involvement with modern olive production covers a continuous period of 15 years. In Argentina, he graduated, after a six-year degree course, as an Agricultural Engineer. Between 1996 and 2001, Leandro has worked for the National Institute of Agricultural Technology specialising in Olives. He has also studied and worked as an invited researcher at the Olive Growing Research Institute of Perugia, Italy and at different Olive Institutes in Andalusia, Spain. In 2001, Leandro moved to Australia and during the last 8 years, he has been providing technical advice to some of the largest olive projects in Australia, as well as conducting applied research in different areas of the industry.

Claudia Guillaume finished her studies in Argentina as a Food Scientist and olive oil specialist. After working for several years in the areas of quality, authenticity and organoleptic testing in a commercial food laboratory, Claudia completed a Post Graduate course in Fats & Oils at the Fats & Oils Institute in Seville, Spain. During this course Claudia extensively studied and researched analytical techniques for oil quality, authenticity and organoleptic tests. In 2006, Claudia moved to Australia to assist with the set up of Modern Olives Laboratory Services and she has been its Technical Manager since then.

Dr. Joshua Johnson is a lecturer at the Werribee campus of Victoria University and has 9 years experience in plant molecular biology research, including extensive work on characterising gene families in plants. This research has been published in a number of papers in international scientific journals and presented at international conferences.

Acknowledgments

Special thanks to Rural Industries Research and Development Corporation for their financial support of this project as well as to the Australian Olive Association and Boundary Bend Limited.

Abbreviations

AOA	Australian Olive Association
AOCS	American Oil Chemists' Society
EVOO	Extra Virgin Olive Oil
E+U	Erythrodiol and Uvaol
IOC	International Olive Council
IUPAC	International Union of Pure and Applied Chemistry
MOLS	Modern Olives Laboratory Services
NATA	National Association of Testing Authorities
RIRDC	Rural Industries Research and Development Corporation
USDA	United States Department of Agriculture

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Executive Summary

What the report is about

This report analyses the horticultural and processing practices that may have an impact on the sterol content and profile of the most important Australian olive varieties. It also provides biochemical and genetic information to allow a better understanding of the dynamics of sterols in olives and olive oil. The information generated by this project aims to address a regulatory barrier and also to maximise the nutritional and health value of Australian olive oils. The development of an Australian Standard for olive oil trade and Australia's position in a number of international forums regarding olive oil regulations will be strongly supported by the outcomes of this report.

Who is the report targeted at?

This report is targeted at the relatively new and actively growing group of olive oil producers and trading companies in Australia. Establishing clear parameters for buying, selling and blending oils that may have problems in meeting international standards for sterols has recently become particularly important. It is also targeted to those members of private or public agencies involved as policy makers for the future Australian and International Standards for olive oil.

Background

Plant sterols have been found effective in lowering elevated cholesterol reporting no adverse health effects and are now being added to a wide range of foods. Ironically, international standards for olive oil set a low limit for phytosterols. Due to natural variations in oils from new growing areas – such as Australia – some Australian oils have higher levels of phytosterols. It has been found that a significant number of samples of varieties cultivated in Australia do not meet international olive oil standards as regards sterols. The total content of sterols and their composition in olive oil seem to be influenced not only by genetic factors but also by management and processing techniques. The Australian olive industry is currently targeting this issue. Research projects led by Dr Rod Mailer comprehensively cover the area of variety and environment characterisation. This new project complements this research by analysing the horticultural and processing practices that may have an impact on the sterol content and profile of the most important Australian varieties, as well as generating biochemical and genetic information for a better understanding of the dynamics of sterols in olive oil.

Aims/objectives

The aim of the report was to improve processing and product quality while meeting national and international standards through supporting consistent production of high quality, healthy and safe olive oil that meets consumer expectations and in which they have confidence. Monitoring season to season experiences in Australia was another important objective of the research as well as elucidating the enzymatic pathways that influence sterol production in olives and to characterise the genes that encode these enzymes.

By knowing the influence of major horticultural and olive oil processing practices on total sterols and their composition in different olive varieties, growers and processors will be better prepared to plan the management and processing of their fruit, minimising the amount of oil that eventually does not meet international criteria and maximising the nutritional value of their product.

Methods used

The evaluation of horticultural and olive oil processing practices on total sterols and their composition was undertaken in commercial groves in Victoria. Fruit from three different varieties (Frantoio, Barnea and Picual) with clearly different sterol profiles were crushed. The horticultural and processing practices evaluated were: Irrigation, fruit size, maturity, malaxing time, malaxing temperature, delays between harvest and processing and storage time.

Three repetitions of each treatment were implemented during the 2007 and the 2008 seasons. The sterols analyses were conducted according to the official method IOC/T.20/N°10/Rev. 1. In addition to the previously detailed and initially planned processing and growing parameters, Modern Olives Laboratory Services conducted further studies associated with the sterol composition of the oil extracted from different parts of the fruit and the sterol characteristics of oils produced from pitted olives in comparison with normal whole olives.

In order to gain an understanding of the biological basis for differences in the sterol content and composition of Australian olive oils, the biosynthetic pathways involved in the synthesis of these compounds were explored at the molecular level. Knowledge of the genes encoding the sterol biosynthetic enzymes in other higher plants was used to isolate the homologous genes from olives. Characterisation of these genes was undertaken to identify any correlation between these genes, their expression and the sterol profiles of different varieties of olive.

Results/key findings

Processing practices had a significant impact on the concentrations of triterpene dialcohols and stigmasterol. In the particular case of the stigmasterol, these results would support the campesterol/stigmasterol ratio as an index of quality of oil. This ratio provides additional information about the general processing conditions and, if utilised in combination with other traditional oil quality chemical parameters, it could provide a good way of comparing the overall quality of different oils produced from the same variety.

The sterol composition of the oils obtained from different fruit tissues supports these conclusions as triterpene dialcohols and stigmasterol tend to be in significantly higher concentrations in the pit/seed and skin/outer flesh obtained oils.

On the other hand, irrigation and fruit characteristics such as maturity and size have a significant effect on β-sitosterol, sitostanol, Δ5-avenasterol and Δ7-avenasterol. Consequently, the relationships between them could potentially be used to determine optimal harvesting times.

Finally, there is a very strong influence of the variety on sterol composition, particularly in the case of certain sterols such as campesterol, stigmasterol, apparent β-sitosterol and total sterols. Based on this variety specificity, it should be possible to include in the current international legislation specific references to those varieties that do not normally comply with the authorised levels for the different sterols.

Australian oils have shown good levels of total sterols and comparatively good Campesterol/Stigmasterol relationships, highlighting their healthy and high quality characteristics. Campesterol levels above most common international standards for certain Australian oils are strongly related to the combination between genetics and environment (phenotype) and they have no relationship with adulterations of any kind or with oil quality issues. No evaluated management or processing practice seems to have contributed to reducing Campesterol levels.

Implications for relevant stakeholders

Based on current and most recognised international regulations on olive oil (IOC, EC and Codex Alimentarius), approximately 40 to 45% of high quality and authentic Australian oils could not be classified as olive oil – largely due to natural levels of campesterol in many Australian extra virgin olive oils being higher than the standards proscribe.

While the importance of sterol analysis for the evaluation of olive oil authenticity should be acknowledged, it is important to highlight that not addressing the natural variations shown by a significant proportion of oils produced around the world is currently being used as a trade barrier.

If we consider that Australia currently produces approximately 15,000 tonnes and it could double this production over the next decade, such trade barriers, unless properly addressed, could represent losses for the industry of up to AU$ 18,000,000 by 2010 and AU$ 30,000,000 per year by 2016.

Over the past years, a number of European scientists have recognised this problem and proposed alternative solutions (Conte, L. 2008 and Conte, L. 2009). Nonetheless, the proposed solution regarding Campesterol levels will not address the problem. Our research in this paper shows that this proposal would be completely ineffective to fix the problem with high Campesterol level oils. Proposed solution referred to a decision making tree where for those oils with campesterol higher than 4.0%, apparent B-Sitosterol has to be higher than 94.0% (instead of 93.0% as it is stated in most standards) to be considered olive oil. As it is explained in the sterols biochemistry paragraph, Campesterol and B-Sitosterol are, mostly, alternative biosynthetic paths. Consequently, when one is higher the other is naturally lower. Achieving both simultaneously is extremely rare. Our research data show that only less than 1% of the oils with campesterol above 4% will have an apparent B-Sitosterol level above 94%. See page 24.

Recommendations

Australian oils have shown good levels of total sterols and comparatively good Campesterol/Stigmasterol relationships, highlighting their healthy and high quality characteristics. Producers should maintain their high quality standards and use these chemical characteristics to promote it. Campesterol levels above most common international standards for certain Australian oils are strongly related to the combination between genetics and environment (phenotype) and they have no relationship with adulterations of any kind or with oil quality issues.

Australian growers and processors should continue focusing on obtaining high quality olive oils by carefully monitoring malaxing temperature and times as well as conducting a prompt processing of the fruit immediately after harvest as no evaluated management or processing practice seems to have contributed to reducing Campesterol levels.

Australian growers, processors, retailers and government should work together towards a modern and consumer oriented olive oil trade standard that reflects the natural chemistry of our oils and addresses not only this problem but a number of other issues present in current international regulations.

Furthermore, the AOA and appropriate Australian government agencies should utilise this information in International Forums while discussing legislation on olive oil. It is important that Australia continues pursuing more appropriate olive oil standards and opposes some of the proposed changes that will wrongly classify a significant proportion of Australian oils.

Introduction

What are phytosterols and why are they good for us?

Phytosterols have similar functions than Cholesterol but different origins. Cholesterol is found in animals and humans while phytosterols occur only in plants. Cholesterol is a very important molecule in animals and humans, serving as a vital constituent of cell membranes and a precursor to various biomolecules. Phytosterols serve similar functions in plants.

Phytosterols are found in vegetables and nuts as well as other foods and are therefore part of our modern day diet but not in the quantities necessary to exert health benefits. Phytosterols differ from cholesterol in small but significant ways. Due to these structural differences, phytosterols are not well absorbed in animals or humans. The vast majority of the phytosterols ingested remain in the gastrointestinal (GI) tract. In healthy humans, absorption is limited to approximately 5% of the total beta-sitosterol and approximately 15% of the campesterol ingested. Campesterol is one of the most important Phytosterols together with Beta-Sitosterol.

Numerous efficacy studies since 1950 have proven that phytosterols are highly effective in lowering cholesterol levels. Phytosterols decrease the transport of cholesterol to absorption sites by excluding it from the transport mechanism. There are two proposed mechanisms by which phytosterols decrease serum cholesterol levels. Both mechanisms are based on the similar physical and chemical properties of phytosterols and cholesterol.

While the effects of phytosterols in lowering cholesterol levels are well studied, there are indications that phytosterols may have additional benefits in areas such as benign prostatic hyperplasia. In addition, there is evidence suggesting that beta-sitosterol can suppress in vivo carcinogenesis. Human prostate cancer cells were studied in vitro comparing the effects of cholesterol vs. beta- sitosterol. It was found that the presence of beta-sitosterol decreased cell growth by 24% and increased the rate at which the cancerous cells died by four-fold.

There are reports of further beneficial effects of phytosterols, alone and in combination with other naturally occurring compounds. Phytosterols are discussed as having possible immune modulatory and anti-inflammatory activities as well as being anti-ulcer and anti-diabetic agents.

What is olive oil and why are sterols used in trade standards?

According to all olive oil regulations around the world “Olive oils are the oils obtained from the fruit of the olive tree to the exclusion of oils obtained using solvents or re-esterification processes and of any mixture with oils of other kinds”.

Different vegetable oils show a large variability of sterol compositions. As olive oil has a fairly stable sterol composition, this enables a relatively reliable detection of any adulterations with other types of vegetable oils. This fraction is determined by gas chromatography and it is not a quality measurement (it doesn’t say if an oil is better or worse), it is an authenticity test (it intends to indicate if it is olive oil or not). The biggest limitation of this method is that the international limits for the different phytosterols have been determined several years ago without considering natural variations originated in new growing areas and from new varieties.

The situation in Australia

It has been previously reported that sterol composition and total sterol content would be affected by cultivar, crop year, degree of fruit ripeness, storage time of fruits prior to oil extraction, processing and by geographical factors (Ranalli, A. and Angerosa, F., 1996; Koutsaftakis, A. et al., 1999; Aparicio, R. and Luna, G., 2002; Ranalli, A. et al., 2002; Boskou et al., 2006). It has been found that a significant amount of samples of largely cultivated varieties in Australia do not meet international standards as regards sterols. The Australian olive industry is currently targeting this problem. Research projects led by Dr Rod Mailer comprehensively cover the area of variety and environment characterisation. This project complements this research by analysing the horticultural and processing practices that may have an impact on the sterol content and profile of the most important Australian varieties as well as generating biochemical and genetic information for a better understanding of the dynamics of sterols in olive oil. As indicated in the above paragraphs, plant sterols have been found effective in lowering elevated cholesterol with no reported adverse health effects, and are now being added to a wide range of foods. The information generated with this project does aim to solve a legislation problem, and also to maximise the nutritional and health value of Australian olive oils.

Recent analyses have identified that Australian olive oils have significant amount of sterols. Some Australian olive oils do not meet international standards for total content of sterols or for certain minor components (Mailer, 2006). The cultivar Barnea oils, in particular, contain up to 5.8% Campesterol, as confirmed by the Australian Government Analytical Laboratories (2004), although the standard requires that the oil must show less than 4.0%. Some cultivar Frantoio oil samples have shown extremely low total sterol levels, barely above or even under the minimum 1,000 ppm established as international limit. It is extremely important to point out that Barnea oil represented 41% and Frantoio oil 26% of the olive oils produced in Australia in 2006 (Ravetti, 2006). Exploratory research conducted by Modern Olives during the past three years (Ravetti, 2006) would indicate that there are some significant correlations between cultural and processing practices and sterol content and composition.

Biological Factors affecting sterol composition

A review of the research that elucidated the sterol biosynthetic pathway of plants by Benveniste (2004) provides a summary of the current state of knowledge about the enzymes and substrates involved in the synthesis of phytosterols, primarily from data generated in the model plant *Arabidopsis thaliana* but can be applied to studying olives. This research has revealed that the pathway is essentially linear from the metabolite cycloartenol through six enzyme catalysed steps to 24-methylene lophenol (Figure 1). Thereafter, the pathway goes through a bifurcation resulting in two separate pathways leading to the formation of sitosterol or campesterol, respectively (Figure 1). Analysis of this pathway reveals that the sterol biosynthetic flux towards sitosterol or campesterol is primarily controlled by two branchpoint enzymes, SAM-24-methylene lophenol-C-24-methyl transferase (SMT2) and C4α sterol-methyl oxidase (SMO2). This suggests that the relative amounts of sitosterol and campesterol are co-regulated by the activity of these two key enzymes and provided the basis for the hypothesis of the current project: that the relative amounts of campesterol and β-sitosterol observed in Australian olive oils is due to amino acid differences in the SMO2 and/or SMT2 enzymes, or in the expression of these enzymes during the development of the fruit. This hypothesis is further supported by direct experimental evidence in *Nicotiana benthamiana* which revealed that the down-regulation of SMO2 led to the accumulation of 24-methylene lophenol and 24-ethylidene lophenol (Figure 1) (Darnet and Rahier, 2004) a result that has led Schaller (2004) to speculate that SMO2 may potentially play a role in determining the relative levels of campesterol and sitosterol in plant tissues. Further evidence generated in tobacco revealed that the upregulation of SMT2 resulted in an increase in sitosterol (and decrease in campesterol) whereas its down-regulation had the opposite effect, ie. increase in campesterol (and decrease in sitosterol) (Schaeffer *et al.*, 2001). Thus, experimental evidence implicates both SMT2 and SMO2 in the determination of relative levels of sitosterol and campesterol in plants.

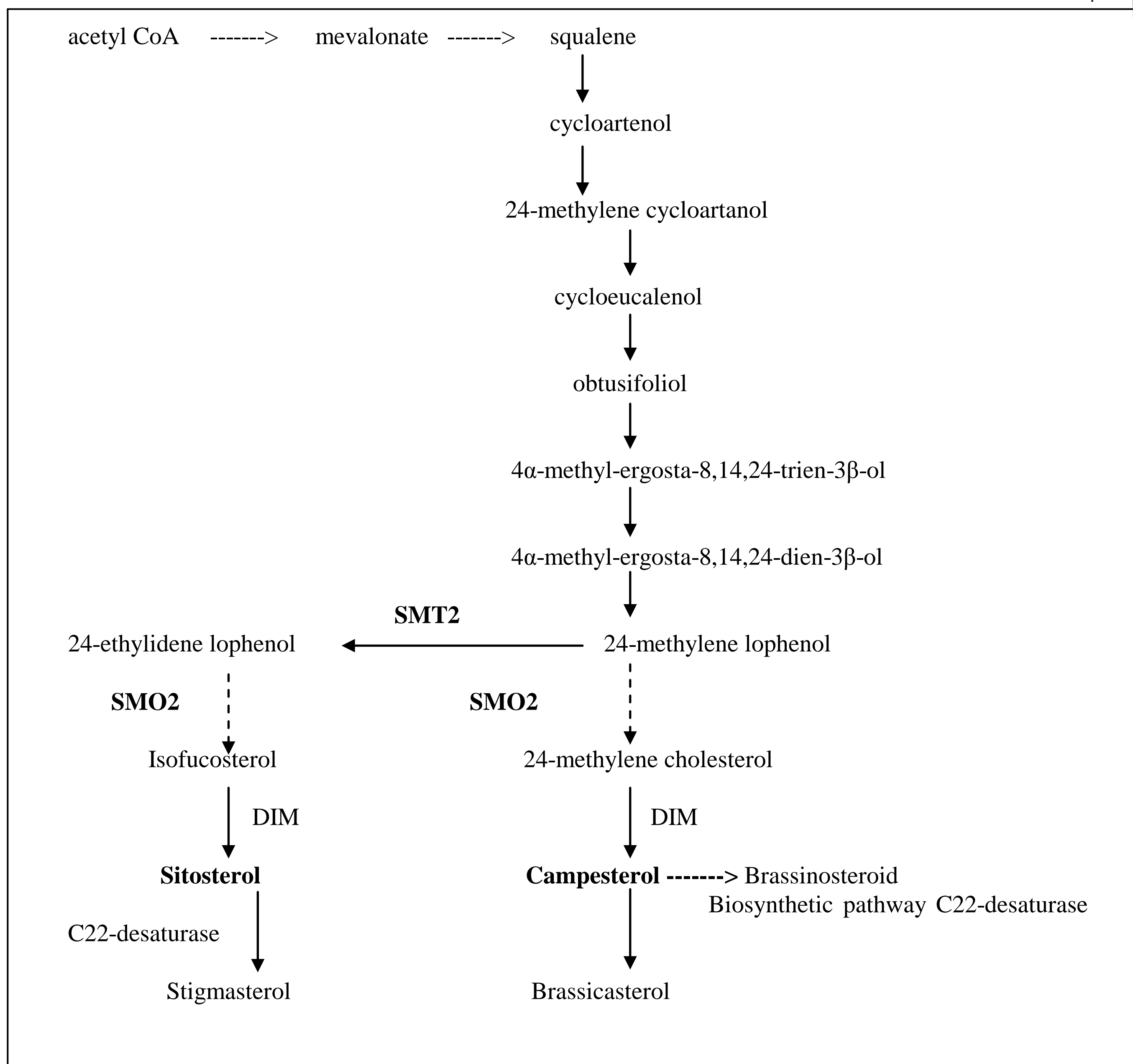

Figure 1 - A simplified outline of the plant sterol biosynthetic pathway

Schematic diagram of the biosynthetic pathway of acetyl CoA to 24-methyl and 24-ethyl sterols. Dashed arrows represent more than one biosynthetic step not indicated here. The key steps catalysed by SMT2 and SMO2 are highlighted in bold.
(Figure adapted from Benveniste, 2004).

Objectives

This work complements previous research by analysing the horticultural and processing practices that may have an impact on the sterol content and profile of the most important Australian varieties, as well as generating biochemical and genetic information for a better understanding of the dynamics of sterols in olive oil. The information generated aims to solve a legislation issue, and also to maximise the nutritional and health value of the Australian olive oils.

By knowing the influence of major horticultural and olive oil processing practices on total sterols and their composition in different olive varieties, growers and processors will be better prepared to plan the management and process of their fruit, minimising the amount of oil that does not meet international criteria, and maximising the nutritional value of their product.

From the biological point of view, assessing the potential role of the different enzymes in determining the relative amounts of campesterol and sitosterol in Australian olive oils, the current research aimed to:

1. Isolate the genes encoding these enzymes from three varieties of olive that differ in their sterol profiles, namely, *Barnea*, *Frantoio* and *Picual*;
2. Sequence these genes to identify any allelic differences that may explain the varying sterol profiles; and
3. Profile the expression patterns of these genes over the development of the fruit to identify any significant differences that may impact the relative sterol concentrations.

Methodology

Modern Olives Laboratory Services

This study was carried out at the Modern Olives Laboratory Services state of the art laboratory at Lara, Victoria. The laboratory staff is well experienced in olive oil research and quality evaluation. The laboratory has ISO 17025 certification through the National Australian Testing Authority (NATA). Claudia Guillaume is an Approved Chemist of the American Oil Chemists' Society (AOCS). MOLS is accredited by the International Olive Council (IOC) for chemical testing of olive oils and by the American Oil Chemists' Society.

Horticultural and Processing Trials

The evaluation of horticultural and olive oil processing practices on total sterols and their composition was undertaken in commercial groves in Victoria. The selected groves are: Boort Estate (Boort, Victoria) and Boundary Bend Estate (Boundary Bend, Victoria).

The management and climatic conditions of each grove during the trial period was recorded. All proposed groves have automatic weather stations, which enabled them to record temperature, humidity, wind speed, radiation and rainfall. Considering that most physiological aspects related to sterol formation and ripening processes in the fruit are related to one or more of those parameters, it is considered that the available information was appropriate for an adequate evaluation of the final results.

The trials have been conducted following a proper statistical design. Fruit from three different varieties (Frantoio, Barnea and Picual) with clearly different sterol profiles were crushed. The significant differences between those varieties that justified their selection are well documented (Ravetti, 2007 & Mailer et al, 2007).

Frantoio: Average total sterol levels of 1,490 ppm and average campesterol of 3.05%.

Barnea: Average total sterol levels of 1,700 ppm and average campesterol of 4.50%.

Picual: Average total sterol levels of 1,500 ppm and average campesterol of 3.40%.

The fruit was processed in an experimental olive oil mill (Abencor®). The Abencor® bench top extraction system provides a fast and inexpensive means to obtain a sample of oil, operating in accordance with the world-wide known similar sampling systems. The full process is developed under the sight of the operator, by faithfully reproducing the work of an olive mill without addition of any solvent. The oil extraction efficiency index attained is very near to the industrial efficiency to be achieved in the plant for most varieties. The quantity of olives needed to get a sample ensures that this volume is fully representative regarding the load analysed. Oil obtained is usually enough to perform organoleptic and quality tests. The Abencor® system has been world-wide used since its development in co-operation with the prestigious Instituto de la Grasa (Fats and oils institute) in Seville, Spain. The processing conditions were those standards for this extraction method apart from the variations applied while evaluating malaxing temperatures and malaxing times.

The horticultural and processing practices evaluated were: Irrigation, fruit size, maturity, malaxing time, malaxing temperature, delays between harvest and process and storage time.

Irrigation: Kc (Crop Factor) of 0.74 during the oil accumulation period (January-April) (Normal treatment); Kc of 0.32 during the oil accumulation period (1/2 X) and Kc of 1.48 during the oil accumulation period (2 X).

Fruit size: Fruit of the different varieties was classified with a table olive fruit grader into three standard sizes. For Barnea: Small (< 2.00 grams), medium (2.00 – 3.00 grams), large (> 3.00 grams). For Frantoio: Small (< 1.40 grams), medium (1.40 – 2.00 grams), large (> 2.00 grams). For Picual: Small (< 2.20 grams), medium (2.20 – 3.20 grams), large (> 3.20 grams).

Maturity: Fruit from the three varieties was harvested at three different times, two to three weeks apart between them. Maturity was measured using the maturity index developed by the CIFA Alameda del Obispo, Spain. The fruit from the early harvest typically showed a MI between 1.00 and 2.00, from the middle harvest between 2.50 and 3.50 and from the late harvest between 4.00 and 5.00.

Malaxing time: Three malaxing times were utilised: 30 minutes (Standard), 15 minutes (1/2 X) and 60 minutes (2 X).

Malaxing Temperature: Three malaxing temperatures were utilised: 25ºC (Standard), 15ºC (Cold) and 35ºC (Hot).

Delays between harvest and process: Three times between harvest and process were applied: Immediate processing (< 12 hours), medium processing (36 - 48 hours) and delayed processing (72 – 84 hours).

Storage time: All samples processed were analysed immediately after processing, 6 months later and 12 months later.

Three repetitions of each treatment have been processed. Each repetition typically consisted of two mixing units of 700 grams of olive paste each.

Repetitions of each treatment were processed during the 2007 and the 2008 seasons. All samples were evaluated by duplicate.

Analytical methodology

The sterols analyses were conducted according to the official method IOC/T.20/Nº10/Rev. 1. The sterol fraction was analysed by an Agilent Technology 6890N GC system, Agilent Technology 7683B series injector with a split inlet and flame ionisation detector managed by Agilent ChemStation. The analytical column was a DB-5 5% phenyl-methyl-siloxane stationary phase (30m x 0.25mm x 0.25µm). The gas chromatographic conditions were as follows: Inlet temperature: 280ºC; oven temperature 267ºC; detector temperature: 290ºC; split ratio: 30:1; amount injected 1:l. Hydrogen was used as gas carrier at a flow rate of 1.2ml/min. Sterols were quantified using 5α-cholestan-3β-ol as internal standard.

The data subjected to a statistical analysis was assessed through an analysis of variance using the SAS version 8.02 (SAS Institute Inc., Cary, NC, USA). Separation of the means was obtained using the least square means test and significant differences will be defined at $P \leq 0.05$. Every aspect was analysed separately. No interactions were evaluated in this project.

Additional trials

In addition to the previously detailed and initially planned processing and growing parameters, Modern Olives Laboratory Services conducted further studies associated with the sterol composition of the oil extracted from different tissues of the fruit (Exocarp or skin; mesocarp or flesh and endocarp or pit/seed) and the sterol characteristics of oils produced from pitted olives in comparison with normal whole olives.

In the first case, fruit from the Barnea variety was carefully peeled with a sharp scalpel removing the skin and external (< 1mm) flesh layer and pitted. The skin, the crushed pit and the rest of the flesh components were weighed separately and then treated with solvent utilising the Soxhlet method in order to obtain the oil present in them.

In the case of the pitted olives, several large samples of Barnea fruit were pitted and processed through the Abencor system in comparison with batches of entire fruit from the same variety. This trial was also conducted over two years.

Biological evaluations

In order to gain an understanding of the biological basis for differences in the sterol content and composition of Australian olive oils, the biosynthetic pathways involved in the synthesis of these compounds were explored at the molecular level. Knowledge of the genes encoding the sterol biosynthetic enzymes in other higher plants was used to isolate the homologous genes from olives. Characterisation of these genes was undertaken to identify any correlation between these genes, their expression and the sterol profiles of different varieties of olive.

In order to do this, fruit and leaf samples have been taken every two weeks from the beginning of the oil accumulation period (January) until the end of the harvest (July). Tissue samples were processed immediately after sampling and immersed in 5x volume of RNAlater solution. The samples were then stored in a refrigerator, or on ice (4C), overnight to allow the solution to permeate the samples, upon which they were stored in a normal freezer (-20C) until analysed in the laboratory.

Analysis of the sterol biosynthesis pathway was initially performed following the methodology proposed and described by Pierre Benveniste (Biosynthesis and accumulation of sterols. Annu. Rev. Plant Biol. 2004. 55:429-57). The recent publication of the results of high throughput sequencing of RNA from olive tissue using next-generation 454 sequencing (Roche) has provided partial sequences of all genes expressed in the olive fruit of the varieties *Coratina* and *Tendellone* (Alagna *et al*. 2009). This data (available at: http://454reads.oleadb.it/) has provided partial sequences putatively encoding SMO2 and SMT2 in olives. This data is currently being used in the isolation of these genes from the varieties *Barnea*, *Frantoio* and *Picual*.

Results

Effect of maturation index on sterol composition

The evolution of sterols and triterpene dialcohols during maturation is presented in table 3. β-sitosterol, sitostanol, Δ5-avenasterol and Δ7-avenasterol are significantly ($P < 0.001$) affected by maturity index. Among them sitostanol is the one most affected (F value of 65.2). β-sitosterol decrease during ripening, while Δ5-avenasterol and Δ7-avenasterol significantly increase. This result agrees with other research (Koutsaftakis, A. et al., 1999). Nonetheless, β-sitosterol sum and campesterol did not change significantly between ripening stages in disagreement with the same research (Koutsaftakis, A. et al., 1999).

Effect of fruit size on sterol composition

Campestanol, stigmasterol, β-sitosterol, sitostanol, Δ5-avenasterol, Δ7-avenasterol and erythrodiol and uvaol are significantly affected by fruit size. While β-sitosterol, sitostanol and erythrodiol + uvaol significantly decrease with fruit size, Δ5-avenasterol and Δ7-avenasterol do the opposite. This comparison is presented in table 4.

Effect of irrigation on sterol composition

The analysis of the effect of irrigation on sterol and triterpene dialcohols concentrations is presented in table 5. 24-methilene cholesterol, stigmasterol, Δ7-stigmasterol, apparent β-sitosterol and Δ7-avenasterol are amongst the significantly affected compounds. It is noteworthy that while stigmasterol and Δ7-stigmasterol decrease with higher levels of irrigation, apparent β-sitosterol significantly increases.

Effect of malaxing time on sterol composition

The malaxing time at the paste preparation stage is a very important parameter of good manufacturing practice. As indicated in table 6, erythrodiol + uvaol were the only components to be significantly affected ($P < 0.001$) by malaxing time while stigmasterol and Δ7-stigmasterol were affected but to lesser extent ($P < 0.01$). All these components tend to increase with more malaxing time.

Effect of malaxing temperature on sterol composition

Similarly to malaxing time, processing temperature is another important parameter during the olive oil manufacturing process. Correspondingly, erythrodiol + uvaol were significantly affected by malaxing temperature and stigmasterol was one of the few sterols being affected (table 7). Once more, these components tend to increase with more malaxing temperature. This is in agreement with other research work (Koutsaftakis, A. et al., 1999). Additionally, the total level of sterols was significantly affected by this processing parameter showing increasing values at higher malaxing temperatures.

Effect of delay between harvest and process on sterol composition

Like in the case of the other two processing parameters evaluation, the delay between harvest and process significantly affected the percentage of erythrodiol + uvaol and stigmasterol (table 8). Both erythrodiol + uvaol and stigmasterol levels increase with longer delays between harvesting and processing.

Effect of the year on sterol composition

The variations of sterols and triterpene dialcohols between the two years are presented in table 9. Most of these compounds are significantly affected by the year, particularly cholesterol, campestanol, stigmasterol, Δ7-stigmasterol, apparent β-sitosterol, Δ5,23-stigmastadienol and Δ5,24-stigmastadienol. Erythrodiol + uvaol levels were also significantly affected by the season.

Effect of the variety on sterol composition

The effect of the variety on sterol and triterpene dialcohols composition is presented in table 10. It is important to point out that the variety has shown the most significant level of effect on the different sterols. This is in line with other authors (Paganuzzi, V., 1987; Aparicio, R., Morales, M. and Alonzo, T., 1997). Only cholesterol and Δ7-campesterol had levels of significance higher than 0.001. Campesterol, β-sitosterol and Δ7-avenasterol were the most affected with F values of 3125, 368 and 451 respectively. Erythrodiol + uvaol were not significantly affected by variety.

As it is indicated in figure 3, all processing practices had a significant impact on the concentrations of triterpene dialcohols and stigmasterol. In the particular case of the stigmasterol, these results would support the campesterol/stigmasterol ratio as an index of quality of an oil as proposed by other authors (Koutsaftakis, A., 1999). Nonetheless, this index could not be used to compare oils of different varieties as a consequence of the strong influence of genetics on campesterol content.

On the other hand, figure 4 shows that irrigation and fruit characteristics such as maturity and size have a significant effect on β-sitosterol, sitostanol, Δ5-avenasterol and Δ7-avenasterol. Consequently, the relationships between them could potentially be used to determine optimal harvesting times.

Finally, figure 5 clearly demonstrates the strong influence of the variety on sterol composition, particularly in the case of certain sterols such as campesterol, stigmasterol, apparent β-sitosterol and total sterols. Based on this variety specificity, it is possible to include in the current legislation specific references to those varieties that do not normally comply with the authorised levels for the different sterols.

Sterol composition in the different fruit tissues

As it is indicated in table 11, the vast majority of the oil (> 75%) comes from the flesh with similar proportions of the remaining oil being contributed by the pit/seed and by the skin/outer layer of flesh (Table 11). Significant differences were observed regarding certain sterols and associated substances. Stigmasterol and total sterols were significantly higher in the oil produced from the pit/seed fraction, while D7 Stigmasterol and Erythrodiol + Uvaol were particularly higher in the skin/outer flesh fraction (Table 12 and graph 6). This difference can explain why the levels of those sterols tend to increase in the final oil produced when processing conditions deteriorate, particularly associated to higher malaxing times, temperatures or time delays between harvesting and crushing. The relatively constant proportion of Campesterol and B-Sitosterol would confirm that those two sterols could not be used as quality indicators and they are relatively little influenced by processing conditions.

Sterol composition from pitted versus entire fruit

No statistically significant differences have been observed in any of the sterols between the oils produced by crushing the entire fruit (conventional method) versus crushing the pitted olives. While this commercial production technique may have an impact on other oil chemical parameters, it failed to produce significant changes in the sterol composition or in the total sterol levels of the final oils (Graph 7).

Results on preservation and RNA isolation methods

The first challenge to overcome in the biochemical aspects of the research was the development of a reliable method of olive tissue preservation at the time of sampling to ensure that the RNA in the tissues was not degraded and accurately reflected the genes expressed in the olive and the relative abundance of the gene transcripts over the course of fruit development. Ordinarily the tissue would be preserved in liquid N_2 at the time of sampling, however this was not practical at the olive grove in Boort. Therefore protocols were developed for sampling and the RNA*later* (Ambion) RNA preservation reagent was utilised to preserve the samples on site at the grove. Multiple RNA isolation techniques were trialed using fruit collected during the 2007 growing season. These methods revealed all samples exhibited differing levels of RNA degradation and led to the development of stricter harvesting and preservation protocols to ensure that RNA-safe practices were followed during the sampling of fruit during the 2009 growing period. These improvements proved successful with high quality RNA isolated from all 8 time points collected during this growing period (Figure 2).

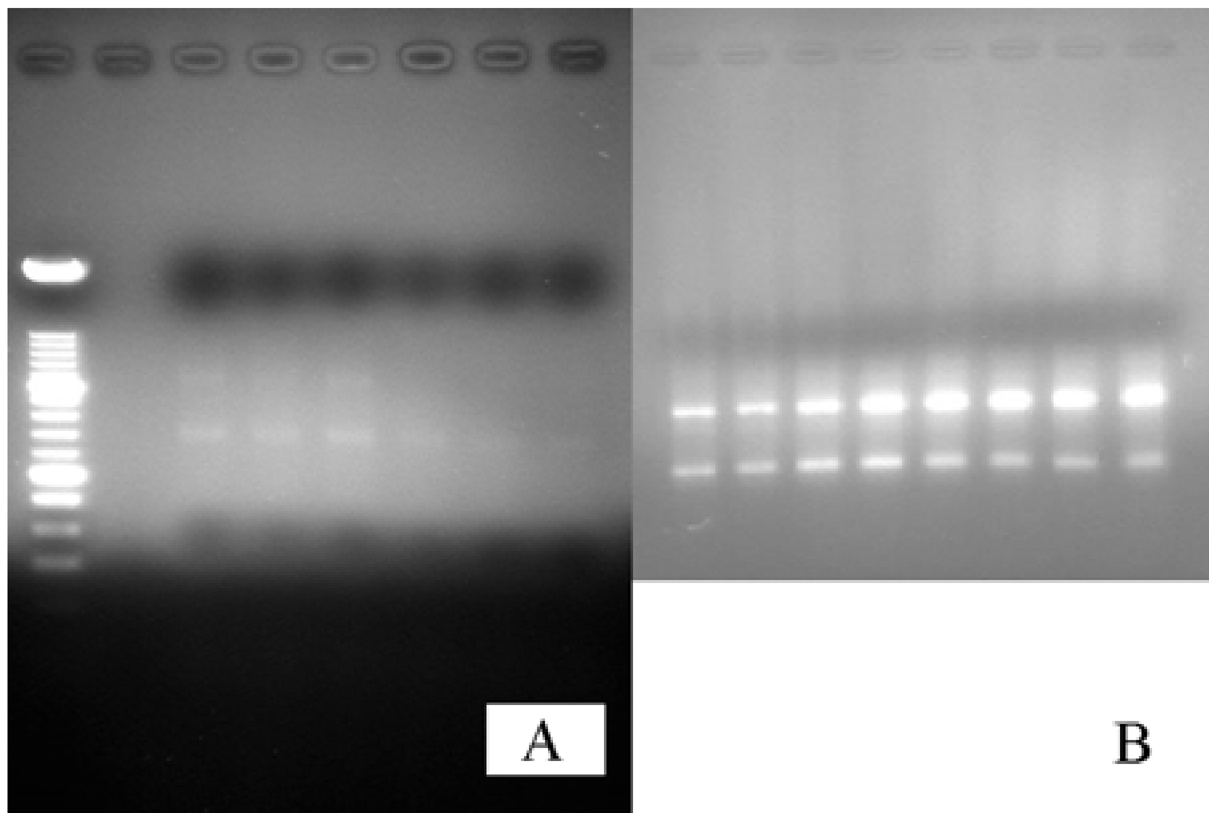

Figure 2 – Assessment of the integrity of RNA isolated from preserved olive tissue

The RNA extracted from eight time points during the development of the fruit (variety *Barnea*) during 2007 (A) and 2009 (B) highlighting the degradation of the 2007 samples.

As no sequence data was available for *SMT2* and *SMO2* genes in olives, initial efforts concentrated on the development of PCR primers based on the sequence of these genes (or partial genes) from related plants, including *A. thaliana*, potato, tomato, tobacco, sunflower represented in the publicly available GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and Plant Gene Indices (http://compbio.dfci.harvard.edu/tgi/plant.html) databases. Conserved sequences in the *SMO2* and *SMT2* genes were used to design consensus primers for the amplification of these genes from olive RNA using reverse transcriptase (RT)-PCR. The results of these efforts are outlined below.

In parallel with the isolation of partial SMT2 and SMO2 genes attempts were made to develop a cDNA library from the RNA isolated during the 2009 growing season (described above). These

efforts only produced low-titre libraries that are of limited use in gene isolation efforts. It is unclear why the olive RNA has proven difficult to clone efficiently in the library; however alternatives to this approach are currently being conducted.

Characterisation of the olive *SMT2* gene(s)

Initial efforts to isolate the SMT2 gene from olive RNA using consensus primers based on the sequence of this gene from other plant species were unsuccessful, probably due to unique sequences in this gene(s) in olives. The screening of the Alagna *et al*. (2009) sequence data identified two sequences that have significant sequence identity to the *SMT2* gene of *A. thaliana* (Table 1). This preliminary result suggests that there are more than one isoform of SMT2 expressed during the development of the olive fruit. Current and future sequencing efforts will determine whether there are any sequence polymorphisms in these genes in different varieties of olive (see below).

Table 1 – Nucleotide and amino acid sequence identities of putative *SMT2* partial genes

	A. thaliana SMT2 cds	OLEEUCl045588	OLEEUCl064665
A. thaliana SMT2 cds(1086bp)	100 (100)	73.9 (84.8)	73.0 (84.3)
OLEEUCl045588* (376bp)	73.9 (84.8)	100 (100)	81.2 (94.1)
OLEEUCl064665* (365bp)	73.0 (84.3)	81.2 (94.1)	100 (100)

* These sequences were identified in the Alagna *et al.* (2009) database.

Characterisation of the olive *SMO2* gene(s)

The initial effort to isolate partial *SMO2* gene(s) from the variety *Barnea* using consensus primers based on *SMO2* gene sequences from other plant species resulted in the successful isolation and sequencing of a 168bp partial sequence (*SMO2-1*). Comparison of this sequence with a putative *SMO2* partial sequence from the Alagna *et al*. (2009) data and the *SMO2* sequence from *A. thaliana* revealed high sequence conservation and interestingly revealed a difference between the olives studied in the Alagna *et al*. (2009) research and the *Barnea* sequence (Table 2). Current sequencing efforts (see below) will determine whether this difference is due to the presence of multiple isoforms of this enzyme in olive, or whether this represents an allelic polymorphism that may impact the activity of SMO2 in *Barnea*.

Table 2 – Nucleotide and amino acid sequence identities of putative SMO2 partial genes

	A. thaliana SMO2 cds	OLEEUCl011741*	*Barnea SMO2-1*
A. thaliana SMO2 cds (783bp)	100 (100)	79.9 (94.8)	82.1 (92.7)
OLEEUCl011741* (234bp)	79.9 (94.8)	100 (100)	89.8 (92.9)
Barnea SMO2-1 (168bp)	82.1 (92.7)	89.8 (92.9)	100 (100)

* This sequence was identified in the Alagna *et al.* (2009) database.

Current sequencing efforts

As described above, the recent availability of olive sequence data (Alagna *et al.* 2009), specifically partial *SMO2* and *SMT2* gene sequences, is being utilized to isolate the entire genes from *Barnea*, *Frantoio* and *Picual* using 5' and 3' RACE techniques. The sequencing of these products will allow the comparison of these genes between the three varieties and identify any differences that may explain the variation in their sterol profiles.

To ensure that no other SMT2 and/or SMO2 isoforms are expressed in the variety *Barnea* total RNA is currently being sequenced using the Illumina/Solexa next generation sequencing method. This will provide data that will identify all of the genes expressed during the development of the olive fruit and provide the sequence data to isolate any additional genes from the varieties *Picual* and *Frantoio* for sequence comparisons.

The data generated from the current 5' and 3' RACE experiments and total RNA sequencing efforts described above will identify any differences in the sequences of the SMT2 and SMO2 enzymes. This sequence data will also be used to develop qPCR probes to assay the expression of the these genes in the developing *Barnea*, *Frantoio* and *Picual* fruit samples from the 2009 and 2010 growing periods. This approach will reveal any differences in the expression of these genes during fruit development that may play a role in determining the different sterol profiles of these varieties.

Table 3

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed from fruit with maturity index of < 2.00, 2.00-4.00 and > 4.00

	< 2	2 - 4	> 4	Std. Err.	F^2	Significance
Cholesterol	0.18 a	0.13 b	0.12 b	0.014	2.055	0.130
24-Methilene cholesterol	0.17 b	0.22 a	0.24 a	0.012	3.375	0.038
Campesterol	3.91 b	3.92 b	4.03 a	0.071	0.300	0.740
Campestanol	0.17 b	0.17 b	0.20 a	0.007	1.930	0.150
Stigmasterol	0.75 b	0.77 b	0.83 a	0.020	1.478	0.230
Δ7-Campesterol	0.22 a	0.08 b	0.11 b	0.016	7.445	0.001
Δ7-Stigmasterol	0.37 a	0.31 c	0.34 b	0.017	1.116	0.330
Apparent β-Sitosterol[3]	93.81 a	93.89 a	93.61 b	0.067	1.613	0.200
Δ5,23-Stigmastadienol	0.10 a	0.05 b	0.11 a	0.021	0.796	0.450
Clerosterol	0.91 b	0.97 a	1.00 a	0.019	2.146	0.120
β-sitosterol	87.00 a	84.99 b	84.58 b	0.217	14.880	0.000
Sitostanol	0.95 a	0.58 b	0.56 b	0.023	65.190	0.000
Δ5-Avenasterol	4.43 b	6.83 a	6.78 a	0.221	16.560	0.000
Δ5,24-Stigmastadienol	0.42 c	0.47 b	0.57 a	0.024	3.701	0.028
Δ7-Avenasterol	0.40 b	0.55 a	0.53 a	0.016	10.700	0.000
Erythrodiol + Uvaol	1.16 a	1.02 b	0.92 c	0.028	6.424	0.002
Total Sterols (in ppm)	1728.99 c	1915.09 a	1853.32 b	27.783	4.105	0.019

[1] Mean sample size = 36. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the maturity index.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 4

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed from fruit of small, medium and large size within each variety

	Small	Medium	Large	Std. Err.	F^2	Significance
Cholesterol	0.12 a	0.13 a	0.14 a	0.012	0.130	0.880
24-Methilene cholesterol	0.17 c	0.22 b	0.28 a	0.013	6.519	0.002
Campesterol	4.12 a	3.85 b	3.85 b	0.081	1.228	0.300
Campestanol	0.25 a	0.14 b	0.18 b	0.013	7.878	0.001
Stigmasterol	0.62 b	0.86 a	0.78 a	0.026	7.764	0.001
Δ7-Campesterol	0.23 a	0.26 a	0.19 a	0.031	0.343	0.710
Δ7-Stigmasterol	0.34 b	0.36 b	0.39 a	0.011	2.147	0.120
Apparent β-Sitosterol[3]	93.66 a	93.83 a	93.48 a	0.087	1.326	0.270
Δ5,23-Stigmastadienol	0.03 a	0.02 a	0.03 a	0.004	1.454	0.240
Clerosterol	1.14 b	1.25 a	1.22 a	0.033	0.935	0.400
β-sitosterol	86.88 a	85.66 b	83.52 c	0.226	28.680	0.000
Sitostanol	0.86 a	0.65 b	0.51 c	0.018	68.650	0.000
Δ5-Avenasterol	4.25 c	5.83 b	7.74 a	0.205	43.360	0.000
Δ5,24-Stigmastadienol	0.60 a	0.57 a	0.55 a	0.031	0.241	0.790
Δ7-Avenasterol	0.51 b	0.47 b	0.73 a	0.023	14.480	0.000
Erythrodiol + Uvaol	1.19 a	1.12 b	0.89 c	0.031	10.330	0.000
Total Sterols (in ppm)	1998.16 a	2002.29 a	1947.13 b	23.947	0.544	0.580

[1] Mean sample size = 36. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the fruit size.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 5

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed from fruit of receiving three different irrigation regimes: ½ X, X and 2 X

	½ X	X	2 X	Std. Err.	F^2	Significance
Cholesterol	0.22 a	0.12 c	0.17 b	0.018	2.636	0.076
24-Methilene cholesterol	0.40 a	0.27 b	0.26 b	0.015	9.700	0.000
Campesterol	4.03 a	3.95 a	3.83 b	0.066	0.844	0.430
Campestanol	0.31 a	0.25 b	0.23 b	0.012	4.364	0.019
Stigmasterol	0.92 a	0.80 b	0.75 b	0.020	7.102	0.001
Δ7-Campesterol	0.15 b	0.24 a	0.16 b	0.018	2.810	0.065
Δ7-Stigmasterol	0.54 a	0.51 a	0.41 b	0.009	24.350	0.000
Apparent β-Sitosterol[3]	92.85 c	93.42 b	93.73 a	0.079	13.200	0.000
Δ5,23-Stigmastadienol	0.16 a	0.08 b	0.09 b	0.018	2.084	0.130
Clerosterol	1.58 a	1.12 b	1.10 b	0.062	7.361	0.001
β-sitosterol	83.96 c	85.29 a	84.69 b	0.203	3.737	0.027
Sitostanol	0.73 a	0.66 b	0.72 a	0.018	1.543	0.220
Δ5-Avenasterol	5.81 b	5.79 b	6.59 a	0.177	2.284	0.110
Δ5,24-Stigmastadienol	0.59 a	0.49 b	0.55 a	0.023	1.927	0.150
Δ7-Avenasterol	0.60 a	0.48 b	0.49 b	0.013	10.530	0.000
Erythrodiol + Uvaol	0.93 a	0.99 a	0.93 a	0.033	0.358	0.700
Total Sterols (in ppm)	1933.71 a	1851.10 b	1992.07 a	29.511	1.954	0.150

[1] Mean sample size = 36. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the irrigation regime during oil accumulation.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 6

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed at malaxing times of 15, 30 and 60 minutes

	15 min		30 min		60 min		Std. Err.	F^2	Significance
Cholesterol	0.15	a	0.14	a	0.13	a	0.015	0.121	0.890
24-Methilene cholesterol	0.26	a	0.25	a	0.22	b	0.007	2.471	0.089
Campesterol	4.00	a	3.99	a	3.85	b	0.062	0.583	0.560
Campestanol	0.23	a	0.20	b	0.19	b	0.011	1.658	0.200
Stigmasterol	0.89	c	0.97	b	1.07	a	0.028	3.490	0.034
Δ7-Campesterol	0.13	a	0.13	a	0.09	b	0.012	1.082	0.340
Δ7-Stigmasterol	0.30	b	0.32	b	0.41	a	0.017	4.198	0.018
Apparent β-Sitosterol[3]	93.58	a	93.55	a	93.60	a	0.057	0.008	0.920
Δ5,23-Stigmastadienol	0.17	a	0.14	b	0.13	b	0.023	0.239	0.820
Clerosterol	0.82	b	0.85	a	0.86	a	0.020	0.317	0.730
β-sitosterol	85.23	a	84.84	b	84.67	b	0.206	0.633	0.530
Sitostanol	0.62	a	0.63	a	0.63	a	0.011	0.004	0.970
Δ5-Avenasterol	6.29	b	6.62	a	6.79	a	0.203	0.522	0.590
Δ5,24-Stigmastadienol	0.45	b	0.47	b	0.52	a	0.029	0.406	0.670
Δ7-Avenasterol	0.49	a	0.46	b	0.48	a	0.014	0.506	0.600
Erythrodiol + Uvaol	0.81	c	1.05	b	1.17	a	0.031	14.810	0.000
Total Sterols (in ppm)	1710.33	b	1813.10	a	1794.17	a	24.160	1.732	0.180

[1] Mean sample size = 36. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the malaxing time.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 7

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed at temperatures of 18ºC, 28ºC and 38ºC

	18ºC		28ºC		38ºC		Std. Err.	F^2	Significance
Cholesterol	0.17	a	0.21	a	0.21	a	0.015	0.764	0.470
24-Methilene cholesterol	0.26	a	0.26	a	0.25	a	0.008	0.208	0.810
Campesterol	3.95	a	3.98	a	3.93	a	0.064	0.004	0.960
Campestanol	0.21	a	0.22	b	0.21	a	0.008	0.213	0.810
Stigmasterol	0.89	b	0.95	b	1.12	a	0.035	4.439	0.014
Δ7-Campesterol	0.13	b	0.09	b	0.18	a	0.013	3.798	0.026
Δ7-Stigmasterol	0.34	b	0.39	b	0.38	b	0.012	1.526	0.220
Apparent β-Sitosterol[3]	93.59	a	93.35	a	93.22	a	0.066	2.706	0.071
Δ5,23-Stigmastadienol	0.23	a	0.24	a	0.28	a	0.034	0.174	0.840
Clerosterol	0.98	a	0.99	a	0.99	a	0.019	0.003	0.970
β-sitosterol	84.21	a	84.05	a	84.33	a	0.191	0.179	0.840
Sitostanol	0.66	a	0.69	a	0.68	a	0.012	0.658	0.520
Δ5-Avenasterol	6.96	a	6.85	a	6.45	a	0.190	0.657	0.520
Δ5,24-Stigmastadienol	0.57	a	0.53	a	0.49	a	0.023	1.047	0.350
Δ7-Avenasterol	0.53	b	0.62	a	0.55	b	0.019	2.044	0.130
Erythrodiol + Uvaol	0.86	c	1.01	b	1.25	a	0.034	13.980	0.000
Total Sterols (in ppm)	1669.97	c	1806.86	b	1924.26	a	25.487	9.656	0.000

[1] Mean sample size = 36. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the malaxing temperature.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 8

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils extracted from fruit within 12 hours of harvesting, 48 hours from harvesting and 120 hours from harvesting

	<12hs	48hs	120hs	Std. Err.	*F*[2]	Significance
Cholesterol	0.08 a	0.08 a	0.06 b	0.009	0.815	0.450
24-Methilene cholesterol	0.25 a	0.25 a	0.23 a	0.007	0.435	0.650
Campesterol	3.90 a	3.88 a	3.90 a	0.063	0.001	0.990
Campestanol	0.21 a	0.17 b	0.15 b	0.006	9.638	0.000
Stigmasterol	0.98 b	1.07 b	1.31 a	0.037	8.241	0.001
Δ7-Campesterol	0.07 b	0.07 b	0.14 a	0.011	5.816	0.004
Δ7-Stigmasterol	0.37 a	0.33 b	0.31 b	0.011	2.568	0.082
Apparent β-Sitosterol[3]	93.56 a	93.63 a	93.42 b	0.045	1.934	0.150
Δ5,23-Stigmastadienol	0.21 a	0.25 a	0.12 b	0.025	2.517	0.086
Clerosterol	0.98 a	0.95 b	0.94 b	0.011	1.084	0.340
β-sitosterol	84.25 a	84.66 a	84.80 a	0.220	0.552	0.580
Sitostanol	0.65 a	0.62 b	0.61 b	0.011	1.018	0.360
Δ5-Avenasterol	6.90 a	6.62 b	6.50 b	0.215	0.303	0.740
Δ5,24-Stigmastadienol	0.58 a	0.52 b	0.47 b	0.026	1.218	0.300
Δ7-Avenasterol	0.58 a	0.54 a	0.48 b	0.017	2.630	0.077
Erythrodiol + Uvaol	1.00 a	1.11 b	1.39 c	0.032	15.670	0.000
Total Sterols (in ppm)	1817.20 a	1802.15 a	1776.41 a	18.0228	0.432	0.650

[1] Mean sample size = 36. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the delay between harvesting and processing.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 9

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed from fruit in two different years

	2007	2008	Std. Err.	*F*[2]	Significance
Cholesterol	0.21 a	0.07 b	0.006	126.500	0.000
24-Methilene cholesterol	0.23 b	0.25 a	0.004	2.974	0.085
Campesterol	3.98 a	3.90 a	0.027	1.617	0.200
Campestanol	0.22 a	0.16 b	0.003	57.680	0.000
Stigmasterol	1.09 a	0.84 b	0.013	72.520	0.000
Δ7-Campesterol	0.16 a	0.08 b	0.005	43.330	0.000
Δ7-Stigmasterol	0.29 b	0.41 a	0.006	91.050	0.000
Apparent β-Sitosterol[3]	93.37 a	93.76 b	0.025	45.300	0.000
Δ5,23-Stigmastadienol	0.33 a	0.01 b	0.011	223.800	0.000
Clerosterol	0.93 b	0.95 a	0.007	0.961	0.330
β-sitosterol	85.16 a	84.44 a	0.087	11.460	0.001
Sitostanol	0.64 b	0.67 a	0.006	2.838	0.093
Δ5-Avenasterol	5.94 b	7.06 a	0.085	30.970	0.000
Δ5,24-Stigmastadienol	0.38 b	0.63 a	0.010	120.100	0.000
Δ7-Avenasterol	0.49 b	0.54 a	0.007	8.120	0.005
Erythrodiol + Uvaol	0.95 a	1.18 a	0.013	62.640	0.000
Total Sterols (in ppm)	1767.92 b	1834.06 a	9.854	7.625	0.006

[1] Mean sample size = 216. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the year.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 10

Sterol and Triterpene Dialcohol Concentrations[1] (values as % total sterols) of oils processed from fruit of three different varieties: Frantoio, Picual and Barnea

	Frantoio	Picual	Barnea	Std. Err.	F^2	Significance
Cholesterol	0.16 a	0.14 b	0.13 b	0.006	1.980	0.140
24-Methilene cholesterol	0.27 b	0.29 a	0.18 c	0.005	67.540	0.000
Campesterol	3.39 c	3.53 b	4.88 a	0.028	3125.000	0.000
Campestanol	0.23 a	0.21 b	0.17 c	0.004	16.760	0.000
Stigmasterol	0.92 b	1.07 a	0.73 c	0.013	76.050	0.000
Δ7-Campesterol	0.12 b	0.16 a	0.16 a	0.008	2.978	0.052
Δ7-Stigmasterol	0.40 a	0.39 a	0.34 b	0.006	9.235	0.000
Apparent β-Sitosterol[3]	93.87 a	93.80 a	92.96 b	0.028	157.700	0.000
Δ5,23-Stigmastadienol	0.21 a	0.15 b	0.05 c	0.010	26.870	0.000
Clerosterol	1.08 a	1.11 a	0.92 b	0.014	18.050	0.000
β-sitosterol	82.57 b	86.09 a	85.94 a	0.088	368.000	0.000
Sitostanol	0.69 a	0.62 b	0.70 a	0.007	16.120	0.000
Δ5-Avenasterol	8.61 a	5.47 b	4.93 c	0.083	451.000	0.000
Δ5,24-Stigmastadienol	0.75 a	0.39 b	0.42 b	0.011	174.600	0.000
Δ7-Avenasterol	0.66 a	0.44 c	0.48 b	0.007	127.100	0.000
Erythrodiol + Uvaol	1.08 a	1.01 b	1.05 a	0.013	2.037	0.130
Total Sterols (in ppm)	1855.44 b	1731.69 c	1968.92 a	10.629	47.510	0.000

[1] Mean sample size = 216. Means followed by the same roman letter within each row do not present significant differences (Duncan's mutiple range test α = 0.05).

[2] *F* tests the effect of the variety.

[3] β-Sitosterol sum = clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5,24-stigmastadienol.

Table 11

Fruit Composition (Oil distribution)

	Weight (% of total)	Oil (% fresh content)	Oil (% of total)	Oil (% of origin)
Skin	7.7%	33.5%	2.6%	12.3%
Flesh	71.0%	22.6%	16.1%	76.4%
Pit/Seed	21.3%	11.2%	2.4%	11.4%
Total	100.0%		21.0%	100.0%

Table 12

Sterol profile of the oils obtained from different parts of the fruit

	Cholesterol	Campesterol	Stigmasterol	D7 Stigmasterol	B Sitosterol	D5 Avenasterol	E+U	Total Sterols
Skin	0.000	4.820	0.990	0.900	89.440	1.580	9.180	1,842.4
Flesh	0.000	5.140	0.740	0.160	89.770	1.750	0.630	2,596.5
Pit/Seed	0.000	4.680	1.210	0.360	88.290	1.530	0.440	4,991.0
Total	0.000	5.020	0.856	0.261	89.441	1.691	1.286	3024.450

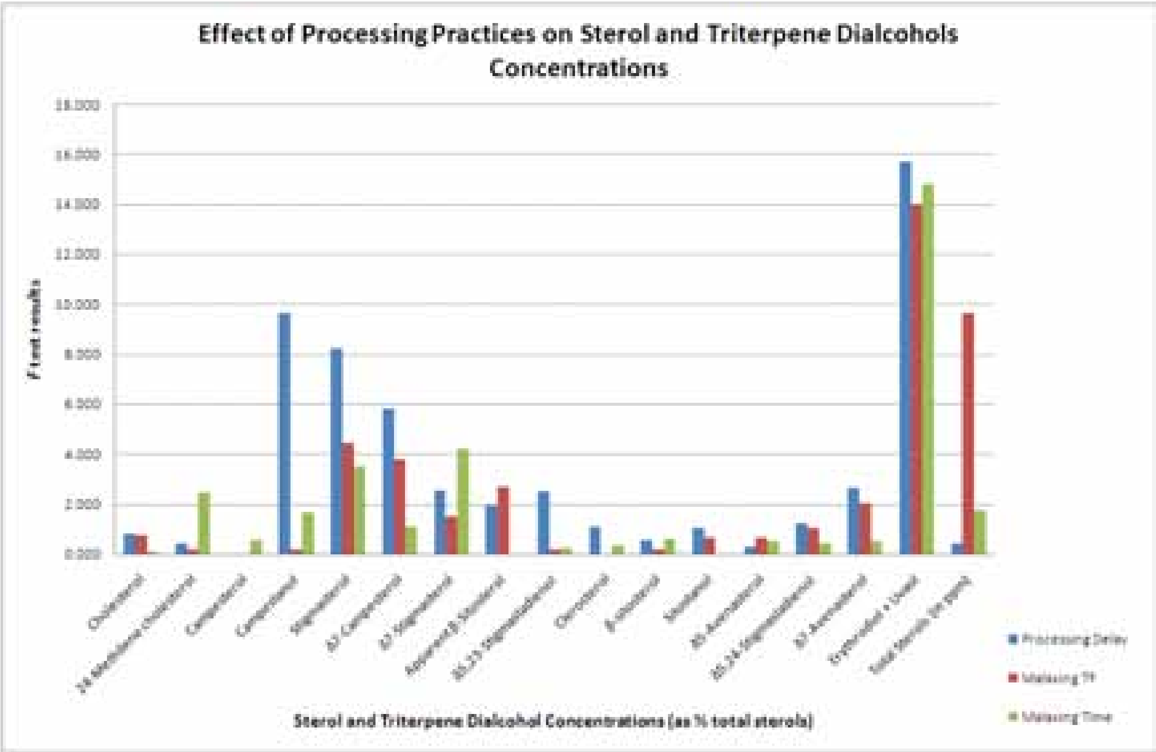

Figure 3

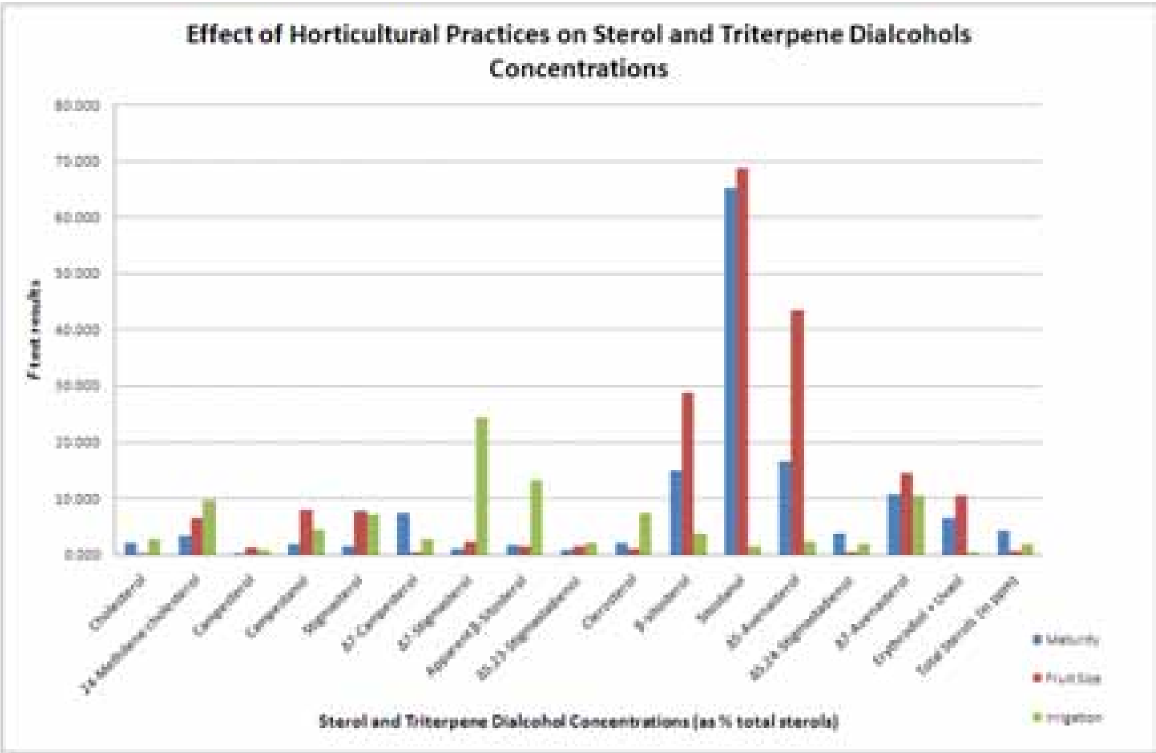

Figure 4

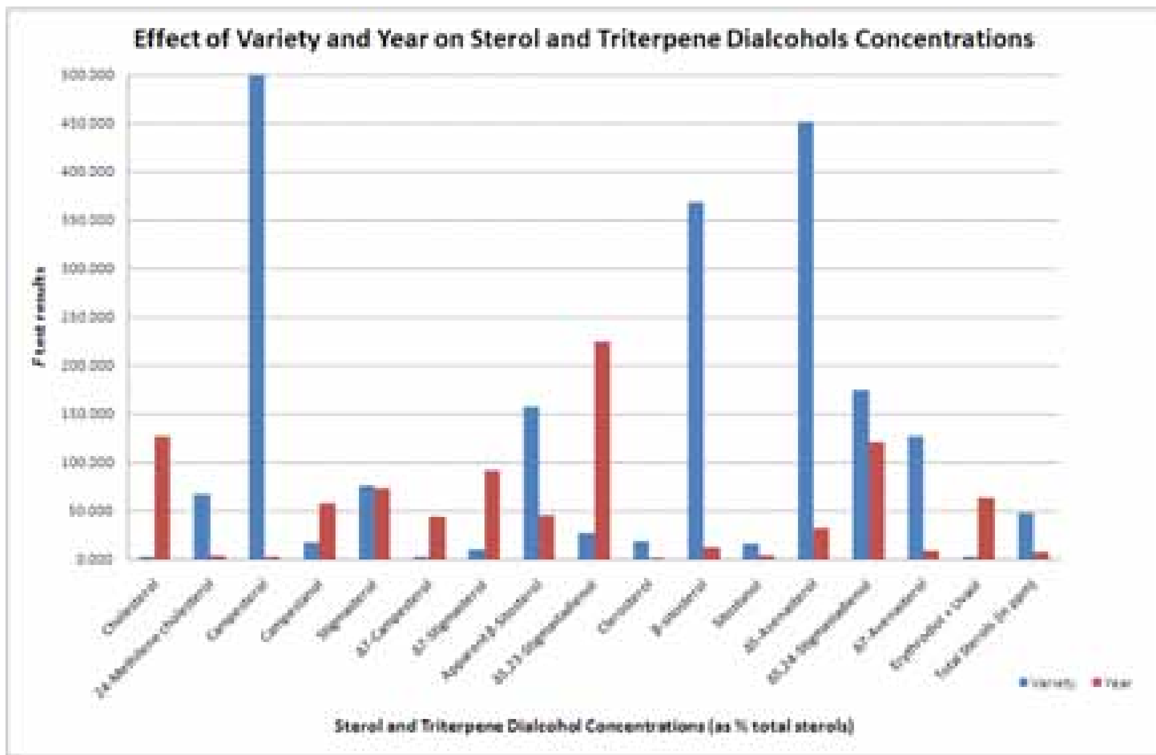

Figure 5

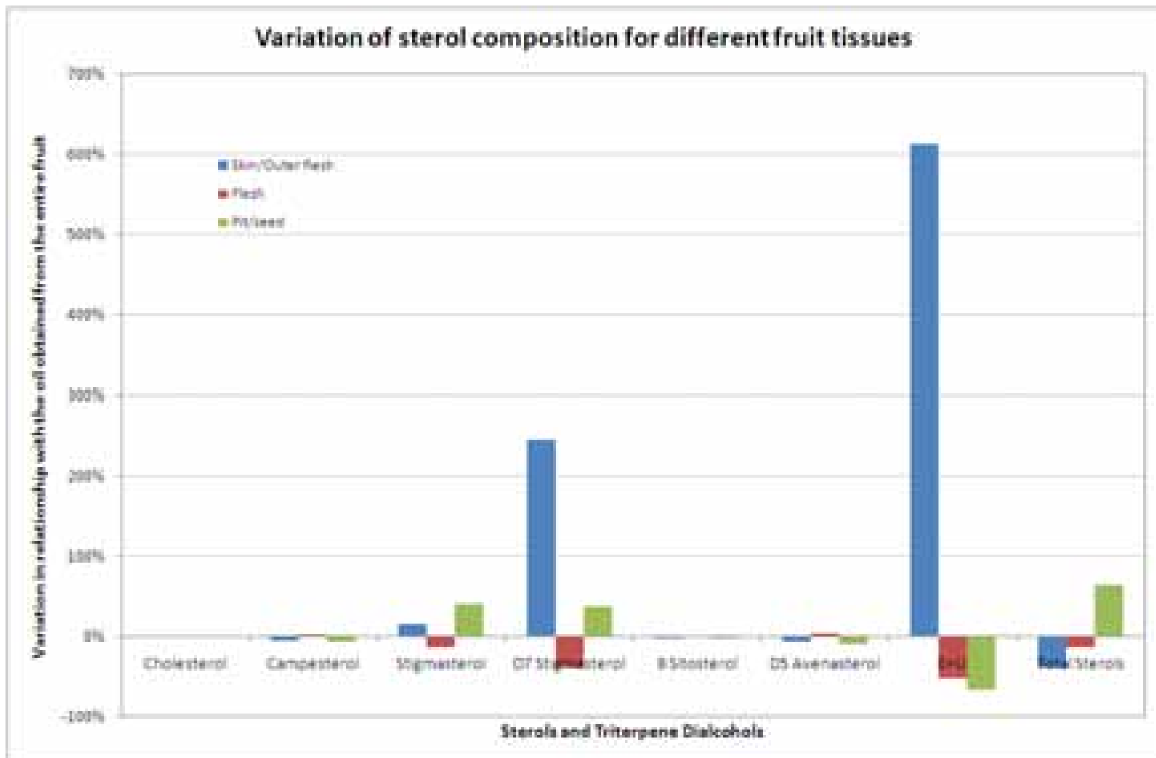

Figure 6

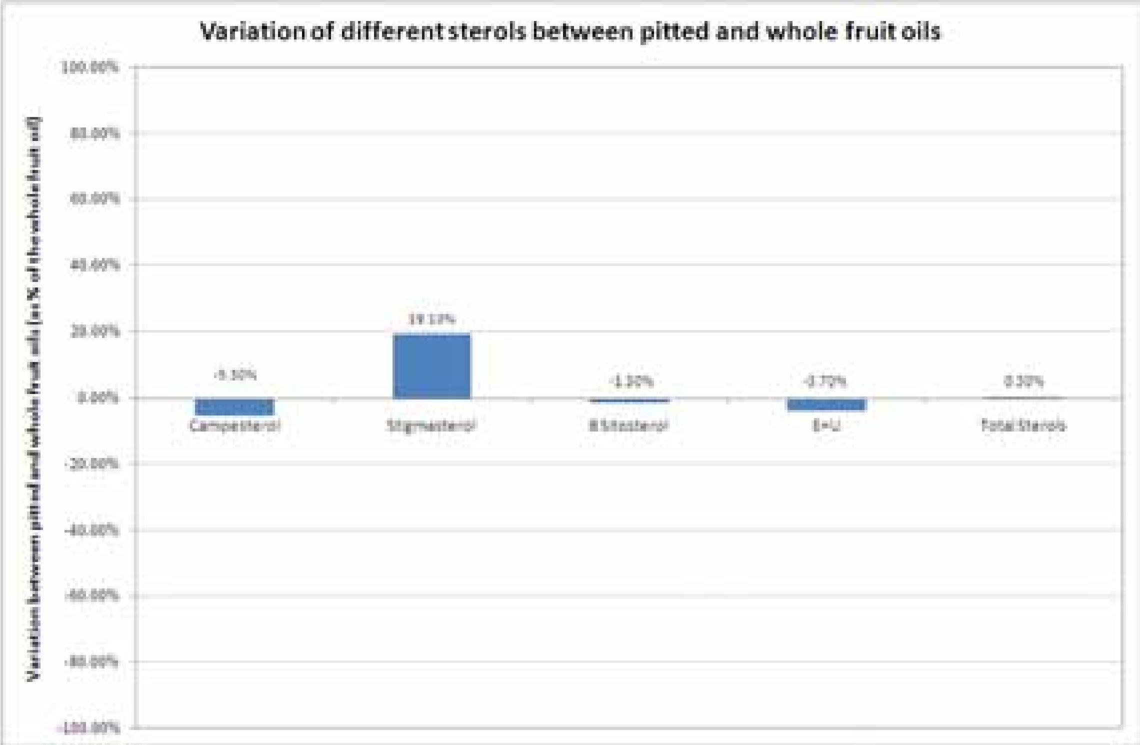

Figure 7

Discussion

Processing practices had a significant impact on the concentrations of triterpene dialcohols and stigmasterol. In the particular case of the stigmasterol, these results would support the campesterol/stigmasterol ratio as an index of quality of oil as proposed by other authors (Koutsaftakis, A., 1999). Nonetheless, this index could not be used to compare oils of different varieties as a consequence of the strong influence of genetics on campesterol content. This ratio provides additional information about the general processing conditions and, if utilised in combination with other traditional oil quality chemical parameters, it could provide a good way of comparing the overall quality of different oils produced from the same variety. The sterol composition of the oils obtained from different fruit tissues support these conclusions as triterpene dialcohols and stigmasterol tend to be in significantly higher concentrations in the pit/seed and skin/outer flesh obtained oils.

On the other hand, irrigation and fruit characteristics such as maturity and size have a significant effect on β-sitosterol, sitostanol, Δ5-avenasterol and Δ7-avenasterol. Consequently, the relationships between them could potentially be used to determine optimal harvesting times.

Finally, it is clear that there is a very strong influence of the variety on sterol composition, particularly in the case of certain sterols such as campesterol, stigmasterol, apparent β-sitosterol and total sterols.

Australian oils have shown good levels of total sterols and comparatively good Campesterol/Stigmasterol relationships, highlighting their healthy and high quality characteristics. Campesterol levels above most common international standards for certain Australian oils are strongly related to the combination between genetics and environment (phenotype) and they have no relationship with adulterations of any kind or with oil quality issues. No evaluated management or processing practice seems to have contributed to reducing Campesterol levels.

Implications

Based on current and most recognised international regulations on olive oil (IOC, EC and Codex Alimentarius), approximately 40 to 45% of high quality and authentic Australian oils could not be classified as olive oil. This is a clear weakness of these current standards, which are failing to recognise the natural variation of olive oils, not only in Australia, but also in many new growing regions and with many new varieties that have been introduced over the past decades.

As it has been clearly demonstrated in this work, high quality and authentic Australian olive oil meets the most important criteria to be considered olive oil: “It is obtained solely from the fruit of the olive tree to the exclusion of oils obtained using solvents or re-esterification processes and of any mixture with oils of other kinds”.

While we acknowledge the importance of sterol analysis for the evaluation of olive oil authenticity, it is important to highlight that not addressing the natural variations shown by a significant proportion of oils produced around the world is currently being used as a trade barrier, or a tool utilised by a number of trading companies to obtain high quality oils at a discounted price.

Ironically, the vast majority of these oils bought at lower prices that do not fit the definition of olive oil, are blended with other olive oils until they reach the legal levels for any given sterol. The blend is sold as olive oil. Based on the regulations that the trading companies are utilising to force a discount price, this should not be allowed as the blend of olive oil with any other oils can never be considered and sold as olive oil.

In well established export markets like Argentina, it is common to have a parallel trading of olive oil that meets IOC standards versus oils that do not meet IOC standards for authenticity. Price differences are usually as high as AU$ 1.00 per kilogram of oil. If we consider that Australia currently produces approximately 15,000 tonnes and it could double this production over the next decade, such trade barriers, unless properly addressed, could represent losses of up to AU$ 18,000,000 by 2010 and AU$ 30,000,000 per year by 2016.

Over the past years, a number of European scientists have recognised this problem and proposed alternative solutions (Conte, L. 2008 and Conte, L. 2009). Nonetheless, the proposed solution regarding Campesterol levels will not address the problem. It has been proposed that the current sterol levels should be compiled as a decision making tree, where an oil with more than 4.0% campesterol would be considered authentic only if the apparent B-sitosterol level is higher than 94% rather than the normal value of 93%.

Our research in this paper shows that this proposal would not address the issue of high Campesterol level oils, and also that it is technically incorrect. Only 1 of the 217 samples analysed (0.46%) with campesterol levels above 4.0% has shown apparent B-sitosterol levels above 94.0%. This is a rather obvious and expected result as there is a quite strong negative correlation between campesterol and apparent B-sitosterol levels (-59.8%). That is, when campesterol levels tend to increase (expressed as percentage of total sterols), the B-sitosterol levels tend to decrease. This negative correlation obtained with the analysis of the 648 samples analysed during this trial is biologically explained by the biosynthetic pathway of sterols presented in figure 1.

Recommendations

Australian oils have shown good levels of total sterols and comparatively good Campesterol/Stigmasterol relationships, highlighting their healthy and high quality characteristics. Campesterol levels above most common international standards for certain Australian oils are strongly related to the combination between genetics and environment (Phenotype) and they have no relationship with adulterations of any kind or with oil quality issues.

Therefore, no evaluated management or processing practice seems to have contributed to reducing Campesterol levels. Australian growers and processors should continue focusing on obtaining high quality olive oils by carefully monitoring malaxing temperature and times as well as conducting a prompt processing of the fruit immediately after harvest.

Based on current and most recognised international regulation on olive oil (IOC, EC and Codex Alimentarius), approximately 40 to 45% of high quality and authentic Australian oils could not be classified as olive oil. Consequently, Australian growers, processors, retailers and government should work together towards a modern and consumer oriented olive oil trade standard that addresses not only this problem but a number of other issues present in current international regulation.

Australia does not have any existing Standard for olive oils and/or olive-pomace oils. The Australian Olive Association Ltd has recently produced and approved an Australian Extra Virgin Code of Practice. The current Food Standards Australia New Zealand (FSANZ) Code makes only a general reference to all edible oils in their section 2.4.1

As explained above, the most widely accepted international standards for olive oils and olive-pomace oils are: Codex Standard for Olive Oils and Olive Pomace Oils - Codex Stan 33-1981 (Rev. 2-2003) and International Olive Council Trade Standard Applying to Olive Oils and Olive-Pomace Oils - COI/T.15/NC Nº 3/Rev. 3 November 2008.

Other relevant standards due to the olive oil and olive pomace oil volumes traded in those countries are: European Commission Regulation (EEC) Nº 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis and subsequent amendments and United States Standards for Grades of Olive Oil - Effective date March 22, 1948 together with their current Proposed United States Standards for Grades of Olive Oil and Olive-Pomace Oil – Release date March 28, 2008.

While a new Australian Standard and the previously listed standards should have a number of areas in common, particularly associated with worldwide recognized analytical methodologies and critical limits, a new Standard should significantly differ in a number of aspects. Some of these differences should include:

- Simpler and clearer commercial denomination of the different categories of olive oils and olive pomace oils in order to avoid the current misleading and confusing terms.
- Review of the range limits for a number of chemical parameters in order to avoid genuine olive oil, particularly Australian, being excluded for its natural variation in composition.
- Introduction of recently developed analytical methods which are capable of detecting modern refining techniques not currently detectable by the older methodologies included in the above standards.

It should be noted that Australia, through the Department of Agriculture, Fisheries and Forestry (DAFF) and with representation from the Australian olive industry, has made several attempts to

introduce such changes to olive oil standards in the Codex Alimentarius Commission Committee on Fats and Oils. Strong opposition from other representatives impeded such changes.

Furthermore, AOA and appropriate Australian government agencies should utilise this information in International Forums while discussing legislation on olive oil. It is important that Australia continues pursuing more appropriate olive oil standards and opposes some of the proposed changes that will wrongly classify a significant proportion of Australian oils.

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Sterols in Australian Olive Oils

— The effects of technological and biological factors —

by Claudia Guillaume, Leandro Ravetti and Joshua Johnson

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Plant sterols have been found to be effective in lowering elevated cholesterol and are now being added to a wide range of foods. Ironically, international standards for olive oil set a low limit for phytosterols. Due to natural variations in oils from new growing areas such as Australia, some Australian oils have higher levels of phytosterols.

The total content of sterols and their composition in olive oil seem to be influenced not only by genetic factors but also by management and processing techniques. The aim of the report was to improve processing and product quality while meeting national and international standards through supporting consistent production of high quality, healthy and safe olive oil that meets consumer expectations and in which they have confidence.

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