Investigation of Quality Parameters in Australian Olive Oils for Authentication

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Abstract

The authentication of extra virgin olive oils (EVOOs) is of importance due to the potential economic impacts on the global market when olive oils are diluted with other edible oils. The International Olive Council stipulates a wide range of methods to authenticate EVOOs, however, these methods are subject to limitations. This research addresses certain weaknesses associated with these methods such as the analysis time, consistency and reliability of the official methods.

A series of techniques and EVOO chemical parameters that could be used to supplement current authentication techniques was investigated. In particular, the "quality" parameters comprising total phenolic content, antioxidant capacity and tocopherol composition of Australian EVOOs diluted with canola, sunflower and rice bran oil were investigated along with the study of the UV and fluorescent spectra of these mixtures. A framework was developed which combines the strengths of the tests methods to propose a scheme to identify an adulterated EVOO.

The total phenolic content measured by the Folin-Ciocalteu assay provides a positive test for the presence of a diluent at concentrations of >5% w/w but is unable to identify the diluent and is non-linear with concentration. The total phenolic content as determined by high-performance liquid chromatography using a diode-array detector that has a similar detection limit to the Folin-Ciocalteu assay and is unable to identify the diluent but provides better linearity at diluent concentrations of >10% w/w. The antioxidant capacity of diluted EVOOs using the radical 2,2 diphenyl-1-picrylhydrazyl (DPPH) is only suitable for rice bran or canola oil diluents at 10% w/w and 20% w/w, respectively but in certain cases can identify the diluent oil in the mixture.

A novel technique in which the UV and fluorescent profiles of the EVOOs mixtures were determined and provided a rapid, non-destructive analysis of the EVOOs. Concurrent scans of both the excitation and emission spectra between 250 and 800 nm enabled the unique identification of EVOOs, canola, sunflower and rice bran oils Furthermore, selected excitation wavelengths of 328 nm and 536 nm were used to identify EVOO that was diluted with 5% w/w sunflower oil.

The total tocopherol concentration can be used to identify 10% w/w mixtures of sunflower and canola oil in EVOO however this parameter is not suitable to identify the presence of rice bran oil. Nonetheless, a strong correlation was found between the compositional changes to α- and γ-tocopherol upon dilution which enabled the detection of diluted EVOOs, and these tocopherol concentrations were found to offer a unique profile for all three diluent oils. For example, a γ-tocopherol concentration exceeding 10 mg kg^{-1} suggests the EVOO is diluted with canola or rice bran oil at a concentration of 5% w/w or 10% w/w, respectively. An α-tocopherol concentration exceeding 178 mg kg^{-1} suggests the EVOO is diluted with sunflower oil at a concentration between 5-10% w/w depending on the EVOO. Overall, the α- and γ-tocopherol profiles were used to develop a decision tree framework to identify and quantitate the diluent oil.

In combination, the above traditional methods used with the novel techniques and the assessment framework developed in this work enable a more robust assessment to be made of the authenticity of EVOOs in the future.

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Declaration by Author

I, Travis Michael Murdoch, declare that the PhD thesis entitled “Investigation of Quality Parameters in Australian Olive Oils for Authentication” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University’s Higher Degree by Research and Procedures

Signature:  Date: 17/12/20

Abbreviations

AAPH	2,2-azobis 2-amdinopropane dihydrochloride
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
AOAC	Association of Official Analytical Collaboration
AOC	antioxidant capacity
AU	absorbance units
CAN	canola oil
CI	confidence interval
DPPH	2,2 – diphenyl -1- picrylydrazyl
ECN42	equivalent carbon content 42
EE	extraction efficiency
EEM	excitation-emission matrix
EVOO	extra virgin olive oil
FA	free acidity
FAMES	fatty acid methyl esters
FC	Folin-Ciocalteu
FCR	Folin-Ciocalteu reagent
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalents
GC-FID	gas chromatography flame ionization detection
HAT	hydrogen atom transfer
HPLC	high performance liquid chromatography
DAD/MS	diode-array detection/mass spectrometer
IOC	International Olive Council
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
ORAC	oxygen radical absorbance capacity
PARAFAC	parallel factor analysis
RBO	rice bran oil
RRF	relative response factor
SAR	standard addition recovery
SET	single electron transfer
SPE	solid phase extraction
SFO	sunflower
TGs	triglycerides
TEAC	Trolox equivalent antioxidant capacity
TLC	thin layer chromatography
TPC	total phenolic content
USDA	United States Department of Agriculture

Table of Contents

Abstract i
Acknowledgements iii
Declaration by Author iv
Abbreviations v
List of Figures viii
List of Tables x
Chapter 1 Introduction 1
1.1 Olive Oils 1
1.2 Olive Oil Production 4
1.2.1 Pressure Extraction 5
1.2.2 Percolation 5
1.2.3 Centrifugation 5
1.3 Olive Oil Authenticity 6
1.4 Research Aims and Objectives 10
1.5 Thesis Outline 12
Chapter 2 Literature Review 14
2.1 Introduction 14
2.2 EVOO Lipids 15
2.2.1 Triglycerides in EVOOs 15
2.2.2 Fatty Acid Methyl Esters in EVOOs 17
2.3 EVOO Phytosterols 19
2.3.1 Measuring Phytosterol Content 20
2.3.2 Factors Influencing Phytosterol Content 22
2.4 EVOO Phenolic Compounds 24
2.4.1 Folin-Ciocalteu Colorimetric Assay of Phenolics 25
2.4.2 HPLC-DAD/MS Analysis of Phenolics 25
2.4.3 Factors Influencing the Phenolic Content of Olive Oils 30
2.5 EVOO Antioxidant Capacity 32
2.5.1 Antioxidant Capacity of EVOOs 33
2.5.2 Factors Influencing EVOO Antioxidant Capacity 35
2.6 Spectrophotometric Methods for EVOO Analysis 36
2.6.1 Ultraviolet Absorbance Measurements 36

2.6.2 Fluorescence Excitation-Emission Matrices....38
2.7 EVOO Tocopherols....41
2.7.1 Tocopherol Composition of Vegetable Oils....42
2.7.2 Factors Influencing Tocopherol Content....45
2.7.3 Tocopherol Measurement to Determine Olive Oil Adulteration....46
2.8 Summary....47
Chapter 3 Materials and Methods....48
3.1 Oil Samples....48
3.1.1 EVOO and Diluent Oils....48
3.1.2 Preparation of Adulterated Oils....48
3.2 Chemicals and Reagents....48
3.3 Analytical Methods....49
3.3.1 Folin-Ciocalteu Assay....49
3.3.2 Total Phenolics by HPLC-DAD....50
3.3.3 DPPH Antioxidant Assay....52
3.3.4 Fluorescence and UV Absorbance Measurements....54
3.3.5 Tocopherol Sample Extraction and Analysis....54
3.4 Method Validation....54
Chapter 4 EVOO Authenticity by Phenolic and Antioxidant Content....58
4.1 Overview....58
4.2 Results and Discussion....58
4.2.1 Total Phenolic Content by Folin-Ciocalteu Assay....58
4.2.2 Total Phenolic Content by HPLC Validation....61
4.2.3 Evaluation of Total Phenolic content *via* HPLC-DAD....62
4.2.4 Validation of DPPH assay for Antioxidant Activity....65
4.2.5 Antioxidant Activity to Determine EVOO Authenticity....67
4.3 Optimized Procedure for Diluent Oil Detection....70
4.4 Summary....71
Chapter 5 Detection of Diluent Oils by Spectrophotometric Methods....73
5.1 Overview....73
5.2 Results and Discussion....73
5.2.1 Fluorescence EEMs of Pure Oils....73
5.2.2 Fluorescence EEMs of EVOO/Sunflower Oil Mixtures....75

5.2.3 EEM Regression to Determine EVOO Authentication 77
5.2.4 UV Absorbance of EVOOs and Mixtures 83
5.3 Summary 87
Chapter 6 Evaluation of Tocopherol Content 88
6.1 Overview 88
6.2 Results and Discussion 88
6.2.1 Quantitative Analysis of Tocopherol Standards by HPLC 88
6.2.2 Recovery of Standard Tocopherols 89
6.2.3 Tocopherol Composition of EVOOs and Diluent Oils 90
6.2.4 Authenticity by Total Tocopherol Content 91
6.2.5 Authenticity by Individual Tocopherols 94
EVOOs Diluted with Sunflower Oil 94
EVOOs diluted with Canola Oil 95
EVOOs Diluted with Rice Bran Oil 99
6.2.6 Optimized Procedure for Diluent Oil Detection 102
6.3 Summary 104
Chapter 7 Proposed Framework for EVOO Authentication 105
7.1 Assessment of Evaluated Methods 105
7.2 Workflow of Methods for Evaluating EVOO Authenticity 107
7.3 Summary 110
Chapter 8 Conclusions and Recommendations 111
8.1 Conclusions 111
8.2 Recommendations for Future Work 114
References 115
Appendix A: Chromatograms 127

List of Figures

Figure 1.1: Fraudulent profit obtained from the sale of EVOO diluted with oils. 4
Figure 1.2: Summary of olive oil production and classifications. 7
Figure 1.3: Conceptual framework developed for this research. 11
Figure 2.1: Structure of the most prevalent phytosterols in EVOOs. 20
Figure 2.2: Example EVOO phenolic HPLC chromatogram 26

Figure 2.3: Optimised method for the extraction of phenolics.....29
Figure 2.4: Structure of α-tocopherol. 42
Figure 3.1: Gallic acid standard curve and associated CI error bars. 50
Figure 3.2: Trolox calibration curve and CI error bars. 53
Figure 4.1: Total phenolic content measured by the FC assay of mixed diluent oils.....60
Figure 4.2: Relationship between TPC and diluent oil concentration.....63
Figure 4.3: Relationship between TPC and diluent oil concentration.....63
Figure 4.4: Absorbance of DPPH in methanol. 65
Figure 4.5: TEAC calibration of pure oils with a mass range of 0.1-1.0 g 66
Figure 4.6: AOC (TEAC) of diluted EVOO#1. 67
Figure 4.7: AOC (TEAC) of diluted EVOO#2…..68
Figure 4.8: Decision tree for TPC and AOC. 71
Figure 5.1: Fluorescence EEMs of pure oils 74
Figure 5.2: EEMs of EVOO#1, sunflower oil, and their mixtures. 76
Figure 5.3: Three-dimensional plot of regression coefficients.....78
Figure 5.4: Scatter plot of regression coefficients *versus* gradient.....78
Figure 5.5: Fluorescence emission spectra for pure EVOO, pure SFO.....79
Figure 5.6: Fluorescence intensity as a function of increasing diluent oil 80
Figure 5.7: Fluorescence intensity as a function of increasing diluent oil content 80
Figure 5.8: Scatter plot of fluorescence data regression coefficients 82
Figure 5.9: Fluorescence emission spectra for pure EVOO, pure SFO.....82
Figure 5.10: Fluorescence intensity as a function of increasing diluent oil content 83
Figure 5.11: UV absorbance spectra for pure EVOO, pure SFO and their mixtures. 84
Figure 5.12: Scatter plot of UV absorbance regression coefficients and gradients.....85
Figure 5.13: UV absorbance as a function of increasing diluent oil content.....86
Figure 5.14: UV absorbance as a function of increasing diluent oil content.....86
Figure 6.1: Chromatogram of α-, β-, δ- and γ-tocopherol standards 89
Figure 6.2: Total tocopherol content of diluted EVOO#1.....92
Figure 6.3: Total tocopherol content of diluted EVOO#2.....92
Figure 6.4: The α-tocopherol content of EVOO diluted with sunflower oil 95
Figure 6.5: Chromatograph of pure canola oil. 96
Figure 6.6: α-tocopherol content in EVOO diluted with canola oil 97
Figure 6.7: Calibration of γ-tocopherol levels in EVOO diluted with canola oil.....98
Figure 6.8: Chromatogram of pure rice bran oil.....100
Figure 6.9: α-tocopherol content of EVOOs diluted with rice bran oil. 100
Figure 6.10: γ-tocopherol content of EVOO and rice bran oil mixtures. 102
Figure 6.11: Framework for detection of diluent oils in EVOO 103
Figure 7.1: Proposed framework for detection of diluent oils in EVOOs. 108

List of Tables

Table 1.1: IOC EVOO Quality Parameters 8
Table 1.2: IOC EVOO Purity Constituents 9
Table 2.1: Olive oil and acceptable ΔECN42 for individual olive oil grades 16
Table 2.2 IOC stipulated FA limits 17
Table 2.3: IOC limits of trans FAs 18
Table 2.4: Limit of detection of various diluent oils *via* TG and FAMEs analyses 19
Table 2.5: Total phytosterol content in different olive oils 20
Table 2.6: Individual phytosterol limits in EVOO 21
Table 2.7: Elution parameters for phenolic compounds 27
Table 2.8: Total phenolic concentrations of European EVOOs 31
Table 2.9: Common antioxidant radicals used in AOC assays 32
Table 2.10: AOC of EVOO measured with different assays 34
Table 2.11: UV absorbance K quality parameters 37
Table 2.12: EEM regions of key EVOO constituents 39
Table 2.13: Summary of tocopherol content in different oils (mg kg^{-1}) 44
Table 3.1: HPLC conditions for determining tocopherol content 54
Table 4.1: Relative response values of HPLC-DAD for TPC standards 61
Table 4.2: Extraction efficiencies of TPC by HPLC 62
Table 4.3: TPC trendline characterises of the relationship between EVOO 62
Table 4.4: Regression analysis of TEAC calibration 68
Table 4.5: AOC of EVOOs with increasing mass of diluent oils 69
Table 6.1: Concentration of tocopherol standards 88
Table 6.2: Standard addition of tocopherol to EVOO#1 diluent mixtures 89
Table 6.3: Calculated %EE values for individual tocopherols 90
Table 6.4: Tocopherols detected in each pure test oil 91
Table 6.5: Total tocopherols of diluted EVOOs 93
Table 6.6: Trendline parameters for total tocopherol calibration curves 94
Table 6.7: Tocopherol composition of EVOOs diluted with canola oil 97
Table 6.8: Regression analysis of EVOO diluted with canola oil 99
Table 6.9: Tocopherol composition of EVOOs diluted with rice bran oil 101
Table 6.10: Regression analysis of EVOO diluted with rice bran oil 102
Table 7.1: Limit of detection values for investigated methods 105

Chapter 1 Introduction

1.1 Olive Oils

Olive oils are classified as oils obtained exclusively from the fruit of olive trees (*Olea eurpaea L*) and the purity of olive oil is predominately affected by the method of its production (Aparicio and Harwood, 2013; Capurso et al., 2018). The high market value of olive oil, reported as *ca* $US 9.5 billion dollars in 2018 (IOC, 2019a), along with the reported health benefits from its consumption can give rise to the addition of lesser quality olive oils or other seed oils to dilute or adulterate pure olive oils (Aparicio and Harwood, 2013). As a result, the regulation of olive oil quality and authenticity is of vital importance to ensure correct labelling of olive oil products as stipulated by the intergovernmental body, the International Olive Council (IOC). The IOC set limits on the constituents of olive oils and proposes several methods for determining the authenticity of an olive oil (IOC, 2018). Within some set limits, the composition of a pure olive oil can provide an indication whether the oil has been subjected to adulteration (IOC, 2018). There are a number of challenges with the determination of olive oil authenticity, however, because several factors associated with the composition of the olive oil can result in false negative or false positive results (Ben-Ayed et al., 2013).

The techniques prescribed by the IOC typically involve chemical and chromatographic methods for the confirmation of olive oil components which include fatty acid methyl esters (FAMES), trans fats and phytosterols (IOC, 2018). These techniques involve extensive measurements for accuracy, repeatability and robustness to ensure the validity of the results. As such, any new technique must also pass the same standards as the current prescribed methods with several years of method validation before implementation and acceptance as a standard method employed by the IOC or other regulatory bodies. This is a major challenge for food analysts and technologists as new techniques require at least equivalent sensitivity to current methods before these will be considered for determining olive oil authenticity. Further challenges also arise from the inherent differences in olive oil constituents due to geographical location and growth conditions across the globe. This can present difficulties in authenticity confirmation since changes in the acceptable range of constituents can provide an avenue for adulteration. Conversely, maintaining strict regulations on olive oil compositions can impact trade as olive oil from certain regions have been shown to fail certain IOC regulated limits (Mailer et al., 2010; Rivera del Álamo et al., 2004; Jabeur et al., 2014).

The importance of olive oil authentication is further highlighted by high profile cases of adulteration of extra virgin olive oils (EVOOs). The European commission publishes an annual report entitled: "*The EU Food Fraud Network and the System for Administrative Assistance - Food Fraud"* which highlights the cases of food fraud reported for that year. These are grouped into ten food groups, of which fats and oils featured first and third most prominent groups in 2018 and 2019, respectively (European Commission, 2019). Furthermore, there have also been several high-profile cases of EVOOs diluted with various edible oils and various surveys of EVOOs which suggest that a significant portion of EVOOs sold to markets both in Australia and Germany do not adhere to IOC standards. Some of these high-profile cases of EVOO diluted with other oils and some of the surveys are listed below:

- In 2010, Spanish EVOOs were diluted with avocado, palm oil and sunflower oil. These diluted EVOOs were produced in a biodiesel factory and sold in both Spanish and Italian markets (Lord et al., 2017).
- Between 2005/2006 76,000 litres of Spanish sunflower oil was disguised as olive oil using preservatives and colourants with only 20% of the final product deemed to be EVOO (Lord et al., 2017).
- In Italy olive pomace oil was exported to the United states and labelled as EVOO (Smith, 2017).
- Europol arrested a large-scale criminal network which adulterated sunflower oil with chlorophyll, beta-carotene and soya oil to give the appearance of EVOO; a total of one million litres of adulterated sunflower oil was seized (Taylor, 2019).
- A series of surveys of 265 EVOOs commercially available in Australia (127 domestic, 138 imported oils) found that 46%, 29% and 42% of the EVOOs surveyed failed to adhere to IOC standards in the years 2008, 2009 and 2010 respectively (Mailer and Gafner, 2020).
- A survey of 266 EVOOs sold in Germany during 2015 found that 33% of EVOOs failed IOC stipulated limits (Mailer and Gafner, 2020).

These cases of EVOO adulteration and the commercially available EVOO which failed to adhere to IOC standards, highlights the need for the on-going investigation of alternative techniques that detect adulteration. This is in particular regard to EVOOs sold to markets as those EVOOs that have failed these standards indicate that either more wide-spread dilution of

EVOOs is occurring than otherwise thought, or that current techniques are falsely identifying pure EVOOs as having been adulterated with other edible oils.

The suitability of a method to determine diluent oils in EVOOs could also be assessed based on a "practical limit of detection". This value being one that is based on the fraudulent profit when a diluted EVOO is sold at EVOO market prices. This exercise combines the cost of EVOO and diluent oils at their respective volumes and subtracts this ratio from the price of EVOOs, and provides the "fraudulent profit" (FP) as a percentage that a diluted EVOO delivers compared to the sale of a pure EVOO (see Equation 1):

$$\text{FP (\%)} = \frac{EVOO_{\$} - (EVOO_{\$} \times EVOO_{V}) + (DIL_{\$} \times DIL_{V})}{(EVOO_{\$} \times EVOO_{V}) + (DIL_{\$} \times DIL_{V})} \quad (1)$$

where $EVOO_{\$}$ and $EVOO_{V}$ are the cost and volume of the EVOO respectively, and $DIL_{\$}$ and DIL_{V} are the cost and volume of the diluent oil, respectively.

This fraudulent profit for three diluent oils (canola, sunflower and rice bran oil) was calculated and plotted against the volume fraction of the diluent oil using data that are currently available (see Figure 1.1). The price of each oil (in $US per kg) sourced from the IOC and United States Department of Agriculture for 2018 are $5.80, $3.41, $1.22 and $0.85 for EVOO, rice bran, sunflower and canola oil respectively (IOC, 2019a; USDA, 2020). The data in Figure 1.1 suggest the price difference between sunflower and canola oil is not significant, until the diluent oil volume faction exceeds *ca*. 0.5 and approximate linearity can be observed up to this volume faction. Conversely, as the volume faction increases above 0.5 a rapid increase in the fraudulent profit is observed and seemingly the fraudulent profit of each diluent oil becomes more distinct, with canola oil delivering the greatest illegal profit. This exercise provides an avenue on what a "practical" limit of detection might entail, for example the identification of the diluent oil used in EVOO adulteration being less important until the diluent faction exceeds, say, 0.5. Figure 1.1 also suggests that it is vitally important that any method is able to detect mixtures of 50% w/w as after his point the value of a fraudulent dilution of EVOOs rapidly increases. This is seemingly supported by high concentration of canola oil diluent observed in some examples of adulterated Spanish EVOOs (Lord et al., 2017).

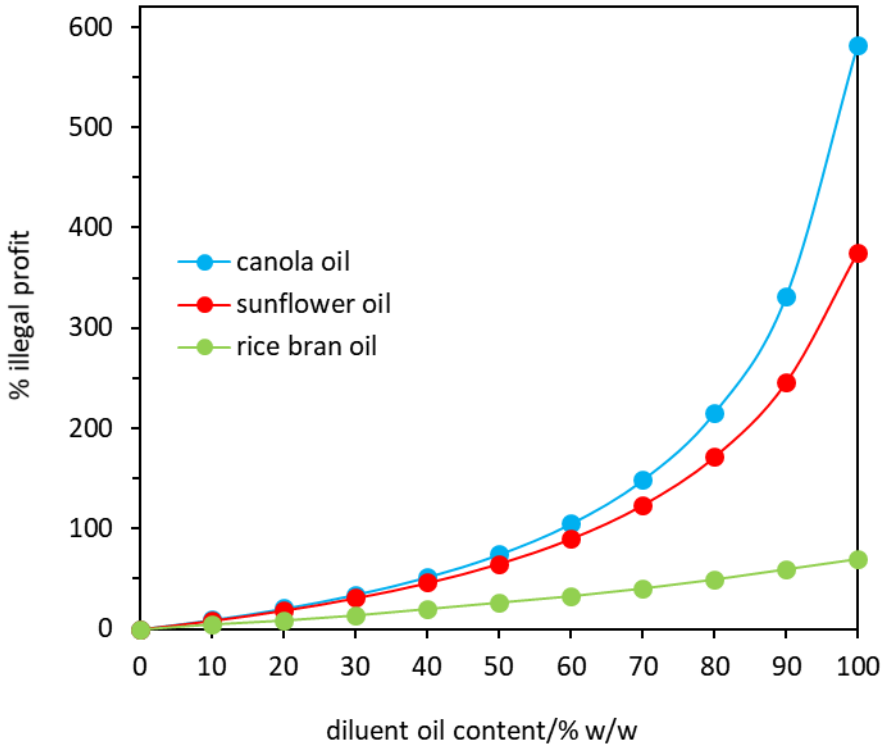


Figure 1.1: Fraudulent profit obtained from the sale of EVOO diluted with oils.

1.2 Olive Oil Production

The production of olive oil involves the grinding of harvested olives into a paste followed by the mechanical extraction of olive oil by various methods (Aparicio and Harwood, 2013; IOC, 2018). Olive oils are classified based on the extraction process with EVOOs defined as the highest quality of oil obtained mechanically without using heat or chemicals (IOC, 2018). Further classification of lower grades of olive oils depends on factors such as mixing of oils after extraction with various olive products (Capurso et al., 2018; IOC, 2018). Oils obtained from olives which exceed various parameters, such as free fatty acid content of 3.3 grams per 100 grams, are deemed inedible and are referred to as "lampante EVOO" (IOC, 2018).

The first stage of olive oil production involves the mechanical crushing of olives into a paste and to prevent degradation of olive oil, harvesting, cleaning and grinding of the olive occurs within 24-36 hours after harvest (Aparicio and Harwood, 2013). Following the crushing of the olives into a paste, several methods can be applied to extract the different grades of oil

including the use of pressure, percolation and centrifugation (Aparicio et al., 2013a; Uceda et al., 2006).

1.2.1 Pressure Extraction

Pressure extraction techniques involve the application of mechanical pressure to remove olive oil from the milled paste which can yield 70-85% oil with the remaining oil contained in the pomace (solid waste) and vegetation water (Aparicio et al., 2013a). Pressure techniques have numerous advantages in the production of olive oil including their simplicity and their ability to limit the amount of residual oil in the pomace and vegetation water. However, pressure techniques involve labour-intensive mechanical devices with little automation being possible and have a relatively low throughput of olives compared to other extraction techniques. Contamination of the oil may also occur from improperly cleaned mats used to catch solid matter which can subsequently affect the quality of the pressed oil (Aparicio et al., 2013a).

1.2.2 Percolation

Percolation is an oil extraction technique that relies on the difference between the surface tension of the oil and the vegetation water. This method involves passing oily pre-coated steel blades though the olive paste and differences in surface tension amongst the oil, pomace and vegetation water results in only the olive oil attaching to the blade which is then removed by scraping the blade in a continuous process. Since this process is performed at ambient temperatures and without the use of filter mats, the potential for contamination is minimised (Di Giovacchino et al., 1994; Aparicio and Harwood, 2013).

1.2.3 Centrifugation

In this process, a centrifugal decanter applies a centrifugal force to separate olive oil from the solid and water phases. Typically, a rotor speed of 3,500 RPM is applied to the olive paste which forces the solid matter out to the sides with the oil retained in the centre. Throughout the process, water is used to wash the olive paste to improve liquid and water phase separation, however the use of water affects can reduce the oil quality due to the solubility of some of the phenolic compounds in the aqueous phase as this reduces the oxidative resistance of the oil and degrades the organoleptic properties of the olive oil with removal of bitterness. The common two-phase centrifugal decanter recovers the water pomace, which can be treated

with solvents to further remove oil from the pomace water which is deemed to be of lesser quality than olive oil (Aparicio and Harwood, 2013).

1.3 Olive Oil Authenticity

The purity of an olive oil, and thus how it is graded, is dependent on the degree of refinement and the mixing the various olive components. As presented in Figure 1.2, an olive oil graded as "extra virgin olive oil" occurs when the oil has been mechanically extracted, with only washing, decanting, centrifugation and filtering permitted. Further classifications of olive oils are based on free fatty acid content, which arise due to hydrolytic degradation of triglycerides (Velasco and Dobarganes, 2002). Any further extraction with other techniques such as chemical solvents and the reuse of previously crushed olives results in lower quality olive oils and influences its composition, such oils are graded as refined olive oil or pomace oil. Furthermore, constituent limits are also set by the IOC for various olive oil constituents, such as phytosterol and FAMEs which are used to assess the purity of an EVOO, in particular when diluted with other edible oils (IOC, 2018).

The confirmation of the authenticity of an olive oil is based on its constituents and is reflected in its quality parameters and purity parameters. The quality parameters provide constitutional information which indicate the quality of the oil, such as the degree of oxidation and various techniques that are used to determine these are presented in Table 1.1. Conversely, the assessment of the purity of constituents entails methodologies with more discrimination power as presented in Table 1.2 (IOC, 2018; Aparicio and Harwood, 2013). The quality extraction techniques methodologies entail HPLC, titration and absorbance determinations whereas purity parameters use various methodologies which include GC-FID with various extraction techniques (IOC, 2018).

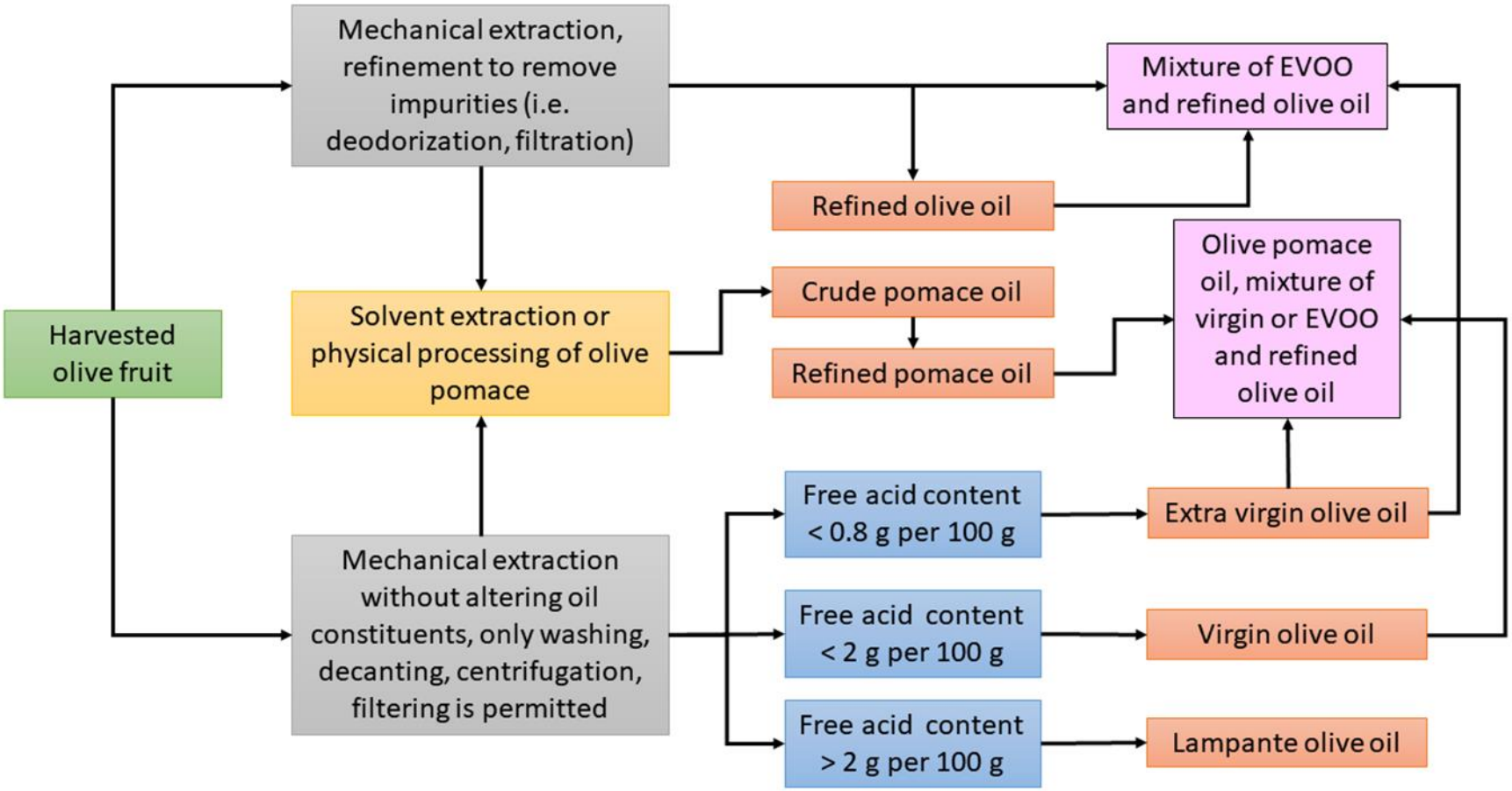


Figure 1.2: Summary of olive oil production and classifications.

The wider literature has reported differences in the concentration of phytosterols, fatty acid methyl esters, tocopherols and phenolic content which correlate to olive variety and growth region; in some cases, the concentration of olive oil constituents have exceeded the IOC stipulated limits. (Guillaume et al., 2011; Flakelar et al., 2015; Jukić Špika et al., 2015; Franco et al., 2014b). For example, a survey found that certain Australian olive oils in NSW exceeded the 4% phytosterol limit set by the IOC standard (Mailer et al., 2010; IOC, 2018). This suggests that either these olive oils were diluted with other vegetable oils or natural variability of phytosterols may cause EVOO to not meet stipulated limits. This factor, and improvements to method efficiencies, encourage the development and validation of alternative techniques for determining the authenticity of olive oils.

Table 1.1: IOC EVOO quality parameters

Quality Parameter	Detectable Oils	Method	Reference
Peroxide value (millieq. peroxide oxygen per kg oil)	N/A (limit of 20, limit of 15 for OO)	titration	IOC (2017d)
Absorbance (K270 nm and K232 nm)	EVOO, VOO, ROO, OO, RPO, PO	UV absorbance	IOC (2019b)
Moisture and volatile matter	LVOO, CPO	gravimetry	IOC (2016)
Insoluble impurities	LVOO	gravimetry	(IOC, 2017c)

Note: CPO (crude pomace oil), EVOO (extra virgin olive oil), LVOO (lampante virgin olive oil), OO (olive oil), PO (olive pomace oil), ROO (refined olive oil), RPO (refined pomace oil), VOO (virgin olive oil).

Alternative techniques to determine olive oil authenticity, such as tocopherol content and fluorescence spectroscopic methods for constituent analysis have been investigated (Chen et al., 2011; Aparicio et al., 2013b; Ben-Ayed et al., 2013), with much of the focus on olive oil produced in the Mediterranean region (Ruiz-Domínguez et al., 2013; López-Cortés et al., 2013; Franco et al., 2014b; El Kharrassi et al., 2017; Bajoub et al., 2015; Bajoub et al., 2016b). The antioxidant properties and health benefits of phenolic compounds and tocopherols present in olive oil have also been investigated (Fuentes et al., 2018; Blekas et al., 2002; Janu et al., 2014).

Table 1.2: IOC EVOO purity constituents

Constituent	Detectable diluent oils	Method	Reference
Fatty acid methyl esters	vegetable oils	methylation of TAGS in hexane, GC-FID determination	IOC (2017b)
Trans fatty acid	EVOO, LVOO, ROO, OO (EVOO+ROO mixture), CPO, RPO, OO+RPO mixture	methylation of TAGS in hexane, GC-FID determination	IOC (2017b)
Individual phytosterol content	vegetable oils	saponification (by reflux condenser), liquid-liquid extraction, TLC separation, GC-FID determination	IOC (2017e)
Total phytosterol content	EVOO, LVOO, ROO, OO (EVOO+ROO mixture), CPO, RPO, PO	saponification (by reflux condenser), liquid-liquid extraction, TLC separation, GC-FID determination	IOC (2017e)
Erythrodiol and uvaol as % of total sterols	EVOO, LVOO, ROO, OO (EVOO+ROO mixture), CPO, RPO, PO	saponification (by reflux condenser), liquid-liquid extraction, TLC separation, GC-FID determination	IOC (2017e)
Wax content	EVOO, LVOO, ROO, OO (EVOO+ROO mixture), CPO, RPO, PO	hexane extraction, silica SPE purification and GC-FID determination	IOC (2017f)
Maximum difference actual/theoretical equivalent carbon number 42	EVOO, LVOO, ROO, OO (EVOO+ROO Mixture), CPO, RPO, PO	hexane extraction, silica SPE purification and HPLC-reflective index determination	IOC (2017g)
Stigmastadienes (mg/kg)	VOO, OO, LVOO	saponification (by reflux condenser), liquid-liquid extraction, silica SPE separation, GC-FID determination	IOC (2017h)
2-glyceryl monopalmitate	EVOO, LVOO, ROO, OO (EVOO+ROO mixture), CPO, RPO, PO	liquid-liquid extraction, silica SPE, pancreatic lipase treatment, GC/FID determination	IOC (2017i)

Note: CPO (crude pomace oil), EVOO (extra virgin olive oil), LVOO (lampante virgin olive oil), OO (olive oil), PO (pomace oil), ROO (refined olive oil), RPO (refined pomace oil), VOO (virgin olive oil).

The natural variability of olive oil constituents, due to regional differences in olives and the scope of testing currently required to confirm the authenticity of an olive oil, presents a gap in the literature particularly with regard to alternative methods for the detection of diluent oils in olive oil. For example, tocopherols and phenolic content, which are deemed as quality parameters by the IOC trade standard (IOC, 2018) could provide a means for detection of diluent vegetable oils. Since it could be suggested that differences in constituents may lead to a viable alternative means to differentiate between a pure EVOO and a diluted EVOO. Furthermore, the role of phenolic and tocopherols with regard to antioxidant capacity (AOC) of an olive oil have been well charactered and the degree of which both contribute to AOC could potentially lead to a method for olive oil authentication. The use fluorescence and UV measurements can also provide an in-depth authentication method, with the use of concurrent excitation emission fluorescence profiles of EVOOs diluent with edible oils. Lastly alternative approaches to determine phenolics in olive oil such as the Folin-Ciocalteu assay, may also provide a means by which olive oil can be authenticated. The prominent aim of this research is the investigation of these constituents and methodologies and evaluate their usage as a means for determining EVOO authenticity.

1.4 Research Aims and Objectives

The aim of this research is to evaluate several olive oil constituents including tocopherols and phenolic compounds, using a range of common and novel instrumental techniques in order to optimise the assessment and evaluation of olive oil adulteration. The main objectives are:

- To develop and validate methods that are able to distinguish between pure EVOOs and diluted EVOO *via* the determination of tocopherol, phenolics, antioxidant capacity, fluorescence, and UV profiles.
- To evaluate these methods and constituents and assess their potential and suitability for olive oil authentication.
- To investigate the relationship between these constituents and how the combined use of their respective methods could overcome the limitations that occur upon their separate use.

To achieve these aims and objectives, the conceptual framework presented in Figure 1.3 was established.

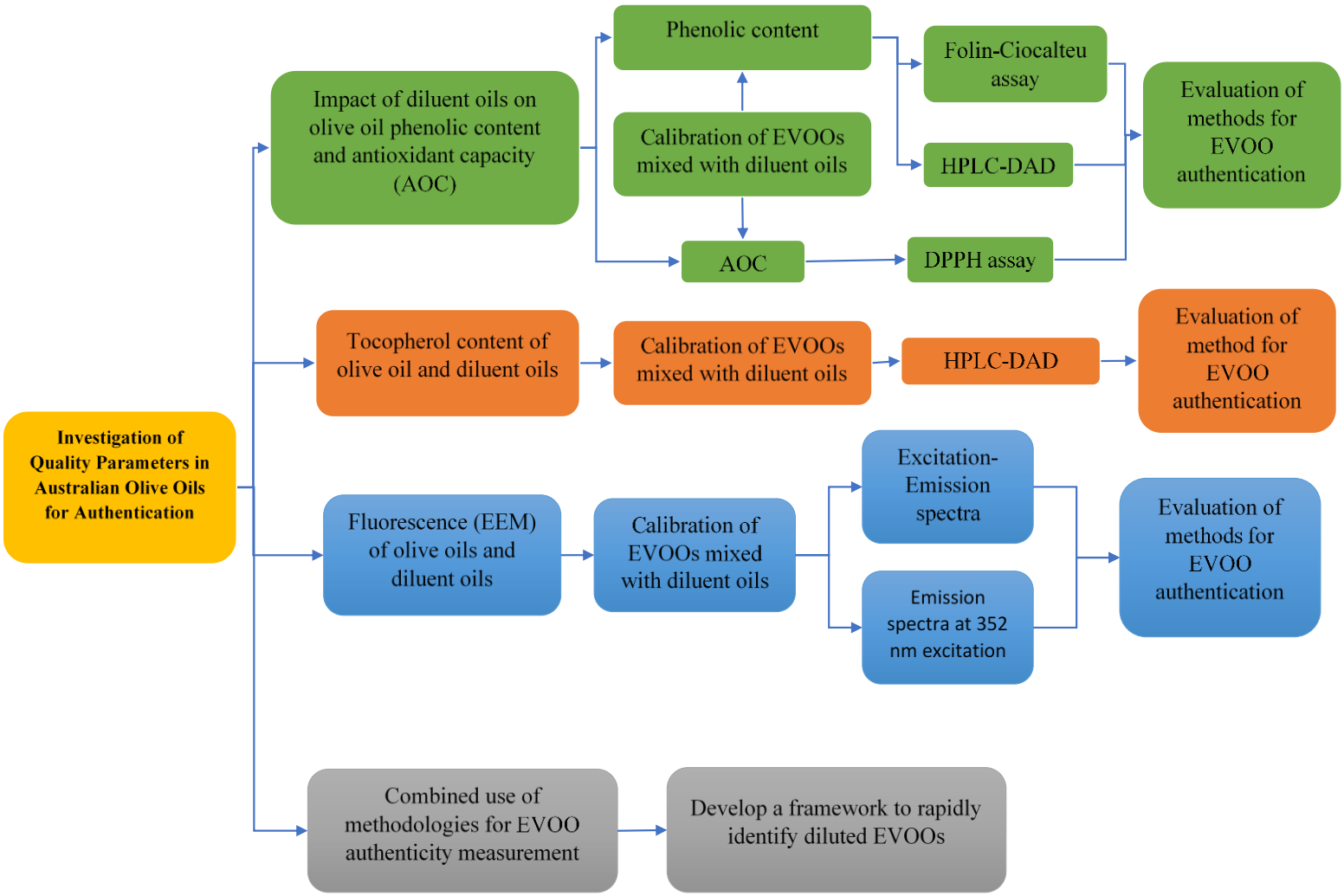


Figure 1.3: Conceptual framework developed for this research.

1.5 Thesis Outline

The thesis encompasses the following chapters as part of the research:

- Chapter 1: Introduction

Introduces the background information in which the production, classification and measurement of olive oil authenticity are introduced. Finally, this section discusses the research aims and objectives of this research and the thesis outline.

- Chapter 2: Literature Review

The literature review presents an extensive discussion of the various constituents that are monitored and assessed in the authentication of olive oil, the methods used for the determination of these and potential means by which EVOO and diluent oils are differentiated.

- Chapter 3: Materials and Methods

The selected EVOOs, diluent oils, and chemicals used in the experiments are listed in this chapter, with a description of all equipment and techniques used in the experiments.

- Chapter 4: Authentication of EVOOs *via* phenolic content and antioxidant capacity

This chapter discusses the validation of three methods for the determination the total phenolic content and the antioxidant capacity of pure EVOOs and those diluted with canola, sunflower and rice bran oil. The methods validated include total phenolic content *via* HPLC-DAD and the Folin-Ciocalteu colorimetric assay for measuring the total concentration of phenolics and the DPPH assay for antioxidant capacity. This chapter evaluates the suitability of these methods for the authentication of EVOOs and in particular, whether the three diluent oils can be detected with each method. The limitations of the methods are discussed, and a decision tree is presented to incorporates all three techniques to overcome these limitations.

- Chapter 5: Evaluation of EVOOs *via* Spectrophotometric Techniques

This chapter investigates the suitability of using three-dimensional fluorescence excitation/emission matrices (EEMs) and UV spectra to evaluate the authenticity of EVOOs diluted with the selected oils. Firstly, the EEMs of pure EVOOs are compared to EVOO mixtures with known concentrations of these diluent oils and the excitation/emission regions which exhibit the highest fluorescence intensity are identified. The intensity of specific excitation and emission wavelengths are further investigated to identity the sensitivity, selectivity, and linearity of this technique which is concurrently performed using UV spectral data.

- Chapter 6: Authentication of EVOOs *via* tocopherol content

This chapter investigations the tocopherol composition of pure EVOOs and EVOOs diluted with selected oil. Firstly, the suitability of the facile extraction and detection *via* HPLC-DAD is confirmed with the standard addition of α-, β-, δ- and γ-tocopherols to EVOOs. Following this confirmation, the total concentration of tocopherols and the compositional differences of the different tocopherol types in pure EVOO and diluted EVOO are compared to identify key parameters for the use of tocopherol as an authentication method. Lastly, these established parameters are used to construct a decision tree that provides a systemic approach to identify a diluted EVOO.

- Chapter 7: Framework for EVOO authentication

This chapter assess the EVOO evaluation methods used in this research with a focus on their suitability for the detection of diluent oils. This assessment of these methods is further extended to the suitability to identify and quantify the diluent oil contained in mixtures. Lastly, this chapter proposes a framework to systemically apply the investigated methods for the authentication of EVOOs.

- Chapter 8: Conclusions and Recommendations

This chapter provides the main conclusions of the research and presents some recommendations for further research.

Chapter 2 Literature Review

2.1 Introduction

The importance of methods to authenticate extra virgin olive oils (EVOOs) has been outlined previously in Chapter 1 . Briefly, this included cases of EVOO adulteration with edible oils and how these EVOO constituents are regulated by the International Olive Council (IOC) trade standard (IOC, 2018). This chapter will discuss the three more prominent IOC methods, namely those that characterize EVOOs on the basis of fatty acid methyl esters, triglycerides and phytosterols to highlight their limitations and the need to further investigate alternative constituents and methods to identify EVOOs diluted with edible oils. Furthermore, this chapter will discuss the range of techniques studied in this research for evaluating the key EVOO constituents of interest (phenolics, antioxidants, tocopherols, and spectroscopic methods). In particular, the methods for determination of the various components, their contribution to EVOO quality, and factors that influence the composition and distribution of these compounds is discussed.

The composition of an EVOOs constituents contributes to both its quality and health benefits, however EVOOs diluted with edible oils will alter the composition of constituents of the mixed oil and thus provide a means by which to detect fraudulent EVOOs. The IOC trade standard presents a list of eleven constituents, each with a set of criteria to determine the purity of olive oil, whilst other parameters provide a measure of the quality of a given olive oil (IOC, 2018) As such, the literature has reported various techniques for the determination of these constituents; which include colorimetric assays and analysis *via* high performance liquid chromatography-diode array detector/mass spectrometry (HPLC-DAD/MS) and gas chromatography-flame ionization detector/mass spectrometry (GC-FID/MS) (Aparicio et al., 2013a; Ben-Ayed et al., 2013).

The prevalent techniques recommended by the IOC for olive oil authenticity include both liquid and gas chromatography where the choice of method is dependent on the constituent of interest (Conte et al., 2019). Gas chromatography is suited for volatile chemical compounds such as fatty acids, wax esters, and phytosterols. Liquid chromatography enables the separation of non-volatile constituents or constituents with poor thermal stability which includes tocopherols and phenolics (Conte et al., 2019; Aparicio et al., 2013a). These chromatographic techniques are typically used to verify EVOO purity and as such, are the most sensitive and

selective for the presence of diluent oils. Other techniques, which involve classical chemistry methods to measure olive oil quality parameters include peroxide value, ultraviolet absorbance, organoleptic properties and insoluble impurities (Conte et al., 2019; IOC, 2018).

The measurement of alternative constituents to determine the authenticity of an EVOO has also been reported in the literature including the evaluation of tocopherols and alternative techniques including fluorescence (Chen et al., 2011; Aparicio et al., 2013b; Ben-Ayed et al., 2013). In addition to the qualitative and quantitative evaluation of the various constituents, the benefits of these compounds have also been widely reported, such as the stability of EVOO due to its antioxidant properties and health benefits provided by phenolic compounds and tocopherols (Rizvi et al., 2013; Cicerale et al., 2009; Dimitrios, 2006; Martín-Peláez et al., 2013). The following sections discuss the various components and functions of EVOOs, their measurement as a means of quality and authenticity determination, and the advantages and limitations of these measurements.

2.2 EVOO Lipids

The IOC stipulates methods to assess the purity of an olive oil and include the determination of triglycerides and fatty acid methyl esters. This section discusses how these evaluations relate to olive oil authentication, as well as their respective advantages and limitations.

2.2.1 Triglycerides in EVOOs

Fatty acids naturally occur as groups of esterified fatty acids, collectively referred to as triglycerides (TGs) and are comprised of a glycol backbone and three fatty acids. These TGs are the major components in olive oils consisting of 95-98% of the total mass of an EVOO. There exists twenty possible TGs present in olive oils and the composition of these is dependent on the EVOO variety, however, only five are present in significant quantities (Aparicio et al., 2013a; Christopoulou et al., 2004; Jabeur et al., 2014; Conte et al., 2019). The predominant TG in olive oil is triolein, that is comprised of three units of oleic acid bound to the glycerol backbone (Aparicio & Harwood 2013; Ben-Ayed et al. 2013).

The IOC stipulates a method for TG determination (IOC, 2017g) which entails a solid-phase extraction using a silica column, with the elution of fatty constituents *via* a n-hexane and diethyl ether mixture. These extracts are volumetrically split equally and are evaporated under

nitrogen with the fatty residues being reconstituted in acetone and n-hexane for further analysis. The n-hexane solution is used for fatty acid methyl ester analysis whilst the acetone solution is used for TGs determinations *via* normal phase-high performance liquid chromatography (NP-HPLC) coupled to a refractive index detector (RID). A calculation can be performed to determine the grade of an olive oil or whether adulteration has occurred which involves the determination of the difference between the theoretical equivalent carbon content (ΔECN42). This calculation is based on the content of fatty acid methyl esters and the experimental ECN42 content which in turn is based on the TG determination. This value, based on the IOC guidelines (IOC, 2018), is able to distinguish between olive oil gradings (i.e. extra virgin, virgin, refined etc.), and a value exceeding ΔECN42 = 0.60 indicates that a diluent oil is present (see Table 2.1).

Table 2.1: Olive oil and acceptable ΔECN42 for individual olive oil grades

Olive Oil	**ΔECN42**
virgin olive oils	≤0.20
lampante virgin olive oil, refined olive oil, olive oil (ROO + VOO)	≤0.30
crude pomace oil	≤0.60
refined olive oil and olive pomace oil	≤0.50

The analysis of TGs on the basis of ΔECN42 values was investigated by Christopoulou et al. (2004). This was to determine whether EVOOs diluted with edible oils (canola, walnut, sunflower, soybean, hazelnut, cotton and mustard) at concentrations of 5% w/w exceeded the limits of ΔECN42 in the IOC trade standard (IOC, 2018). This study found the IOC method was not suited to identify an EVOO diluted with mustard, hazelnut, almond or peanut oil at the 5% w/w level and could therefore not provide a LOD for these diluent oils. Conversely, rapeseed and canola oil exceeded IOC limits at concentrations of 4% and 3% w/w, respectively. Finally, TG analysis was able to detect EVOO diluted with sunflower, soybean, cotton and corn oil at concentrations of 1% w/w. The suitability of evaluating TGs *via* ΔECN42 for EVOO authentication was also studied by Jabeur et al. (2014), who suggest that the required mass of soybean, corn and sunflower oil that exceed the ΔECN42 of 0.60 is 3% w/w. An alternative method reported by Salghi et al. (2014), incorporated HPLC coupled to evaporative light

scattering detectors for the determination of argan oil diluted with sunflower, soybean and olive oil up to a level of 5% w/w and was reported to offers similar detection limits for EVOOs.

2.2.2 Fatty Acid Methyl Esters in EVOOs

Fatty acids (FAs) are the dominant constituents of olive oils and are present as esterified mono-, di-, and triglycerides rather than free fatty acids. The official method for the determination of FAs in EVOO involves the methylation of the FAs followed by chromatographic separation by GC-FID (IOC, 2017f). The methylation of olive oil samples removes the glycerol backbone liberating the free fatty acid methyl esters (FAMEs) that are analysed individually to profile the oil. The relative fatty acid limits stipulated by the IOC are presented in Table 2.2.

Table 2.2: IOC stipulated FA limits[#]

FA Common Name	Lipid Number	IUPAC Name	Regulation Limit (% w/w FAME)
myristic acid	C14:0	hetradecanoic acid	≤0.03
palmitic acid	C16:0	hexadecenoic	7.50 - 20
palmitoleic acid	C16:1	9-hexadecanoic	0.30 - 3.50
margaric acid	C17:0	heptadecanoic acid	≤0.40
margaric acid	C17:1 10c	cis-10c heptadecanoic acid	≤0.6
stearic acid	C18:0	octadecanoic acid	0.50-5.00
oleic acid	C18:1 9c	cis-9-octadecanoic	55.00-83.00
linoleic acid	C18:2	cis-9-octadecanoic	2.50-21.00
α-linolenic acid	C18:3	9,12,15-octadecatrienoic acid	≤1
arachidic acid	C20:0	icosanoic acid	≤0.60
gadoleic acid (eicosenoic)	C20:1	*cis*-9-Eicosenoic acid	≤0.5
behenic acid	C22:0	docosanoic acid	≤0.20
lignoceric acid	C24:0	tetracosanoic acid	≤0.20

#Trade standard applying to olive and olive pomace oils (IOC, 2018)

The methylation of TGs to the form of FAMEs has seen extensively studied in edible oils since the 1960s and as such, are well understood (Aparicio et al., 2013a). The IOC has stipulated limits for FAs in olive oils as the levels of these can be used as indicators of the presence of diluent oils (IOC, 2018). As the production of olive oil has no effect on the fatty acid content of the oil, no regulatory limit is given by the IOC for individual olive oil grades.

Thus, measurement of FAMEs is unable to identify olive oils that are adulterated with lesser grade oils (IOC, 2018; Conte et al., 2019). Furthermore, adulterant oils high in oleic acid content, such as rapeseed and soybean oils, are difficult to identify with this method.

The FA composition can be categorized further with the determination of *trans* isomers of key fatty acids which include oleic, linoleic and linolenic acids which are used to discriminate between olive oil grades (Table 2.3). If these *trans* isomers are present at levels above the regulated limits, this may indicate the adulterant is a refined olive oil or other refined seed oil (Aparicio et al., 2013a; Ben-Ayed et al., 2013).

Table 2.3: IOC limits of trans FAs[#]

Olive oil grade	oleic acid trans isomers (% fatty acid w/w)	linoleic acid + α-linoleic acid trans isomers (% fatty acid w/w)
extra virgin olive oil (EVOO)	≤0.05	≤0.05
lampante virgin olive oil	≤0.10	≤0.10
refined olive oil (ROO)	≤0.20	≤0.30
olive oil (EVOO +ROO)	≤0.20	≤0.30
olive pomace oil	≤0.40	≤0.35

#Trade standard applying to olive and olive pomace oils (IOC, 2018)

The analysis of fatty-acid methyl esters (FAMEs) constituents of EVOOs was investigated by Jabeur et al. (2014) who studied mixtures of corn, soybean and sunflower oils with Tunisian EVOOs over two harvest periods. The olive oils were diluted with up to 10% w/w of each diluent oil and were characterized by their FAMEs, TG, and phytosterol content. The results of the FAMEs analysis suggested that only soybean oil was able to be detected due to the increased the linoleic acid content and required a mass ratio of 5% w/w to exceed IOC FAME limits. The corn and sunflower oils presented similar FAMEs compositions profiles to the EVOOs and were therefore unable to be detected, however, the methods involving the analysis of TGs and phytosterols were able to distinguish between EVOOs diluted with these oils (Jabeur et al., 2014). This suggests that although FAMEs analysis can potentially identify diluent oils, it is limited to diluent oils where the FAMEs composition differs to that of the EVOO. A summary of the limits of detection for several diluent oils investigated in the literature are presented in Table 2.4.

Table 2.4: Limit of detection of various diluent oils *via* TG and FAMEs analyses

Constituent Group	Extraction Method	Instrument	Diluent oil	Constituent of interest	Limit of Detection % (w/w)	Reference
TGs	silica solid phase extraction, hexane solvent	HPLC-RID	sunflower, soybean, cotton, corn, walnut, safflower	ΔECN42	1	Christopoulou et al. (2004)
			sesame		2	
			canola		4	
			soybean		3	Jaber et al. (2014)
			corn		3	
			sunflower		1	
		HPLC-evaporative light scattering	argan	TGs	5	Salghi et al. (2014)
FAMEs	trans-esterification in methanol, silica solid phase extraction	GC-FID	soybean	linolenic acid	2	Christopoulou et al. (2004)
			canola		5	
			walnut		1	
			mustard		1	
	trans-esterification in methanol	GC-FID	soybean	linolenic acid	3	Jaber et al. (2014)
	trans-esterification in methanol	GC-FID	sesame	linolenic acid	10	Rohman and Che Man (2012)

2.3 EVOO Phytosterols

The minor constituents of olive oil comprise the final 1-2% w/w of the oil and are collectively referred to as the unsaponifiable fraction. However, these constituents contribute greatly to the measure of the quality of an EVOO and provide some of the well-established health benefits (Ben-Ayed et al., 2013; Conte et al., 2019). Several factors influence the concentration of these constituents such as the olive variety and growth region (Kycyk et al., 2016; Mailer et al., 2010), the production and refinement of the oil (Capurso et al., 2018), and the age of the oil (Jukić Špika et al., 2015). The IOC has stipulated methods and limits for several minor constituents of EVOO, such as phytosterols (IOC, 2018), which are used to indicate the quality of the oil. For example the quality of an EVOO is determined *via* the analytical measurement of total phytosterol content, whilst individual phytosterol

characterization can potentially provide a means to assess the purity or the authenticity of the oil (Jukić Špika et al., 2015).

2.3.1 Measuring Phytosterol Content

Phytosterols are natural compounds that are present in plants and are similar in structure to that of cholesterol, only differing in the functional groups and the side chains that are present on the main structure (Lagarda et al., 2006).The most prevalent phytosterols in EVOOs are in the apparent β-sitosterol fraction (≥93% w/w) and campesterol (≤4% w/w) with the respective structures presented in Figure 2.1 (IOC, 2018).

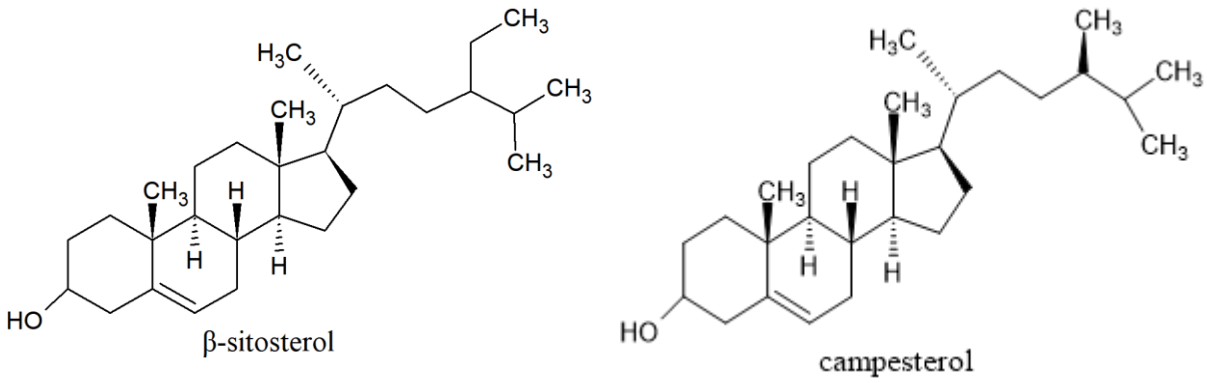


Figure 2.1: Structure of the most prevalent phytosterols in EVOOs.

The phytosterol concentration in olive oils typically exceeds 1000 mg kg^{-1} although olive pomace oils typically exhibit greater concentrations as shown in Table 2.5. The concentration of phytosterols in olive oil may therefore be used for authenticity determination based on differences in the total sterol content and the composition of individual phytosterols (IOC, 2018; Jabeur et al., 2014).

Table 2.5: Total phytosterol content in different olive oils

Olive Oil	Limit (mg kg^{-1})
EVOO	≥1000
refined olive oil	≥1000
olive oil	≥1000
crude olive pomace oil	≥2500
refined olive pomace oil	≥1800
olive pomace oil	≥1600

#Trade standard applying to olive and olive pomace oils (IOC, 2018)

Certain individual sterols are also regulated by the IOC and are used to identify olive oils diluted with other edible oil adulterants, as presented in Table 2.6 (IOC, 2018). An EVOO

phytosterol profile which differs from these limits could be presumed to be adulterated with a diluent oil.

Table 2.6: Individual phytosterol limits in EVOO#

Individual phytosterol content	phytosterol limit (% w/w)
cholesterol	≤0.5
brassicaterol	≤0.1
campesterol	≤4.0
stigmasterol	≤4.0
δ-7-stigmastenol	≤0.5
apparent β-sitosterol	≥93.0

Note: the apparent β-sitosterol fraction includes δ-5-avenasterol, δ-5,23-stigmastadienol, clerosterol, sitostanol and δ-5-24 stigmatadienol.
#Trade standard applying to olive and olive pomace oils (IOC, 2018)

The prescribed IOC method (IOC, 2017e) entails extraction of phytosterols *via* the saponification of olive oil which liberates the sterols from the oil matrix. This is followed by a liquid-liquid extraction and separation of the phytosterol fraction from other constituents with normal phase thin-layer chromatography (TLC). Finally phytosterol volatility is improved with silanization of the final extract before analysis by GC-FID (IOC, 2017e). As such, the IOC method requires significant time and labour investment, particularly with regard to the TLC separation. This is due to the necessity to pre-treat the TLC plates, carefully load them with 200 µL of the unsaponifiable fraction, and the subsequent development time required for a large TLC plate (Srigley et al., 2015; IOC, 2018). Further concerns may arise when the phytosterol content is present at low concentrations which my result in poor separation of the phytosterol fraction on the TLC plate and poor extraction repeatability (Srigley et al., 2015). Deposition of excess saponifiable fraction on the plate, lack of development time and removal of the sterol band present on the silica may also contribute to inadequate separation of phytosterols and other constituents in the olive oil (Aparicio et al. 2013b; Nestola & Schmidt 2016). These difficulties and high labour investment of the prescribed IOC method have prompted the development of alternative techniques that deliver this important fingerprint of phytosterol types and contents in EVOOs with the concurrent improvements in extraction techniques.

A simplified phytosterol quantification method which reduces the laborious nature of the IOC method has been proposed by Mathison and Holstege (2013). This method replaces the reflux condensers with in-tube saponification and the liquid-liquid extraction is also carried

out in-tube. The subsequent separation of the phytosterol fraction that is typically performed using normal phase TLC is replaced with silica solid phase extraction (SPE). The GC-FID condition utilized in this method correlates to the conditions stipulated by the IOC phytosterol method, in which a 5 %w/w diphenyl polysiloxane GC column and oven temperatures to elute phytosterols of 260 °C are used for chromatographic separation (Mathison and Holstege, 2013; Gül and Şeker, 2006; Fernández-Cuesta et al., 2013).

The replacement of TLC separation with silica SPE has also been widely used and has the advantage of maintaining sensitivity, reducing labour time and removing errors due to the use of silica TLC plates (Cunha et al., 2006; Nestola and Schmidt, 2016; Mathison and Holstege, 2013). A further advantage of SPE includes automation via on-line SPE and has resulted in the shortening of the analysis time for the phytosterol content in EVOOs by 2 h (Nestola & Schmidt 2016). A comparative study between the IOC method and an improved alternative for phytosterol content measurement in EVOO was reported by Mathison and Holstege (2013) which compared both methods for sensitivity and selectivity (IOC, 2017e). The improved method fortified EVOO samples with known concentrations of stigmasterol and β-sitosterol. This method recovered 88% and 84% of stigmasterol and β-sitosterol respectively, compared with the IOC method that recovered 61% and 65% of stigmasterol and β-sitosterol, respectively. Thus, the IOC standard method may present an expanded analytical error for the analysis of phytosterol content in EVOO due to its significant decrease in recovered phytosterols which could be presumed to arise due to the loss of phytosterols throughout the extraction process. A direct comparison of endogenous phytosterols in 34 EVOOs via both the IOC method and the improved method presented comparable results with R^2 values of both methods were between 0.91 and 0.97. The improved method therefore offers reduced labour time, increased throughput and potentially reduces the loss of phytosterols during extraction.

2.3.2 Factors Influencing Phytosterol Content

The composition of phytosterols in edible oils offers a unique fingerprint which can be used to analytically identify a diluted EVOO. Both the total phytosterol concentration and relative concentrations of the individual phytosterols are useful for identifying olive oils that have been adulterated with other plant oils (Srigley et al., 2015; Jabeur et al., 2014). For example, Jabeur et al. (2014) diluted EVOO test oils with known concentrations of soybean, sunflower and corn oil and established the required mass of adulterant to exceed IOC limits. The apparent β-sitosterol fraction decreased with the addition of sunflower and corn oil and

exceeded the IOC limit when the EVOOs were diluted with 5% and 10% w/w mixtures respectively. Conversely, the concentration of Δ7-stigmastenol increased with diluent addition and required 1% w/w and 10% w/w of sunflower and soybean oil respectively to exceed the IOC limit (Jabeur et al., 2014). This apparent success of the authentication *via* phytosterol content suggests the possibility of using several key phytosterol indicators to determine olive oil purity and identify adulterated oils.

One limitation in measuring phytosterols for the authentication of EVOOs is the natural variance in phytosterols that occurs due to year of harvest, olive cultivar and the growing region. For example, the phytosterol campesterol has been observed to exceed the IOC limits in some pure EVOOs due to natural variance (Guillaume et al., 2011; Rivera del Álamo et al., 2004; Kycyk et al., 2016; Fernández-Cuesta et al., 2013). Rivera del Álamo et al. (2004) measured the phytosterol content of Cornicabra EVOOs from a single region in Spain over four harvest seasons. This study found the concentrations of β-sitosterol and campesterol varied between 91.9-95.0% w/w and 3.42-4.50% w/w respectively over the course of the seasons. Although the respective means of β-sitosterol and campesterol (i.e. 93.5% and 4.01% w/w respectively) are withing the stipulated limits set by the IOC, the upper and lower limits of the concentration range could potentially result in the false identification of the EVOOs as adulterated (IOC, 2018; Rivera del Álamo et al., 2004). This has also been observed for certain Australian EVOOs produced in New South Wales, which also exhibited campesterol levels above the IOC limits of 4% w/w of phytosterols (Mailer et al., 2010).

This natural variability in phytosterol content of EVOOs has also been reported in the literature for both olive cultivar and location of the olive grove (Skiada et al., 2019; Giuffrè and Louadj, 2013; Haddada et al., 2007; Mansouri et al., 2015; Rivera del Álamo et al., 2004; Kycyk et al., 2016). The phytosterol content of 43 olive cultivars, from various regions such as Greece, Spain, Italy, Turkey, Croatia and USA, was presented by Kycyk et al. (2016). The range of total phytosterol content for all tested oils was between 848 and 2378 mg kg^{-1}, thus the phytosterol content of certain EVOOs tested was less than the IOC (2018) phytosterol limit (≥1000 mg kg^{-1}). This variability was also observed with certain individual phytosterols including β-sitosanol which presented a range between 89-96% w/w of all phytosterols in the surveyed EVOOs. The measured levels of campesterol were between 2.4 and 5.1% w/w of total phytosterols. This indicates that some of the investigated EVOOs fall outside phytosterol limits for β-sitosanol and campesterol (≥93% and ≤4% respectively). These included seven of the 43

EVOOs which fell below the IOC stipulated limit of β-sitosanol and five EVOOs which exceeded the IOC campesterol limit (IOC, 2018; Kycyk et al., 2016). The natural variance of phytosterols content arising from differences in the olive cultivar and growth region has also been observed in the wider literature, and highlights that this variance can lead to a EVOO incorrectly failing the purity criterion of the IOC trade standard (Giuffrè and Louadj, 2013; Haddada et al., 2007; Mansouri et al., 2015; López-Cortés et al., 2013; Cunha et al., 2006; Skiada et al., 2019).

These studies have highlighted the limitations of using of phytosterol measurements including the laborious extraction of phytosterols and the necessity of two separate analysis to calculate ΔECN42. Although phytosterol profiles present a unique fingerprint for individual oils and thus a sensitive means to authenticate an EVOO diluted with other oils, concerns arise due to the natural variability of phytosterol content arising from harvest season, olive variety and olive grove region as these have been shown in certain cases to lie outside stipulated limits of phytosterols. This may potentially result in EVOOs being falsely labelled as adulterated; thus, supplement testing is required for alternative confirmation methods. These could include certain quality parameters, such as phenolic, antioxidant capacity, tocopherols or spectrometry.

2.4 EVOO Phenolic Compounds

The phenolic compounds of an EVOO contribute to both its oxidative resistance and nutritional benefits (Cicerale et al., 2009; Czerwińska et al., 2012). Similar to the measurement of phytosterols, the analysis of phenolic compounds in olive oils as a measure of purity has been seemingly limited due to natural variability, particularly between growth regions (Bajoub et al., 2016a; Arslan et al., 2013). Nonetheless, their use as a measure of the quality of an EVOO, in particular the contribution of phenolics to antioxidant capacity, has been widely studied (Fuentes et al., 2018; Blekas et al., 2002; Krichene et al., 2010; Janu et al., 2014; Franco et al., 2014a; De Leonardis et al., 2013; Kalogeropoulos and Tsimidou, 2014; Czerwińska et al., 2012; Cecchi et al., 2013; Okogeri and Tasioula-Margari, 2002). The determination of phenolic content of edible oils *via* both HPLC-DAD and colorimetric assays is frequently reported in literature (Fuentes et al., 2012; Alessandri et al., 2014; Reboredo-Rodríguez et al., 2016; Zullo and Ciafardini, 2008). Determinations by HPLC-DAD are common and the IOC has provided a prescribed method for the analysis of a wide range of phenolics present in olive oils (IOC, 2017a). Other techniques have also been reported such as alternative extraction techniques based on solid-phase and liquid-liquid extraction, as well as other types of

instrumentation or analysis such as the use of mass spectrometry (MS) coupled to HPLC and the common Folin-Ciocalteu colorimetric assay.

2.4.1 Folin-Ciocalteu Colorimetric Assay of Phenolics

The Folin-Ciocalteu (FC) assay is a colorimetric method that utilises the FC reagent (FCR) which acts as a reducing agent and as such, is a measure of antioxidants which include phenolics (Singleton et al., 1999). The measurement of antioxidants using the FC assay is commonly expressed in terms of equivalence to the phenolic compound gallic acid, with the results referred to as gallic acid equivalents (GAE) (Singleton et al., 1999; Reboredo-Rodríguez et al., 2016; Alessandri et al., 2014; Garcia et al., 2013).

The FC assay measures phenolics *via* a hydrogen transfer from phenolics to the FC reagent (FCR) in an alkaline medium (Reboredo-Rodríguez et al., 2016; Fuentes et al., 2018; Alessandri et al., 2014). The typical FC assay entails extraction of reducing substrates in a polar solvent such as methanol/water mixtures followed by a pH adjustment to 10 which corresponds to the typical pK_a value of phenolics. This results in the uptake of oxygen by the phenolate ions and the subsequent dissociation of hydrogen ions (Singleton et al., 1999). The released hydrogen ions bind to the FCR which generates a blue chromophore that is assessed by measuring its absorbance at 750 nm which, in turn, is correlated to the amount of antioxidants, predominantly phenolics, present in the oil sample (Ballus et al., 2015; Singleton et al., 1999; Reboredo-Rodríguez et al., 2016; Fuentes et al., 2018; Alessandri et al., 2014; Georgé et al., 2005; Garcia et al., 2013). The IOC presents the measurement of phenolics as a quality parameter for EVOOs, thus the FC assay is a more rapid, simplified technique compared to determination *via* HPLC-DAD to estimate total phenolic content (TPC). However, the FC assay is non-specific to phenolics and may respond to other antioxidants, so it is therefore only suitable as a means to estimate the TPC of an EVOO. Nonetheless, the FC assay has been shown to correlate well with phenolic composition determined using HPLC-DAD/MS analysis (Garcia et al., 2013; IOC, 2018; Alessandri et al., 2014).

2.4.2 HPLC-DAD/MS Analysis of Phenolics

The extraction of phenolics in EVOO prior to HPLC analysis typically involves the removal of the compounds in a polar solvent such as methanol, ethanol and water or methanol/water mixtures (Morelló et al., 2004; Becerra-Herrera et al., 2014; Tasioula-Margari and Tsabolatidou, 2015). The IOC presents a standard method for the extraction of phenolics

from olive oils whereby 2 g of EVOO is diluted in 80% (v/v) MEOH:H_2O and syringic acid is added as an internal standard (IOC, 2018; IOC, 2017a).The quantification of total phenolics is established *via* HPLC-DAD with UV absorbance at 280 nm and is based on the response of the internal standard and all phenolic constituents. These phenolics are identified based on their elution order and retention time relative to the internal standard. Figure 2.2 presents an example chromatogram with the peaks numbered in accordance with the relative retention times of typical phenolics as per the IOC method that are presented in Table 2.7 and (IOC, 2017a).

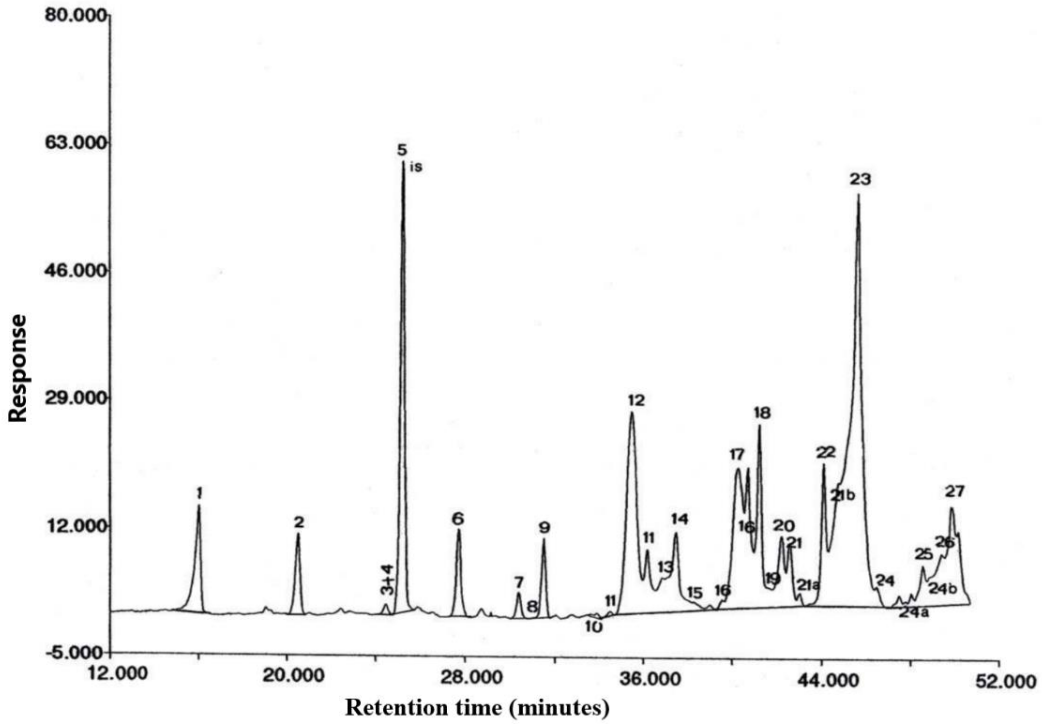


Figure 2.2: Example EVOO phenolic HPLC chromatogram.
#Determination of Biophenols in Olive Oils by HPLC (IOC, 2017a).

Table 2.7: Elution parameters for phenolic compounds[#]

Peak number	Phenolic compounds	Relative retention time/min	UV absorbance wavelength/nm
1	hydroxytyrosol	0.62	230-280
2	tyrosol	0.8	230-275
3	vanillic acid	0.96	260
4	caffeic acid	0.99	325
5	syringic acid (internal standard)	1	280
6	vanillin	1.1	310
7	para coumaric acid	1.12	
8	hydroxytyrosyl acetate	1.2	232-285
9	ferulic acid	1.26	325
10	ortho-coumaric acid	1.31	
11	decarboxymethyl oleuropein aglycone, oxidised dialdehyde form	-	235-280
12	decarboxymethyl oleuropein aglycone, dialdehyde form	1.45	
13	oleuropein	1.48	
14	oleuropein aglycone, dialdehyde form	1.52	
15	tyrosyl acetate	1.54	
16	decarboxymethyl ligstroside aglycone, oxidised dialdehyde form	1.63	235-275
17	decarboxymethyl ligstroside aglycone, dialdehyde form	1.65	232.28
18	pinoresinol	1.69	270
19	cinnamic acid	1.73	235-275
20	ligstroside aglycone, dialdehyde form	1.74	235-280
21	oleuropein aglycone, oxidised aldehyde and hydroxylic form	-	255-350
22	luteolin	-	235-280
23	oleuropein aglycone, aldehyde and hydroxylic form	1.79	235-275
24	ligstroside aglycone, oxidised aldehyde and hydroxylic form	-	230-340
25	apigenin	1.98	255-350
26	methyl-luteolin	-	255-350
27	ligstroside aglycone, aldehyde and hydroxylic form	2.03	235-275

#Determination of Biophenols in Olive Oils by HPLC (IOC, 2017a)

To normalize the instrument response of the UV detector, a relative response factor (RRF) is calculated based on two standards measured at specific concentrations. These standards are syringic acid (15 mg kg^{-1}) and tyrosol (30 mg kg^{-1}) and the acceptable RRF value when using these standards is 5.1 ± 0.4. The use of an internal standard, in syringic acid equivalents, provides a method to calculate total phenolic content without requiring standards for each individual phenolic (IOC, 2017a). Although this IOC prescribed method is well

established as a quality determinant for olive oils, other methods for measuring phenolic content have also been proposed for both EVOOs and other oils which offers improved sample clean-up, and subsequently better HPLC resolution by the removal of interfering constituents (Tasioula-Margari and Tsabolatidou, 2015). These techniques differ in extraction technique and include liquid-liquid extraction, which entails the removal lipid constituents with n-hexane (Becerra-Herrera et al., 2014; Tasioula-Margari and Tsabolatidou, 2015; Wang et al., 2017).

An alternative method for phenolic extraction from EVOOs was developed by Tasioula-Margari and Tsabolatidou (2015), which established an efficient and robust procedure as shown schematically in Figure 2.3. Firstly, a comparison of two extraction solvents was performed with neat methanol and an 80% (v/v) methanol/water mixture which showed no significant difference in phenolic recovery. However, methanol was found to provide a more robust extraction because the methanol/water mixture formed emulsions between the EVOO and aqueous phase. Therefore, to remove the phenolics from the EVOO, three volumes of 5 mL of methanol was mixed with the oil, and the methanol layer was separated from the oil and removed under nitrogen. The phenolic residue was reconstituted with one of three polar solubilization solutions which were a methanol/water mixture (50:50 v/v), acetonitrile or dimethylformamide (DMF). The suitability of these solutions was confirmed by the recovery of phenolic standards and it was found that the methanol/water mixture only recovered 69% of the phenolics. The acetonitrile solution resulted in a more satisfactory recovery of 90%, and the DMF solution resulted in the greatest recovery of 95% of the phenolics. This improvement in recovery for acetonitrile and DMF can be attributed to their less polar nature whereby these solvents are able to extract the less polar phenolic compounds present in the oils (Tasioula-Margari and Tsabolatidou, 2015; Becerra-Herrera et al., 2014).

The final phase of the extraction process was the removal of non-polar residues *via* a liquid-liquid extraction with 15 mL of n-hexane, in three separate 5 mL volumes. The 50/50% (v/v) methanol/water and DMF solutions were separated from the n-hexane wash and injected directly into the HPLC-DAD/MS. Conversely, the acetonitrile solution was removed under nitrogen and the phenolic residues were reconstituted with methanol/water. The phenolic content of the solution was than determined using a C18 HPLC column coupled to a UV spectrometer set at 280 nm. This chromatographic separation has also been employed by the IOC methodology (IOC, 2017a) and other published literature (Alessandri et al., 2014; Bajoub et al., 2016a; Farrés-Cebrián et al., 2016; Tasioula-Margari and Tsabolatidou, 2015; IOC, 2017a).

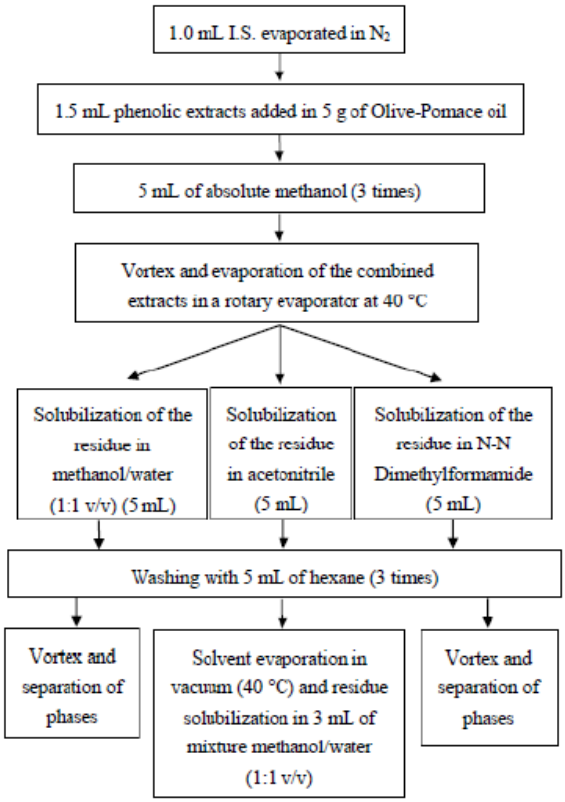


Figure 2.3: Optimised method for the extraction of phenolics (Tasioula-Margari and Tsabolatidou, 2015).

The detection of phenolic compounds was commonly achieved using HPLC-DAD whereby chromatographic separation is performed on a C18 column using an acidified aqueous phase such as 0.2% (v/v) phosphoric acid and a 50% (v/v) methanol/acetonitrile organic phase. Typically, a steady increase in the organic ratio occurs throughout chromatographic separation to elute the less polar phenolics (Alessandri et al., 2014; Becerra-Herrera et al., 2014; Farrés-Cebrián et al., 2016). The coupling of MS to reverse-phase HPLC has also been used to measure the phenolic content of vegetable oils and has the advantage of producing an expanded detection of phenolic constituents and greater power of peak identification (Tasioula-Margari and Tsabolatidou, 2015). Due to the lack of standards for many phenolic compounds, HPLC-MS provides an added benefit of identification of phenolic compounds in vegetable oils using appropriate reference libraries. Conversely, a potential disadvantage of HPLC-DAD measurements is the lower sensitivities to several phenolic compounds which exhibit emissions at lower energy wavelengths. For example, luteolin and apigenin present increased sensitivity at 340 nm, with the latter losing resolution at 280 nm thereby potentially limiting the sensitivity

of the IOC method if an EVOO contains greater concentrations of luteolin or apigenin (Tasioula-Margari and Tsabolatidou, 2015; IOC, 2017a).

2.4.3 Factors Influencing the Phenolic Content of Olive Oils

The two common techniques are employed to determine phenolic content in EVOOs, namely the FC assay and HPLC-DAD/MS, have been used to report factors which contribute to the composition of phenolics in EVOOs(Tasioula-Margari and Tsabolatidou, 2015; Fuentes et al., 2018). These factors include the region of growth, harvest season, and olive cultivar (Cicerale et al., 2009; Malheiro et al., 2015; Fuentes et al., 2018; Cecchi et al., 2013; Bajoub et al., 2016a). An example of the variability of phenolics due to growth region and harvest season was reported in the study of Bajoub et al. (2016a). This study investigated 136 EVOOs of the monovarietal *Picholine Marocaine* in Morocco between 2013 and 2014. The characterization of these EVOOs included the identification of twenty-nine phenolics, which included the twenty major phenolic compounds in EVOO, and the nine isomers of oleuropein and ligstroside aglycone *via* HPLC-MS. The majority of the phenolic compounds detected in all samples were from the group of secoiridoids whose concentrations ranged between 507 to 744 mg kg^{-1} (Bajoub et al., 2016a). Furthermore, there was also a significant difference between the phenolic profile of oils harvested between consecutive crops, in particular the secoiridoid fraction, which increased from 744 to 1224 mg kg^{-1} between the 2013/2014 harvest seasons. These differences in phenolic contents are also observed throughout the globe as presented in Table 2.8 (Kesen et al., 2014; Arslan et al., 2013; Fuentes et al., 2018) and suggests that the phenolic composition are influenced by both the growth region and the crop season.

Clearly the measurement of phenolic content is an accepted and widely used method for the determination of EVOO quality. However, given the aforementioned limitations, some caution should be used when employing this method for authenticity evaluation. This is particularly important for EVOOs obtained from different regions, cultivars or seasons and highlights the importance of obtaining adequate and samples for thorough benchmarking purposes. The measurement of phenolic content as a measure of EVOO authenticity is explored in this thesis to demonstrate its applicability and the potential limitations.

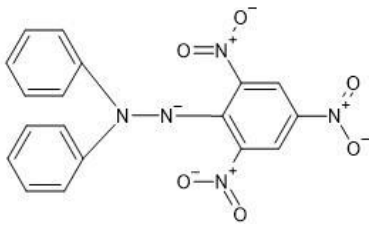
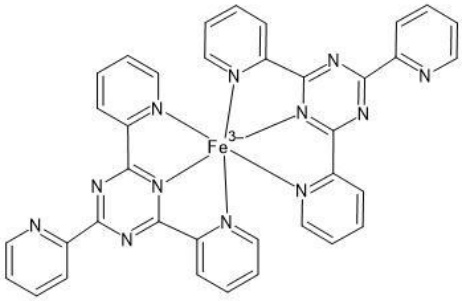
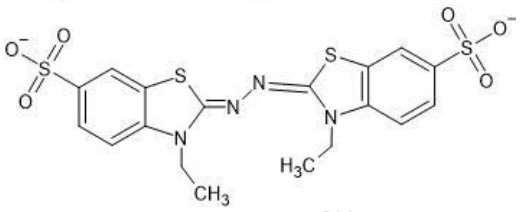
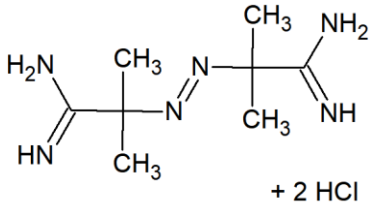
Table 2.8: Total phenolic concentrations of European EVOOs

Region/country	Detection method	Total phenolic content/ mg kg^{-1}	Harvest year	Cultivar/s	Reference
Morocco	HPLC-MS	507-744	2013	Picholine Marocaine	Bajoub et al. (2016a)
		532-1224	2014		
Italy and Spain	FC assay	117-687	2015	N/D	Reboredo-Rodríguez et al. (2016)
Mediterranean	HPLC-MS/MS	234-2212	2018	Picual	Criado-Navarro et al. (2020)
		88-2603	2019	Picual	
		118-2595	2018	Hojiblanca	
		1.8-2090	2019	Hojiblanca	
Sierra de Segura/Spain		157-201	2012	Picual	
Oli de Mallorca/Spain	HPLC-MS/MS	111-199	2012	Picual	Becerra-Herrera et al. (2018)
Priego de Córdoba/Spain		92-138	2012	Hojiblanca	
		94-132	2012	Picudo	
Italy	HPLC/DAD	282-404	2002-2008	Frantoio, Correggiolo, Leccino, Moraiolo, Seggianese, Taggiasca	Alessandri et al. (2014)
	FC assay	118-250 GAE			
Turkey	HPLC/DAD	20-70	2007	Sarıulak	Arslan et al. (2013)
Spain	HPLC-MS	58-190	2014	Sarıulak	Garcia et al. (2016)
Italy	FC assay	138-700 GAE	2016	Coratina	Squeo et al. (2018)
Italy	FC assay	138-278 GAE	2018	Colozzese, Barone di Monteprofico, Cellina di Nardò Cornola, Ogliarola di Lecce, Orniella, Oliva Grossa, Spina	Negro et al. (2019)
Ancona/Italy	HPLC/DAD	153-396	2019	Leccino, Frantoio, Maurino, Moraiolo, Pendolino, Carboncella, Piantone di Falerone, Rosciola, Sargano di Fermo, Marzio, Coratina	Di Lecce et al. (2020)
Peru	HPLC-MS	10-425	2013	Not disclosed	Gilbert-López et al. (2014)

2.5 EVOO Antioxidant Capacity

In edible oils, free radicals generally arise from the peroxidation of lipids due to oxygen, light and heat (Stefanoudaki et al., 2010; Issaoui et al., 2011). The antioxidant capacity (AOC) of oils and other food products is due to the presence of specific antioxidants which bind with free radicals present in the food matrix (Huang et al., 2005; Plank et al., 2012). The determination of AOC was typically achieved *via* the use of a free radical generator, of which four are commonly used as shown in Table 2.9. These free radical generators, under correct experimental conditions, release a radical which is quenched *via* binding with antioxidants in the oil (Moniruzzaman et al., 2011; Plank et al., 2012; Ballus et al., 2015).

Table 2.9: Common antioxidant radicals used in AOC assays

Assay	Radical generator	Mechanism	Structure
DPPH	2,2-diphenyl-1-picrylhydrazyl	SET	
ferric reducing antioxidant power (FRAP)	2, 4, 6-tripyridyl-s-triazine	SET	
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid	HAT	
ORAC	2,2′-azobis(2-amidinopropane) dihydrochloride	HAT	

The AOC measurements can be classified into one of two mechanisms: (1) the single electron transfer (SET) mechanism and (2) the hydrogen atom transfer (HAT) mechanism. In the case of the SET mechanism, the direct transfer of an electron to the radical generator occurs and the reduced radical generator changes colour. This is monitored spectrophotometrically by measuring the absorbance of the extract at a selected wavelength. The HAT mechanism measures the total AOC with a fluorescent probe which competes with antioxidants to reduce the radical generator. The AOC is subsequently quantified by monitoring fluorescence kinetic curves (Moniruzzaman et al., 2011; Huang et al., 2005). The AOC of an edible oil is typically quantified by direct comparison to a standard of a known antioxidant, typically 6-hydroxy-2,5,7,8-tetramethylchroman-2-caboxylic acid, a vitamin E analogue and commonly referred to as Trolox. This equivalence measurement is referred to as the Trolox equivalent antioxidant capacity (TEAC) in the literature (Plank et al., 2012; Carrasco-Pancorbo et al., 2005; Zullo and Ciafardini, 2008).

2.5.1 Antioxidant Capacity of EVOOs

The different AOC assays vary in their mechanism of action and as such, these will provide a different AOC value as presented in Table 2.10. The Oxygen Radical Absorbance Capacity (ORAC) assay is an example of an AOC assay *via* the HAT mechanism. The ORAC assay generates peroxyl radicals with the 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) radical generator, and in turn the peroxyl radicals react with the fluorescent probe fluorescein. As fluorescein is oxidized, it loses fluorescence, and these are measured kinetically using a fluorescence spectrophotometer. Conversely, the presence of antioxidants hinders this loss of fluorescence, as peroxyl radicals bind to these antioxidants instead of the fluorescein probe (Moniruzzaman et al., 2011; Huang et al., 2005). This confers a greater measurement of fluorescence at excitation 490 nm and emission 514 nm and quantified against a known antioxidant (Fuentes et al., 2018). However, several drawbacks arise from the use of the ORAC assay as a means to quantify AOC, such as the prolonged time taken to perform the measurement (30-80 min) and the reproducibility of the method as fluorescein emission is dependent on pH of the extract (Litescu et al., 2014).

Table 2.10: AOC of EVOO measured with different assays[#]

Method	μmol L^{-1} TEAC
FRAP	3-118
ABTS	378-1367
DPPH	68-942
ORAC	131-665

#(Ballus et al., 2015)

An example of the SET mechanism includes the 2,2-diphenyl-1-picrylydrazyl (DPPH) assay which measures AOC with the direct reduction of DPPH with a single election transfer from an antioxidant (Plank et al., 2012; Huang et al., 2005). The analysis of AOC *via* DPPH entails the extraction of antioxidants in a polar medium which is typically methanol. A known concentration of DPPH is added to this solution and the extract is heated to *ca*. 35˚C to enable DPPH to scavenge antioxidants *in vitro* for 30 min (Ballus et al., 2015; Zullo and Ciafardini, 2008). The initial DPPH solution presents as a deep blue coloured solution in methanol and once reduced it fades to colourless, which is monitored directly *via* its absorbance at 517 nm. Therefore, the AOC of the foodstuff is related to the inverse of the DPPH absorbance and is typically quantified against the antioxidant Trolox (Moniruzzaman et al., 2011; Plank et al., 2012). This facile method simplifies the mechanism for the measurement of AOC as a fluorescent probe is not required, unlike other SET determinations. As such, the DPPH assay is widely used to assess the AOC of olive oils among many other applications (Plank et al., 2012; Carrasco-Pancorbo et al., 2005; Zullo and Ciafardini, 2008). Although the DPPH assay is a rapid technique, some difficulties may arise due the steric composition of DPPH. As the active site is situated amongst three aromatic rings, smaller antioxidants are able to bind to DPPH more readily. Such smaller antioxidants may therefore produce a greater DPPH scavenging capacity which will imply a greater *in vitro* antioxidant capacity (Xie and Schaich, 2014). Furthermore, some antioxidants which are highly reactive to transient lipid peroxides may react slowly or are inert to DPPH and as such the method can present a negative bias for certain antioxidants (Huang et al., 2005). The rate of reaction between DPPH and antioxidants is also highly variable and the time for reaction completion between the antioxidants and DPPH needs to be considered (Plank et al., 2012; Carrasco-Pancorbo et al., 2005; Ballus et al., 2015; Zullo and Ciafardini, 2008).

2.5.2 Factors Influencing EVOO Antioxidant Capacity

The AOC of an EVOO arise from the presence of phenolics and tocopherols, with the former providing most of the antioxidant activity (Fuentes et al., 2018; KamaI-Eldin and Appelqvist, 1996). Furthermore, individual phenolics impart their own antioxidant properties, thus the AOC of an EVOO depends not only on the concentration of phenolics but also on the composition of individual phenolics (Zullo and Ciafardini, 2008). As discussed previously, several assays have been developed to measure AOC but since these utilize different radical generators, the AOC of one assay cannot be compared to that of another assay. Thus, the AOC of EVOOs, or indeed any antioxidant or food, can vary depending on the assay that is used (Plank et al., 2012; Ballus et al., 2015; Zullo and Ciafardini, 2008).

The correlation between phenolics and AOC has been well established in the literature, in which both the total contribution of phenolics and the individual contribution of certain phenolics have been established (Dimitrios, 2006; Czerwińska et al., 2012; Kesen et al., 2014). An example of the correlation between total phenolic content and AOC was presented by Kesen et al. (2014) who measured the AOC of Turkish EVOOs using two radical generators (DPPH and ABTS) and the TPC *via* HPLC-MS. The results are consistent with those discussed previously (Section 2.4.3), in which the phenolic content was found to vary with the growth region and harvest season (Alessandri et al., 2014; Bajoub et al., 2016a; Reboredo-Rodríguez et al., 2016). The AOC of these EVOOs was found to vary almost linearly with the total phenolic content using each of the DPPH and ABTS assays.

Individual phenolics offer unique contributions to the total AOC of an edible oil and among these, luteolin, caffeic acid, elenolic acid and tyrosol impart some of the largest antioxidant activities (Czerwińska et al., 2012; Zullo and Ciafardini, 2008). A study of the AOC of EVOOs conducted by Zullo and Ciafardini (2008) investigated the relative AOC of neat caffeic acid, oleuropein, gallic acid and hydroxytyrosol by performing DPPH assays. The investigation revealed that individual phenolics exhibit certain degrees of AOC and of the four phenolics studied, gallic acid was found to exhibit the greatest AOC at concentrations greater than 5 ppm. Furthermore, the AOC increased by 1.7 TEAC units per ppm of gallic acid, whereas caffeic acid and oleuropein exhibited an increase of 0.8 and 0.4 TEAC units per ppm, respectively. Although the phenolic compound oleuropein is one of the major phenolics found in olive oils, it exhibits limited AOC but its importance in olive oil is fundamental to the taste of olive oils (Bajoub et al., 2015). However, when oleuropein is subjected to hydrolysis, it is

converted to hydroxytyrosol which presents a significant increase in antioxidant capacity from 5 to 25 TEAC units per ppm (Zullo and Ciafardini, 2008).

The AOCs of other edible oils also differ from those of EVOOs as presented by Janu et al. (2014) who investigated edible oils including coconut, sunflower, rice bran, groundnut, sesame and mustard oil. The AOCs of these vegetable oils were assessed with both the DPPH and ABTS assays and the oils with high phenolic content, such as groundnut, coconut and rice bran oil, were found to exhibit the greatest AOC. Conversely, the more refined vegetable oils, such as sesame oil and sunflower oil, exhibited lower AOC. A comparison was made to assess the sensitivity between the DPPH and ABTS assays and it was found that the DPPH assay resulted in the highest scavenging activity of all oils with the exception of rice bran oil. This suggests that the DPPH assay typically provides a greater sensitivity to antioxidants compared to the ABTS assay.

The variability in AOC of edible oils, and the relative ease of measuring AOC may provide an alternative means to detect adulteration in EVOOs. One such AOC analytical method, the DPPH assay, will be investigated as part of this thesis.

2.6 Spectrophotometric Methods for EVOO Analysis

The spectrophotometric analysis of olive oils has been presented in the literature as a means to authenticate olive oil mixtures (IOC, 2019b; Lia et al., 2018; Durán Merás et al., 2018). Furthermore, the IOC stipulates a UV absorbance measurement at 270 nm as a means measure of the extent of olive oil oxidation and to identify an foodstuffs *"Protected designation of origin"* (Martelo-Vidal and Vázquez, 2016; Tsiaka et al., 2013; Aparicio-Ruiz et al., 2018; Wang et al., 2016; Goncalves et al., 2014). Lastly, excitation emission spectrometric measurements have been used for the authentication of olive oils, the classification of olive oils based on total phenolic content, and to investigate changes in phenolic and chlorophyll composition during oxidation (Lia et al., 2018; Durán Merás et al., 2018; Squeo et al., 2018; Domínguez Manzano et al., 2019; Ali et al., 2018).

2.6.1 Ultraviolet Absorbance Measurements

The IOC stipulates a quantitative method *"spectrometric investigation in the ultraviolet"* (IOC, 2019b) to assess olive oil quality with absorbance measurements at 270 nm or 268 nm, (in cyclohexane or isooctane respectively) and the absorbance at 232 nm are also

measured. These absorbance measurements are used to calculate the specific extinction coefficient (K) measured at the 268 and 232 nm wavelengths (IOC, 2019b; Aparicio et al., 2013a). A further stipulation for olive oil quality *via* UV absorbance entails the difference in extinction coefficients (ΔK) surrounding the upper absorbance wavelengths at K268 nm or K270 nm (IOC, 2019b). For EVOO extracts in cyclohexane, this is accomplished with the measurement of K values at 270, 266 and 274 nm and the resulting value of ΔK is calculated. The stipulated limits of EVOO quality obtained using this technique are presented below in Table 2.11 (IOC, 2018; IOC, 2019b).

Table 2.11: UV absorbance K quality parameters

Wavelength/nm	EVOO	VOO	ROO	OO	ROPO	OPO
K270	<0.22	≤0.25	≤1.25	1.15	≤2.00	≤1.70
K232	≤2.50	≤2.60				
ΔK	≤0.01	≤0.01	≤0.16	≤0.15	≤0.20	≤0.18

Note: EVOO = virgin olive oil, VOO = virgin olive oil, OO = olive oil, ROPO = refined olive oil and OPO = olive pomace oil.

The investigation of the specific extinction maxima in the ultraviolet of olive oils is typically reserved as a method to assess the quality of an olive oil. The UV absorbance measurement of olive oil at 270 nm has been shown to increase during storage, which is attributed to the increase of secondary oxidation products which absorb at this wavelength (Stefanoudaki et al., 2010). This study investigated EVOOs over a 15-month period and found the absorbance at 270 nm increased under atmospheric conditions from 0.15 to 0.25, which exceeds the IOC maximum stipulated limit at this wavelength of 0.22 (IOC, 2018; Stefanoudaki et al., 2010). This suggests that whilst autoxidation should be considered when the quality of an EVOO is investigated *via* UV absorbance, this is seemingly only of concern for EVOOs which exceed 15 months storage time. Similarly, a more recent study was conducted by Aparicio-Ruiz et al. (2018) in which EVOO was stored for over 18 months and measured three quality parameters, namely free acidity (FA), peroxide value (PV) and UV absorbance. This study found an increase in the absorbance at 232 nm with a plateau reached after 6-14 months of storage. This followed by a rapid increase in the absorbance up to 2.3 within 18 months, whereas the absorbance at 270 nm increased until 12 months of storage followed by a decrease for the remaining storage time. The corresponding PV remained at 8 mEq O_2 for 8 months, increased to 14 mEq O_2 at 12 months, and decreased back to 8 mEq O_2 after 18 months of

storage (Aparicio-Ruiz et al., 2018). This suggests that the UV absorbance correlates to the level of peroxides as it follows a similar trend throughout storage period. Overall, these factors limit the usefulness of absorbance measurements as means to authenticate olive oils after 12 months of storage.

2.6.2 Fluorescence Excitation-Emission Matrices

The acquisition of a fluorescence excitation-emission matrix (EEM) is a spectroscopic technique in which both the excitation and emission wavelengths are measured to produce a three-dimensional excitation (X) emission (Y) intensity (Z) spectrum or "map". This spectroscopic method commonly utilizes the analysis of excitation and emission wavelengths between 200-800 nm. Utilizing EEMs also provides a suitable way in which to apply chemometric analysis, in particular *via* parallel factor (PARAFAC) analysis, to identity key regions of fluorescence and deconvolute datasets into various clusters (Ali et al., 2018; Squeo et al., 2018; Domínguez Manzano et al., 2019). These various regions of excitation and emission have been attributed to the constituents present in olive oils, as presented in Table 2.12. The EEM technique has been used in various studies as a means to identify adulterated EVOOs with either olive oils of lesser quality, or other edible oils (Ali et al., 2018; Durán Merás et al., 2018; Lia et al., 2018). Furthermore, fluorescence EEMs have been investigated as a means to directly identify olive oils with high and low total phenolic content whilst further studies have also investigated changes in EEM profiles during thermal and photooxidation of olive oils (Domínguez Manzano et al., 2019).

Table 2.12: EEM regions of key EVOO constituents

Constituent	Excitation Region (nm)	Emission Region (nm)	Reference
phenolic compounds	270 - 320	310 - 350	Squeo et al. (2018) Domínguez Manzano et al. (2019)
oxidation products	350	441 - 486	Ali et al. (2018)
pigments (chlorophylls and pheophytins)	370 - 420	660 - 690	Squeo et al. (2018) Ali et al. (2018)
carotenoid	350	515	Ali et al. (2018)
tocopherols	270 - 310	300-340	Squeo et al. (2018) Squeo et al. (2018) Ali et al. (2018)

Fluorescence EEMs have been used by Durán Merás et al. (2018) to assess the feasibility of detecting EVOOs diluted with lesser quality olive oils and olive pomace oils. This was accomplished with two sets of ten EVOO samples that were diluted with 5%, 10% 16% and 33% w/w olive oil and olive pomace oil. The authors scanned two regions, excitation 270-310 nm and emission 300-340 nm, which correspond primarily to tocopherol and phenolic constituents in the oils. The second region of interest, the excitation range 370-420 nm and emission range 669-690 nm, correlates to the pigments chlorophyll and pheophytin (Squeo et al., 2018). Both the tocopherol/phenolic and chlorophyll/pheophytin regions exhibit changes to fluorescence as EVOOs were diluted with either olive oil or olive pomace oil. In addition, an increase in the fluorescence intensity was observed in the tocopherol and phenolic region when EVOOs were diluted with olive pomace oil at concentrations of 5 %w/w. However, this region was not suitable for detection of olive oil diluent as no measurable change occurred when this oil was added (Durán Merás et al., 2018). The chlorophyll and pheophytin region were observed to decrease with the addition of either olive pomace or olive oil and could be observed at 10 %w/w mixtures for both diluents and suggests the presence of chlorophyll maybe used as an indicator of adulteration of EVOO with olive oil or olive pomace oil (Durán Merás et al., 2018).

The characterization of constituents *via* EEMs also provides a qualitative means to characterize EVOOs based on their phenolic composition. This was investigated by Squeo et

al. (2018) who evaluated sets of EEM data to distinguish between 52 Italian EVOOs with either high or low total phenolic content. The EVOOs were grouped as per by EU Regulation No. 432/2012 (European Commission, 2012) which sets a low limit for the declaration of health benefits derived from phenolics, namely 250 mg kg^{-1} of total phenolics in EVOO. The phenolic content of these EVOO samples, ranging between 130-700 mg kg^{-1}, was determined *via* the Folin-Ciocalteu assay and compared to EEM data collected in the fluorescent region λ_{ex} = 270-320 nm and λ_{em} = 300-350 nm and the data were further characterized *via* PARAFAC analysis. Similar to the data reported previously (Durán Merás et al., 2018), this study found two key fluorescence signatures in wavelength regions of λ_{ex} = 270-310 nm and λ_{em} = 300-340 nm (tocopherols and phenolics), and λ_{ex} = 370-420 nm and λ_{em} = 660-690 nm (chlorophyll and pheophytin). The PARAFAC modelling of these regions exhibited correlation between the chlorophyll and pheophytin region and TPC, however, if EEM was used as means to profile phenolic content, it would provide overestimation of TPC (Squeo et al., 2018). This bias can be seemingly removed with a targeted approach with an upper wavelength limit λ_{em} = 600 nm which excludes pheophytin (Squeo et al., 2018) and improved the robustness of the PARAFAC model. This study highlights the applicability for EEM to selectively identify fluorescent constituents in edible oils and suggests EEM maybe an alternative method to identify EVOOs diluted with other edible oils.

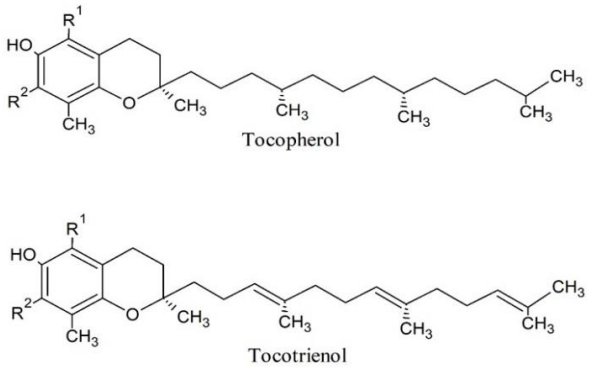
While the study of an entire EEM spectrum provides constitutional information for EVOOs, a more selective approach may provide a simpler and more rapid method to distinguish between pure and diluted EVOOs (Squeo et al., 2018; Ali et al., 2018). An example of the use of fluorescence EEMs to detect sunflower diluent oils in EVOOs was investigated by Ali et al. (2018). In this study, a single λ_{ex} = 350 nm wavelength, with emission intensities monitored between 380 nm and 700 nm, was applied to mixtures of EVOO and sunflower oils (5-95% w/w), as well as the pure oils. As the sunflower oils contained high concentrations of polyunsaturated fatty acid which offer limited oxidative resistance, these presented a broad, intense emission peak over the 447-489 nm region with a maximum at 469 nm. This emission region is typically correlated with peroxides resulting from the oxidation of lipids (Ali et al., 2018; Goncalves et al., 2014), and is also exhibited by EVOOs but at only half the intensity compared to sunflower oils. This reduced intensity may be attributed to the inherit oxidative resistances of EVOOs, *via* tocopherols, phenolic constituents and the lipid composition of the EVOOs (Issaoui et al., 2011). An increase in intensity was observed over this range when the EVOOs were mixed with 20% w/w sunflower oil diluent (Ali et al., 2018). As these emissions

are due to oxidative products, the robustness of the method with regard to the age of olive oil would need to be further studied as the technique could overestimate the sunflower content in older EVOOs. The longer wavelength emission at λ_{em} = 673 nm (i.e. chlorophyll) which appears as an intense peak in EVOO is absent in sunflower oil which does not contain this pigment. Thus, a decrease in fluorescence emission at this wavelength can only offer a qualitative indicator of dilution, whereas a more positive response may be obtained by monitoring the region related to the oxidation products in the sample. In the same study, the discrimination between pure EVOO and EVOO diluted with sunflower oil was also investigated using principal component analysis (PCA). The results suggested that across the entire emission spectrum, the majority of dissimilarities arise from oxidative products which depend on differences in fatty acid content and the antioxidant constituents present in the mixtures (Ali et al., 2018). The decrease in intensity of tocopherol, phenolics and chlorophyll regions due to dilution of EVOO with sunflower oil also provided an indicator of EVOO adulteration.

2.7 EVOO Tocopherols

Certain edible oils including EVOOs contain tocopherols and to a lesser extent tocotrienols. These are comprised of a series of lipid-soluble compounds collectively referred to as tocochromanols or by the collective term Vitamin E (Aguilar-Garcia et al., 2007; El Kharrassi et al., 2017). Not unlike other chemical components, the Vitamin E content of vegetable oils and the relative concentrations of Vitamin E derivatives are dependent on the originating plant (Franco et al., 2014b; Rizvi et al., 2013). Tocopherols and tocotrienols exhibit antioxidant activity, preventing lipid peroxidation and maintaining quality of edible oil throughout storage, as well as imparting health benefits to the consumer (Aguilar-Garcia et al., 2007; Blekas et al., 1995; Duthie et al., 2016; Okogeri and Tasioula-Margari, 2002).

The nomenclature and activity of tocochromanols are based the composition and location of the R groups bound to the phenol ring in the main structure. There are four primary forms of tocopherol and tocotrienols that naturally occur in plants, namely the α-, β-, γ-, and δ-forms. The most active tocopherol, α-tocopherol, contains a methyl group bonded to both R^1 and R^2 groups. The functional groups present in the structures of all tocopherols and tocotrienol forms are presented in Figure 2.4.



Form	R^1	R^2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 2.4: Structure of α-tocopherol.
(Rizvi et al., 2013)

The tocopherol concentration can vary widely in different olive oils, with reported values ranging between 15 and 400 mg kg^{-1} (Velasco and Dobarganes, 2002; Franco et al., 2014b). The measurement of tocopherols is therefore not currently used as means to authenticate EVOO according to the IOC (2018). However, the IOC does stipulate a maximum limit of 200 mg kg^{-1} for α-tocopherol in refined olive oils that olive oil producers may add, to restore any natural tocopherol lost in the refining process (IOC, 2018).

2.7.1 Tocopherol Composition of Vegetable Oils

The measurement of tocopherol composition in edible oils has been reported using methods primarily based on HPLC (Bakre et al., 2015; Blekas et al., 1995; El Kharrassi et al., 2017). Both normal phase (NP) and reverse phase (RP) HPLC are commonly used to separate tocopherols with the elution order of the different types of tocopherols dependent on the column phase (Duthie et al., 2016; Flakelar et al., 2017). The elution of tocopherols *via* NP-HPLC is typically in the order of α-, β-, δ-, then γ-tocopherol, which is reversed in the case of RP-HPLC. Both techniques provide significant resolution of these compounds whereby the detection of tocopherols is achieved with UV excitation (at 296 nm) and emission (at 330 nm) using DAD or by MS. Detection with DAD offers a more rapid technique due to higher flow rates being possible whereas MS offers greater sensitivity but the lower flow rate extends the required analysis time (Duthie et al., 2016; Grilo et al., 2014). Tocopherols, which are lipid soluble, require a relatively simple extraction from vegetable oils by dilution in n-hexane. As such, methods for the analysis of tocopherols are relatively rapid due to the facile extraction and HPLC analysis (Flakelar et al., 2017; Franco et al., 2014b; Grilo et al., 2014; Jukić Špika et al., 2015; Chen et al., 2011; Rastrelli et al., 2002).

The analysis of tocopherol content in various EVOOs and other oils has been widely reported using HPLC with either DAD or MS (Grilo et al., 2014; Flakelar et al., 2015; Flakelar et al., 2017; El Kharrassi et al., 2017). As presented in Table 2.13, α-tocopherol is the predominant tocopherol in olive oil whereas β-, γ- and δ-tocopherol are not present except in the case of olive oils from Morocco which can contain 7.6 and 4.4 mg kg^{-1} of β- and γ-tocopherols, respectively. Canola oil exhibits similar α-tocopherol levels to olive oil, whereas γ-tocopherol is also present and at similar concentrations to that of α-tocopherol (Grilo et al., 2014; Flakelar et al., 2015; Flakelar et al., 2017). Since canola oil contains a significant concentration of γ-tocopherol and the total tocopherol content exceeds that of olive oil, it may be expected that the tocopherol content of an olive oil diluted with canola oil could potentially be used as an indicator of adulteration. Similarly, sunflower oils exhibit greater concentrations of α-tocopherol than olive oil at 432 mg kg^{-1} compared to 88-261 mg kg^{-1} respectively (Grilo et al., 2014). As EVOOs typically contain low concentrations of γ-tocopherol, the detection of this compound in a sample could potentially indicate the presence of canola oil in a case of suspected adulteration.

Table 2.13: Summary of tocopherol content in different oils (mg kg^{-1})

Edible oil	Tocopherol type				Detection technique	Region	References
	α	β	δ	γ			
Canola	120.3±4.2	N.D.	N.D.	122.3±7.9		Brazil	Grilo et al. (2014)
Sunflower	432.3±86.6	N.D.	N.D.	92.3±9.5	RP-HPLC-DAD	Brazil	Grilo et al. (2014)
Corn	173±82.3	N.D.	N.D.	173±82.3		Brazil	Grilo et al. (2014)
Soybean	71.3±6.4	N.D.	N.D.	273.3±11.1		Brazil	Grilo et al. (2014)
Canola	476±45.9	N.D.	15.4±1.04	663±48.1	NP-HPLC-MS/MS and DAD	Australia	Flakelar et al. (2017)
Olive	261±41.3	N.D.	N.D.	N.D.		Australia	Flakelar et al. (2017)
Canola	263±46	N.D.	8.94±3.2	378±44	NP-HPLC-MS/MS	Australia	Flakelar et al. (2017)
Olive	88.01±0.06	7.57±0.03	N.D.	4.42±0.04	RP-HPLC-differential refractometer	Morocco	El Kharrassi et al. (2017)
Argan	5.86±0.04	0.12±0.00	4.04±0.15	4.42±0.04		Morocco	El Kharrassi et al. (2017)
Cactus pear seed	1.27±0.02	36.89±0.16	3.77±0.07	58.07±0.10		Morocco	El Kharrassi et al. (2017)
EVOO	76-157	1-9	N.D.	1.1-25	NP-HPLC-DAD/MS	Italy, Spain, Portugal	Dugo et al. (2020)
EVOO	79-151	N.D.	N.D.	3-16	NP-HPLC-DAD	Greece	Mikrou et al. (2020)
EVOO	368-768	N.D.	N.D.	5.5-13		Croatia	Jukić Špika et al. (2015)
EVOO	69-481	N.D.	N.D.	N.D.	NP-HPLC-DAD	Australia	Mailer et al. (2010)

Note: N.D. = not detected

2.7.2 Factors Influencing Tocopherol Content

The tocopherol content and concentration of EVOOs is dependent on factors similar to those that affect phenolic content and include olive variety. These include growing conditions, storage time, growing region and ripeness of the olive at the time of processing (Jukić Špika et al., 2015; Mikrou et al., 2020; Dugo et al., 2020). The change in tocopherol content during harvesting of two olive varieties from two different growing region was investigated by Jukić Špika et al. (2015). Both EVOO cultivars were grown at two growing regions Kastela and Sestanovac regions in Croatia and each of these olive varieties (Leccino and Oblica) exhibit different compositions of tocopherols. The most significant loss of tocopherols occurred between the first and second harvests, a period of two weeks and the α-tocopherol concentration in particular decreased by *ca.* 30% over a three-month period (Jukić Špika et al., 2015).

In general, the variability of total tocopherols and that of individual tocopherols (α- and γ-tocopherol in particular) in different oil types is a result of both the cultivar and harvest period (see Table 2.13). Further literature has highlighted the importance of growth region in relation to tocopherol content as suggested by Dugo et al. (2020) and Mikrou et al. (2020) who investigated Italian oils and Greek oil respectively. The ranges of individual tocopherol contents presented in Table 2.13 for both studies confirm the variability of tocopherols observed in other studies, including the low concentrations of β-tocopherol which are also present in some Moroccan olive oils (El Kharrassi et al., 2017).

Based on the analysis of EVOO tocopherols *via* principal component analysis, Dugo et al. (2020) observed clusters that typically did pertain to specific Italian growing regions, however same regional clusters did overlap. This pattern of multivariable clusters could also be observed in the study presented by Mikrou et al. (2020) who investigated two olive varieties (Koroneiki and Kolovi) in three regions of Greece. The statistical difference between the two EVOO varieties was not significant for α-tocopherol, whilst the region of growth seemingly played a more substantial role in the EVOO α-tocopherol content. Conversely, the γ-tocopherol content did exhibit significant variability based on the EVOO variety, with a range of 3 to 16 mg kg^{-1}. This suggests the concentration of γ-tocopherol might provide a more suitable indicator of diluted EVOO as the endogenous concentration and variability are less than that of α-tocopherol. However, the variability

of α- and γ-tocopherol highlights a potential drawback for the use of tocopherols for authentication purposes and may indicate the need for a comprehensive library which catalogues the expected concentration range, location and cultivar that could be consulted for diluent identification.

2.7.3 Tocopherol Measurement to Determine Olive Oil Adulteration

The use of tocopherol analysis as a specific measure to evaluate the presence of diluent oils in EVOOs was reported by Chen et al. (2011). In this study, a screening technique was developed using RP-HPLC with DAD detection for evaluating the levels of the various tocopherols as well as the α- to β/γ-tocopherol ratio (i.e. α/β ratio) in a range of vegetable oils. The study hypothesized that since EVOOs are comprised of prominently α-tocopherol, the presence of β/γ-tocopherol in a test sample could suggest that a EVOO has been diluted with an edible oil that contains these tocopherols (Chen et al., 2011; Grilo et al., 2014). Although variability was observed for the α/β ratio in the pure EVOOs studied, it was found that peanut, hazelnut, corn and soybean oils all exhibited a lower ratio than the EVOOs. In one sample of "pure" EVOO, an α/β ratio of 11.3 was determined which was consistent with that of an adulterated EVOO. In addition, δ-tocopherol was detected in this sample which further suggests this EVOO was adulterated since δ-tocopherol was not present in any other EVOO sample.

The average α/β ratios of the pure oils were found to be 22.8, 13.8, 2.69 and 12.1 for EVOO, sunflower, hazelnut and peanut oils, respectively (Chen et al., 2011). The standard deviation of the EVOO (5.5) was used to identify the lowest α/β ratio applicable to EVOO which suggested that a α/β ratio of 17 or lower may indicate a potential dilution of olive oil with a vegetable oil. The EVOO and diluent oil mixtures were used to identify the minimum percentage weight of added oil which correlates to this value and it was found that the proposed limit of detection (LOD) for these edible oils were 1.5%, 3% and 15% for peanut, hazelnut and sunflower oils, respectively. It could be suggested that this LOD may be underestimated as only a single standard deviation was used. An expanded standard deviation, being twice the reported value in the study would increase the LOD, however would also reduce the possibility of falsely identifying a pure EVOO as being adulterated. Some oils, such as sunflower oil, exhibit similar tocopherol composition to EVOO where α-tocopherol is the major component, and thus may be difficult to detect with this technique. However, this strategy highlights the potential of measuring

tocopherol content as a means to identify Australian EVOOs which have been diluted with edible oils, which will be investigated as part of this research.

2.8 Summary

The authentication of EVOOs has been extensively studied and offers methods that are able to identify diluted EVOOs, however, many of these techniques suffer limitations to their effectiveness. Many of these methods are laborious, require extractions, derivatisation, dilution, sample clean-up and advanced analytical instrumentation. This not only adds to the costs associated with the authentication process, but also introduces method errors at various stages of the analysis. In addition, some methods have been shown to falsely identify pure oils as being adulterated.

These limitations in current authentication methodologies have led to same in literature to investigate alternative methods, such as ratios between tocopherols in diluted oils and fluorescence techniques which have employed advanced chemometric techniques, such as PARAFAC. The use of ratios between tocopherols, while effective in some cases, is limited to diluent oils which differ significantly in tocopherol composition compared to EVOOs. While advanced data analysis, such as PARAFAC, are not readily available to industry and are often difficult to generate with limited samples. Their use however highlight that alternative methods can offer suitable detection of diluents in EVOOs.

Furthermore, the literature has highlighted the natural variability observed in EVOO components which include phenolics, antioxidant methods and certain tocopherols, which should be considered when developing novel alternatives to olive oil authentication. These considerations are important as constituents which are highly variable, while seeming effective in a case-by-case basis, may give arise to expanded method error which limits the useful of these components. To address the limitations of current olive oil authentication techniques, this research aims to investigate a range of "quality" olive oil components which may provide supplemental methods to identify diluted EVOOs.

Chapter 3 Materials and Methods

3.1 Oil Samples

3.1.1 EVOO and Diluent Oils

Two commercially available Australian extra virgin olive oils (EVOOs) and three diluent oils: sunflower, rice bran, and canola oil, and were selected on the basis of their cost. The value of each diluent oil was $0.20 per 100 mL for canola and sunflower oil, whilst rice bran oil was valued at $0.70 per 100 mL, compared to an average price of $2.10 per 100 mL of pure EVOO (pricing as at 15/10/2018).

3.1.2 Preparation of Adulterated Oils

Each of the pure EVOOs were diluted with each diluent oil and this was accomplished gravimetrically by altering the ratio of the mass of diluent oil to the mass of EVOO. For example, a 20% w/w mixture of diluent oil in EVOO was prepared by mixing 10 g of diluent oil and 40 g of EVOO. The mixture was homogenized in a mixing tumbler for 30 min at 20 rpm and stored in the dark at 4°C. Mixtures of the diluent oils in EVOO were made with the range of dilutions ranging between 5% and 80% w/w. Furthermore, pure EVOOs and pure diluent oils were also included in the analyses to ensure a complete calibration range and each pure oil was analysed in triplicate (n = 3). Two methods used a limited set of calibrations, which include the Folin-Ciocalteu (FC) assay and fluorescence analysis. The FC assay test sample range was limited to mixtures between 5 - 20 %w/w and the pure edible oils. This limitation is due to the limited space on the 96 well plate. The fluorescence analysis of pure and diluted edible oils is limited to mixtures between 5 – 50 %w/w.

3.2 Chemicals and Reagents

The FC assay uses the following reagents and equipment: Folin-Ciocalteu's phenol reagent with respect to 2 M acid (Sigma-Aldrich), 7.5% w/w, sodium carbonate (Sigma-Aldrich, ≥99.0%) in deionized water, methanol (Merck, preparative chromatography grade), 80% v/v de-ionised water/methanol mixture ($MeOH/H_2O$) and gallic acid (Sigma-Aldrich, ≥97.5%, 200 mg L^{-1} in de-ionized water). The equipment included a centrifuge capable of reaching speeds of 3000 rpm and a BMG Labtech

Fluostar plate reader with coupled spectrometer capable of measuring absorbance at 750 nm (Fuentes et al., 2012; Georgé et al., 2005; Reboredo-Rodríguez et al., 2016).

The following reagents were used in the HPLC-DAD determination of total phenolic content (TPC): methanol (Merck, preparative chromatography grade), de-ionized water (Milli-Q) and n-hexane (Merck, for gas chromatography). The n-hexane was used as the solvent for liquid-liquid extraction (LLE). Several phenolic standards were used throughout the method evaluation to confirm the retention order. These were: syringic acid (Sigma-Aldrich, ≥95%), tyrosol (Sigma-Aldrich, ≥98%), ferulic acid (Sigma-Aldrich, ≥98%), caffeic acid (Sigma-Aldrich, ≥98%), and apigenin (Sigma-Aldrich, ≥95%).

The DPPH assay, based on the method proposed by Plank et al. (2012), utilized the following reagents: methanol (Merck, preparative chromatography grade), 2,2 diphenyl-1-picrylhydrazl (DPPH) (Sigma-Aldrich ≥95%) and Trolox, a vitamin E analogue (Sigma-Aldrich ≥97%). The equipment used for this analysis included, a water bath (Ratek, SWB20D) set at 35˚C and spectrophotometer (Agilent, Cary 50 UV-Vis).

The following four tocopherol standards were obtained from Sigma-Aldrich, Australia and were used to identify and quantify tocopherol content in EVOO and diluent oils: α-tocopherol (≥ 96%), β-tocopherol (≥ 96%), δ-tocopherol (≥ 90%) and γ-tocopherol (≥ 96%). The solvent, *n*-hexane (Merck) was used to extract tocopherols from the oil samples. The HPLC solvents used are 10 % (v/v) isopropyl in heptane (mobile phase A) and n-heptane (mobile phase B).

3.3 Analytical Methods

3.3.1 Folin-Ciocalteu Assay

The FC assay was accomplished as follows, an oil sample of 2 g was dissolved in 5 mL of 80% v/v MeOH/H_2O and homogenised with a RATEK VM1 vortex mixer at 400 rpm for 30 s then centrifuged. Extracts were filtered with a hydrophilic 0.45 µm syringe filter (Advantec, hydrophilic PTFE) to remove particulates from the solution. On a 96-well plate, 25 µL of filtered extract and 115 µL of de-ionized water were added to each sample well. Additional reagents of 100 µL of sodium carbonate and 12 µL of Folin-Ciocalteu reagent were automatically injected into the sample wells by on-board injector

needles in the Fluostar plate reader. Two incubation periods were used to ensure reaction completion. The first was a 5 min incubation after the sodium carbonate was added and the second was a 90 min incubation after the addition of the FC reagent. Finally, the absorbance was measured and compared to a gallic acid standard curve (Figure 3.1). The total phenolic content was calculated as gallic acid equivalents (GAE) (Fuentes et al., 2012; Georgé et al., 2005; Reboredo-Rodríguez et al., 2016).

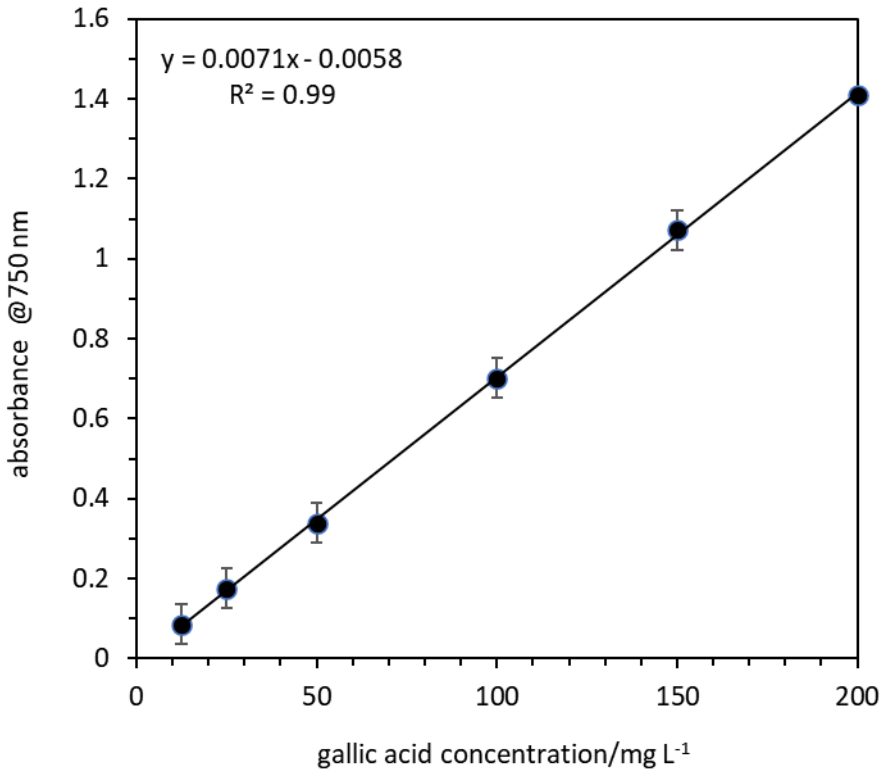


Figure 3.1: Gallic acid standard curve with associated CI error bars (*n* = 3, p = 0.05).

3.3.2 Total Phenolics by HPLC-DAD

The phenolic content was based on the extract method proposed by Tasioula-Margari and Tsabolatidou (2015) and chromatographic conditions proposed by the IOC phenolic method (IOC, 2017a).. The equipment included a nitrogen evaporator (Dionex, SE-500) and an Agilent 1200 HPLC-DAD. Chromatographic separation was achieved using an Agilent C_{18} Eclipse column (4.6 mm × 150 mm × 3.5 µm) and a binary pump and detection by HPLC-DAD *via* UV absorbance at 280 nm. Solvent A was a mixture of 0.2% v/v orthophosphoric acid (Sigma-Aldrich ≥85% v/v) in de-ionized water and solvent B was a 50% v/v methanol/acetonitrile mixture. The applied solvent gradient was

as follows: 96:4% A:B for 40 min, a gradual change to 50:50% A:B over 5 min, then back to 96:4% A:B for a total runtime of 50 min**.** The TPC content of oils are determined *via* absorbance at 280 nm.

The TPC of EVOO and EVOO mixtures was extracted as follows: firstly, 5 g of oil and 500 μL of the internal standard (15 mg L^{-1} syringic acid in methanol) was added to a 50 mL extraction tube and diluted with 5 mL of methanol. The extraction tubes were homogenised with a RATEK VM1 vortex mixer at 400 rpm for 30 s and centrifuged at 250 rpm for 2 min, after which the methanol supernatant was removed. This process was repeated twice, and the extracts combined to achieve a total methanol extraction volume of 15 mL. The combined methanol supernatant was removed under nitrogen before the TPC residue was reconstituted in acetonitrile. A liquid-liquid extraction was used with two 5 mL volumes of hexane to remove any oil residue. Both volumes of hexane were discarded, and the acetonitrile solution was evaporated under nitrogen. Finally, the phenolic residue was dissolved in 3 mL of 80% v/v MeOH/H_2O and was filtered through a hydrophilic 0.45 μm filter (Merck, Millex-FH PTFE) before being injected into the HPLC-DAD instrument and the phenolic analytes separated under the previously described chromatographic conditions.

The relative response factor (RRF) for syringic acid and tyrosol was determined using Equation (3.1):

$$\mathrm{RRF} = \mathrm{RF_{syr}}/\mathrm{RF_{tyr}} \tag{3.1}$$

where RF_{syr} and RF_{tyr} are the response factors for syringic acid and tyrosol respectively which are calculated using Equation (3.2):

$$\mathrm{RF_{std}} = \mathrm{A_{std}}/\mathrm{m_{std}} \tag{3.2}$$

where A_{std} is the peak area for syringic acid or tyrosol and m_{std} is the mass of the corresponding syringic acid or tyrosol standard (IOC, 2017a).

The TPC of the oil, measured in units of mg kg^{-1}, was determined using Equation (3.3):

$$TPC = \frac{TPA \times 1000 \times RRF \times m_{syr}}{A_{syr} \times m_{oil}} \quad (3.3)$$

where *TPA* is the sum of the phenolic peak areas, m_{syr} is the mass of syringic acid in the internal standard, A_{syr} is the area of the syringic acid internal standard peak, and m_{oil} is the mass of the oil sample.

3.3.3 DPPH Antioxidant Assay

A 1000 mg L^{-1} stock solution of DPPH (Sigma-Aldrich, ≥95%) was made up in de-ionized (Milli-Q) water. From this stock solution, a 33 mg L^{-1} DPPH solution was prepared by dilution with methanol. For the AOC quantification of pure EVOOs, diluent oils, and EVOO/diluent oil mixtures, a set of Trolox standards was prepared from a 1000 mg L^{-1} stock solution which was serially diluted to give a final standard concentration range between 1.5 and 25 mg L^{-1}. All solutions were prepared freshly for each analysis.

The oil extracts were prepared by diluting 0.5 g of oil in 10 mL of methanol. The extracts were homogenised with a RATEK VM1 vortex mixer at 1700 rpm for 30 s before the supernatant was collected for analysis. Methanol extracts were added to a prepared 33 mg L^{-1} DPPH solution, at a ratio of 1 mL to 3 mL respectively and were incubated for 30 min at 35°C. The background absorbance at 517 nm was measured using methanol as the reference solution and was used to correct for any baseline methanol absorbance, before the measurement of the DPPH oil extracts and standards. A control DPPH solution, containing no standard or extract, was prepared with 1 mL of methanol diluted in 3 mL of DPPH. The absorbance of this solution was measured in order determine the initial DPPH absorbance before reaction with antioxidants in the test standard and samples. The collected data were used to calculate the percentage of DPPH reacted (%*DPPH*) in accordance with Equation (3.4)

$$\%\text{DPPH} = 1 - \left(\frac{A_s}{A_c}\right) \times 100 \qquad (3.4)$$

where A_s is the absorbance of the test sample and A_c is the absorbance of the control sample (Plank et al., 2012).

The AOC activity *via* DPPH assay is reported as Trolox equivalents, where a 25 mg L^{-1} Trolox standard produces an absorbance of 0.06, whereas unreacted DPPH produces an absorbance of 0.68. An example standard curve is presented in Figure 3.2, with associated confidence interval error bars (n = 4, p = 0.05). The absorbance of the Trolox was determined using the same method as above. Briefly, this method involved the measurement of a 25 mg L^{-1} DPPH solution reacted with a known concentration of Trolox and was used to construct calibration plots of %*DPPH* against the corresponding concentration of Trolox. These values of %*DPPH* of the test samples were then calculated with reference to the Trolox standard curve, and the results presented as mg kg^{-1} Trolox equivalent antioxidant capacity (TEAC).

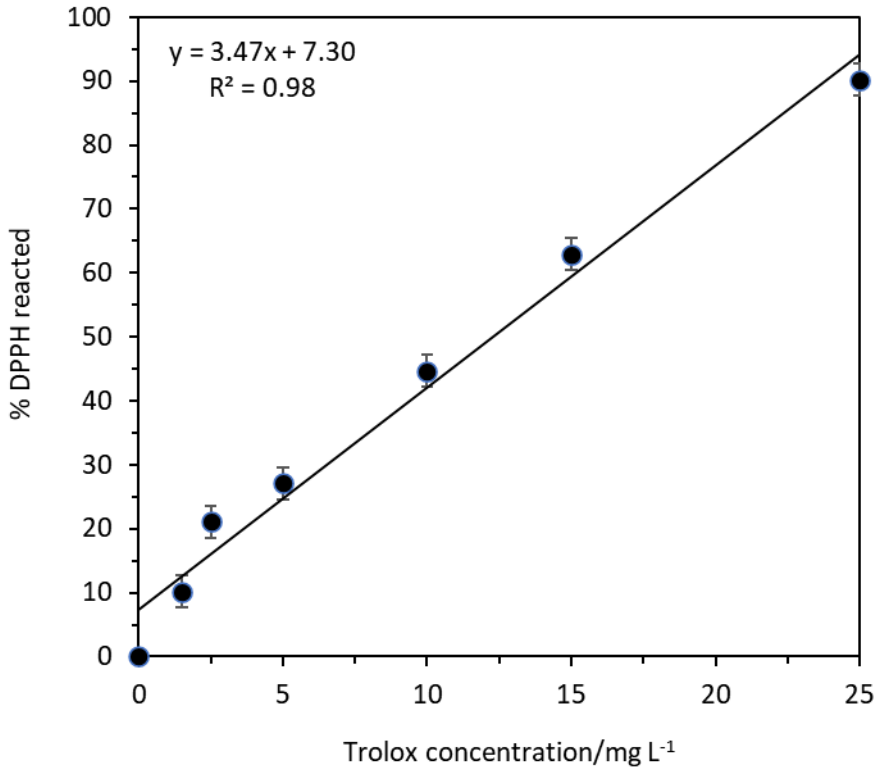


Figure 3.2: Trolox calibration curve and CI error bars (n = 4, p = 0.05).

3.3.4 Fluorescence and UV Absorbance Measurements

Fluorescence measurements were performed using a Horiba Aqualog fluorescence spectrophotometer (Horiba, Japan) capable of recording both absorbance and fluorescence intensities. The EVOO and diluent oil samples were prepared as discussed in Section 3.1.2. The fluorescence profiles of pure oils and a representative set of EVOO#1 diluted with sunflower oil (5 to 50% w/w) were recorded directly from test oils without solvent dilution. The EEMs were recorded by measuring the fluorescence intensities across the excitation and emission wavelength range 250 nm to 800 nm at an interval of 4 nm. The UV absorbance was recorded simultaneously over the same wavelength range.

3.3.5 Tocopherol Sample Extraction and Analysis

To extract the tocopherols from the oils and mixtures, 1 g of oil was diluted in 10 mL of *n*-hexane and homogenised with a RATEK VM1 vortex mixer at 400 rpm for 30 s. Sample extracts were then filtered through an Advantec 0.45 µm hydrophilic PTFE filter prior to injection of 10 µL of extract into a Thermofisher Ultimate 300 system HPLC system with diode array detector as per the chromatographic conditions presented in Table 3.1.

Table 3.1: HPLC conditions for determining tocopherol content

Column	Phenosphere NH2 3µ150 x 4.6 mm
Mobile Phase A	10% isopropyl alcohol in heptane
Mobile Phase B	Heptane
Gradient	A:15 B:85
Flow rate	1 mL min^{-1}
Excitation	296 nm
Emission	326 nm

3.4 Method Validation

This section discusses the approach this research used to assess the suitability of individual methods and the resultant framework. Briefly, each method was validated to assess its suitability for the constituent of interest based on four key parameters. These validation parameters are defined as a method's robustness, sensitivity, linearity and selectivity (Ellison and Williams, 2012; Peters et al., 2007). The *robustness* of a method is its susceptibility to small changes on repeat analyses, such as changes in mobile phase

composition or minor differences in solution preparation which results in a change of the analytical result. The *robustness* of a method is able to be determined with the repeat analysis of a test sample (Peters et al., 2007; Ellison and Williams, 2012). The method *sensitivity* relates to its ability to detect small changes in the concentration or presence of analytes of interest and is reflected in the gradient of the calibration curve. Thus, in this study, the *sensitivity* of a method relates to the changes to analytes which corresponds to deviations in the EVOO profiles due to the presence of diluent oils. For example, a decrease in total phenolic content of an EVOO indicates the presence of a diluent oil and its corresponding *sensitivity* relates to the minimum weight of diluent oil at which a detectable change in the total phenolic content occurs. Both the sensitivity and selectivity of the method were assessed statistically with the calculation of confidence intervals (CI). The CI values are calculated based on the repeat analysis (n = 3) of EVOO#1, EVOO#2 and pure diluent oils. As the CI values of both EVOOs exceeded those of the pure diluent oils, these were applied to each mixture.

The *selectivity* of the method relates to its ability to distinguish between analytes of both EVOO and diluent oil and interferences from the sample matrix and relates to the ability of the method to determine the identity of a diluent oil. The method *linearity* is a measure of its ability to produce a result that is directly proportional to the concentration of the analyte. For example, a doubling of the concentration of the analyte should correspond to a doubling of the concentration of the diluent oil and the linearity is reflected in the coefficient of determination of the corresponding calibration curve (R^2).

The sensitivity and selectivity of a method determines the percentage by weight (% w/w) of a diluent oil which is detectable, referred to as the *limit of detection* (LOD). Thus, a method which offers greater sensitivity and selectivity decreases the corresponding LOD of the diluent oil. In this study, this is reflected in the calculation of the confidence interval with a precision of 95% (p = 0.05) of the pure EVOO and the calculated confidence intervals calculated in accordance with Equation 3.5 (Peters et al., 2007; Ellison and Williams, 2012). The acceptance criterion for LOD is the first datum of a diluted test sample which is outside the confidence interval.

$$\mathrm{CI} = \mathrm{Z} \times \frac{\sigma}{\sqrt{n}} \tag{3.5}$$

where *Z* is the z-score (Greenland et al., 2016) (calculated at p = 0.05%, z score = 1.96)), σ is the standard deviation, and *n* is the number of replicates (*n* = 3).

As the replicate oils contain the same analyte concentration, any deviation amongst replicates is presumably due to method error, which is measured with the calculated confidence intervals (CI). As diluent oil is added, the reported analyte would be expected to deviate, and the minimum percentage of diluent oil needed to reproducibly measure this deviation (which lies outside of CI range of the pure EVOO response) corresponds to the limit of detection for the method. To ensure the measured concentration of diluent oil exceeds the method error, the CI value of the EVOO replicates (*n* = 3, p = 0.05) was calculated and applied to each datum point as error bars. If the proceeding datum point lies outside the error bars, then the LOD can be assigned to that value (Peters et al., 2007; Ellison and Williams, 2012). The method sensitivity also requires a *limit of quantification* (LOQ) and which also relates to both the method CI and linearity. Thus, the LOQ is the first data point at which linearity is observed and does not overlap the previous data point with regard to its CI.

The *extraction efficiency* (EE) of the method is the measure of any change in analyte concentration during extraction (Peters et al., 2007; International Organization for Standardization, 2019). This is determined *via* a comparison of three separate extractions, namely the test oil with standard additions (Extract A, EA), test oil (Extract B, EB), and the standard(s) added to the extraction solution without any test sample (Extract C, EC). The standard recovery is the comparison of all three solutions, as presented in Equation (3.6) (Peters et al., 2007):

$$\mathrm{EE}\ (\%) = \frac{\mathrm{EA} - \mathrm{EB}}{\mathrm{EC}} \times 100 \tag{3.6}$$

The range of acceptable EE is in accordance with the guidelines stipulated by the Guidelines for Standard Method Performance Requirements AOAC Official Methods of Analysis (2016). The expected recovery of an analyte is a function of its concentration, which in the case of concentrations between 10 mg kg^{-1} to 100 µg kg^{-1} an %EE between 80 - 110% should be observed.

The decision trees developed for each set of methods were designed to address the limitations of each method. This was typically due to either poor selectivity or linearity of a method, such as that observed with both TPC methods and which was alleviated with the addition of an alternative method. Lastly, Chapter 7 proposes a decision tree which incorporates all the findings of this research and in addition to addressing limitations of the individual methods, presents an improved workflow. This was accomplished with consideration of certain individual methods sample throughput, which could offer screening for EVOO adulteration. In this research, two methods could be considered as high throughput methods and include the FC assay, with its ability to concurrently measure 96 samples and EEM fluorescence with its rapid measurement of spectral profiles.

Chapter 4 EVOO Authenticity by Phenolic and Antioxidant Content

4.1 Overview

A potential approach for the determination of EVOO authenticity involves the determination of phenolic content and the resulting AOC of the oil. In this chapter, methods for evaluating changes in phenolic content of two diluted Australian EVOOs were determined using both the FC assay and HPLC-DAD analyses. Furthermore, the AOC *via* DPPH assay of diluted EVOOs was measured as a means to further explore the use of AOC as an authenticity indicator. The aim of this chapter is thus to establish a methodology that integrates these three methods with a view to discriminating between pure EVOO and EVOOs that have been diluted with canola, sunflower and rice bran oils,

4.2 Results and Discussion

This chapter investigates three methods: (i) total phenolic content (TPC) *via* Folin-Ciocalteu assay, (ii) TPC *via* HPLC and (iii) antioxidant capacity (AOC) *via* DPPH assay of two EVOOs which were diluted with canola oil, sunflower oil and rice bran oil. The *sensitivity*, *selectivity* and *robustness* of these method are assessed to determine the *limit of detection* (LOD) and *limit of quantification* (LOQ) for each method and if the method is able to distinguish pure EVOOs and EVOO mixtures with sunflower oil, canola oil and rice bran oil diluents.

4.2.1 Total Phenolic Content by Folin-Ciocalteu Assay

The investigation and validation of the FC assay for olive oil adulteration involved the analysis of pure and diluted EVOOs. These test samples were diluted with known masses of canola, sunflower and rice bran diluent oils mixed with two Australian EVOOs at 5%, 10%, 15% and 20% w/w. The limit of detection was assessed based on the average FC response (n = 3) of EVOO#1 and EVOO#2 and the datum point of the EVOO diluted with diluent oil which lies outside the CI range.

As presented in Section 3.4, the CI value (p = 0.05) of the EVOO test sample was calculated to determine the *sensitivity* of the method with respect to diluent oil. The TPC was determined using the FC assay (see Figure 4.1) with associated CI value error bars.

Both EVOO#1 and EVOO#2 TPC content are above 1000 mg kg^{-1} GAE, whereas sunflower oil, canola oil and rice bran oil all contained significantly lower TPC content than the EVOOs, namely between 265 and 163 mg kg^{-1} GAE. As such, it would be expected that as the diluent oils were added to EVOOs, a corresponding linear decrease in the TPC would also occur, as phenolic content has been found to correspond with the response to the FC reagent (Alessandri et al., 2014; Fuentes et al., 2012; Reboredo-Rodríguez et al., 2016; Singleton et al., 1999). However, as shown in Figure 4.1 a linear decrease in the TPC was not observed, instead a single decrease of *ca* 50% was observed for all 12 diluted mixtures of EVOO and the decrease was seemingly independent of the amount of diluent oil. The observed behaviour cannot be explained by a simple dilution effect of the phenolics but is possibly due to changes in phenolic composition or other constituents that arise from the addition of the diluent oils (Everette et al., 2010).

The determinations of TPC by FC assay offers a means for the identification of EVOOs adulterated with diluents. As shown in Figure 4.1 the TPC of diluted EVOOs decreases compared to pure EVOOs and the TPC response of 5% w/w diluent lies outside the EVOO datum point and method error, which suggests this is the LOD. However, the mixtures of EVOO diluted with all three diluent oils present a similar TPC response and a lack of linearity between the mixtures is observed. As these responses suggest the FC assay lacks the specificity to identify either the diluent oil or the concentration, no further measurement of increased diluent oil was recorded above 20% w/w. Therefore, the use of the FC assay seems to be limited to a qualitative approach which can identify if an EVOO has been adulterated with canola, sunflower, or rice bran oil. However, it appears the assay cannot be used to identify and/or determine the concentration of the diluent oil. Other approaches to determining the phenolic content such as chromatographic determinations may provide better discriminatory power between EVOO and EVOO containing diluent oils, as explored later in this chapter.

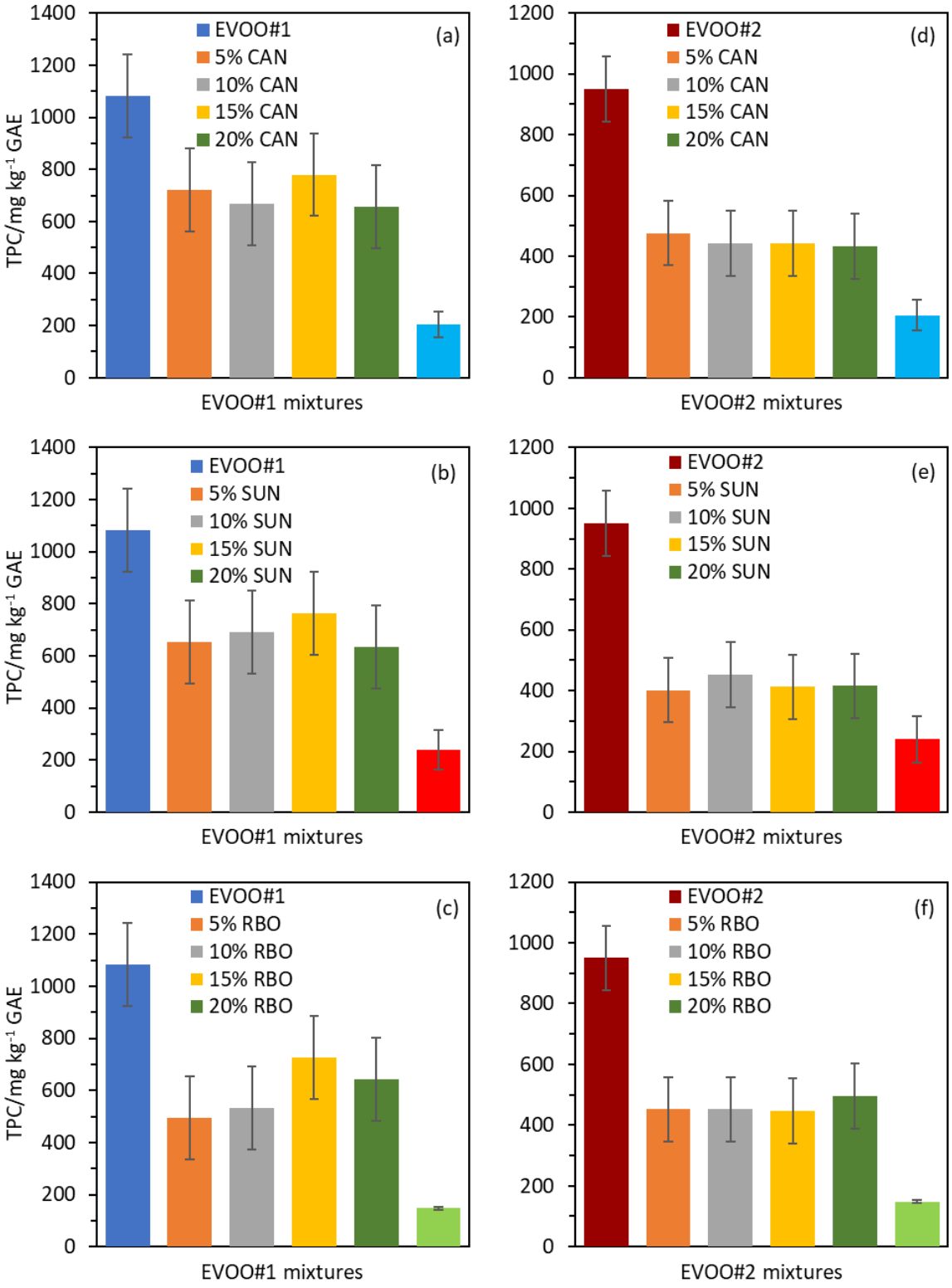


Figure 4.1: Total phenolic content measured by the FC assay of mixed diluent oils (a) EVOO#1 and canola oil, (b) EVOO#1 and sunflower oil, (c) EVOO#1 and rice bran oil, (d) EVOO#2 and canola oil, (e) EVOO#2 and sunflower oil and (f) EVOO#2 and rice bran oil CI values are 159 and 106 for EVOO#1 and EVOO#2, respectively. The CI values for diluent oils are 76, 50 and 6 for sunflower, canola and rice bran oil, respectively.

4.2.2 Total Phenolic Content by HPLC Validation

The HPLC-DAD total phenolic method validation was performed by investigating two parameters, namely the *relative response factor* (RRF) and *extraction efficiency* (%EE). Firstly, the RRF was calculated as described in Section 3.3.2 in accordance with the IOC *"Determination of biophenols by HPLC"* method (IOC, 2017a). The RRF compares the instrument responses to both syringic acid and tyrosol in order to normalize any instrumental variability. Examples of the calculated syringic acid and tyrosol *response factors* (RFs) and the calculated RRF values are given in Table 4.1 and indicate that the RRF value were acceptable when compared to the prescribed range of 4.7-5.5 (IOC, 2017a). The %EE was calculated as described in Section 3.4 and in accordance with the literature (Peters et al., 2007; Ellison and Williams, 2012). The extraction efficiencies for TPC by HPLC are presented in Table 4.2.

Table 4.1: Relative response values of HPLC-DAD for TPC standards

Standard	**Response area**	**Injected standard ($\mu g\ mL^{-1}$)**	**RF**
syringic acid	1836.5	30	61.2
tyrosol	180.1	15	12.0
RRF			5.1

The %EE of the method measures the difference of a known concentration of standards added to oil matrices before the method extraction and measures if any loss of analyte has occurred throughout the extraction. The %EE of TPC by HPLC was assessed with the standard addition of five standards: tyrosol, ferulic acid, caffeic acid, vanillic acid and apigenin at 15 $mg\ kg^{-1}$. As the method error acts on each test sample independently, variabilities can arise in the extraction efficiencies and this explains the greater than 100% extraction efficiencies observed in Table 4.2. Other researchers use %EE as a quality control measure for the chromatographic analysis of TPC and have reported acceptable extraction efficiencies between 80% and 120% (Becerra-Herrera et al., 2014; Pereira et al., 2013). Furthermore, as the difference between the experimental and theoretical standard recovery is a measure of precision which is also expressed in the CI value, it can be presumed that the error in the standard recovery is also reflected in the CI values for the EVOOs (Peters et al., 2007). As such the %EE values obtained in this

work are in the acceptable range stipulated by the AOAC Official Methods of Analysis (2016) guidelines, all standard addition analytes can be considered to be accounted for after analysis by HPLC-DAD.

Table 4.2: Extraction efficiencies of TPC by HPLC

Sample	TPC of Oil (*n* = 3)	Total TPC of oil and standards	Extraction efficiency %
canola	0	42	108
rice bran	0	38	97
sunflower	0	40	103
EVOO#1	250	290	103
EVOO#2	203	244	105
standard recovery solution	0	39	100

Standard deviation for EVOO#1 and EVOO#2 was 1.25 and 1.62, respectively. The CIs for EVOO#1 and EVOO#2 were 1.41 and 1.83, respectively.

4.2.3 Evaluation of Total Phenolic content *via* HPLC-DAD

The validated method was evaluated for its suitability to detect diluent oils in the EVOO samples and calibration curves for the TPC concentration in both EVOO#1 and EVOO#2 were constructed. As the trendline characteristics for EVOO diluted with all three diluent oils were similar, the calibration curve for sunflower oil with its associated error bars was subsequently selected to represent the other two diluent oils. Therefore, Figure 4.2 and Figure 4.3 presents the calibration plots for diluent oils in EVOO#1 and EVOO#2, respectively. The method error was calculated as the CI associated with repeated pure EVOO analyses ($n = 3$, $p = 0.05$) and the corresponding regression analyses for these curves are shown in Table 4.3.

Table 4.3: TPC trendline parameters for diluted EVOO#

Olive Oil	Diluent Oil	Trendline gradient mg kg^{-1} %w/w	Coefficient of determination (R^2)
EVOO#1	RBO	2.57	0.97
EVOO#1	CAN	2.45	0.99
EVOO#1	SFO	2.50	0.97
EVOO#2	RBO	2.00	0.98
EVOO#2	CAN	2.08	0.99
EVOO#2	SFO	2.05	0.99

#diluent oil concentration 0 – 100 %w/w

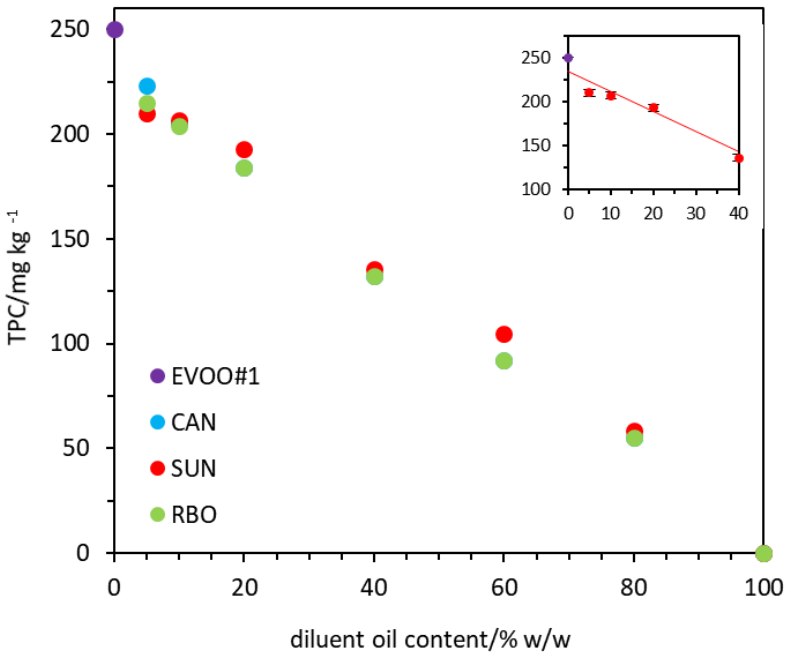


Figure 4.2: Relationship between TPC and diluent oil concentration for EVOO#1 where inset shows trendline for EVOO#1 and sunflower oil mixtures (error bars are the calculated CI values (CI = 1.41, p = 0.05, *n* = 3).

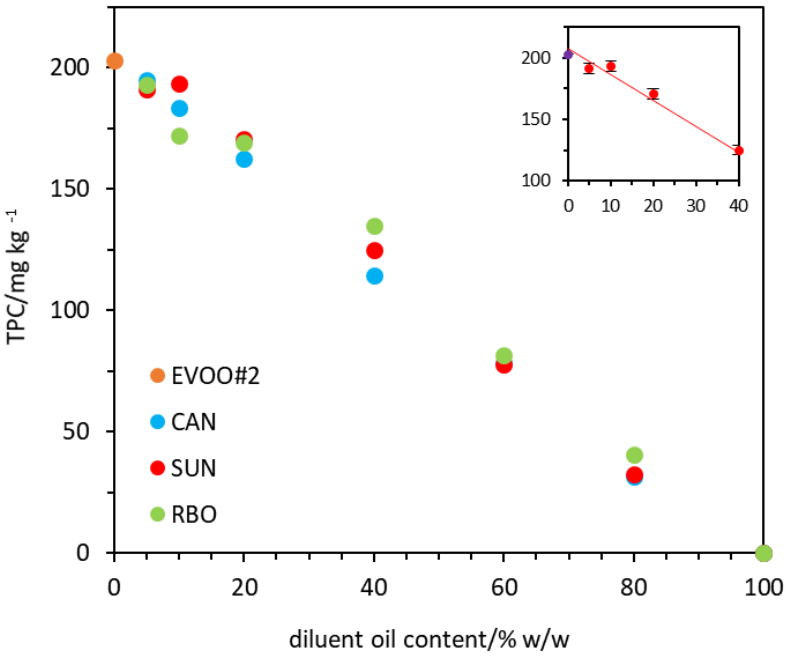


Figure 4.3: Relationship between TPC and diluent oil concentration for EVOO#2 where inset shows trendline for EVOO#2 and sunflower oil mixtures (error bars are the calculated CI values (CI = 1.83, p = 0.05, *n* = 3).

The calibration curves presented in Figure 4.2 show a linear decrease in the TPC concentration for EVOO #1 when diluted with sunflower, canola and rice bran oil. The first datum point to exceed the CI range of the pure EVOO is the 5 %w/w sunflower mixture, which suggests this is the LOD for TPC *via* HPLC-DAD. The TPC responses exhibit a plateau for mixtures <10 %w/w, which limits the LOQ for this method as shown in Figure 4.2. This renders the method of TPC determination by HPLC as being a semi-quantitative method only if used to detect diluent oil concentrations below 10% w/w of sunflower oil, canola oil or rice bran oil.

The regression analyses of the calibration curves, as presented in Table 4.3 shows a strong correlation between the TPC concentration and the concentration of diluent oil in the mixtures (R^2 = 0.97-0.99) and supports the notion that the TPC concentration can be used to detect the presence of diluent oils. Furthermore, the trendline gradients were found to be consistent among rice bran, canola and sunflower oils. Since no detectable phenolics were present in any of the pure diluent oils and a consistent linear decrease in the TPC concentration is observed as the diluent concentration is increased in all cases. However, since no detectable difference in TPC was observed for the EVOO samples diluted with canola, sunflower, or rice bran oil, it can be suggested that the determination of TPC by HPLC lacks specificity to identity which diluent oil is present in the mixture.

Although the results of this study have demonstrated the potential use of TPC determination to investigate EVOOs diluted with edible oils for the purposes of authenticity measurement, there are several limitations to this method. Firstly, the method lacks selectivity as no unique TPC marker was observed for any of the three diluent oils. Secondly, whilst the LOD was found to be 5% w/w for all three diluent oils, the LOQ of diluent oil is limited to mixtures >10% w/w diluent oil in each case. Lastly, the reported variability of TPC in EVOOs in the current study and in the literature, may give rise to expanded method error (Bajoub et al., 2016a; Cerretani et al., 2009; Janu et al., 2014; Kalogeropoulos and Tsimidou, 2014). However, as phenolic constituents contribute to the AOC of EVOOs, the use of AOC assays may provide improved sensitivity and/or selectivity to diluent oils as discussed later.

4.2.4 Validation of DPPH assay for Antioxidant Activity

The development and validation of the AOC assay that utilizes DPPH radical species is based on the method *"Determination of Antioxidant Activity in Foods and Beverages by Reaction with 2,2'-Diphenyl-1-Picrylhydrazyl (DPPH)"* (Plank et al., 2012). Firstly, the absorbance of DPPH at concentrations from 2.5 to 80 mg L^{-1} was measured to establish a DPPH concentration which presents a suitable initial absorbance before the addition of antioxidants (see Figure 4.4). As the concentration of DPPH increases, a concomitant increase in absorbance is also observed that ranges between 0.1 to 2.5 absorbance units (AU) for DPPH concentrations of 2.5 to 80 mg L^{-1}, respectively. A solution of DPPH of 25 mg L^{-1} provides the optimal absorbance of 0.9 AU and decreases to *ca* 0.7 AU after a 30 min incubation at 35°C. The compound Trolox, a common vitamin E analogue, is the standard for this method that is commonly reported in the literature (Amiri, 2010; Plank et al., 2012; Zullo and Ciafardini, 2008) and was used to quantify the AOC of EVOOs prepared as per Section 3.3.3. The Trolox standard provides strong linearity and is suitable for quantifying AOC based on the percentage of DPPH reacted under the test conditions. The AOC of pure EVOOs and those diluted with sunflower oil, canola oil and rice bran oil were therefore expressed in units known as "Trolox equivalent antioxidant capacity" (TEAC).

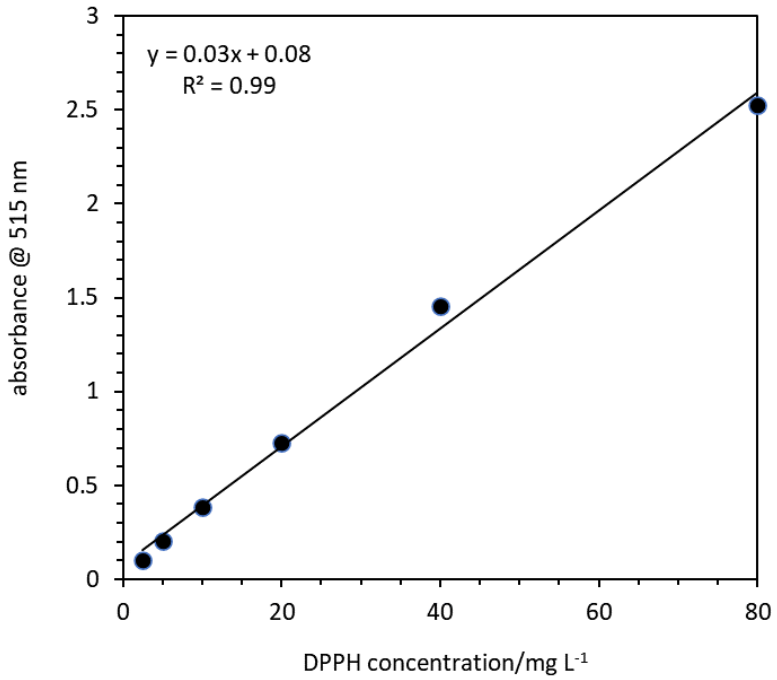


Figure 4.4: Absorbance of DPPH in methanol.

After the determination of an appropriate DPPH concentration, the next step in the DPPH assay validation is to establish a suitable experimental mass of test sample, whose response falls within the linear response range for the TEAC assay. This was accomplished with a series of pure EVOO#1, sunflower, canola and rice bran oil test samples, over a range of masses (0.1 to 1.0 g, n = 3). The %DPPH reacted were plotted against the mass of the pure oil samples as shown in Figure 4.5. A comparison of the AOC values of each oil, shows that both canola oil and rice bran oil have greater AOC compared to EVOO#1, whereas the AOC of sunflower oil is comparable to those of EVOOs. This lack of discriminatory power between sunflower oil and EVOO, suggests the method of AOC determined by DPPH assay is unable to distinguish between these two oils and hance was not further investigated. Seemingly the increase of test sample mass which exceed 0.5 g oil seemingly only marginally increases the AOC of the oil, as many of these mass present overlapping CI errors. Conversely, except for rice bran oil, 0.1 g of test sample presents a minimal response to AOC, which suggests this mass is not suitable to measure AOC of these edible oils. For all subsequent experiments, a mass of 0.5 g is selected as this ensures a suitable response between the DPPH radical and antioxidants present in the test samples.

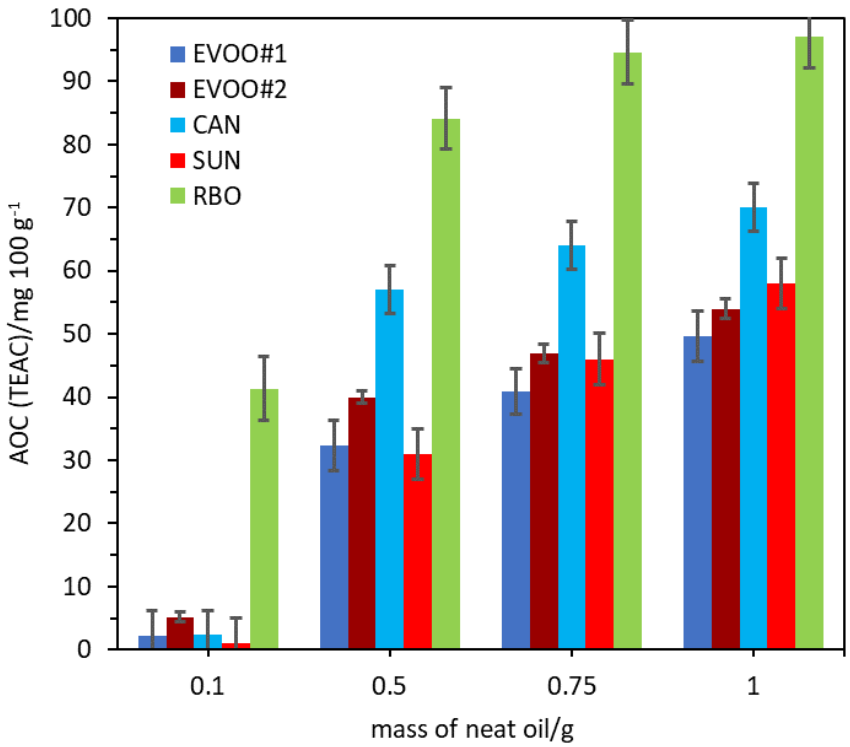


Figure 4.5: TEAC calibration of pure oils with a mass range of 0.1-1.0 g associated error bars are CIs (n = 3, p = 0.05). The CI values are 4.0, 1.0, 3.8, 4.0 and 5.0 for EVOO#1 EVOO#2, canola, sunflower and rice bran oil, respectively.

4.2.5 Antioxidant Activity to Determine EVOO Authenticity

The evaluation of the AOC of EVOOs and diluent oil mixtures utilized the same methods with regard to robustness, sensitivity and selectivity as described earlier (see Section 3.4). Since pure samples of sunflower oil and EVOO#1 previously produced similar AOC values, sunflower oil was not investigated. The calibration curves for EVOO#1 and EVOO#2 diluted with canola oil and rice bran oil are presented in Figure 4.6 and Figure 4.7 respectively whereas Table 4.4 presents the trendline properties of these calibration curves.

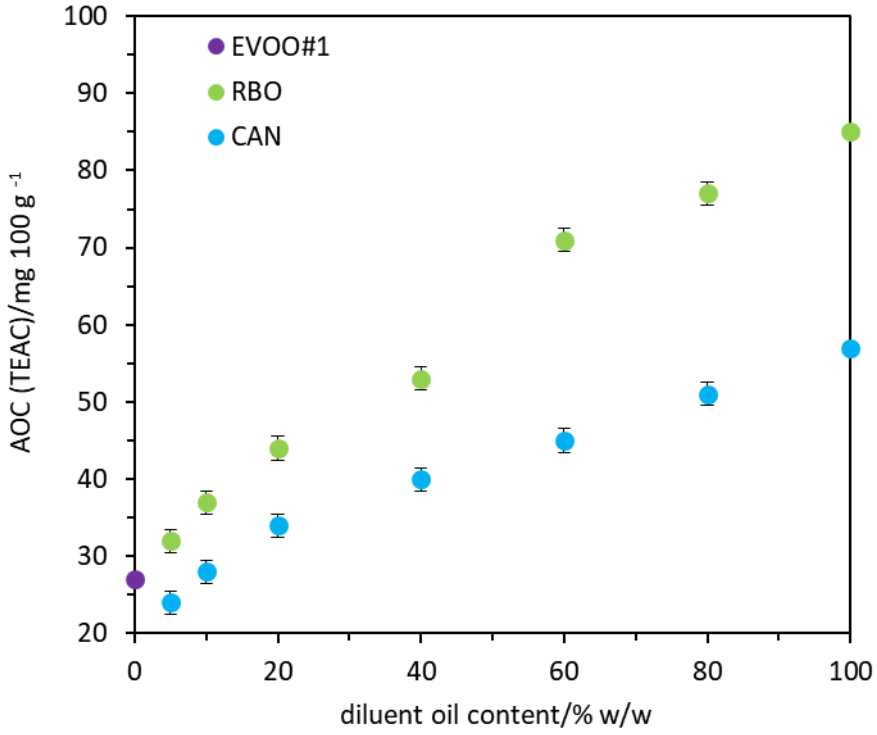


Figure 4.6: AOC (TEAC) of diluted EVOO#1
where CI values (CI = 1.44, p = 0.05, n = 3) are presented as the error bars.

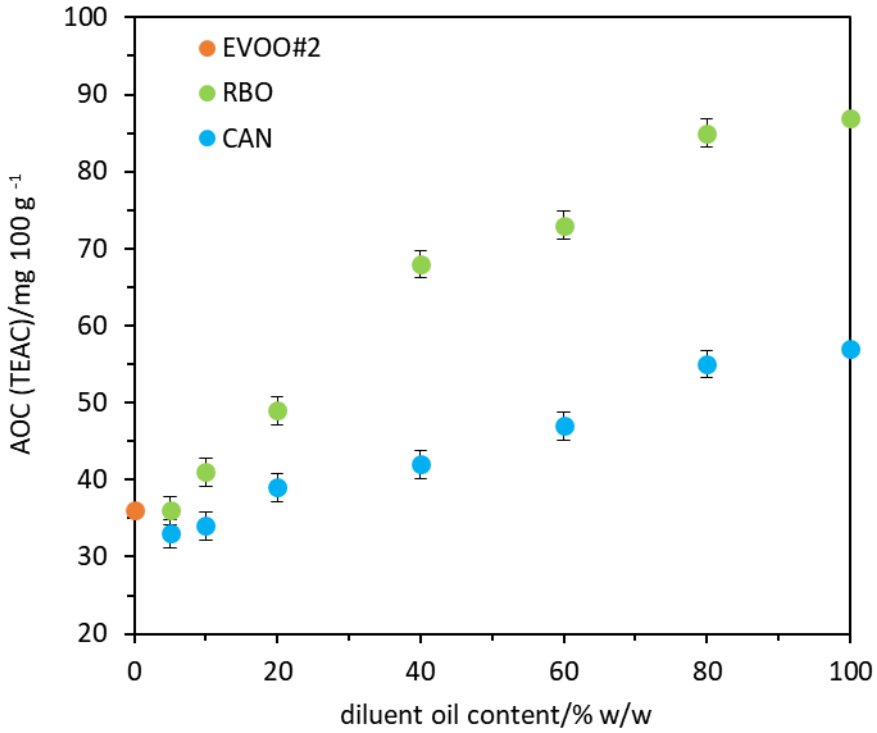


Figure 4.7: AOC (TEAC) of diluted EVOO#2
where CI values (CI = 1.82, p = 0.05, n = 3) are presented as the error bars.

Table 4.4: Regression analysis of TEAC calibration

Olive Oil	Diluent Oil	Gradient ΔTEAC/diluent oil%(w/w)$^{-1}$	Coefficient of determination (R^2)	LOD (% w/w)
EVOO#1	RBO	0.024	0.97	5
	CAN	0.012	0.98	20
EVOO#2	RBO	0.022	0.98	10
	CAN	0.007	0.86	20

As presented in Figure 4.6 and Table 4.5 both EVOO#1 and EVOO#2 exhibit comparable TEAC values of 27 and 36 mg 100 g^{-1} TEAC respectively, and their AOC increased with the addition of canola and rice bran diluent oils. The AOC increase arises from the contributions of the antioxidants present in both rice bran and canola oil (Ballus et al., 2015; Czerwińska et al., 2012; Zullo and Ciafardini, 2008). When compared to EVOO the AOC of rice bran and canola oil were 314% and 211% of EVOO#1 AOC, respectively. It is likely that other constituents in the diluent oils contribute to the

antioxidant properties of canola and rice bran oil, which reflect the elevated AOC measured by the TEAC assay.

The LOD of the method, as discussed in Section 3.4 was determined by identifying the first datum to exceed the CI range of the pure EVOO. The LOD for rice bran oil is 5% w/w and 10 % w/w for EVOO#1 and EVOO#2, respectively. In the case of canola oil, no detectable change in TEAC occurred up to 20% w/w of canola oil, limiting the LOD to mixtures which exceed 20% w/w. The higher LOD of canola oil diluent by DPPH may be due to the smaller AOC of canola oil than that observed with rice bran oil. This was also highlighted by the sensitivity of the method, as reflected by the gradient (see Table 4.4) where the gradient of canola oil is *ca* 50% of that of the rice bran oil. This difference in sensitivity allows the identification of an unknown diluent oil mixed with EVOO in some cases. A sample of EVOO diluted with 20% w/w canola oil presents a similar AOC to an EVOO diluted with 10% w/w rice bran oil, and this trend continues across the calibration curves. This hinders the identification of these two diluent oils up to concentrations of 55 mg/100 g TEAC after which the greater AOC of rice bran oil is more prevalent. It could be suggested that as canola oil provides greater fraudulent profit (see Section 1.1) canola oil would typically be the diluent in this case. Lastly, a TEAC of <25 mg $100g^{-1}$ would suggest the oil is either a pure EVOO or an EVOO diluted with sunflower oil and in this case a diluted EVOO can be identified with either of the TPC methods.

Table 4.5: AOC of EVOOs with increasing mass of diluent oils

	TEAC/mg 100 g^{-1}			
	EVOO#1		**EVOO#2**	
Diluent Oil % (w/w)	**RBO**	**CAN**	**RBO**	**CAN**
0	27	27	36	36
5	32	24	36	33
10	37	28	41	34
20	44	34	49	39
40	53	40	68	42
60	71	45	73	47
80	77	51	85	55
100	85	57	85	57

Note: n = 3, EVOO#1 standard deviation and CI was 1.28 and 1.44, respectively. The EVOO#2 standard deviation and CI was 1.60 and 1.81, respectively.

As reported in the literature, other AOC assays such as the ORAC assay could provide alternative means for diluent oil detection (Franco et al., 2014a; Janu et al., 2014; Kalogeropoulos and Tsimidou, 2014). Further study and validation of these alternative approaches may result in the improved discrimination between sunflower and EVOO or lower the LOD value for canola oil. The literature also reports the link between phenolic content and its contribution to increased AOC (Alessandri et al., 2014; Blekas et al., 2002; Tasioula-Margari and Tsabolatidou, 2015). However, as diluent oils were added (see Section 4.2.3) a decrease was observed in TPC content which is contrary to that expected given the AOC. This may be attributed to the complex nature of the measurement of antioxidants and AOC assays (Huang et al., 2005), or other constituents contributing to the AOC of mixtures, such as tocopherols (Duthie et al., 2016). Nonetheless, with the oils studied, there exists the potential to use TPC and AOC of olive oils to identify the presence of canola, sunflower or rice bran diluent oils.

4.3 Optimized Procedure for Diluent Oil Detection

This chapter has presented a proposed procedure for the detection of diluent oils, with determination of EVOO adulteration *via* measurement of phenolic content and AOC. Whilst the proposed methods individually suffer several limitations in their usage, the combined use of both TPC, (either by HPLC or FC assays) and AOC by DPPH assay, alleviates some of these difficulties. To this end, a decision tree was constructed as presented in Figure 4.8.

The proposed procedure can be used to determine whether any of the evaluated EVOOs were diluted with canola, sunflower or rice bran oil. Firstly, the TPC of EVOOs by the FC assay or the HPLC-DAD method is determined, with a value of <930 mg kg^{-1} TEAC or <195 mg kg^{-1} respectively indicating that a diluent oil is present. If the EVOO is suspected to be adulterated, the AOC of the oil should be determined with the DPPH assay. This assay provides the selectivity between one of three possible diluent oil groups. A test sample with an AOC of ≥56 mg kg^{-1} indicates a rice bran diluent oil, whereas a value <56 mg kg^{-1} indicates either canola or rice bran oil is the diluent oil. Lastly, a TEAC value <25 mg 100 g^{-1} indicates that sunflower oil is the diluent. As the TPC determined by FC assay offers poor selectivity and poor linearity with respect to suspect diluent oils and the lack of discriminatory power between EVOOs and sunflower oil *via* the DPPH

AOC assay, the quantification of diluent oil should be based on the TPC that has been determined *via* the HPLC-DAD method. This investigation of TPC *via* the FC assay, TPC *via the* HPLC and AOC *via* the DPPH assay has highlighted the potential of their combined use to authenticate olive oils. In combination, the three methods offer a broader characterisation of adulterated EVOOs than the use of a single method only.

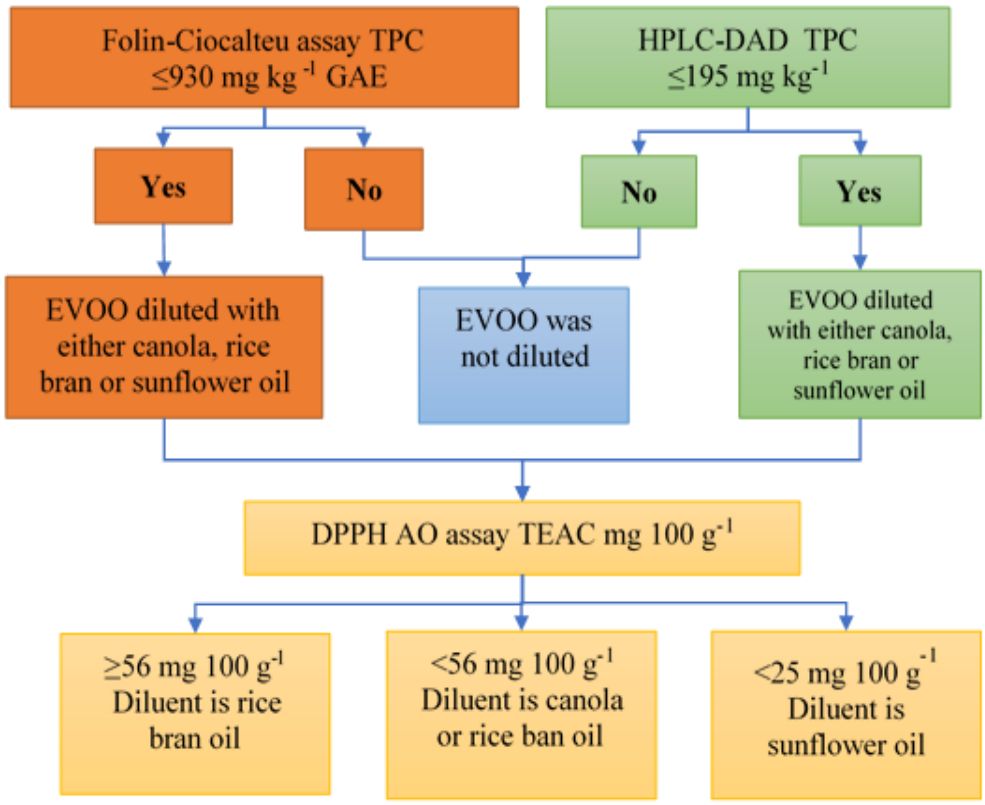


Figure 4.8: Decision tree for TPC and AOC.

4.4 Summary

This chapter has explored the phenolic content, as determined by both the FC assay and HPLC-DAD methods, to identify canola, sunflower and rice bran diluent oils in two Australian EVOOs. The FC assay provides a suitable qualitative screening method, as it is able to clearly detect EVOOs that contain 5% w/w diluent. However, the FC assay is unable to identify the diluent oil or determine its concentration. The determination of TPC by HPLC is also able to detect an EVOO diluted with concentrations of diluent oil and offers a means to quantify diluent oils at concentrations >10% w/w. Moreover, the

DPPH assay offers improved selectivity between the diluent oil, with the limitation that it suffers from a high LOD of >20% w/w in the case of canola diluent oils and is unable to discriminate between sunflower oil and EVOO. With these method limitations, a procedure for the combined use of all three methods has been proposed. The suggested approach offers a suitable LOD of 5% w/w when either the FC assay or HPLC DAD techniques are used and a LOQ of >10% w/w with HPLC-DAD. While the AOC assay provides limited selectivity to identify which of the diluent oils is present.

Chapter 5 Detection of Diluent Oils by Spectrophotometric Methods

5.1 Overview

Spectroscopic techniques have been widely used to investigate edible oils, including authenticity studies of extra virgin olive oils (EVOOs) (Lia et al., 2018; Squeo et al., 2018). The use of UV/visible absorbance spectra alone or in tandem with other techniques can be used to discriminate oils based on their region of origin among other parameters (Martelo-Vidal and Vázquez, 2016). Fluorescence techniques are also widely used and components such as chlorophyl and phenolics can be readily detected and quantified (Squeo et al., 2018; Domínguez Manzano et al., 2019; Ali et al., 2018). Fluorescence excitation-emission matrices (EEMs) with the associated parallel factor analysis (PARAFAC) for chemometric analyses have also been used to study EVOOs (Squeo et al., 2018; Domínguez Manzano et al., 2019; Lia et al., 2018; Durán Merás et al., 2018; Ali et al., 2018). However, advanced data analysis techniques not readily available to industry and are often difficult to implement on large data sets (Durán Merás et al., 2018; Nikolova et al., 2014; Ali et al., 2018; Domínguez Manzano et al., 2019; Lia et al., 2018; Squeo et al., 2018).

This chapter explores an approach to evaluating EVOOs directly using fluorescence EEM and concurrently recorded UV absorbance data to provide an initial screening of an adulterated olive oil followed by analysis of selected data sets for the purpose of quantification. A novel and simplified regression approach for analysing the fluorescence and UV absorbance data is presented.

5.2 Results and Discussion

5.2.1 Fluorescence EEMs of Pure Oils

In order to assess the potential for using fluorescence EEMs for detecting EVOO adulteration, it is important to benchmark the neat oils. This enables the acquisition of a considerable amount of fluorescence data that can be used to identify specific oils and to determine the various regions that may relate to specific components in a variety of oils. The EEM plots of the pure EVOOs, canola, sunflower and rice bran oil are presented in Figure 5.1. The fluorescence EEMs of the pure oils exhibit several "hotspots" which are associated with fluorophores such as chlorophyll, peroxides and other oxidation products (Squeo et al., 2018; Lia et al., 2018; Ali et al., 2018). The EEMs of both EVOO#1 and EVOO#2 exhibit strong and

broad fluorescence intensities across the excitation range between 300 and 675 nm and the corresponding narrower emission range between 650 and 750 nm. The distinct hotspots in the λ_{ex} = 350-360 nm and λ_{em} = 673-675 nm region relate to chlorophyll and pheophytin, both of which are highly prevalent in EVOOs (Squeo et al., 2018; Ali et al., 2018). A weaker fluorescence region can also be observed around the λ_{ex} = 350 and λ_{em} = 450-575 nm which arises from peroxides and oxidation products (Squeo et al., 2018).

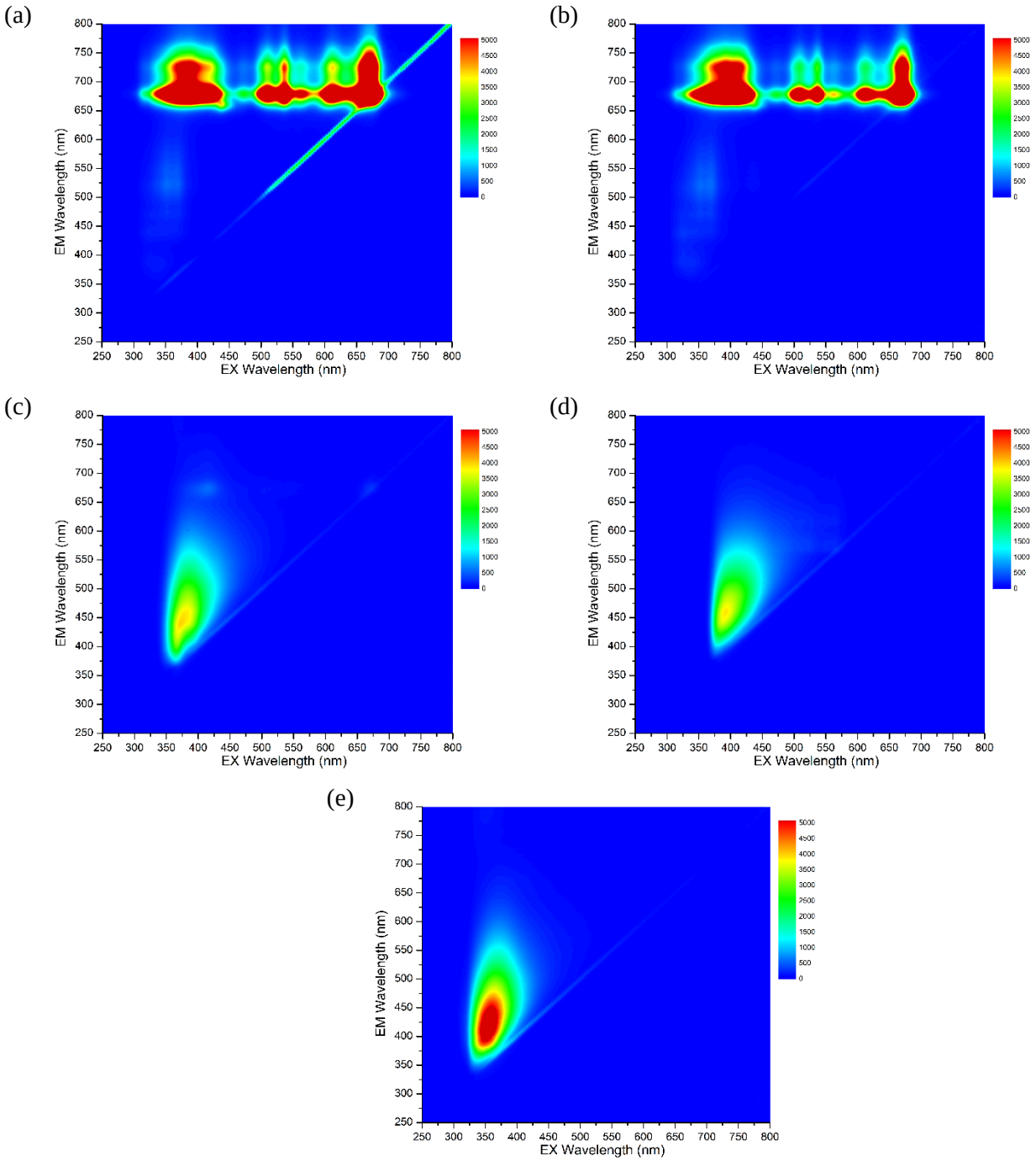


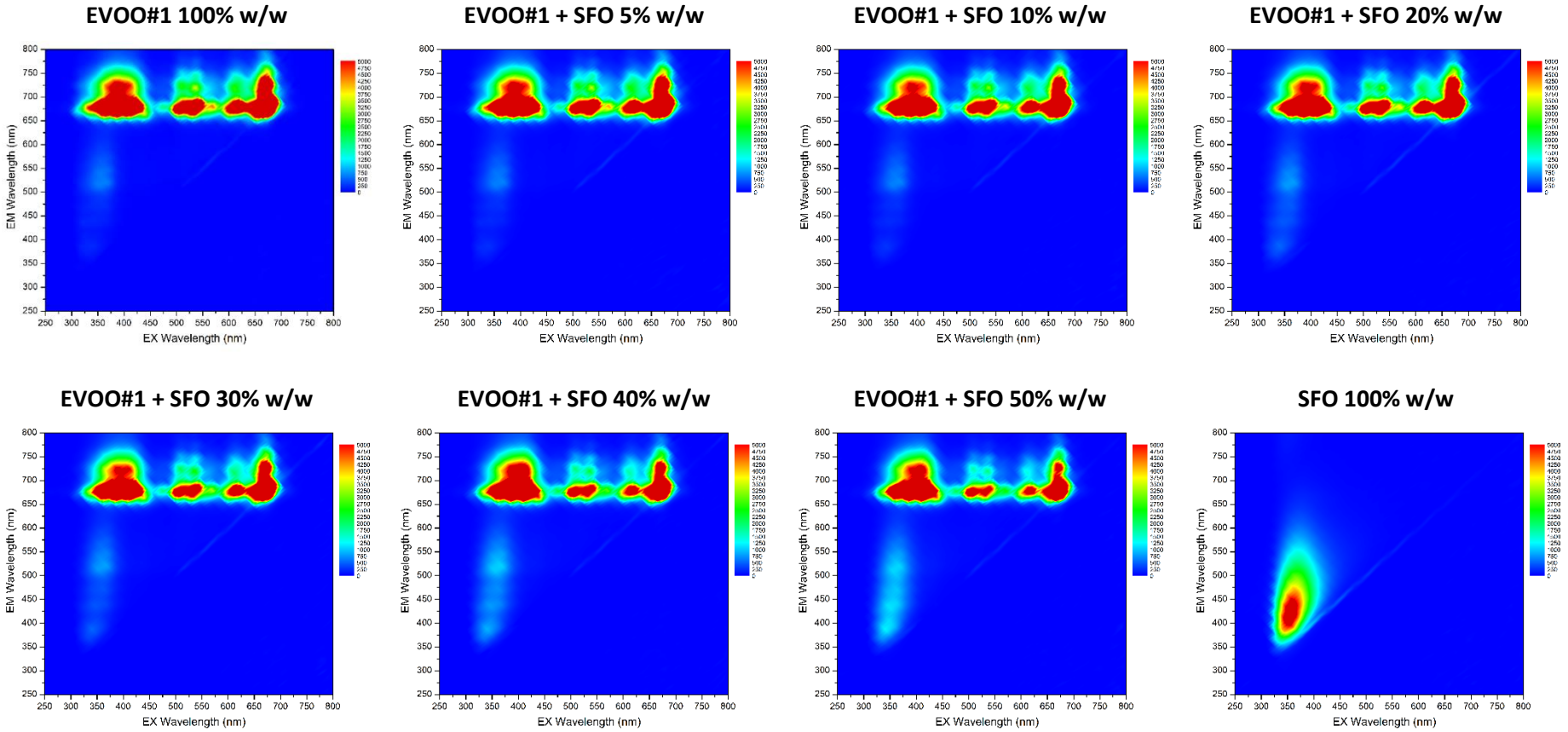
Figure 5.1: Fluorescence EEMs of pure oils
(a) EVOO#1, (b) EVOO#2, (c) canola, (d) rice bran, and (e) sunflower oil.

The EEM spectra of canola, rice bran and sunflower oils clearly differ significantly from those of the pure EVOOs. Firstly, the high intensity region observed in the EVOO emission range 650-750 nm is not present in the rice bran oil, with only very small regions of much lower intensity centred at λ_{ex} = 420 nm and λ_{em} = 675 nm for both the canola and sunflower oils. This suggests the presence of very low levels of fluorescent pigments and/or chlorophyl in these seed-based oils (Ali et al., 2018), whereas these are the dominant fluorescent species in the EVOOs. The major characteristic of the canola, sunflower and rice bran oil samples is a distinct teardrop shape with high fluorescence intensities in the regions λ_{ex} = 325-450 nm and λ_{em} = 350-550 nm. The location of the teardrop shape appears to differ among all samples.

5.2.2 Fluorescence EEMs of EVOO/Sunflower Oil Mixtures

The distinct differences in the fluorescence profiles of the EVOOs and the diluent oils suggests the potential for using fluorescence profiling to detect diluted EVOOs. Thus, the EEMs of mixtures of EVOO#1 and sunflower oil obtained directly and without solvent dilution were recorded and are presented in Figure 5.2. Based on the visual inspection of the EEMs it is evident that as sunflower oil is added to the EVOO, the fluorescence intensity in the emission range 650-750 nm decreases and this is consistent with a decrease in the concentration of chlorophyll fluorophores. Although this is a subtle decrease, particularly at lower concentrations of the diluent oil, it is more evident at concentrations of sunflower oil greater than 20% w/w. A concurrent increase in the fluorescence intensity is observed over the emission range 350-550 nm which is consistent with the presence of peroxides (Ali et al., 2018) and is unique to the diluent oils. Overall, the change in these regions suggests that fluorescence EEMs could potentially be used to qualitatively screen for diluent oils.

Figure 5.2: EEMs of EVOO#1, sunflower oil, and their mixtures.



5.2.3 EEM Regression to Determine EVOO Authentication

The acquisition of EEM profiles generates a significant number of data points, depending on the wavelength increments used to record the individual spectra. For example, the EEMs presented in Figure 5.1 and Figure 5.2 are comprised of more than 35,000 individual data points. To establish any correlation between one or more spectra requires advanced chemometric techniques such as PARAFAC analysis which deconvolutes EEMs to obtain the spectra as discreet data sets (Domínguez Manzano et al., 2019; Lia et al., 2018; Durán Merás et al., 2018; Ali et al., 2018). However, PARAFAC analysis requires considerable user input to select the number of components, and handle issues with scattered/missing data, noise and outliers (Andersen and Bro, 2003). In addition, there are issues associated with validation of the models used to derive the analyses, and interpretation of the PARAFAC outputs can be challenging.

To address this, an approach was developed to simplify the comparison of the separate EEMs based on the content of the diluent oil using a regression analysis of the complete data sets. The fluorescence intensity of each (λ_{ex}, λ_{em}) data pair were collated from the EEMs shown in Figure 5.2 to obtain six columns (for 0 to 100% diluent oil) and 35,520 rows of data. A regression analysis was applied to each row of data and the corresponding gradient was calculated. Figure 5.3 shows a 3D plot of the resulting R^2 values (z) as a function of the excitation (x) and emission (y) wavelengths. It is clear from this plot that that the majority of the data are not well correlated, however, there are several clustered regions where the data show good correlations and the R^2 values are >0.98. The data were then sorted in order from highest to lowest R^2 value and those that were <0.98 were excluded. There were a total of 2043 data sets with R^2 values >0.98, which is equal to 5.8% of the total data set. This suggests that there are a significant number of individual (λ_{ex}, λ_{em}) pairs that correlate with increasing levels of diluent oil in the EVOO. Figure 5.4 presents a scatter plot of the R^2 values >0.98 and the corresponding gradients of the linear regression for the data set. It is evident from this plot that the majority of the data present negative gradients with only 0.7% of the data presenting a positive gradient. Since the data were regressed based on increasing diluent oil content, a negative gradient is indicative of dilution of EVOO and thus the corresponding (λ_{ex}, λ_{em}) pairs are most likely related to the fluorescence of the EVOO components.

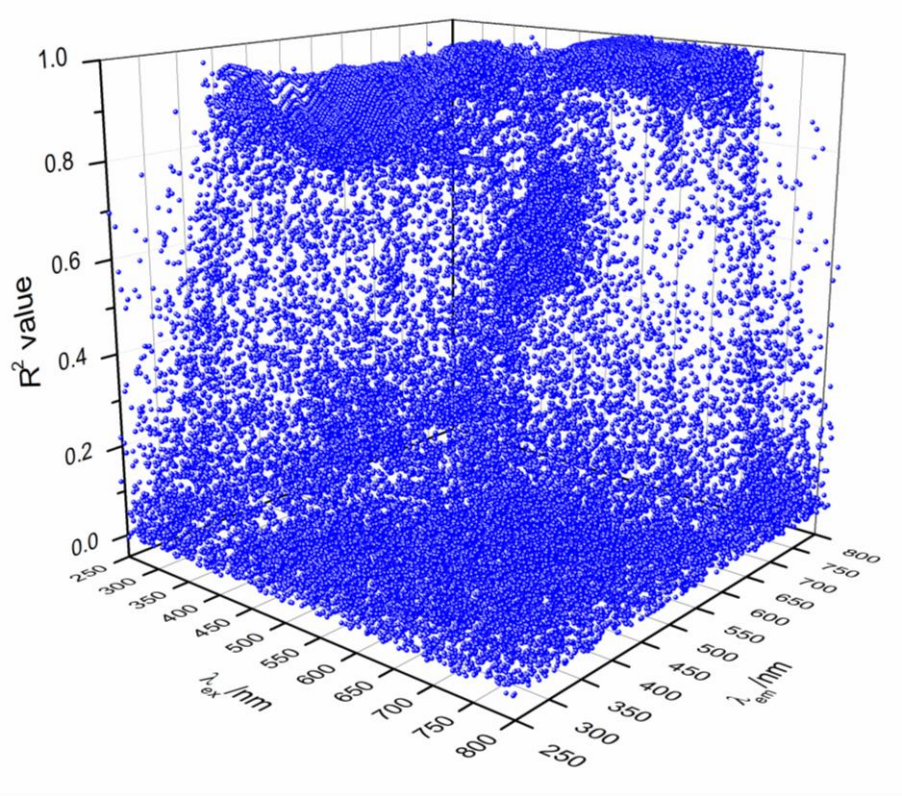


Figure 5.3: Three-dimensional plot of regression coefficients as a function of λ_{ex} and λ_{em}.

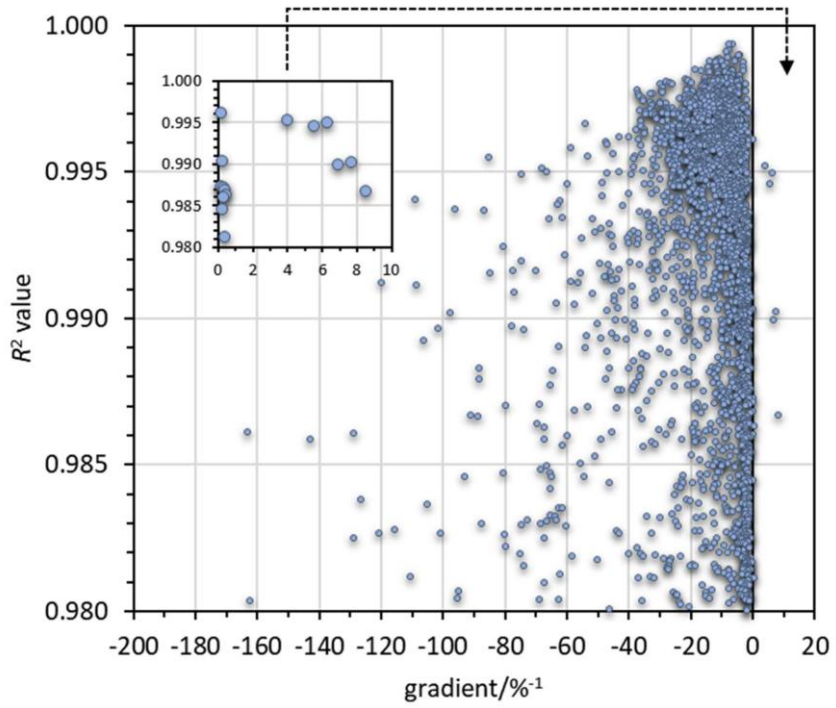


Figure 5.4: Scatter plot of regression coefficients *versus* gradient.

Figure 5.5 shows the fluorescence emission spectra of EVOO#1, sunflower oil and their mixtures at λ_{ex} = 536 nm. It is evident that the large peak at λ_{em} = 684 nm decreases with increasing sunflower oil diluent and Figure 5.6 shows the calibration curve of these data as a function of diluent oil content. In this case, the gradient is highly negative with a very high R^2 value and suggests λ_{ex} = 536 nm and λ_{em} = 684 nm offers an apparent *limit of detection* (LOD) of 5% w/w. Figure 5.7 shows further examples of calibration plots of fluorescence intensity with increasing sunflower oil content for selected data sets where the R^2 value >0.99 and the gradients are also negative. In all of these examples, the λ_{ex} is in the range of 530-560 nm and the λ_{em} is in the range of 680-750 which are clearly within the highly fluorescent region of the EVOO and correspond to the chlorophyll chromophores in the oil (Guimet et al., 2005; Ali et al., 2018). This confirms that the negative gradient is correlated with dilution of the coloured pigments in EVOO. Therefore, if the oils were suitably benchmarked, a decrease in fluorescence intensity in these wavelength regions would qualitatively indicate possible adulteration with the potential for quantitative assessment with adequate calibration.

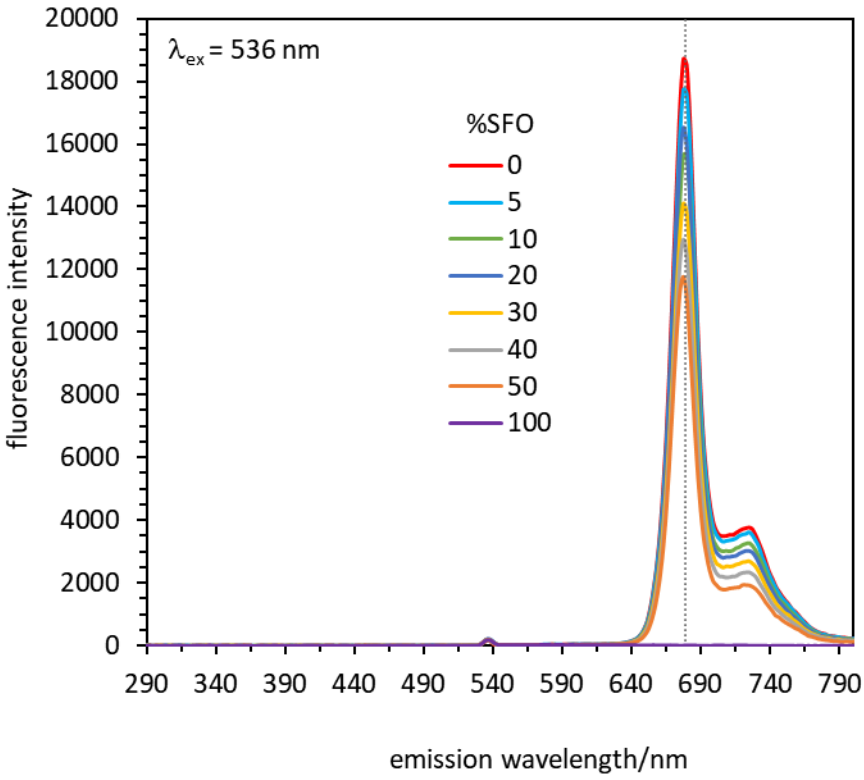


Figure 5.5: Fluorescence emission spectra for pure EVOO, pure SFO and their mixtures.

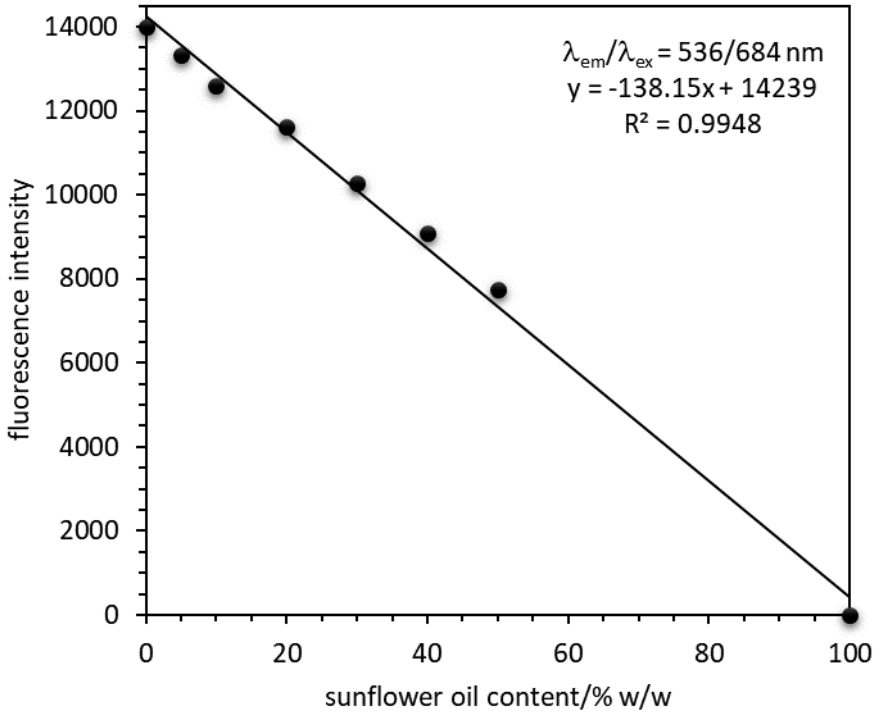


Figure 5.6: Fluorescence intensity as a function of increasing diluent oil concentration for highly negative gradient.

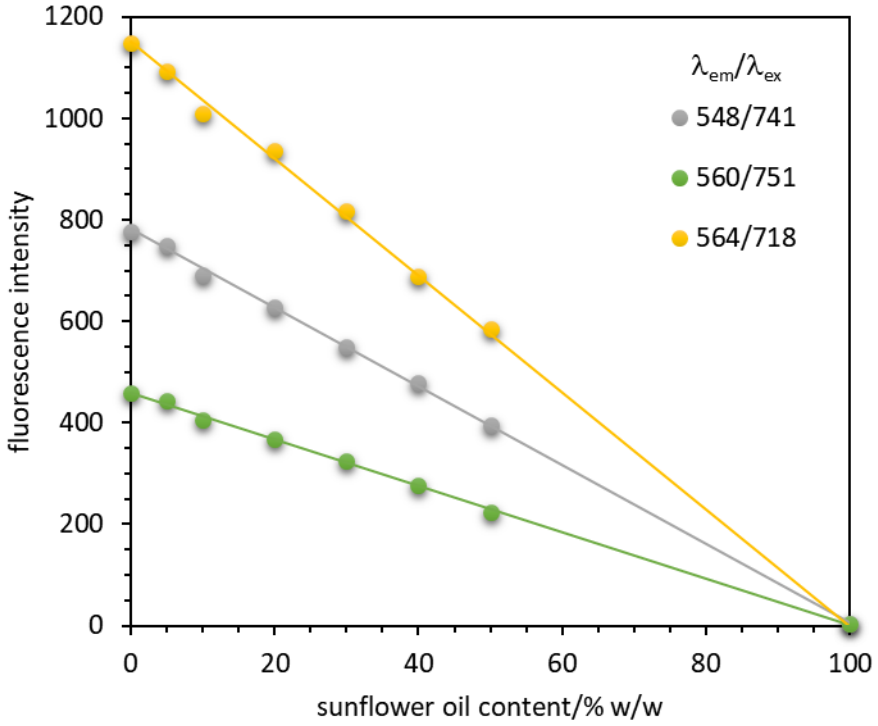


Figure 5.7: Fluorescence intensity as a function of increasing diluent oil content for negative gradients.

Figure 5.8 shows the scatter plot of the R^2 values >0.98 and the corresponding positive gradients obtained from the linear regression analysis. The values of the gradients are clustered in two general regions, one where the values are <1, and the other where the values are between 4 and 9. Figure 5.9 shows the fluorescence emission spectra of EVOO#1, sunflower oil and their mixtures at λ_{ex} = 328 nm as an example of a high R^2 value and a highly positive gradient obtained from the regression analysis. In this case, high R^2 value corresponds to a fluorescence intensity that is convoluted within a larger peak which demonstrates the complexity of the fluorescence data. However, it also demonstrates the applicability of this approach to extract correlations without the need for deconvolution or more intricate chemometrics. Examples of the calibration curves of fluorescence intensity as a function of increasing sunflower oil content for selected data sets where the R^2 value >0.99 and the gradient is positive are shown in Figure 5.10. In each of these examples, the increase in fluorescence intensity indicates an increase in concentration of the diluent oil and since this particular fluorophore is not present in EVOO, it may offer a further indication that a diluent oil is present in an adulterated mix. In these cases, the λ_{ex} is constant at 328 nm and the λ_{em} is in the range 350-360 nm which may correspond to the presence of phenolics and tocopherols (Milanez et al., 2017; Ali et al., 2018). In particular, the fluorescence intensity at λ_{ex} = 328 nm and λ_{em} = 360 nm seemingly offers an apparent LOD of 5-10% w/w. Based on these results, it is evident that this approach could readily be expanded to evaluate other oils and diluents that have been investigated in the literature (Lia et al., 2018; Durán Merás et al., 2018). Moreover, the apparent sensitivity and selectivity could be improved by altering the fluorescence spectra acquisition parameters including integration time and wavelength intervals.

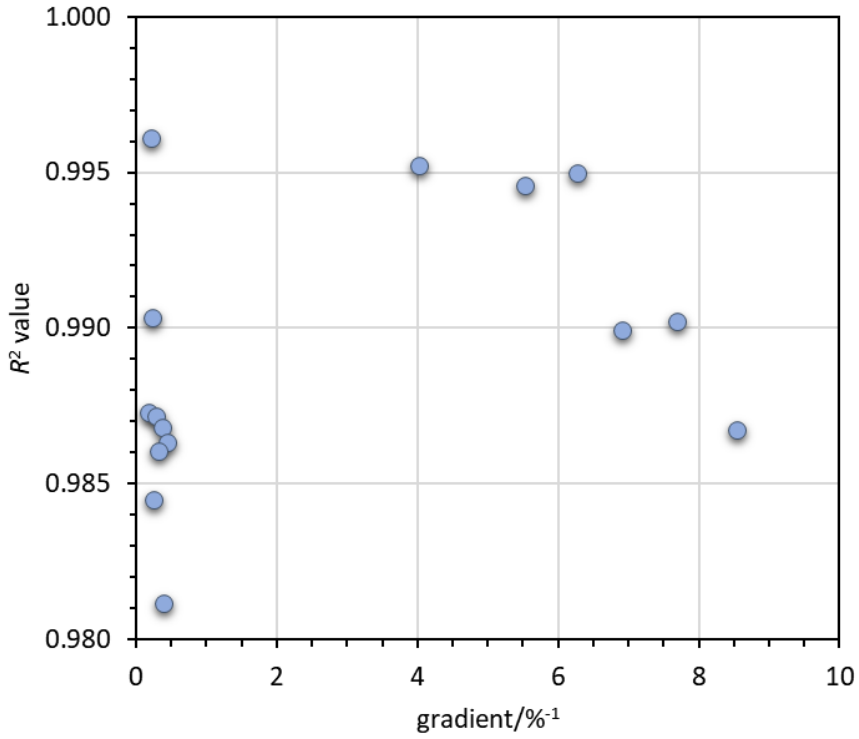


Figure 5.8: Scatter plot of fluorescence data regression coefficients *versus* positive gradients.

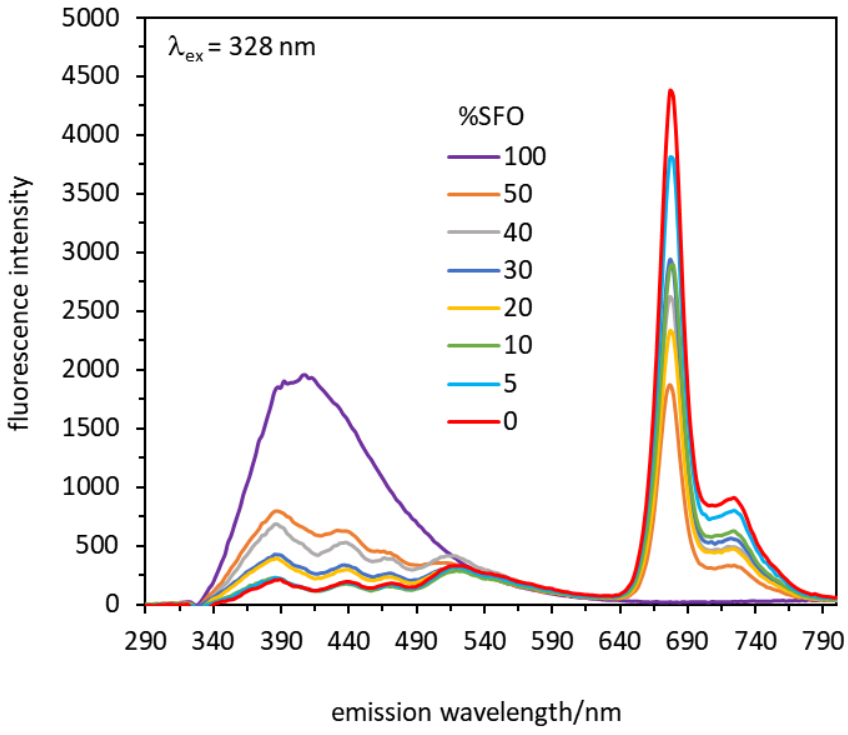


Figure 5.9: Fluorescence emission spectra for pure EVOO, pure SFO and their mixtures.

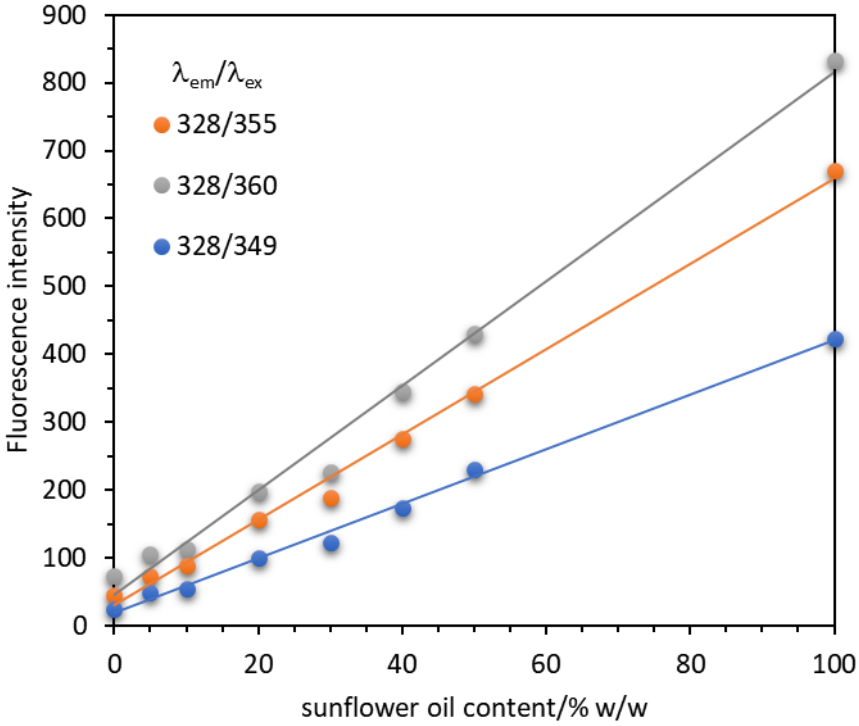


Figure 5.10: Fluorescence intensity as a function of increasing diluent oil content for positive gradients.

5.2.4 UV Absorbance of EVOOs and Mixtures

When EEM data are recorded using instruments such as the Horiba Aqualog, UV absorbance data are acquired simultaneously over the same excitation wavelength range. The same regression approach was applied to these data in order to identify any wavelengths where the absorbance maxima may be used to discriminate adulterated EVOOs but in this case, the data sets are comprised of six columns and 129 rows of data. Typically, UV absorbance data are acquired to identify and quantify coloured compounds within oils and in the case of EVOO, this will include the highly pigmented chlorophylls (Martelo-Vidal and Vázquez, 2016). Figure 5.11 shows the UV absorbance spectra of EVOO#1, sunflower oil and their mixtures which clearly shows differences between the pure oils. In the case of EVOO#1, multiple peaks are observed centred at 416, 456, 484, and 672 nm with several minor peaks. It is also clear that these major peaks decrease in intensity as sunflower oil is added to the EVOO.

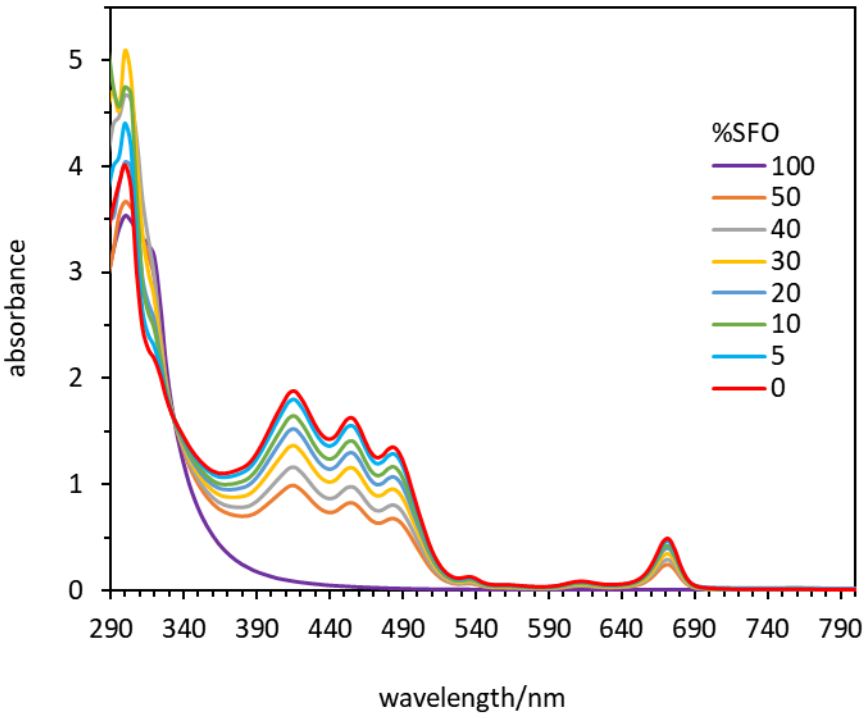


Figure 5.11: UV absorbance spectra for pure EVOO, pure SFO and their mixtures.

The regression analysis applied to the data resulted in a series of R^2 values and linear gradients which are presented in Figure 5.12. Similar to the scatter plot of the R^2 values and linear gradients obtained for the fluorescence data (Figure 5.4), most of the gradients are negative with only six positive gradients. The inset in Figure 5.12 shows that there is only a single positive gradient where the R^2 value is >0.98. Furthermore, this wavelength corresponds to that of the fluorescence data for the same conditions of high R^2 value and positive gradient.

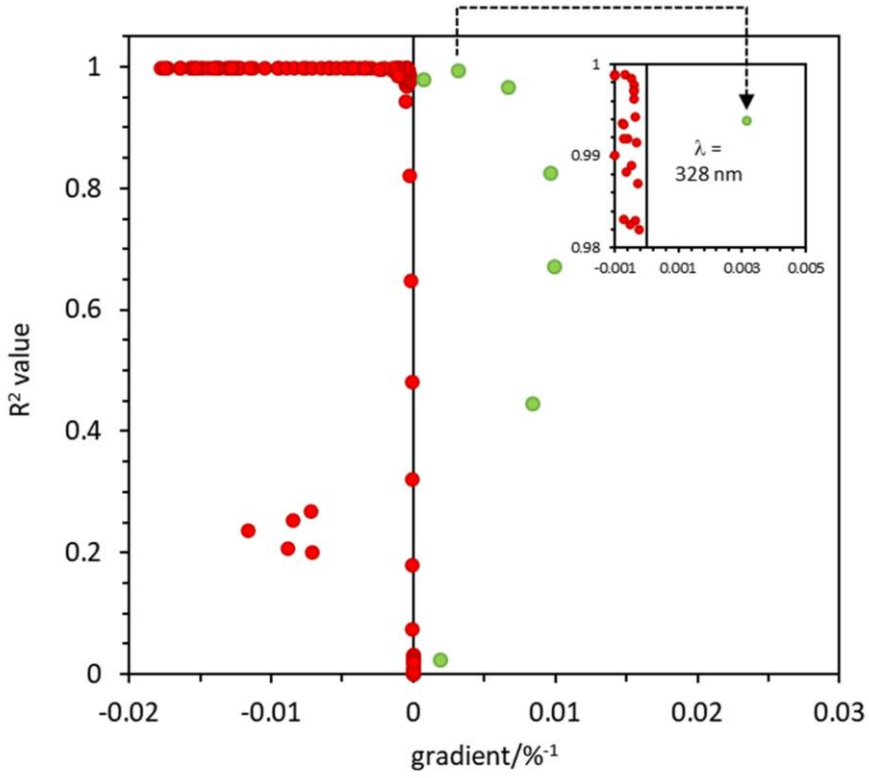


Figure 5.12: Scatter plot of UV absorbance regression coefficients and gradients.

Figure 5.13 and Figure 5.14 show examples of the calibration curves of UV absorbance as a function of increasing sunflower oil concentration where the R^2 value >0.99 and the gradient is negative and positive, respectively. In the case of the negative gradients, the wavelengths are associated with chlorophyll pigments and thus demonstrate a dilution of the EVOO. In the case of the positive gradient, and similar to the fluorescence data, the wavelength may be associated with α-tocopherol as this component is shown in the HPLC-DAD method to absorb UV light at 326 nm. However, the absorbance values are relatively high and the pure EVOO demonstrates a strong absorbance at this wavelength. This strong fluorescence signal of EVOO is reported in literature to correspond to peroxide and has demonstrated to decrease in intensity after 12 months of storage and limits the usefulness of UV measurements after this interval (Stefanoudaki et al., 2010; Aparicio-Ruiz et al., 2018). The suitability of using UV absorbance may therefore be less viable than fluorescence, although it may offer a qualitative technique to indicate potential adulteration for suitably calibrated/benchmarked EVOOs. However, the IOC stipulates the use of UV absorbance at 268 nm and 232 nm and is seemingly

effective in identifying less quality olive oils in samples diluted in isooctane (IOC, 2019b). This suggests the limitation of this strong absorbance may be of concern, particularly when samples are tested without using a solvent.

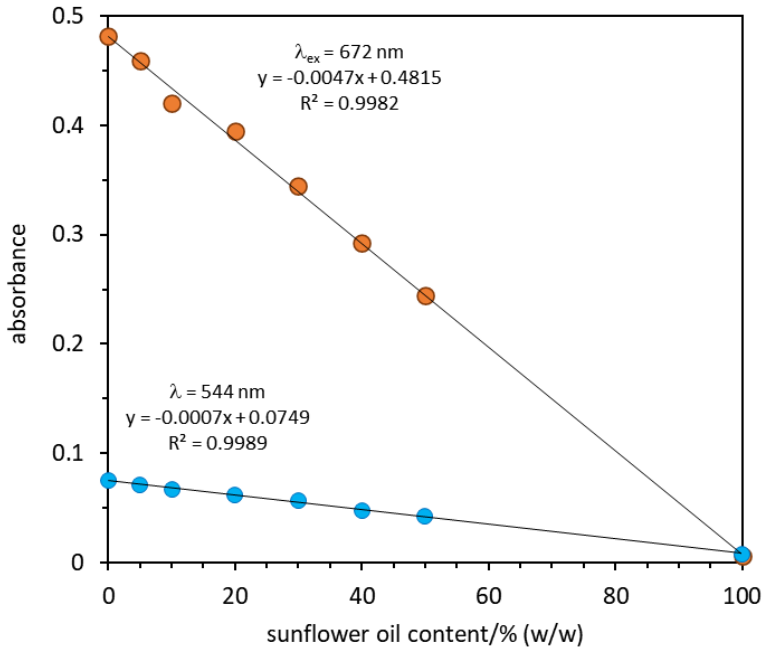


Figure 5.13: UV absorbance as a function of increasing diluent oil content for negative gradients.

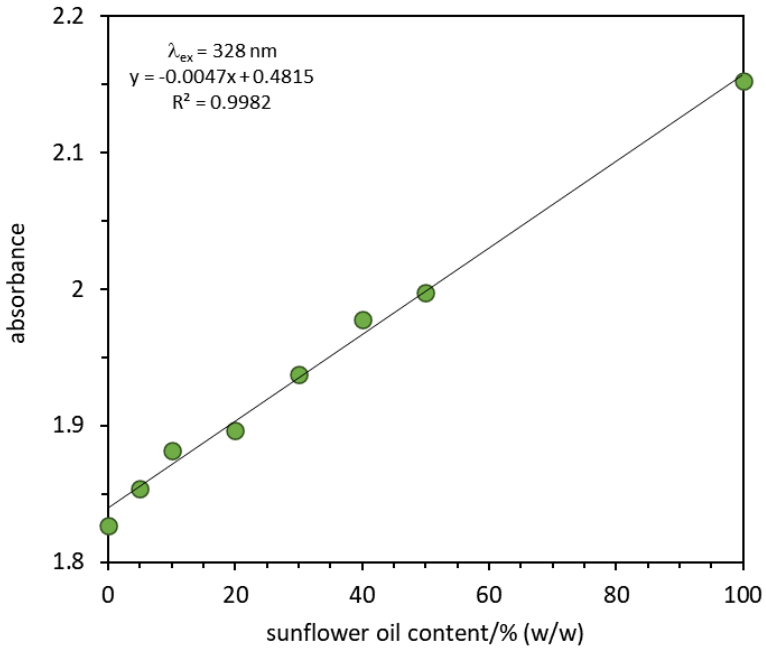


Figure 5.14: UV absorbance as a function of increasing diluent oil content for positive gradient.

5.3 Summary

This chapter has investigated the suitability of using fluorescence EEMs and UV absorbance measurements to discriminate between a pure EVOO and diluted samples. The method, based on linear regression of the complete EEM and UV absorbance data sets, demonstrated an alternative approach to evaluating large data sets with minimal data processing. The method was able to rapidly identify correlations between the emission/excitation and UV absorbance wavelengths and the composition of the diluted oils. The resulting calibrations were classified based on the gradients obtained for the highest regression coefficients where negative gradients indicated a dilution of the EVOO, and positive gradients resulted from the presence of the diluent oil. Overall, the method shows some promise for further development as a quantitative technique. However, the immediate benefit of this approach as a qualitative measure of potential adulteration is apparent. The rapid and direct analysis, without the need for extraction or other chemical treatment, clearly favours the acquisition of EEMs and fluorescence data for routine evaluation of EVOOs.

Chapter 6 Evaluation of Tocopherol Content

6.1 Overview

The variabilities in tocopherol forms and content of different edible oils suggest that extraction and quantification of tocopherols could be developed into a viable quantitative method providing a suitable calibration is implemented for each type of diluent oil. This chapter investigates the extraction and measurement of the tocopherol contents of two Australian extra virgin olive oils (EVOOs) diluted with canola, sunflower and rice bran oil. The aim of this chapter is to evaluate a tocopherol method to identify the diluent oil and the concentration of the respective diluent oil.

6.2 Results and Discussion

6.2.1 Quantitative Analysis of Tocopherol Standards by HPLC

To characterise the elution of the tocopherol standards by HPLC-DAD α-, β-, δ-, and γ-tocopherol standards were prepared at two concentrations as shown in Table 6.1 and these standards were then analysed *via* absorbance 326 nm and with the chromatographic conditions presented in Section 3.3.5. As shown in Figure 6.1, the chromatographic resolution of all four tocopherols is acceptable and the analysis was rapid with the final standard, δ-tocopherol, eluting at 6.1 min. The sensitivity is also acceptable as all tocopherols were detected at concentrations ≥3 mg L^{-1}.

Table 6.1: Concentration of tocopherol standards

	Tocopherol standard (mg L^{-1})			
Standard	**α**	**β**	**δ**	**γ**
1	3	2	1	2
2	8	4	2	4

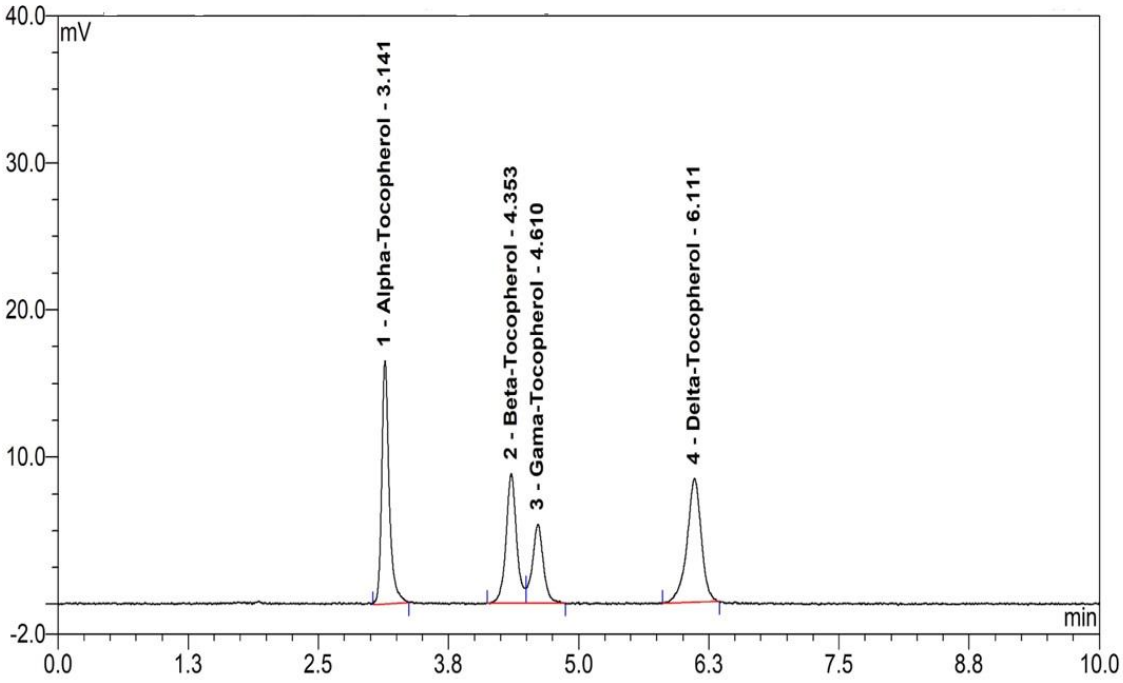


Figure 6.1: Chromatogram of α-, β-, δ- and γ-tocopherol standards (3, 2, 1 and 2 mg L^{-1} respectively).

6.2.2 Recovery of Standard Tocopherols

The recovery of standard tocopherols with calculation *via extraction efficiency* (%EE) provides an indicator of any method error associated with the measured tocopherol content as described previously in Chapter 3 (see Section 3.4). The standard recovery is calculated using the analytical results of three extracts: (i) a test sample, (ii) the test sample with an added known concentration of tocopherol standards, and (iii) a third extract of standards in solution. This is assessed by addition of known concentrations of α-, β-, γ- and δ-tocopherol to diluted EVOO#1 test samples which were mixed with 5% w/w diluent oils (see Table 6.2) and the calculated %EE are presented in Table 6.3.

Table 6.2: Standard addition of tocopherol to EVOO#1 diluent mixtures

Standard	Standard concentration (mg L^{-1})
α-tocopherol	50.3
β-tocopherol	26.1
γ-tocopherol	14.6
δ-tocopherol	29.2

Table 6.3: Calculated %EE values for individual tocopherols in EVOO and EVOO blends.

	Tocopherol			
Diluent mixture % w/w	**α**	**β**	**γ**	**δ**
EVOO#1 95% CAN 5%	105	97	107	90
EVOO#2 95% SFO 5%	105	95	107	87
EVOO#1 95% RBO 5%	119	95	114	95
EVOO#2 100%	111	93	107	90
Average (n = 4)	110	95	109	91
Standard deviation	7	2	4	3
CI (n = 4, p = 0.05)	7	2	3	3

The recoveries ranged between 87% and 119% for all four tocopherols in the EVOOs and diluent oil mixtures. The average recovery across all tocopherols was 101 ± 2%, however, α- and γ-tocopherol consistently presented elevated recoveries with an average recovery of 110 ± 7% and 109 ± 3% respectively. The method variance independently acts on each test sample which explains the >100% recoveries observed for both α- and γ-tocopherol (Peters et al., 2007). Conversely, lower average recoveries of β- and δ-tocopherol (<100%) can be attributed to a loss of standard during the extraction and although this is a potential drawback of this extraction method, it is acceptable since neither of these tocopherols is present in any of the edible oils in this study. Recovery efficiencies are commonly used in the literature as a quality check and whereas EVOO diluted with SFO exceeds AOAC %EE guidelines, the literature has reported ranges between 80-120 %EE as being acceptable (Becerra-Herrera et al., 2014; Pereira et al., 2013; AOAC Official Methods of Analysis, 2016).

6.2.3 Tocopherol Composition of EVOOs and Diluent Oils

The composition of tocopherols in each of the EVOO and diluent oils presents a unique tocopherol profile as summarised in Table 6.4 and these are consistent with other published studies (Bakre et al., 2015; Chen et al., 2011; Flakelar et al., 2015; Flakelar et al., 2017). All five oils in this study contained α-tocopherol and sunflower oil presented a much higher concentration of α-tocopherol compared to all other oils. Furthermore, γ-

tocopherol was present in canola and rice bran oil but not sunflower oil. A small amount of γ-tocopherol was found in EVOO#2, which is often found in EVOOs from olives harvested later in the season (Jukić Špika et al., 2015). This small concentration of γ-tocopherol present in EVOO#2 will increase the limit of detection for diluent oils, as this contributes to the measurement uncertainty of the method. These differences in concentration suggest that the method is appropriate to quantitate EVOOs diluted with canola, sunflower and rice bran oil.

Table 6.4: Tocopherols detected in each pure oil

Edible oil	α-tocopherol (mg kg^{-1})	Standard deviation	CI $n = 3$ p = 0.05	γ-tocopherol (mg kg^{-1})	Standard deviation	CI $n = 3$ p = 0.05
EVOO#1	162	3.5	3.9	N.D.		
EVOO#2	132	4.9	5.5	N.D.		
CAN	258	0.52	0.59	417	0.65	0.73
SFO	748	0.68	0.78	N.D.		
RBO	65	0.33	0.36	65	0.24	0.27

Note: N.D. = not detected

6.2.4 Authenticity by Total Tocopherol Content

The suitability of evaluating tocopherol content for the detection of diluent oils in EVOO was investigated by measuring the total tocopherol content in the two EVOOs. The EVOOs were diluted with sunflower, canola and rice bran oil and calibration curves for each diluted EVOO were constructed. The total tocopherol contents of EVOO#1 and EVOO#2 were 169 mg kg^{-1} and 132 mg kg^{-1} respectively, and thus a variance of 37 mg kg^{-1} between these EVOOs was observed. This increases the method error for authentication by total tocopherols and increases the LOD of the analytical method. The total tocopherol content for the EVOOs diluted with canola or sunflower oils shows an upward linear response with increasing diluent oil content as shown in Figure 6.2 and Figure 6.3 for EVOO#1 and EVOO#2, respectively. In the case of rice bran oil, a slight decrease in total tocopherol content with increasing diluent oil is observed and reflects the relatively low levels of total tocopherols measured in this oil (see Table 6.5).

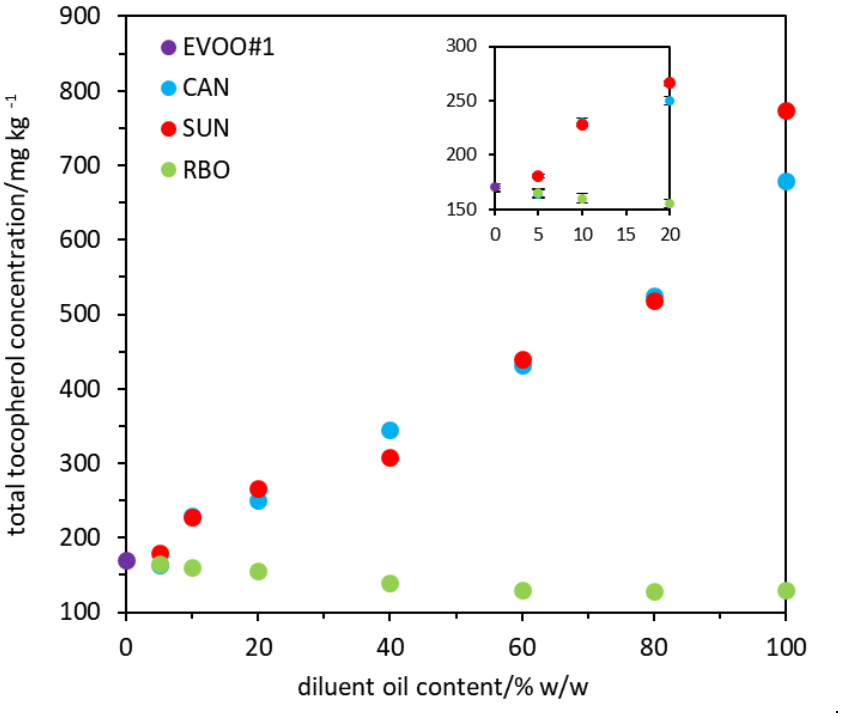


Figure 6.2: Total tocopherol content of diluted EVOO#1 containing rice bran oil, canola oil and sunflower oil, where inset is mass range 0-20% w/w error bars reflect CI value (CI = 3.9, p = 0.05, *n* = 3).

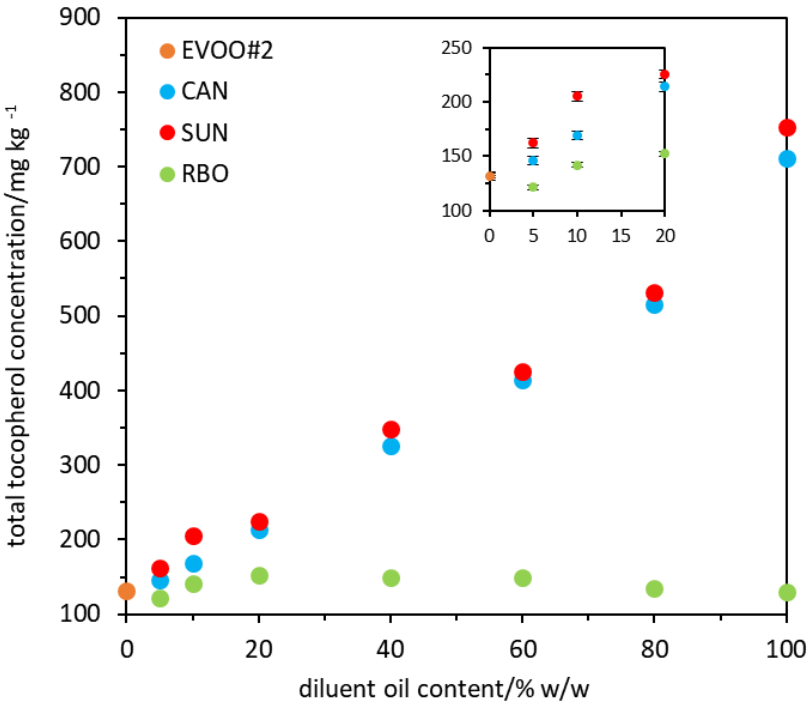


Figure 6.3: Total tocopherol content of diluted EVOO#2 containing rice bran oil, canola oil and sunflower oil, where inset is mass range 0-20% w/w error bars reflect CI value (CI = 5.5, p = 0.05, *n* = 3).

Table 6.5: Total tocopherols of diluted EVOOs

Diluent oil% w/w	CAN		SFO		RBO	
	EVOO#1/ mg kg^{-1}	EVOO#2/ mg kg^{-1}	EVOO#1/ mg kg^{-1}	EVOO#2/ mg kg^{-1}	EVOO#1/ mg kg^{-1}	EVOO#2/ mg kg^{-1}
0	169	132	169	132	169	132
5	164	146	180	162	180	162
10	230	169	227	205	228	205
20	250	214	266	225	266	225
40	344	326	307	349	308	349
60	432	414	439	425	439	425
80	524	515	518	532	518	532
100	679	711	774	753	774	753

Note: n = 3, EVOO#1 standard deviation and CI was 3.5 and 3.9, respectively. EVOO#2 standard deviation and CI was 4.9 and 5.5, respectively.

The trendline parameters obtained for both EVOO samples and diluent oils are presented in Table 6.6 and the linearity of the calibration curves is acceptable for both sunflower oil and canola oil. This suggests strong agreement between the increase in total tocopherols and the mass of diluent oils. In the case of rice bran oil, the correlation is poor with a R^2 = 0.05 and 0.18 for EVOO#1 and EVOO#2 respectively, suggesting that the total tocopherol content lacks the sensitivity to identify EVOOs diluted with this oil. Total tocopherol content is clearly influenced by the addition of both canola and sunflower oils. However, difficulty in the use of total tocopherol content for identification of diluted EVOOs lies in the natural variability of olive oils. For example, if EVOO#2 were adulterated with sunflower or canola oil by *ca.* 7% w/w, the tocopherol concentration would be similar to EVOO#1. Due to this variance, a suitable limit of 178 mg kg^{-1} of total tocopherols could be applied for identification, which corresponds to a concentration of *ca.* 10% w/w for EVOO#2. This suggests that a targeted approach, in which a total tocopherol value is used to determine the presence of diluent oil, would present a larger method error for the identification and quantification of diluent oils. The variance of the total tocopherol content in EVOO has also been reported by Velasco and Dobarganes (2002) and Franco et al. (2014b). Therefore, a more suitable approach is needed for diluent oil detection, and this may be achieved by identifying the individual tocopherols present in the samples.

Table 6.6: Trendline parameters for total tocopherol calibration curves for EVOOs diluted with different oils

Olive Oil	Diluent Oil	Trendline gradient mg kg^{-1} % w/w $^{-1}$	Coefficient of determination (R^2)
EVOO #1	rice bran	-0.4	0.05
EVOO #1	canola	4.9	0.97
EVOO #1	sunflower	5.1	0.94
EVOO #2	rice bran	0.1	0.18
EVOO #2	canola	5.2	0.94
EVOO #2	sunflower	4.9	0.81

6.2.5 Authenticity by Individual Tocopherols

The assessment of individual tocopherols was performed with the same extraction and HPLC-DAD method described in Section 3.3.5. It was observed that small additions of diluent oils either increased or decreased the concentration of α- and γ-tocopherol in the oil mixtures. The method error of each individual tocopherol analysis was determined as the calculated CI values of EVOO#1 and EVOO#2 which are 3.9 and 5.5 mg kg^{-1} respectively (n = 3, p = 0.05). This section presents the suitability of profiling the individual tocopherol content for the identification of EVOOs diluted with the selected diluent oils.

EVOOs Diluted with Sunflower Oil

The sunflower oil sample was only comprised of α-tocopherol and its concentration was nearly five times greater than both EVOOs (see Figure 6.4). The evaluation of α-tocopherol therefore was able to detect diluent sunflower oil whereby high concentrations of α-tocopherol may indicate the presence of sunflower oil in a suspected adulterated EVOO. Since sunflower oil contains no other tocopherol analogues, the analysis of total tocopherol content should provide a similar linearity, sensitivity and selectivity as that obtained for α-tocopherol. A direct comparison of the α-tocopherol response between EVOOs and the test samples diluted with 5% w/w sunflower oil shows an overlap of CI error between EVOO#2 and the test sample diluted with 5% w/w sunflower oil. Conversely, the 5% w/w mixture of EVOO#1 lies outside the method error, which suggests the LOD for α-tocopherol determination using the sunflower oil diluent lies between 5% and 10% w/w. Although the sunflower sample used in this study

contained no other tocopherol analogues, it has been reported that sunflower oils produced in Brazil also contain γ-tocopherol which could further be evaluated as an indicator of adulteration (Grilo et al., 2014). As both EVOO#1 and EVOO#2 exhibit different concentrations of α-tocopherol, there is a degree of sample error for the calculation of sunflower oil concentration *via* α-tocopherol analysis. This sample error can be estimated with the calculation of α-tocopherol in EVOO#1 with the calibration curve of EVOO#2. The average difference between the theoretical diluent concentration and the experimental concentration is *ca* 7% which corresponds to the sample error.

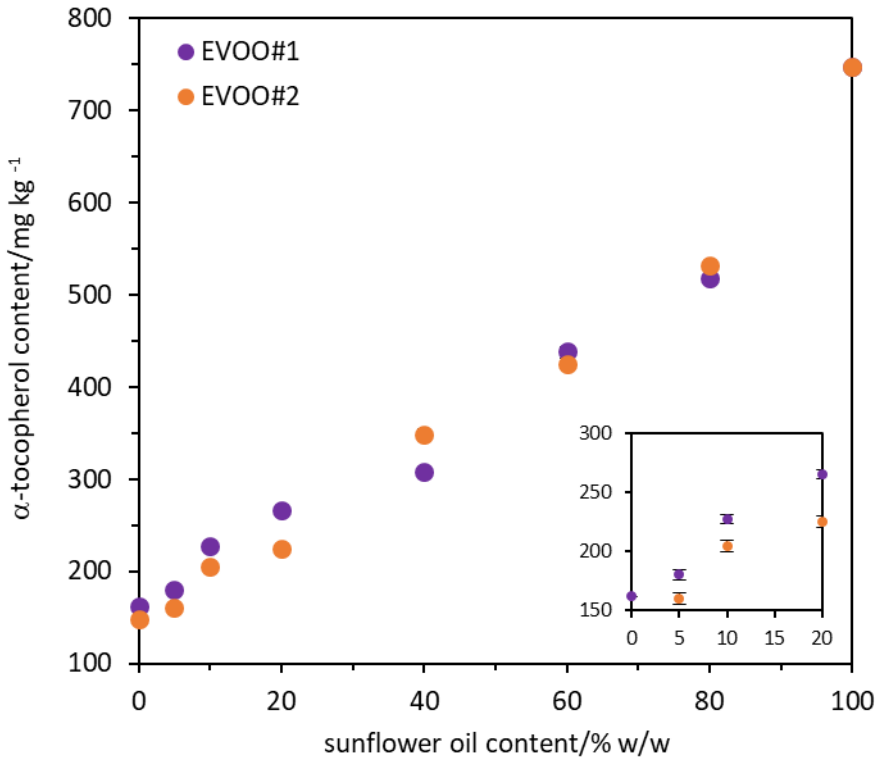


Figure 6.4: The α-tocopherol content of EVOO diluted with sunflower oil where inset is 0-25% w/w mass range with associated error bars (CI value n = 3, p = 0.05).

EVOOs diluted with Canola Oil

The analysis of the tocopherol content in canola oils *via* HPLC-DAD (see Figure 6.5) found both α- and γ-tocopherols to be present. Therefore, both of these tocopherols were evaluated with a view to detecting canola oil diluent in the tested EVOO samples. As presented in Figure 6.6 and Table 6.7, the tocopherol composition of both EVOOs

diluted with canola oil demonstrated an increase in α-tocopherol concentration as the mass of canola diluent oil increased. However, the relatively low change in the α–tocopherol concentration with increasing mass of canola oil diluent resulted in overlapping method errors until the level of canola oil reached *ca.* 40% w/w in both the EVOO#1 and EVOO#2 test samples. This limits the detection of using α-tocopherol for quantification for EVOOs diluted with canola which exceed 40% w/w.

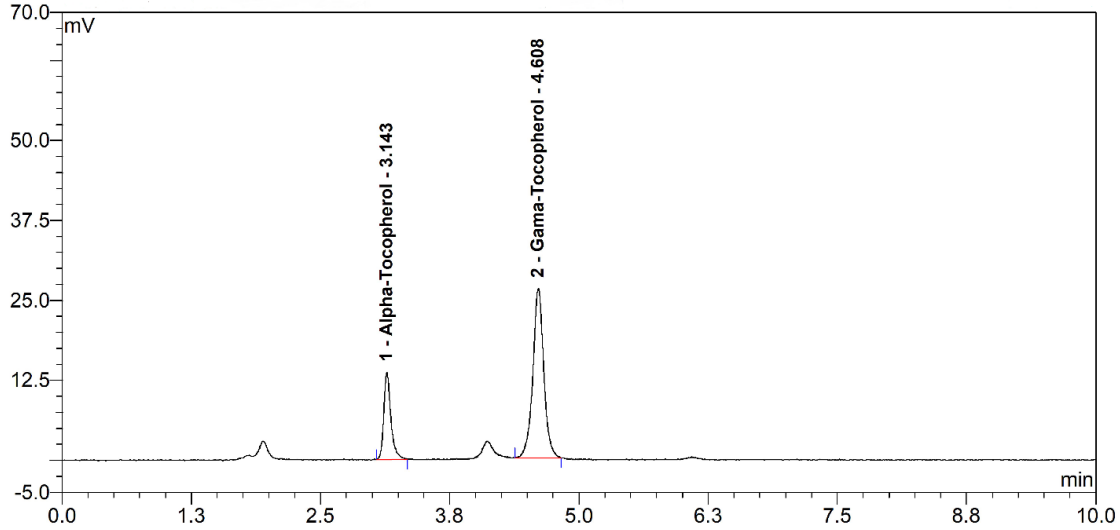


Figure 6.5: Chromatograph of pure canola oil.

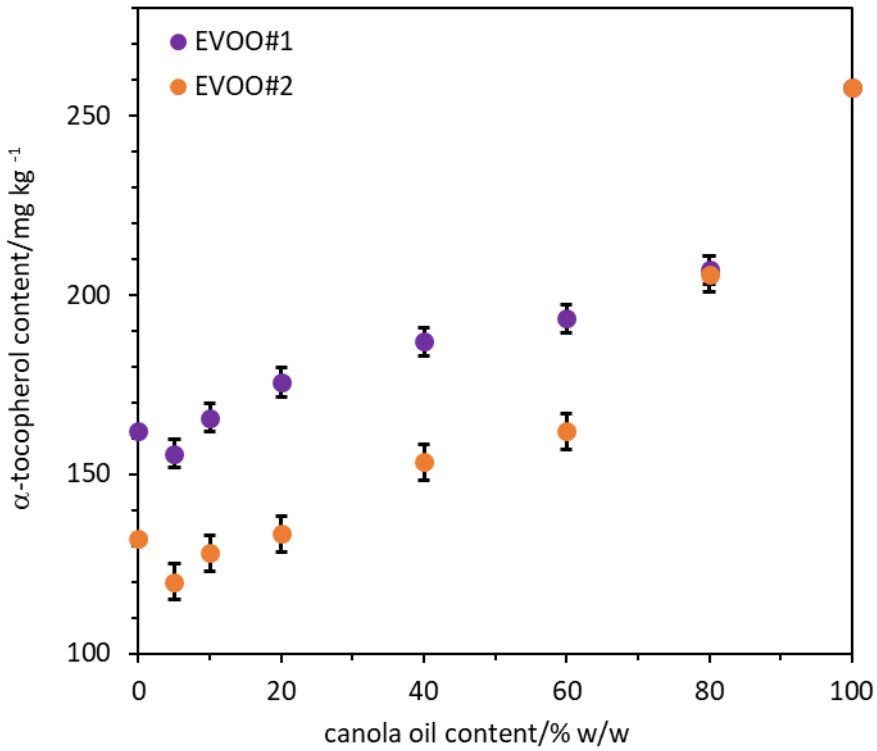


Figure 6.6: α-tocopherol content in EVOO diluted with canola oil associated error bars reflect CI values of EVOO#1 and EVOO#2, 4 and 5 mg kg^{-1} respectively.

Table 6.7: Tocopherol composition of EVOOs diluted with canola oil

Diluent oil % w/w	α-tocopherol content/ mg kg^{-1}		γ-tocopherol content/ mg kg^{-1}	
	EVOO #1	EVOO #2	EVOO #1	EVOO #2
0	162	132	6	0
5	156	120	25	25
10	166	128	42	40
20	176	133	74	81
40	187	153	157	173
60	193	162	239	252
80	207	206	318	310
100	258	258	446	446

Note: *n* = 3, EVOO#1 standard deviation and CI was 3.5 and 3.9, respectively. EVOO#2 standard deviation and CI was 4.9 and 5.5, respectively.

Since canola oil also contains γ-tocopherol, this may provide an improved indicator of potential adulteration. Furthermore, since the pure EVOO samples present low concentrations of γ-tocopherol, this reduces the method uncertainty due to endogenous γ-tocopherol content. The γ-tocopherol analysis therefore offers a more sensitive approach to detect canola diluent in EVOO as shown in Figure 6.7 and Table 6.8. Since the EVOO test samples contain no or limited concentrations of γ-tocopherol, the evaluation of this component enables the detection of canola oil diluent at a level of 5% w/w and is clearly quantifiable across the entire calibration range. The relatively low level of γ-tocopherol found in EVOO#1 had no impact on the sensitivity and selectivity to EVOOs diluted with canola oil as the γ-tocopherol response of both diluted EVOOs remained outside the method error for the entire calibration curve. The improved response is reflected in the trendline parameters for γ-tocopherol determination including the trendline gradient and R^2 values compared to those of α-tocopherol in EVOOs diluted with canola oil (see Table 6.8). Other studies of canola oil have reported similar α- and γ-tocopherol contents which suggests the broader applicability of using these as indicators for canola oil dilution of EVOOs (Flakelar et al., 2015; Grilo et al., 2014).

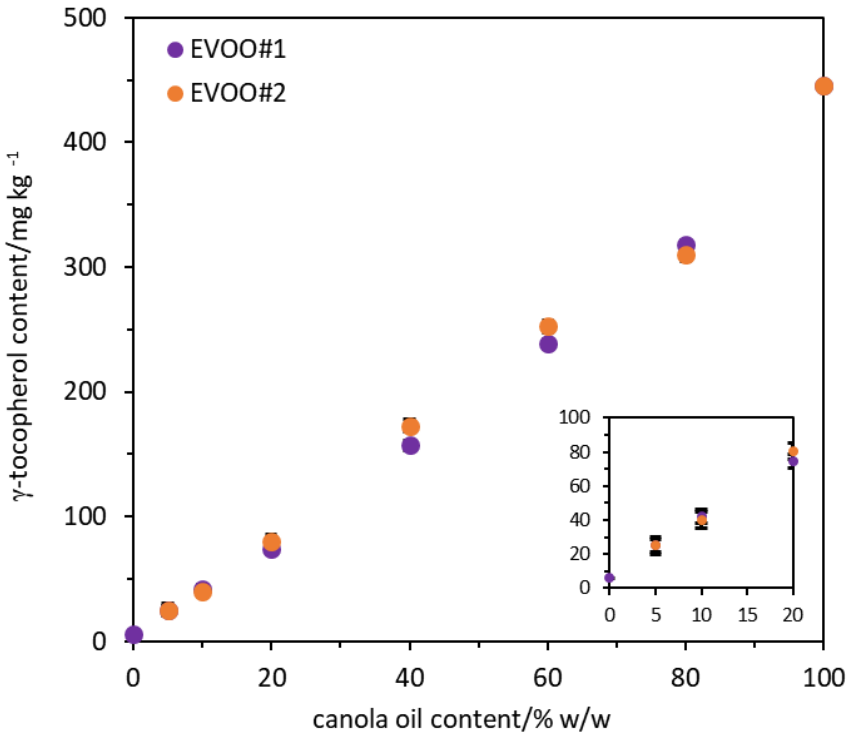


Figure 6.7: Calibration of γ-tocopherol levels in EVOO diluted with canola oil where inset is 0-25% w/w mass range with associated error bars (CI value *n* = 3, p = 0.05).

Table 6.8: Regression analysis of EVOO diluted with canola oil

Olive Oil	Tocopherol	Gradient (method sensitivity) mg kg^{-1} per g $100g^{-1}$	Coefficient of determination (R^2)
EVOO#1	α	0.84	0.89
EVOO#2	α	0.90	0.90
EVOO#1	γ	4.1	0.99
EVOO#2	γ	4.2	0.99

EVOOs Diluted with Rice Bran Oil

As discussed in Section 6.2.3, the measurement of the total tocopherol content of rice bran oil was not able to adequately discriminate amongst mixtures of the selected EVOOs. The rice bran oil is comprised of a near equal mix of α- and γ-tocopherol of 65 and 57 mg kg^{-1} respectively as presented qualitatively in the chromatogram shown in Figure 6.8 and Table 6.9. Thus, as rice bran oil is added to EVOO, it could be presumed that a decrease of α-tocopherol and an increase of γ-tocopherol would occur. The plot presenting the relationship between the α-tocopherol composition and rice bran diluent oil content is shown in Figure 6.9 and it is evident that the α-tocopherol concentration of EVOO#1 decreases as the concentration of rice oil bran increases. However, the first datum to exceed the CI range of the pure EVOO#1 is the 20% w/w mixture, which suggests a LOD of 20% w/w for rice bran oil diluent using α-tocopherol content. The relationship between α-tocopherol and rice bran oil content in EVOO#2 presents a similar response, however the required mass of rice bran needed to exceed the method error is 40% w/w and is seemingly due to the smaller difference between the α-tocopherol content of EVOO#2 and that of the rice bran diluent oil. This difference highlights a recurring concern with the use of α-tocopherol for identification of sunflower, canola and rice bran oils in which the natural variability of α-tocopherol contributes to an increased method uncertainty. The difficulty in establishing a baseline concentration lies with this natural variability and is reflected in the range of LOD presented with α-tocopherol and those reported in the literature (Chen et al., 2011; Franco et al., 2014b; Grilo et al., 2014). This large source of error can potentially be diminished if another tocopherol analogue, such as γ-tocopherol, is used to identify diluent oils.

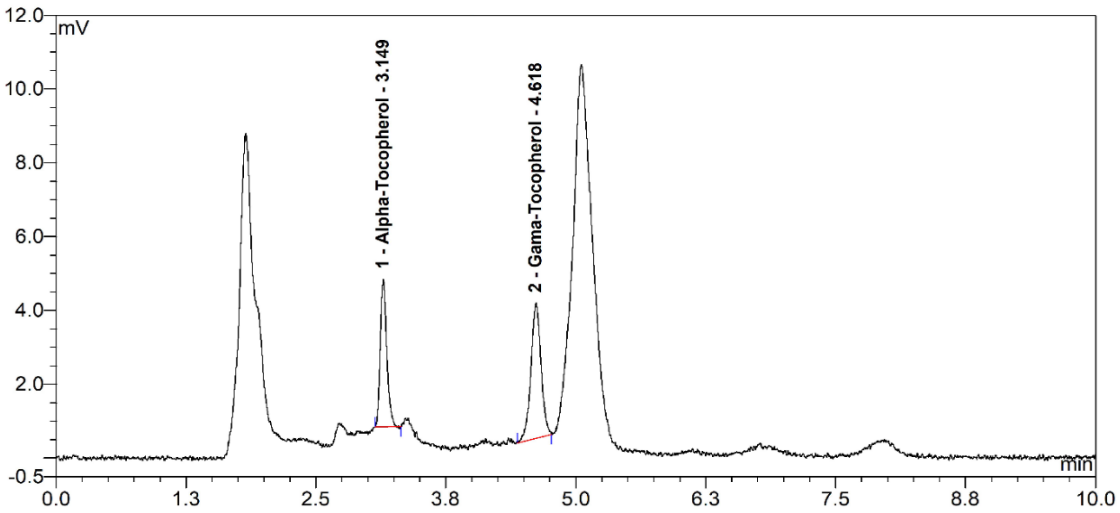


Figure 6.8: Chromatogram of pure rice bran oil.

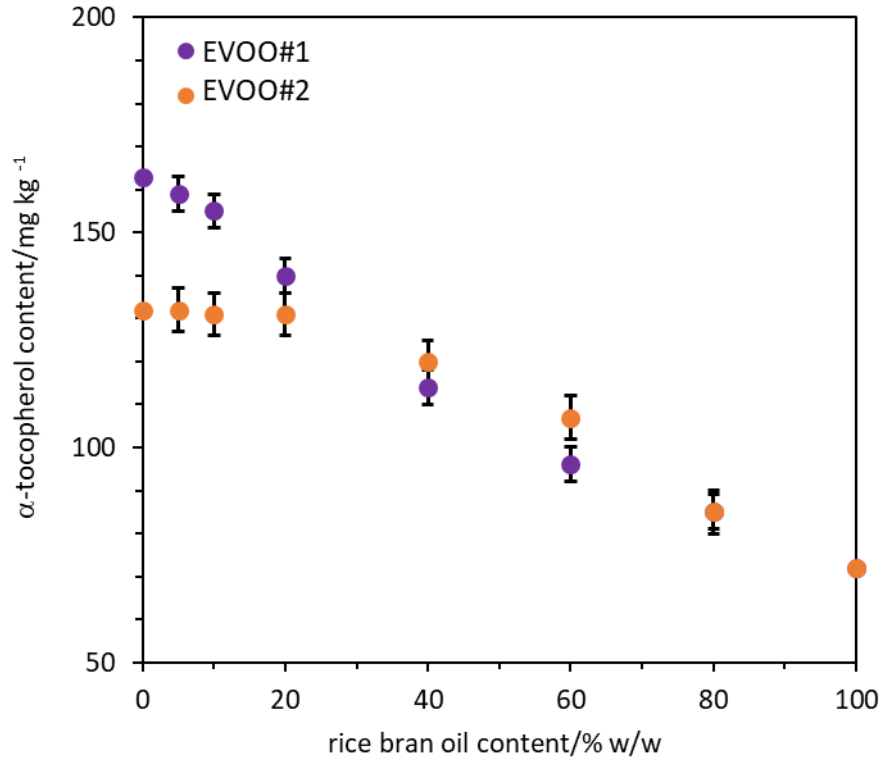


Figure 6.9: α-tocopherol content of EVOOs diluted with rice bran oil.

Table 6.9: Tocopherol composition of EVOOs diluted with rice bran oil

Diluent oil % w/w	α-tocopherol content/ mg kg^{-1}		γ-tocopherol content/ mg kg^{-1}	
	EVOO #1	EVOO #2	EVOO #1	EVOO #2
0	162	132	6	0
5	159	114	6	7
10	152	126	11	13
20	140	132	16	20
40	114	120	26	29
60	96	107	34	42
80	85	86	44	48
100	65	65	57	57

Note: n = 3, EVOO#1 standard deviation and CI was 3.5 and 3.9, respectively. EVOO#2 standard deviation and CI was 4.9 and 5.5, respectively.

Rice bran oil also contains γ-tocopherol, thus a calibration curve of this component with increasing levels of rice bran oil were constructed and presented in Figure 6.10. These results demonstrate an improved calibration curve compared to those of α-tocopherol and the total tocopherol content due to the absence of γ-tocopherol in EVOO#1. Moreover, similar R^2 values and method sensitivities were determined for both EVOO samples (see Table 6.10). Since low concentrations of γ-tocopherol were present in EVOO#1, this potentially increases the LOD for the determination of rice bran oil diluent in this and similar EVOOs using this component as the indicator. Given the natural levels of γ-tocopherol in EVOO#1 (i.e. 6 mg kg^{-1}) and the corresponding CI method error (i.e. 4 mg kg^{-1}), the minimum concentration of γ-tocopherol to indicate the EVOO has been diluted with rice bran oil is 10 mg kg^{-1}, which corresponds to a mixture of *ca.* 10 % w/w.

Table 6.10: Regression analysis of EVOO diluted with rice bran oil

Olive Oil	Tocopherol	Gradient (method sensitivity) mg kg^{-1} per g 100 g^{-1}	Coefficient of determination (R^2)
EVOO#1	α	0.61	0.95
EVOO#2	α	0.95	0.98
EVOO#1	γ	0.55	0.97
EVOO#2	γ	0.62	0.99

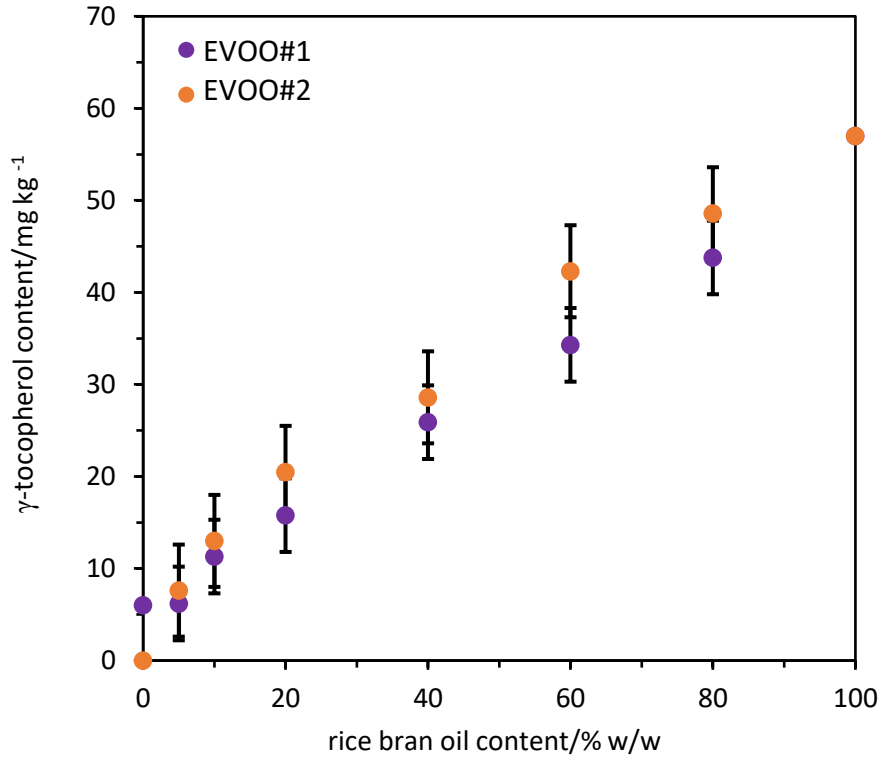


Figure 6.10: γ-tocopherol content of EVOO and rice bran oil mixtures.

6.2.6 Optimized Procedure for Diluent Oil Detection

The analysis of both total tocopherols and individual tocopherols and the subsequent generation of calibration curves presents a method to discriminate between pure EVOOs and EVOOs diluted with sunflower, canola and rice bran oils. Based on the evaluation of these methods, a decision tree was constructed as shown in Figure 6.11. The first key parameter to be determined is the γ-tocopherol concentration for the suspect mixture. This parameter is potentially able to distinguish between a pure EVOO and

EVOO diluted with canola or rice bran oil. Based on the two EVOOs used in this study, a typical LOD would be greater than 10 mg kg^{-1} γ-tocopherol. As the γ-tocopherol concentration of EVOO diluted with 5% w/w canola oil is similar to that of EVOO diluted with 40% w/w rice bran, both tocopherols should be used for identification. Any EVOOs which exceed this γ-tocopherol limit should then have their α-tocopherol content quantified. If both values of α-tocopherol and γ-tocopherol are >140 mg kg^{-1} and >25 mg kg^{-1} respectively, it could be suggested the EVOO is diluted with canola oil, otherwise the diluent is rice bran oil. For an EVOO which contains <10 mg kg^{-1} of γ -tocopherol, the α-tocopherol of the oil should be quantified, which if >178 mg kg^{-1} suggests sunflower diluent oil is present. In the case where the concentration of α- tocopherol was >178 mg kg^{-1} and no γ-tocopherol is detected, it may be concluded that no diluent oil is present. This proposed framework identifies a diluted EVOO at 10% w/w for rice bran, sunflower oil and 5% w/w for canola oil diluent.

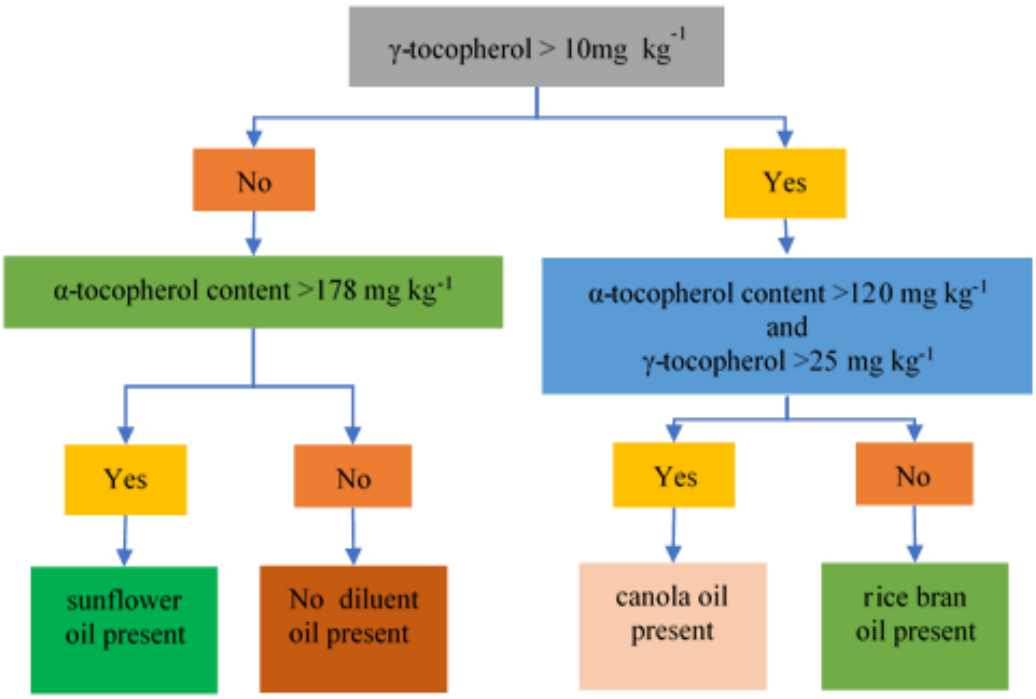


Figure 6.11: Framework for detection of diluent oils in EVOO using tocopherol content.

6.3 Summary

This chapter has presented an investigation of the presence of tocopherols in EVOO to detect potential diluent oils in adulterated EVOO samples with a facile heptane extraction and instrumental detection *via* NP-HPLC-DAD. The total tocopherol content can be used to identify EVOOs diluted with canola and sunflower oil at 10% w/w. Conversely, total tocopherols are not suitable for rice bran diluent oil due to similarities between the pure EVOOs and pure rice bran oil. However, the composition of individual tocopherols provides an improved means to discriminate between the pure EVOOs and those which are diluted with canola, sunflower or rice bran oil. as these diluent oils exhibit different concentrations of α- and γ-tocopherol. EVOOs which are diluted with canola or rice bran oil can be identified *via* an increase in γ-tocopherol, with a limit of detection of 5% w/w and 10% w/w, respectively. The greater α–tocopherol concentration in sunflower oil increases the α–tocopherol content of a diluted EVOO with an LOD of 5% w/w. Finally, as the tocopherol composition differs between the diluent oils, it is possible to systemically identify which diluent oil is present *via* the decision tree proposed as one of the outcomes of this research.

Chapter 7 Proposed Framework for EVOO Authentication

7.1 Assessment of Evaluated Methods

This research has highlighted the use of various analytical techniques to evaluate extra virgin olive oils (EVOOs) components and parameters which may supplement current authentication methods. In general, these methods have demonstrated the ability to distinguish between pure EVOOs and EVOOs diluted with canola, sunflower or rice bran oils at acceptable *limit of detections* (LODs) as shown in Table 7.1. When these LODs are compared to the estimated fraudulent profit presented in Chapter 1 (see Figure 1.1), the measurement of total phenolic content (TPC), fluorescence intensity and tocopherol content can all be used to detect a diluent oil at *ca* 10% w/w, a concentration that is consistent with a conceivable motivation to obtain a fraudulent profit.

Table 7.1: Limit of detection values for investigated methods

Method	Detection	LOD-SFO % w/w	LOD-CAN % w/w	LOD-RBO % w/w
FC assay	Colorimetric	5	5	5
TPC	HPLC-DAD	5	5	5
DPPH assay	Colorimetric	N.D.	20	10
fluorescence	Qualitative EEM	20	20	20
fluorescence	λ_{ex} = 536 nm λ_{em} = 684 nm	5	N.M.	N.M.
fluorescence	λ_{ex} = 328 nm λ_{em} = 360 nm	5	N.M.	N.M.
UV absorbance	λ_{max} = 328 nm	5	N.M.	N.M.
total tocopherols	HPLC-DAD	10	10	N.D.
α- tocopherol	HPLC-DAD	10	40	40
γ - tocopherol	HPLC-DAD	10	10	5

Note: N.D. = not detected, N.M. = not measured

Other method parameters, such as the selectivity and linearity, are also of importance in assessing the suitability of an authentication method. However, these are secondary to the primary role of any authentication method which is the detection of diluent oils at a low mixture concentration and with small method error. As demonstrated in this research, the method error which arises from replicate analyses is typically marginal. However, the presumptive EVOO sample error which is typically derived from the natural variability of EVOO components may significantly contribute to this sample error.

The limitations of the investigated methods in this research are as follows:

(i) The Folin-Ciocalteu assay was able to detect diluent oils at low concentrations, however this method lacks the selectivity to identify which diluent was present and lacks linearity to quantitate the concentration of the diluent oil in the mixture.

(ii) Measurement of TPC *via* HPLC offers a LOD between 5% and 10% w/w and the concentration of the diluent can be determined (limit of quantification) for mixtures which exceed 10% w/w. However, this method is not suited for the identification of the diluent, due to the lack of selectivity for each diluent. Furthermore, the TPC of EVOOs can vary significantly, which contributes to an expanded sample error.

(iii) The AOC as determined *via* the DPPH assay were less sensitive to diluent oils in those EVOOs that were evaluated and as such this parameter was unsuitable for determining the presence of sunflower oil in particular. The DPPH assay offers limited selectivity but may offer a qualitative indication of adulteration.

(iv) Fluorescence profiles, in particular the use of EEM profiles, offers a non-destructive, rapid and potentially portable method to evaluate EVOOs. A rapid qualitative approach can be used to identify a potentially diluted EVOO at low concentrations. Seemingly, two fluorescent regions offer a qualitative method to identify diluted EVOOs that involves the detection of a decrease of the observed fluorescent intensity at λ_{ex} = 575, λ_{em} = 675 nm or an increase in the emission intensity at λ_{ex} = 350, λ_{em} = 400-500 nm range. A suspected diluted EVOO can be further confirmed and quantified using the highly correlated and sensitive wavelengths as shown in Table 7.1. The measurement of UV absorbance, concurrently with fluorescence or using standalone equipment, can also be used qualitatively and quantitatively. However, the use of UV absorbance measurements alone was typically less sensitive than that observed in combination with fluorescence measurements, which limits its usefulness to a more qualitative approach.

(v) The tocopherol composition of an EVOO using HPLC-DAD provides an excellent advanced instrumental technique. In particular, the individual tocopherol composition can be used as a method to characterize and quantify the diluent oil based on the unique α- and γ- tocopherol profiles of diluted EVOOs. The major advantage of this method compared to others in this research was that certain

individual tocopherols, in particular γ-tocopherol, are present in very low concentrations in EVOOs compared to the diluent oils, which limits the sample method error.

7.2 Workflow of Methods for Evaluating EVOO Authenticity

Based on the effectiveness and limitations of the methods evaluated in this research, a proposed workflow framework was constructed as shown in Figure 7.1. This framework proposes a systemic approach in using the key methods investigated in this research to identify EVOOs diluted with low concentrations of diluent oils (5% w/w). The approach of this framework is to first identify whether an EVOO has been diluted and, in many cases, this may be sufficient for any evaluation. However, the concentration and identity of the diluent oil may be useful information to determine, particularly with regard to collecting evidence for a criminal litigation.

As shown in this research, while the antioxidant capacity (AOC) *via* DPPH assay can be used to identify the diluent oil, it is limited to identifying only canola oil or rice bran oils. Therefore, sunflower oil can be identified as the diluent only if another method is used to distinguish between pure EVOO and EVOO diluted with sunflower oil. Furthermore, since portions of the AOC calibration curves for canola oil and rice bran oil overlap, this suggests that the DPPH assay cannot be used to identify the presence of canola oil specifically and the results will only suggest that either canola or rice bran oil is the diluent. As the DPPH assay offers this limited selectivity it should only be used if the tocopherol composition cannot be determined.

The TPC *via* HPLC-DAD offers an approach to detect diluted EVOOs at low concentrations and can be used to quantitate the diluent oil content in evaluated oils. However, it suffers from poor selectivity and from many of the same limitations that arise with current EVOO authentication methods. Firstly, the extraction of phenolics from EVOOs is more laborious compared to other methods, such as tocopherol analysis. The TPC in EVOOs also exhibits a natural variability which potentially expands the method error. As other investigated methods in this research offers comparable sensitivity, such as the FC assay and fluorescence EEMs, the TPC should only be used if these methods are not available.

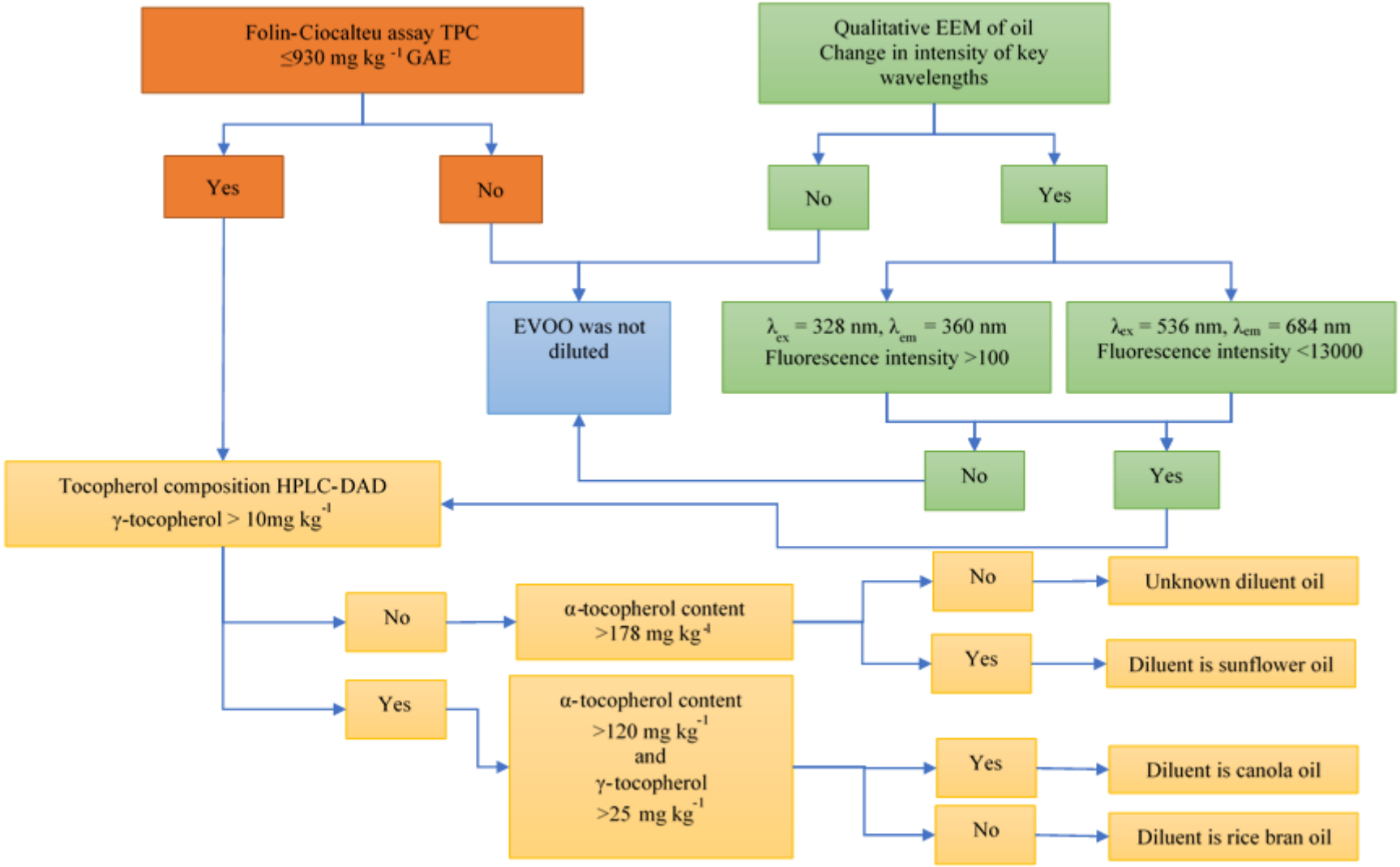


Figure 7.1: Proposed framework for detection of diluent oils in EVOOs.

The first key qualitative parameter to be determined is the TPC measured using the FC assay or alternatively, by directly and rapidly recording the fluorescence EEM spectrum. In the case where the TPC content of an oil >930 mg kg^{-1} measured using the FC assay, the EVOO is potentially unadulterated. Further examination by fluorescence can be performed and if the qualitative analysis of an EEM spectrum of a known EVOO presents a decrease in fluorescence intensity at λ_{ex} = 575, λ_{em} = 675 nm or an increase in the λ_{ex} = 350, λ_{em} = 400-500 nm. This decrease in the latter fluorescent region may indicate that dilution has occurred with an oil that does not contain chlorophyll. Further interrogation of the fluorescence at wavelengths λ_{ex} = 328, λ_{em} = 360 nm and λ_{ex} = 536, λ_{em} = 684 nm where the fluorescence intensities are >100 or <13000 respectively, may suggest the EVOO is diluted with sunflower oil. Clearly these intensities are relative to known EVOOs that should be suitably calibrated. If no changes are observed, then no diluent oil is deemed to be present, and the sample may be an unadulterated pure EVOO. If the aim of the investigation is only to determine if an EVOO is diluted, either the FC assay or fluorescence EEM spectrum could be used as a qualitative confirmation of dilution. However, neither of these methods provides a means to selectively identify which diluent is present without further development.

If either the FC assay or EEM spectrum indicates an EVOO is diluted and further investigation is required, the tocopherol profile (type and composition) should be determined to both identify the diluent oil that is present and quantify its concentration. The identity of the diluent oil can be determined based on its unique tocopherol profile whereby an oil with elevated concentrations (>10 mg kg^{-1}) of γ-tocopherol indicates either canola or rice bran oil. Furthermore, the identity of the diluent oil can be determined from the concentration of both α- and γ-tocopherol in the mixture. An elevated concentration of α- and γ-tocopherol, >25 and >120 mg kg^{-1} respectively, is indictive of a canola oil diluent in the mixture, otherwise the diluent oil is rice bran oil. Both rice bran and canola oils should be quantified based on their respective γ-tocopherol calibration curves as shown in Section 6.2.5. Conversely, an oil with elevated concentrations of α-tocopherol (>178 mg kg^{1}) is suggestive of sunflower oil diluent, and since the increase in α-tocopherol is proportional to the concentration of sunflower oil, quantification can be achieved. It should be noted that the difference in α-tocopherol between EVOO#1 and EVOO#2 tested in this research gives rise to an expanded sample error, presenting an

overestimation of the sunflower oil content in EVOO#1 by *ca* 7% w/w. However, as an EVOO is identified as diluted by either the FC assay or its EEM spectrum, this will not impact the LOD of this assessment framework, which can clearly identify test mixtures of 5% w/w in all cases.

7.3 Summary

The establishment of alternative methods for EVOO authentication is of increasing global importance due to the current limitations of current official methods. This framework provides a systemic approach to not only rapidly detect EVOOs diluted with canola, sunflower or rice bran oil but in many cases is able to determine both the concentration and the identity of a diluent oil and is able to identify test mixtures that were adulterated at 5% w/w in all cases. In some cases, the positive presence of a diluent oil determined by EEM spectroscopy and/or the FC assay may be a sufficient outcome. If the investigation requires that the diluent oil be identified and its concentration quantified, the individual tocopherol compositions can be evaluated since the EVOOs and diluent oils are comprised of unique tocopherol profiles.

Chapter 8 Conclusions and Recommendations

8.1 Conclusions

The development of alternative techniques for EVOO authentication is important to address the growing global challenges of EVOO adulteration in which the fraudulent practice of adulteration can result in potential monetary gains to criminals. The need for analytical methods that are more rapid and reliable than the current official methods is apparent and in response to this need, the research presented in this thesis aimed to critically assess a variety of techniques to supplement the current official methods used by international bodies, specifically the International Olive Council (IOC). These IOC methods, whilst seemingly effective in certain cases to detect diluent oils in EVOOs, are limited due to the requirement to use several methods to encompass all potential diluents and in some cases, these have been demonstrated to falsely identify a pure EVOO as having been adulterated. In particular, due to regional variations, some Australian EVOOs may fail authentication testing based on these IOC protocols.

In this investigation, six analytical techniques were explored to identify and quantify a range of EVOO chemical constituents and parameters for their ability to detect Australian EVOOs that were diluted with canola, sunflower oil or rice bran oil. Firstly, the total phenolic content (TPC) of the pure oils and mixtures was determined using both the Folin-Ciocalteu (FC) assay and HPLC-DAD analysis. The FC assay presented similar TPC for all EVOO diluent mixtures which suggests it lacks the selectivity to identify which diluent oil is present in a mixture. Furthermore, the FC assay also exhibited poor linearity between TPC and the concentration of diluent oil thus diminishing the applicability of the assay to quantify a diluted EVOO. However, its rapid nature and high throughput suggests the FC assay could be used as an initial screening technique to indicate that an EVOOs may be diluted with another oil at relatively low concentrations, in which a clear decrease in the FC response occurs at 5% w/w for all diluent oils that were tested. The TPC was also investigated using HPLC-DAD, however, this method also lacks the selectivity to distinguish between the different diluent oils. Similar to the FC assay, the TPC measured using HPLC-DAD was able to identify a diluted EVOOs at low concentrations of diluent with a limit of detection (LOD) of 5% w/w, and the TPC was found to be proportional to the concentration of the diluent oil. However, since no unique phenolic markers are prevalent, the TPC *via* HPLC-DAD method cannot be used

to identify which diluent is present in a mixture. The diluent oil is potentially quantifiable by measuring the TPC *via* HPLC-DAD, with a *limit of quantification* (LOQ) of 10% w/w of diluent. A further limitation of this method relates to the natural variability of phenolics observed in both test EVOOs, which were also evident from other reports in the literature, and subsequently expands the method error.

The measurement of antioxidant capacity (AOC) using the DPPH assay was investigated and found to be effective in identifying diluted EVOOs only in certain cases. In the case of EVOO mixtures with sunflower oil, this method lacks the sensitivity to identify the diluent in the EVOO, as the AOC of pure EVOO and sunflower oil were similar. Conversely, the AOC of EVOO diluted with rice bran oil or canola oil exhibits linearity with respect to the diluent oil concentration and the diluent concentration was found to be adequately quantified at concentrations above the respective LOD values which are 10% and 20% w/w, respectively. The identity of a diluent oil can potentially be determined based on the AOC response since each diluent oil offers unique AOC. It can be suggested that a measured AOC value that is >55 mg 100 g^{-1} may indicate that the EVOO is diluted with rice bran oil. Lower AOC values may indicate the presence of either canola or rice bran oil. If there is little or no difference in the measured AOC in comparison with a known EVOO, this may indicate that no other oil is present. However, the absence of sunflower oil could be further validated using with one of the other methods that are presented in this research, such as the FC assay or fluorescence.

The UV absorbance as well as fluorescence excitation and emission spectra of the pure EVOOs and diluent oils were measured between 250-800 nm and all three diluent oils present a strong fluorescent signal in the phenolic and oxidation product regions that were absent in the EVOOs. However, only pure EVOOs present a strong signal over the excitation range 300-750 nm and emission range 600-700 nm emission region which represent chlorophylls, whereas all three diluent oils presented similar spectral responses. Sunflower oil was deemed to be representative of the other oils and further evaluated over the concentration range 5-50% w/w in mixtures with EVOO#1. A data analysis method, based on linear regression of the complete fluorescence EEM and UV absorbance data sets, was able to rapidly correlate the fluorescence intensities and UV absorbance data with the composition of the diluted EVOO. Evaluation of the resulting gradients and regression coefficients are able to identify and quantify dilution of the EVOO with sunflower oil at levels of 5% w/w with either λ_{ex} = 536, λ_{em} = 684 nm or λ_{ex} = 328 nm,

λ_{em} = 360 nm. Concurrent analysis UV absorbance (λ_{max} = 328 nm) also offers quantitation of diluents which exceed 5% w/w, however as pure EVOOs strongly absorbs UV in this region, this approach may be more appropriate as a semi-quantitative method. Although this method shows promise for further development as a quantitative technique, it offers an immediate benefit as a rapid and direct qualitative measure of potential EVOO adulteration.

The total tocopherol content of a diluted EVOO measured *via* HPLC-DAD offers a means to detect canola and sunflower oils at concentrations of 10% w/w, however, this approach lacks the specificity to identity which of these diluent oils is present. Since the EVOOs and rice bran oil appear to exhibit similar concentrations of total tocopherols, this method is unable to identify EVOOs containing this diluent oil. A further investigation of the individual tocopherol composition of the tested EVOOs found that these only contain α-tocopherol and low concentrations of γ-tocopherol. Moreover, a unique profile of tocopherols was observed for each of the investigated diluent oils. An increased concentration of α-tocopherol in EVOOs indicates that the EVOO was diluted with sunflower oil in mixtures at concentrations >10% w/w. Although pure canola oil also contains α-tocopherol, the levels are similar to that found in the pure EVOOs and therefore, the LOD is 40% w/w. When compared to α-tocopherol, the γ-tocopherol content of the EVOOs diluted with canola and rice bran oil provides improved LOD with values of 5% and 10% w/w, respectively. Overall, the unique tocopherol profiles of these diluent oils provided a means to systematically identify and quantify the diluent oil in the EVOO mixtures tested.

The investigation of a range of EVOO and diluent oil constituents and parameters has demonstrated the potential of evaluating TPC, AOC, UV absorbance, fluorescence and tocopherols to detect the presence of diluent oils in mixtures. In some cases, such as the DPPH AOC assay and certain tocopherols, the LOD was found to be less suitable compared to other methods in this study. These methods could be used collectively as suite of methods, if the analyst wishes to identify and quantitate an EVOO which has been adulterated with canola, sunflower or rice bran oil. Furthermore, the methods in the research can supplement the current official methods as these methods can rapidly identify diluent oils without the laborious extraction and extended workflow typically observed with current EVOO authentication techniques. This research has proposed a novel framework which uses the TPC, fluorescence spectroscopy, and the composition of

tocopherols to systemically identify the dilution of EVOOs with low concentrations (i.e., 5% w/w) of canola, sunflower or rice bran oil.

8.2 Recommendations for Future Work

This research has evaluated the suitability of six methods for the authentication of EVOOs diluted with canola, sunflower or rice bran oil including: FC assay; TPC *via* HPLC-DAD; AOC *via* DPPH assay; concurrent fluorescence excitation/emission and UV absorbance; and tocopherols *via* HPLC-DAD. These techniques, whilst seemingly effective in many cases, do suffer several limitations which could be addressed in future work.

The spectroscopic techniques investigated in this research could be further evaluated by measuring other diluent oils and EVOOs. Since these techniques offer a rapid, and direct evaluation of EVOOs, these analyses could also be routinely performed and developed into a comprehensive database of EVOO spectra. In particular, the measurement of fluorescence EEMs would offer an initial point of reference for identifying potential adulteration which could initiate further investigation in accordance with the proposed novel framework.

Future work to address limitations in all detection methods explored in this work could also include a substantive survey of domestic and international EVOOs for their TPC, AOC and tocopherol content to establish a baseline for each parameter and with appropriate statistical approaches and apply this to all future determinations of EVOOs. This may address the current limitations arising from the variance in the tested parameters due to region, harvest year, climate, and other changes to EVOO composition after the survey is complete. This survey of EVOOs would also confirm the robustness of this framework as a wider range of commercially available EVOOs would be investigated. Alternatively, a database of the composition and quality parameters of pure EVOOs procured directly from the olive grower/oil producer grove could be constructed and routinely updated. This database of EVOOs, grouped by their region of growth, cultivar and harvest year, could also be expanded to include data derived from other traditional EVOO authentication techniques such as TAGs, FAMEs and phytosterol content. Along with the data obtained from the suite of methods explored in the current research, the combined database would constitute a very powerful tool that could be used to ensure greater confidence in the authenticity of olive oil that is obtained from anywhere on the globe.

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Appendix A: Chromatograms

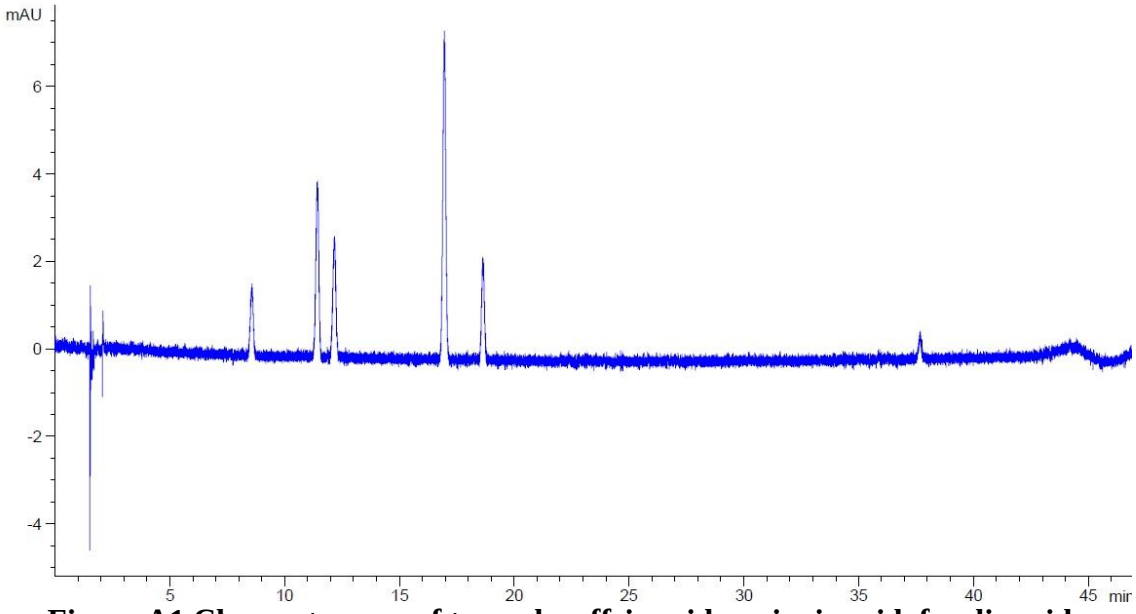

Figure A1 Chromatogram of tyrosol, caffeic acid, syringic acid, ferulic acid, coumaric acid and apigenin at 2.5 mg L^{-1}

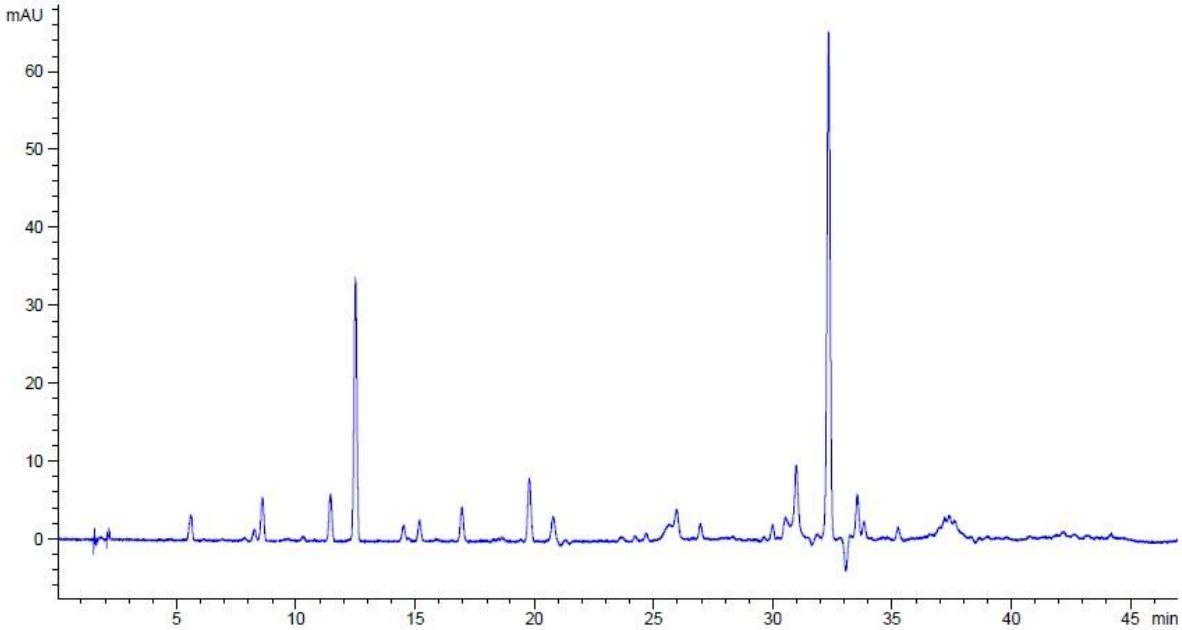

Figure A2 pure EVOO#1 chromatogram

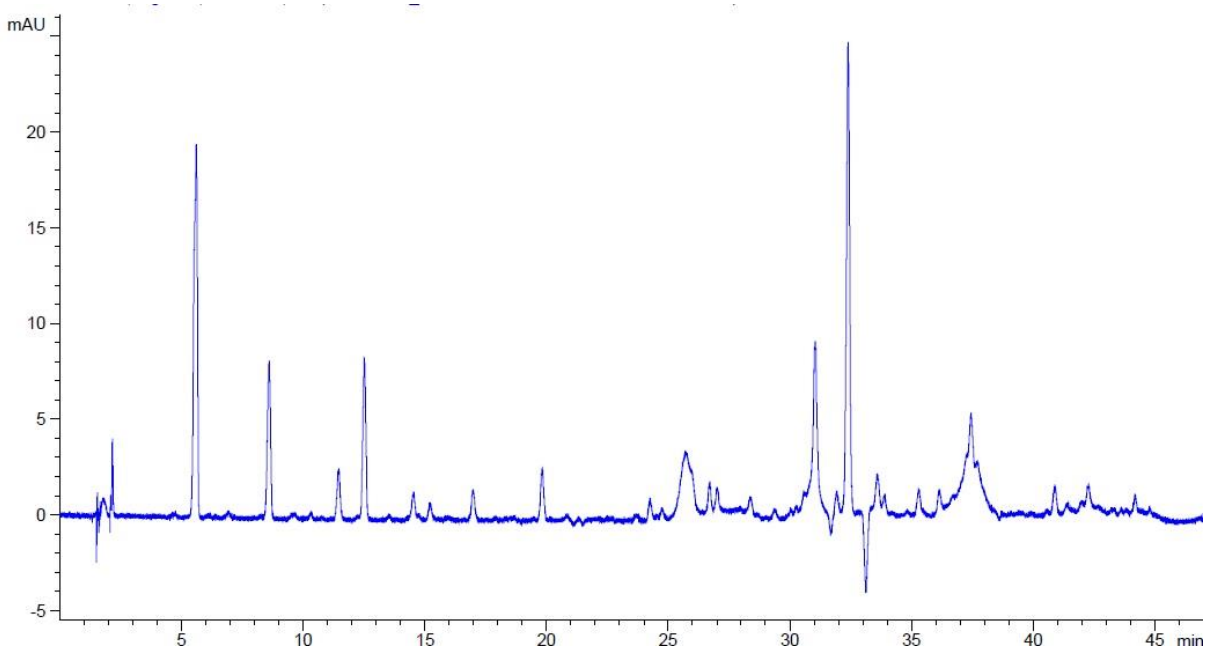

Figure A3 pure EVOO#2 Chromatogram

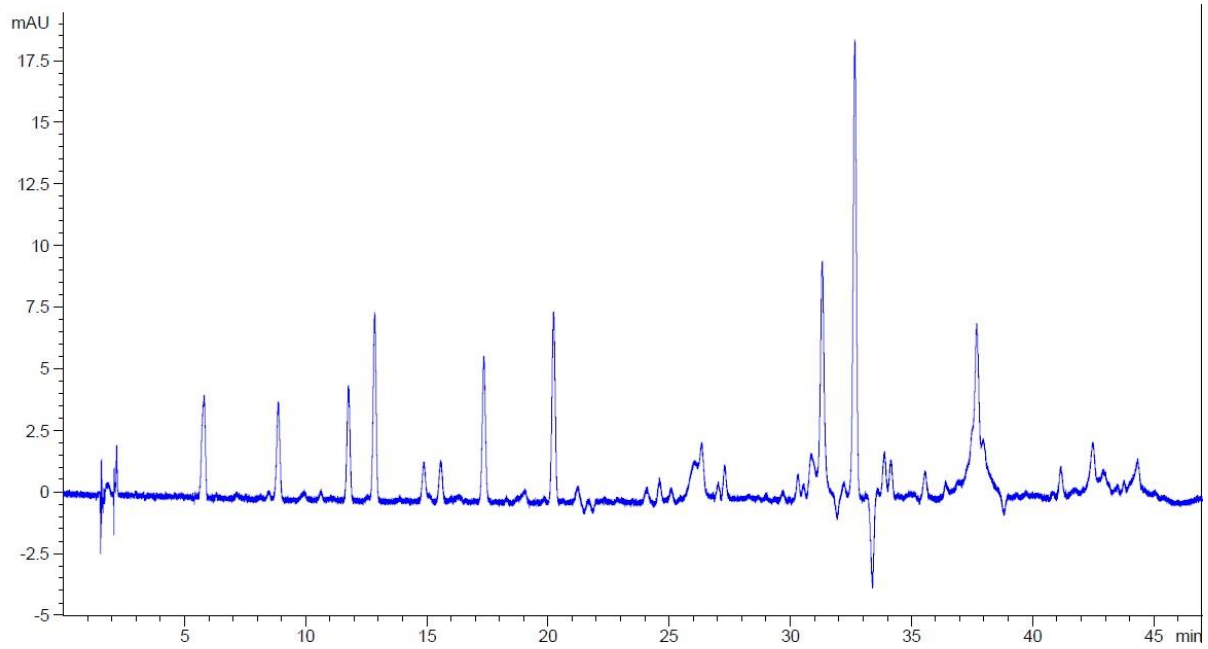

Figure A4 EVOO#1 diluted with 10% w/w rice bran oil

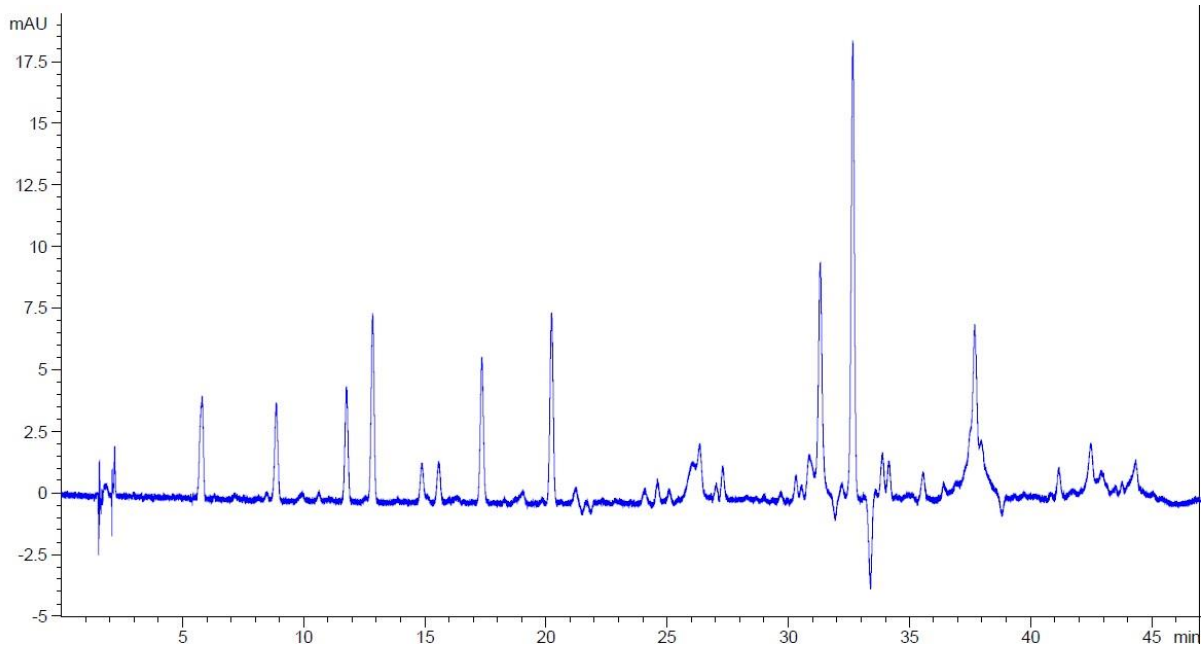

Figure A5 EVOO#2 diluted with 10% w/w rice bran oil

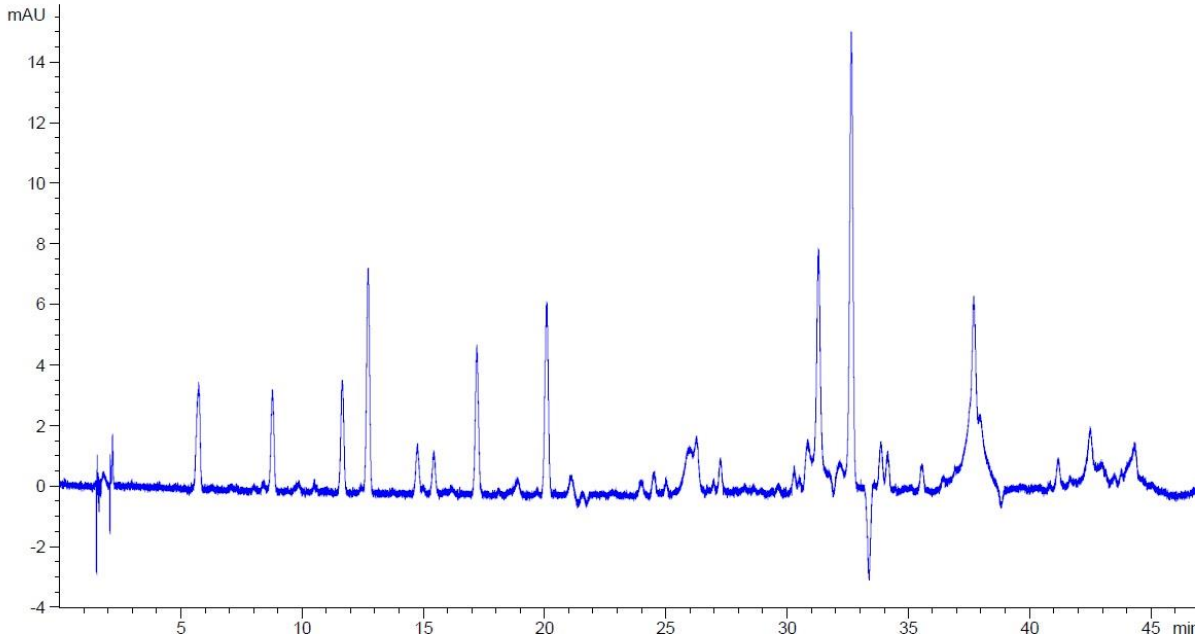

Figure A6 EVOO#1 diluted with 20% w/w rice bran oil

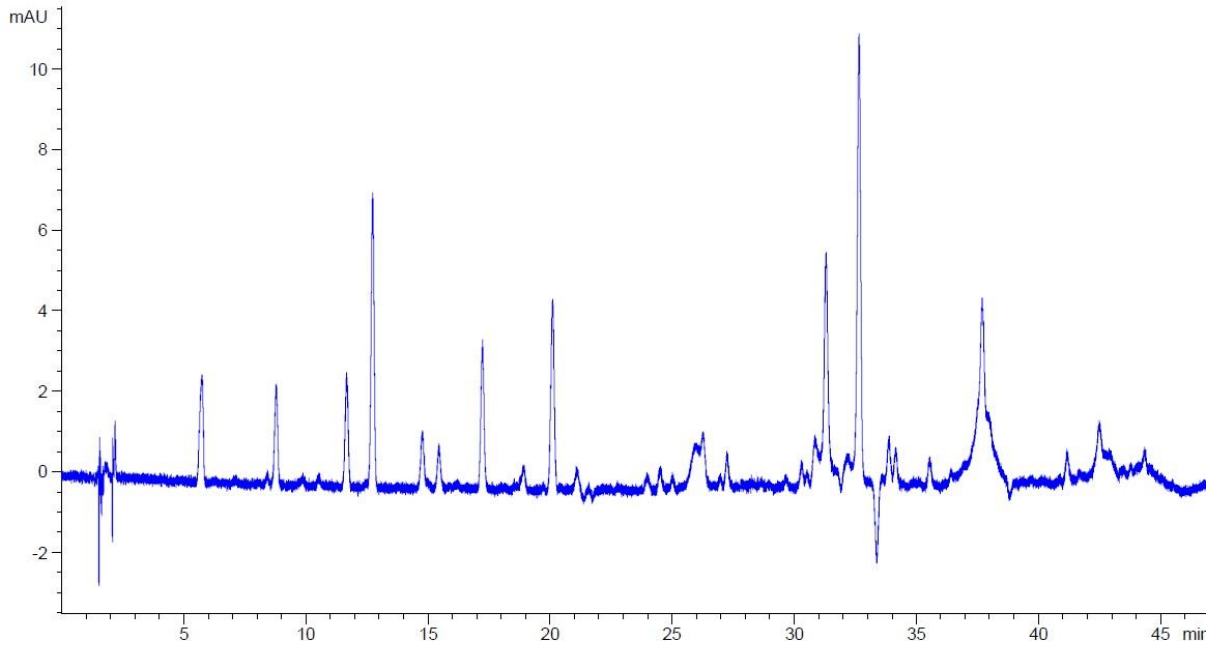

Figure A7 EVOO#1 diluted with 40% w/w rice bran oil

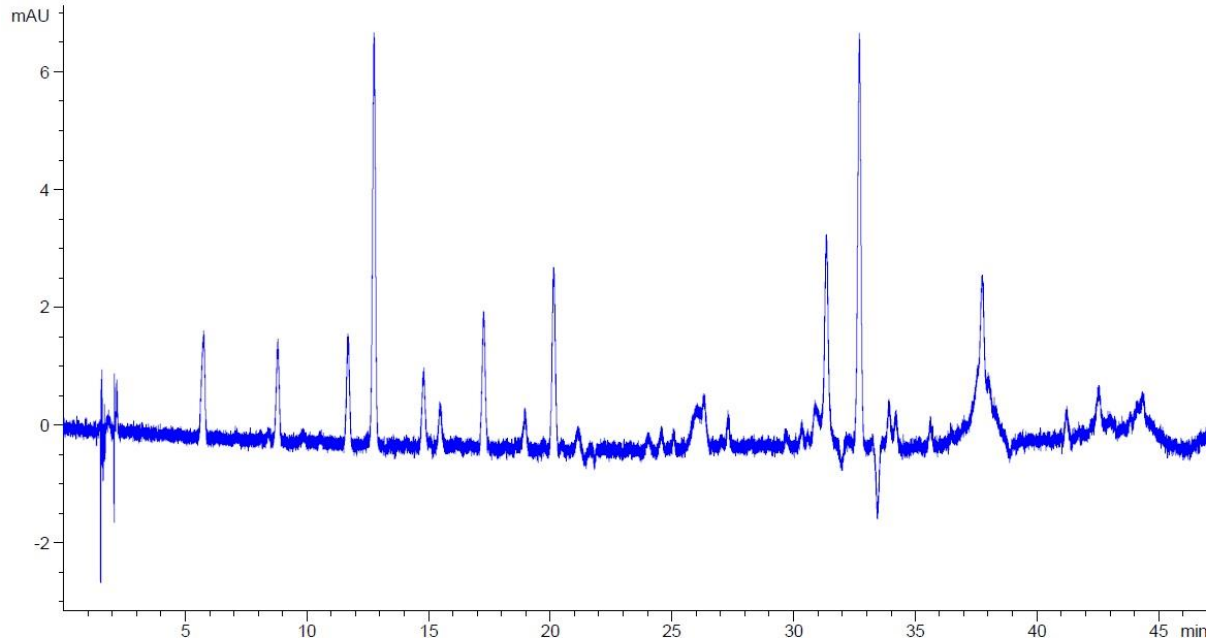

Figure A8 EVOO#1 diluted with 60% w/w rice bran oil

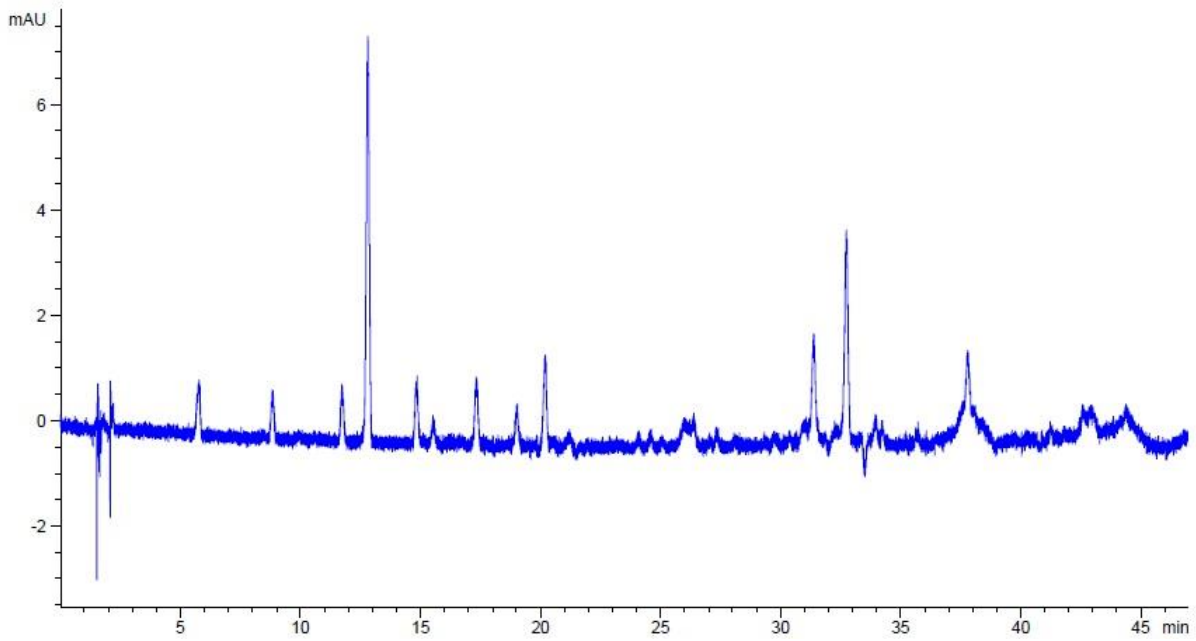

Figure A9 EVOO#1 diluted with 80% w/w rice bran oil

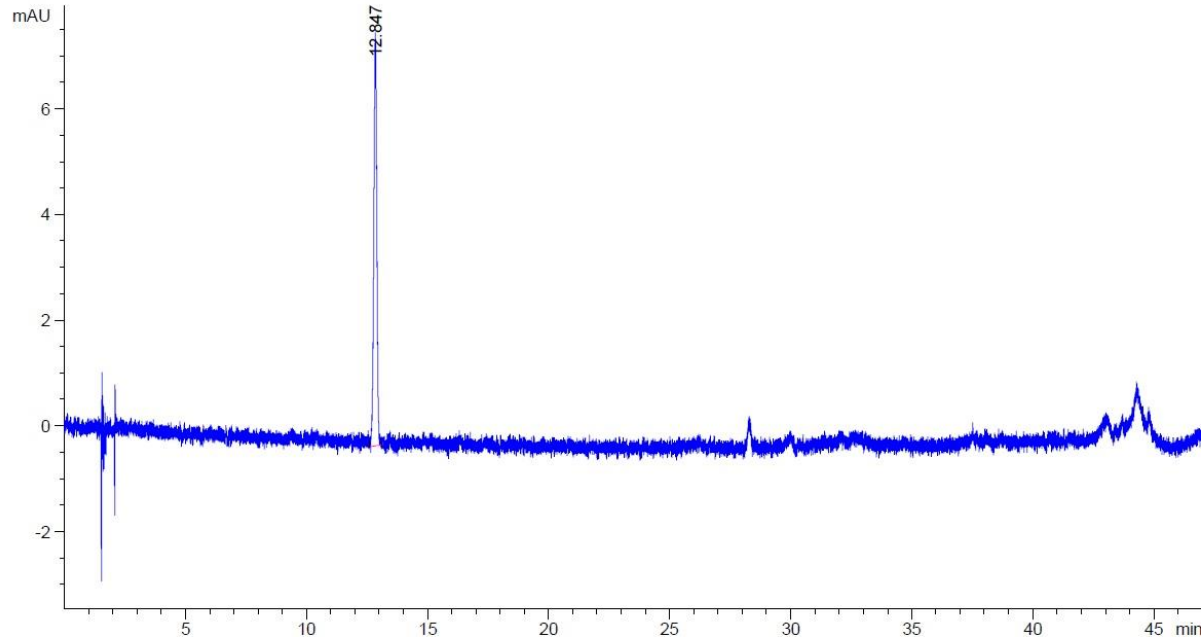

Figure A10 pure rice bran oil

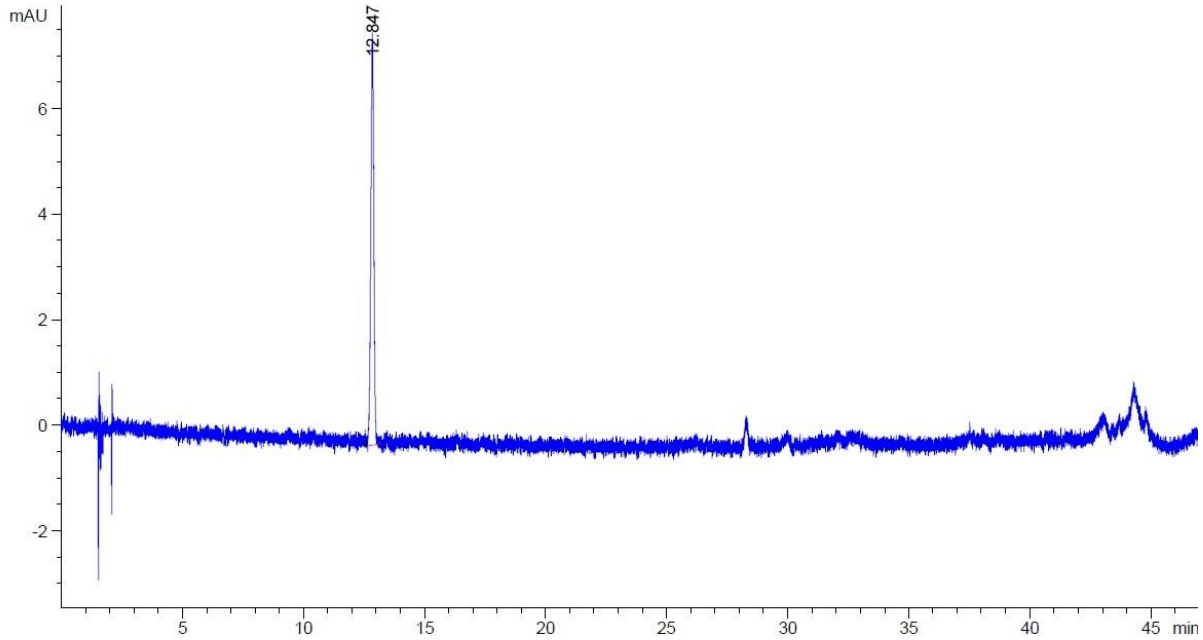

Figure A11 pure sunflower oil

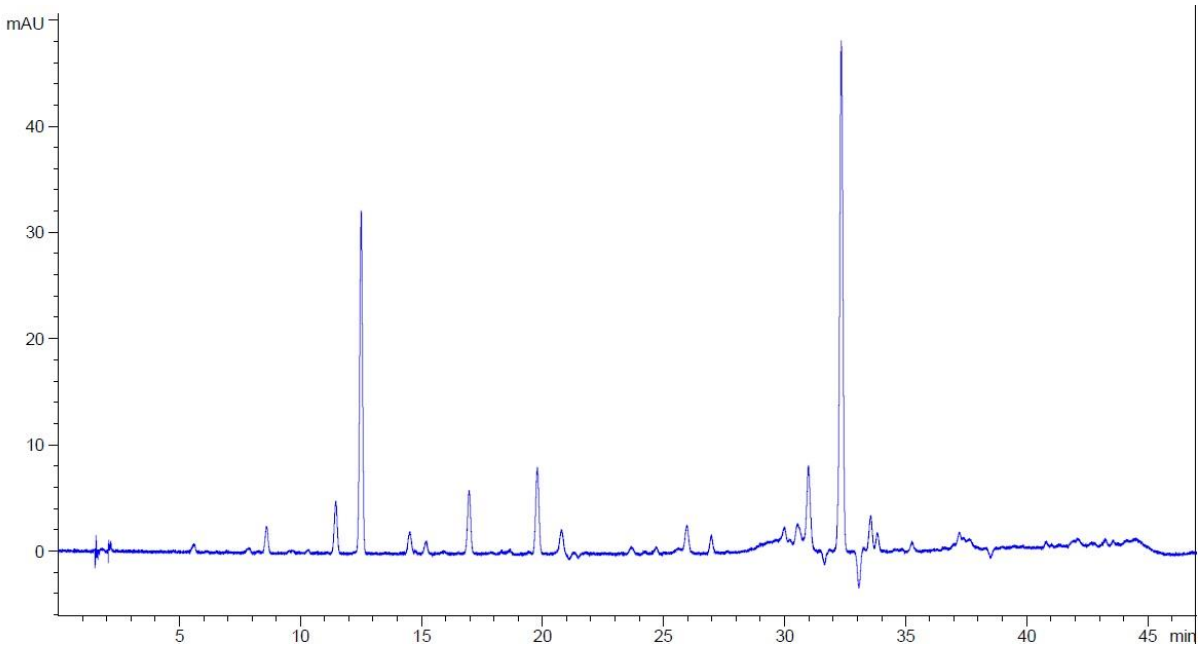

Figure A12 EVOO#2 diluted with 10% w/w sunflower oil

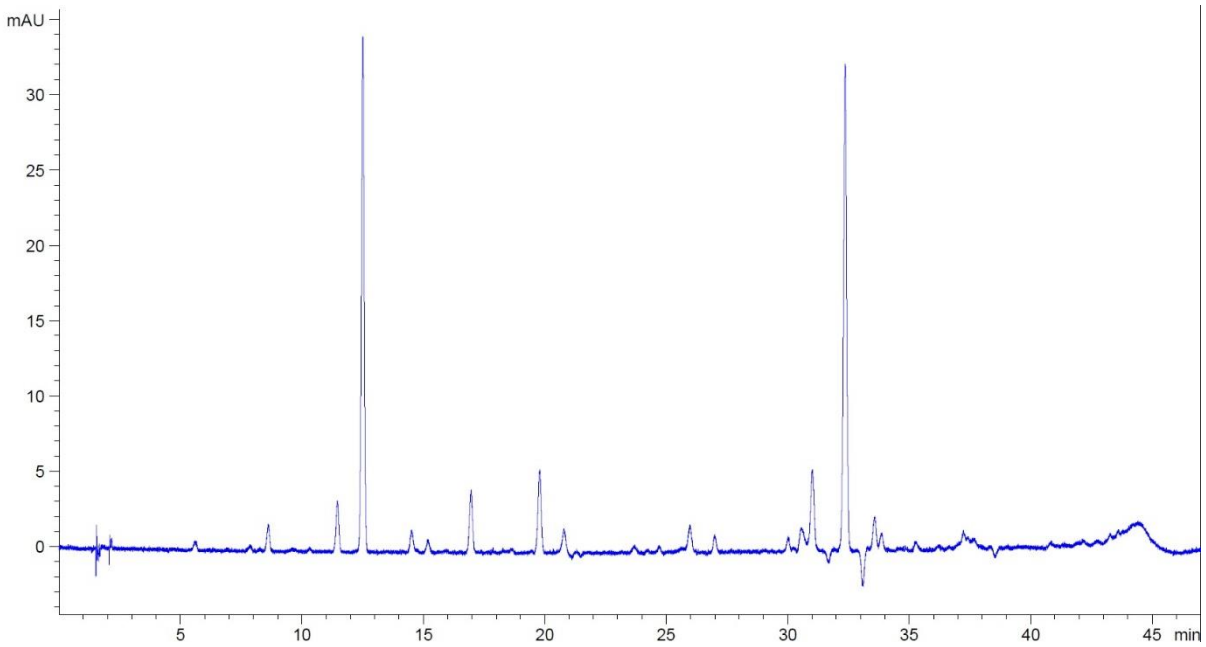

Figure A13 EVOO#2 diluted with 20% w/w sunflower oil

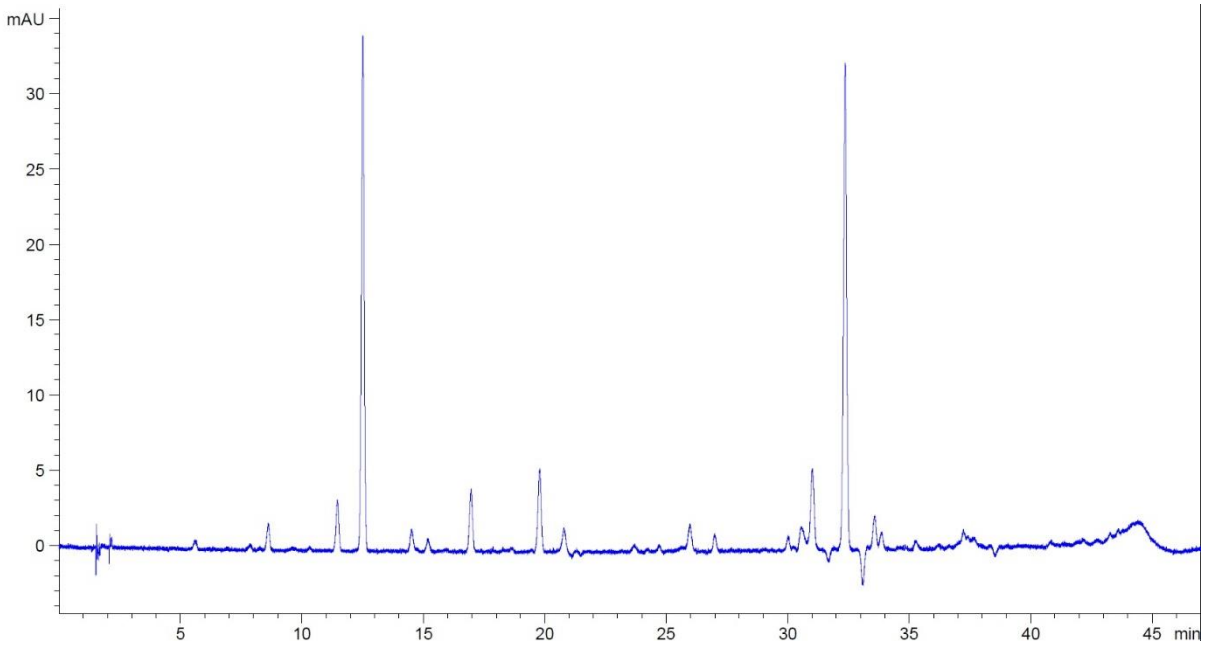

Figure A14 EVOO#2 diluted with 40% w/w sunflower oil

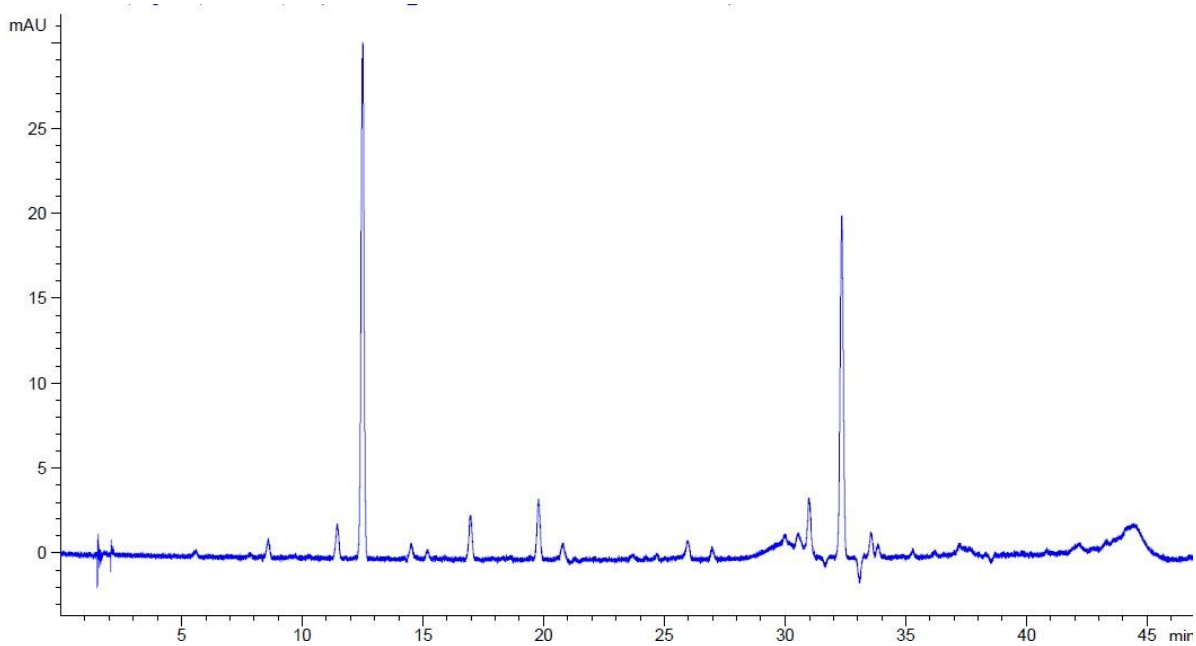

Figure A15 EVOO#2 diluted with 60% w/w sunflower oil

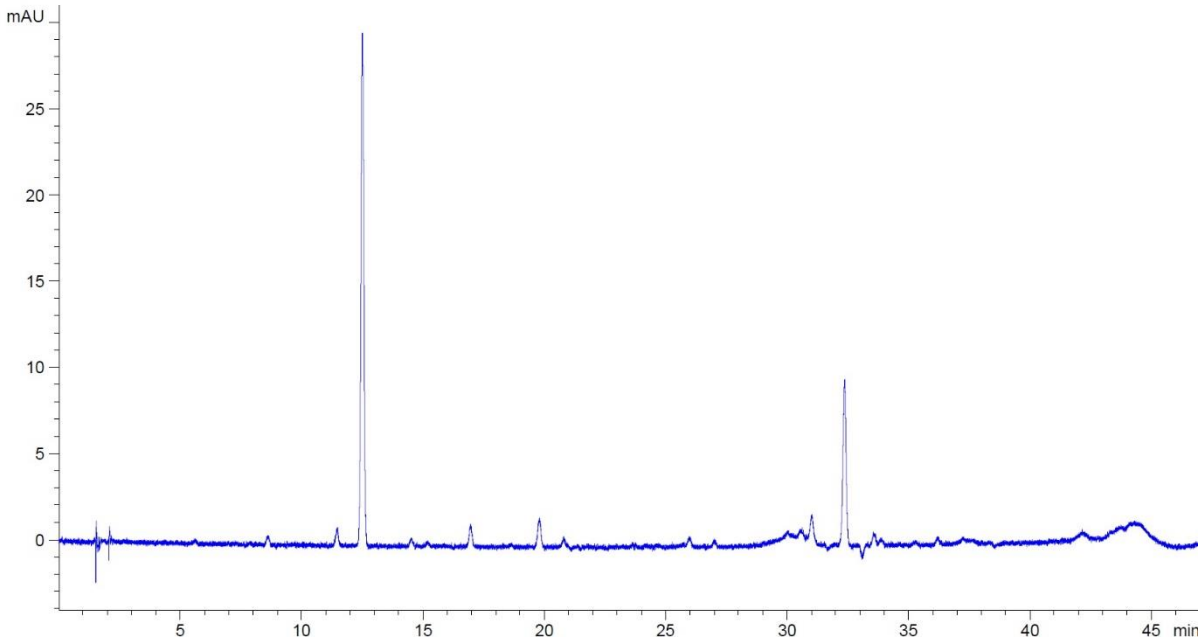

Figure A16 EVOO#2 diluted with 80% w/w sunflower oil

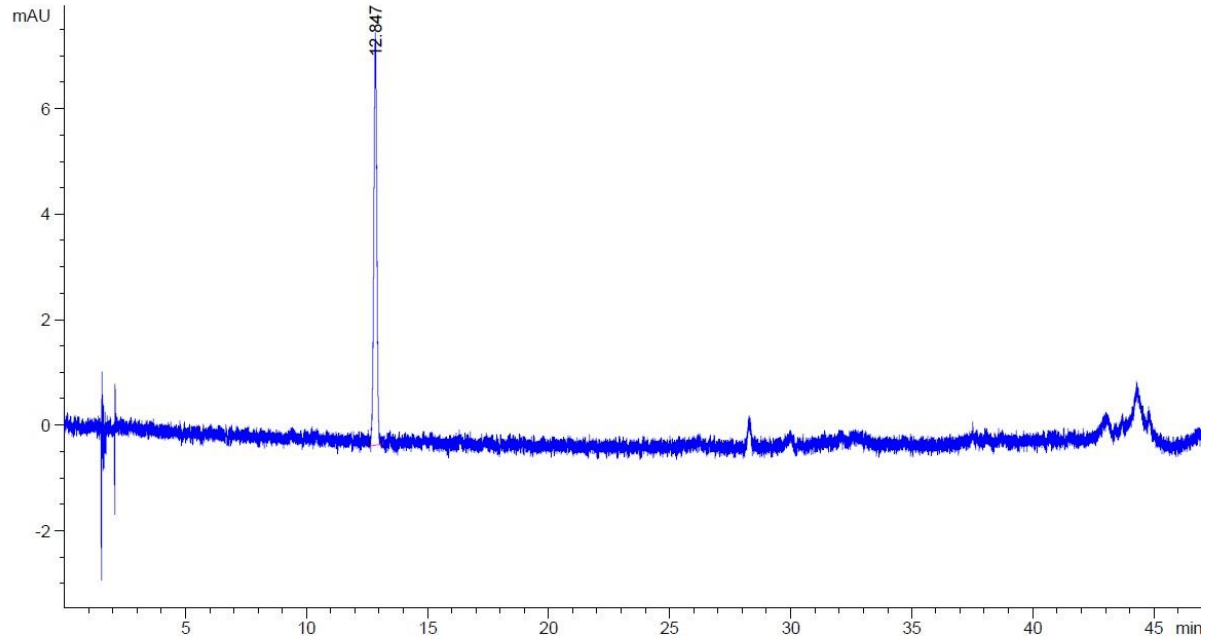

Figure A17 pure canola oil

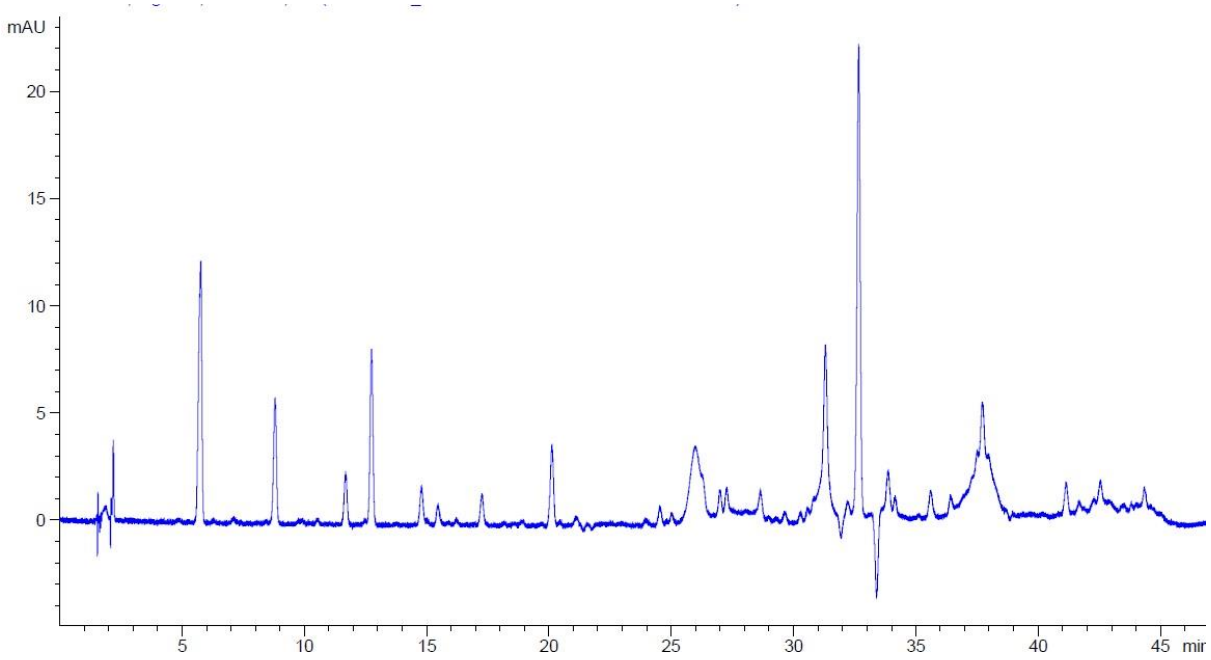

Figure A18 EVOO#2 diluted with 10% w/w canola oil

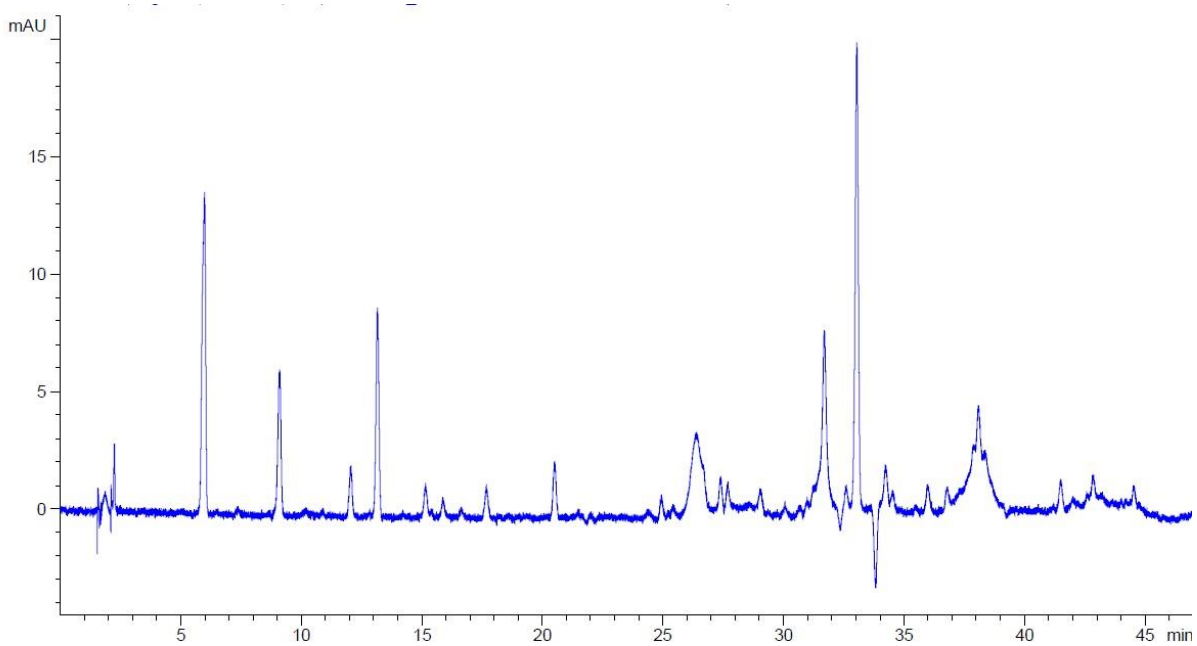

Figure A19 EVOO#2 diluted with 20% w/w canola

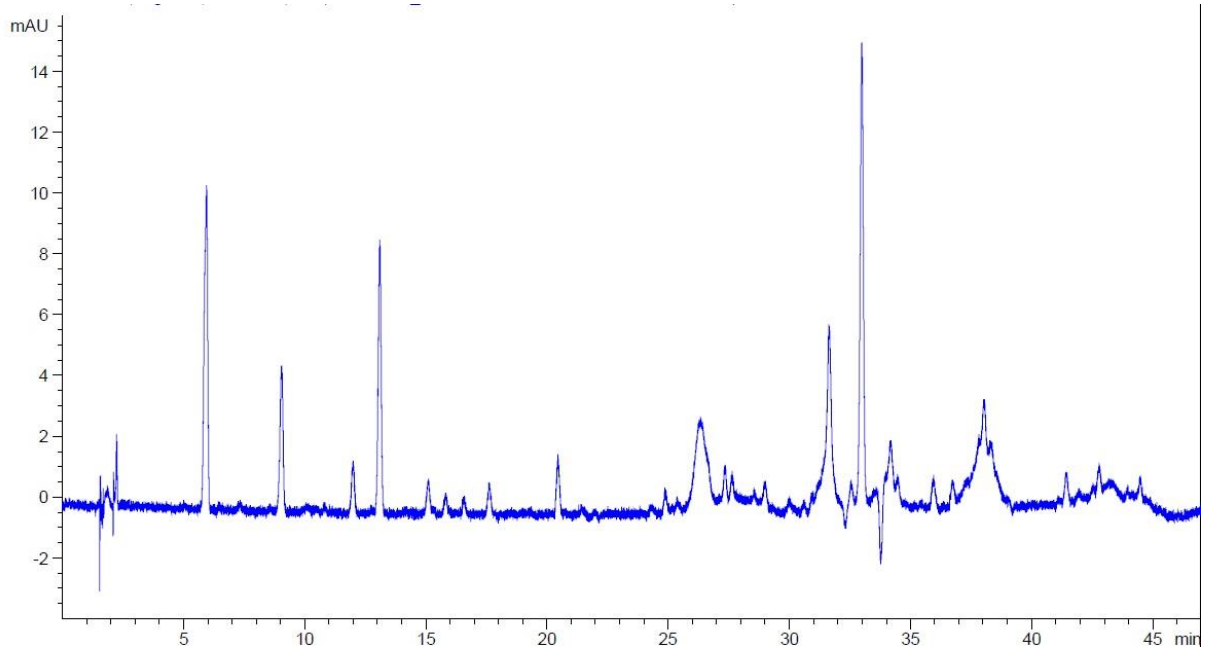

Figure A20 EVOO#2 diluted with 40% w/w canola

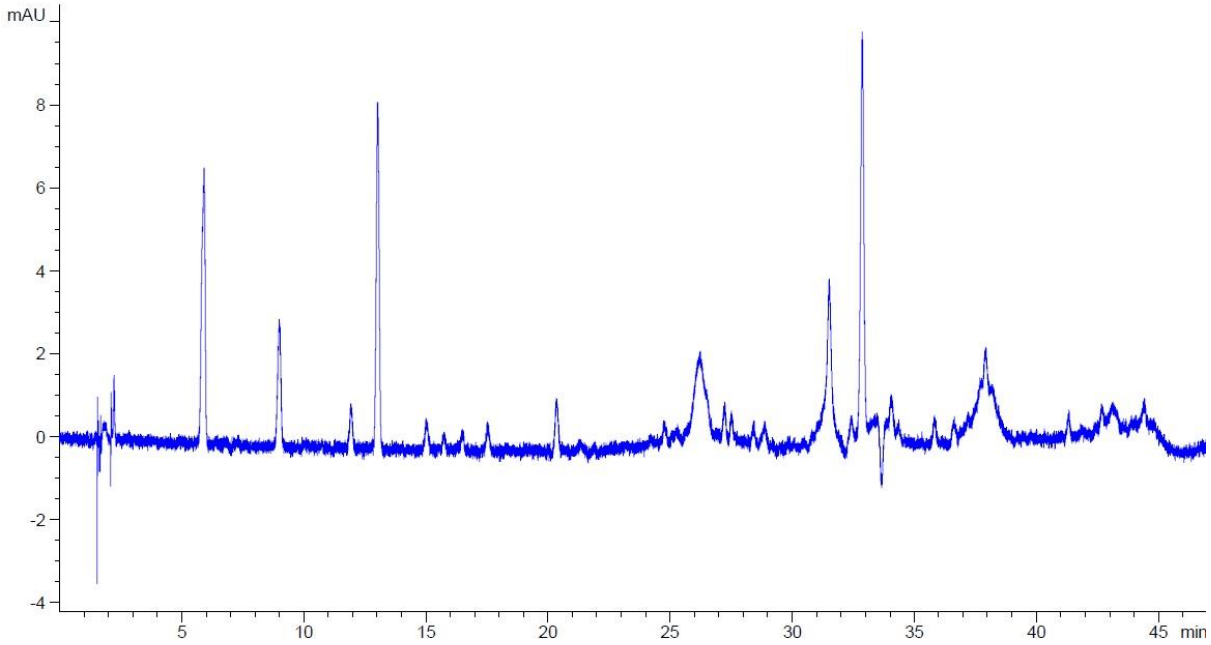

Figure A21 EVOO#2 diluted with 60% w/w canola

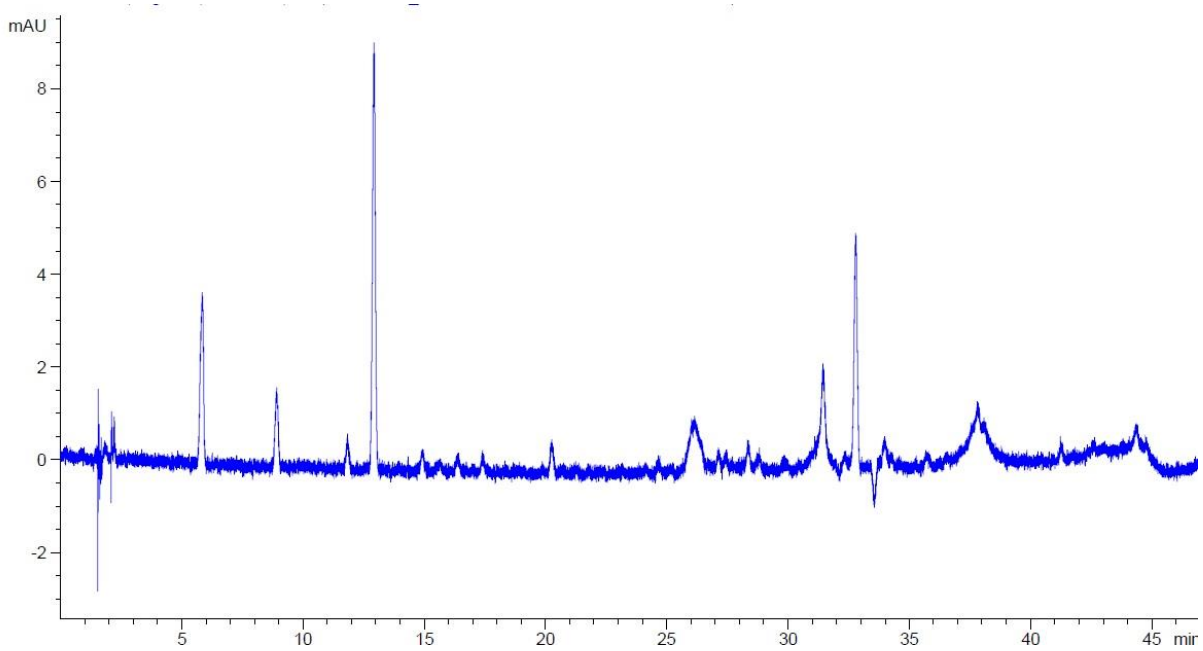

Figure A22 EVOO#2 diluted with 80% w/w canola

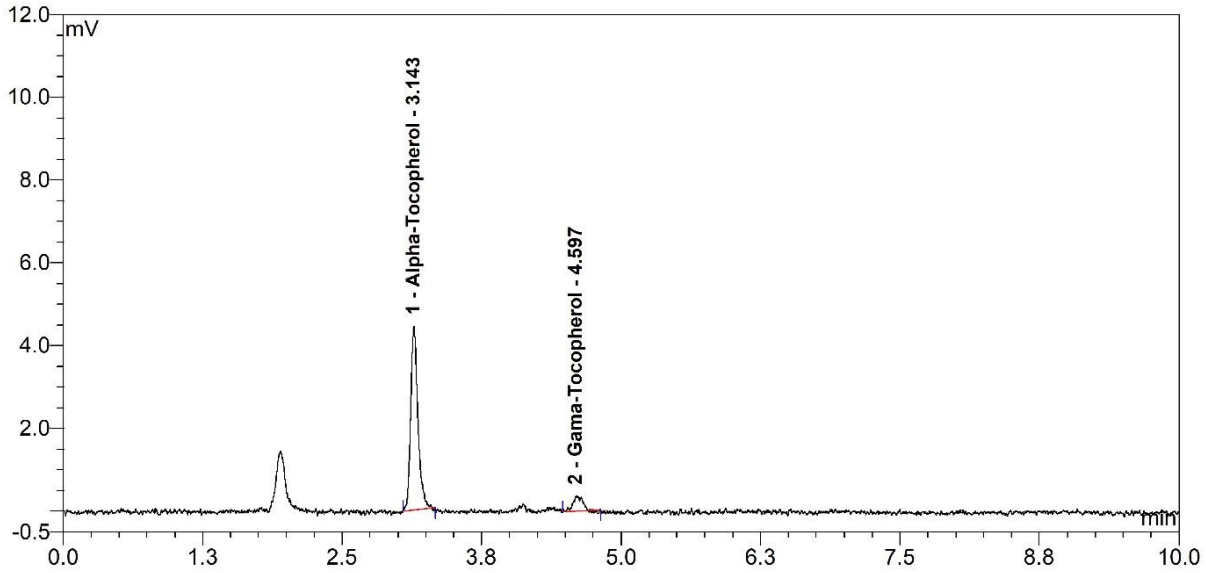

Figure A23 pure EVOO#1 Chromatogram

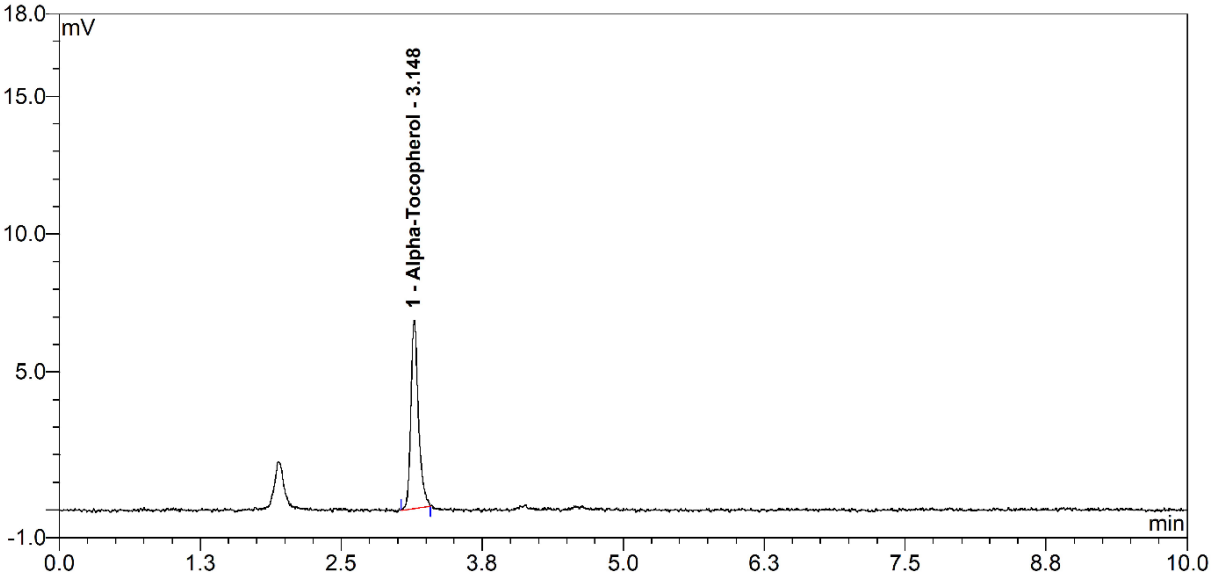

Figure A24 pure EVOO#2 Chromatogram

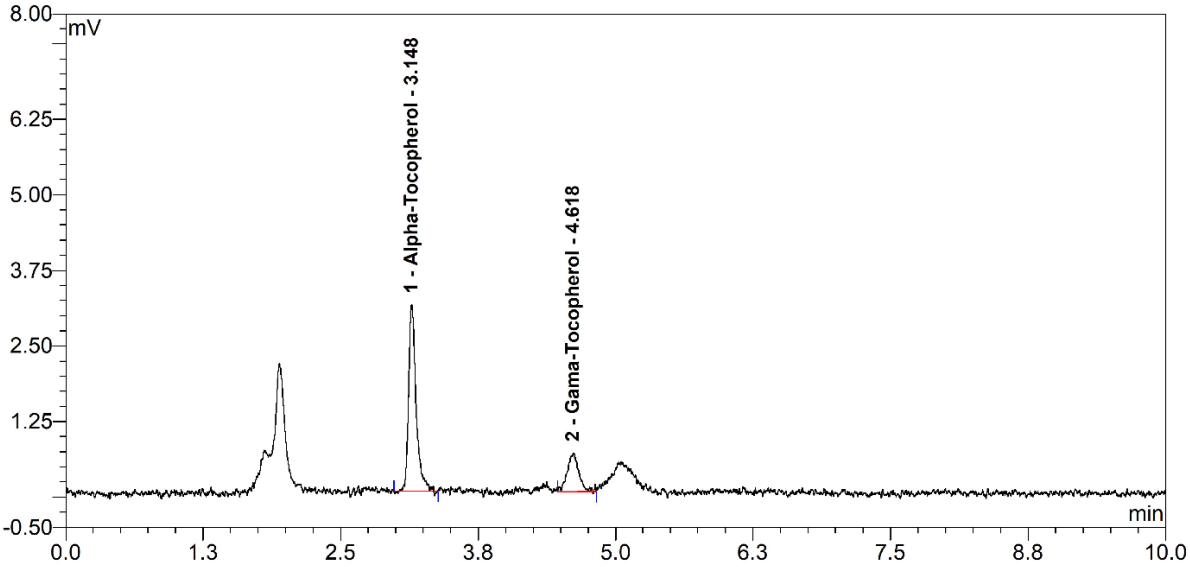

Figure A25 EVOO#1 diluted with 10% w/w rice bran oil

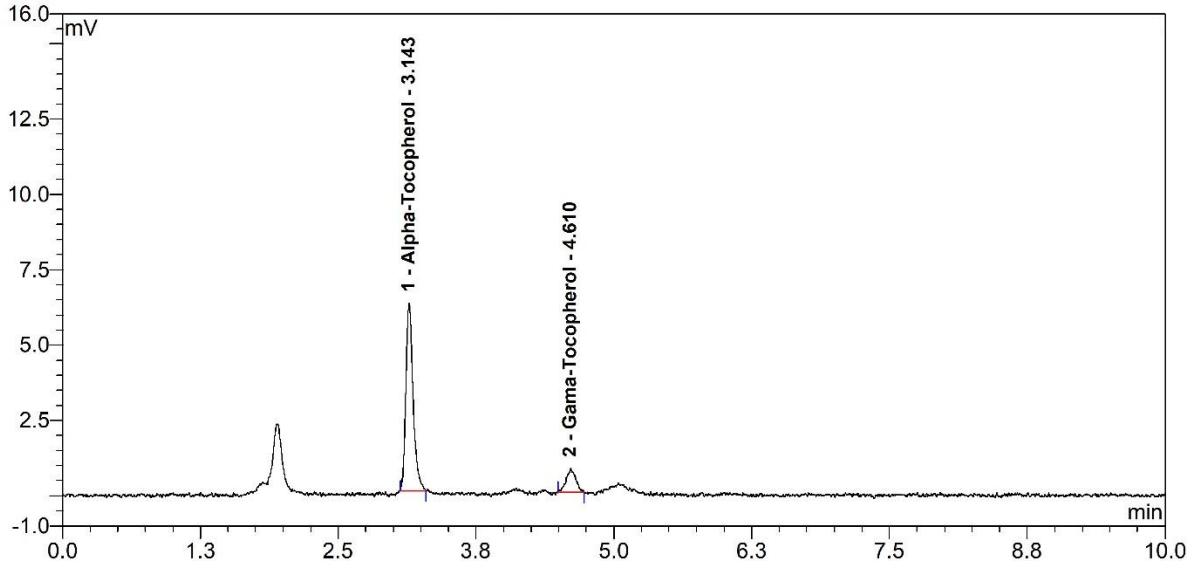

Figure A26 EVOO#2 diluted with 10% w/w rice bran oil

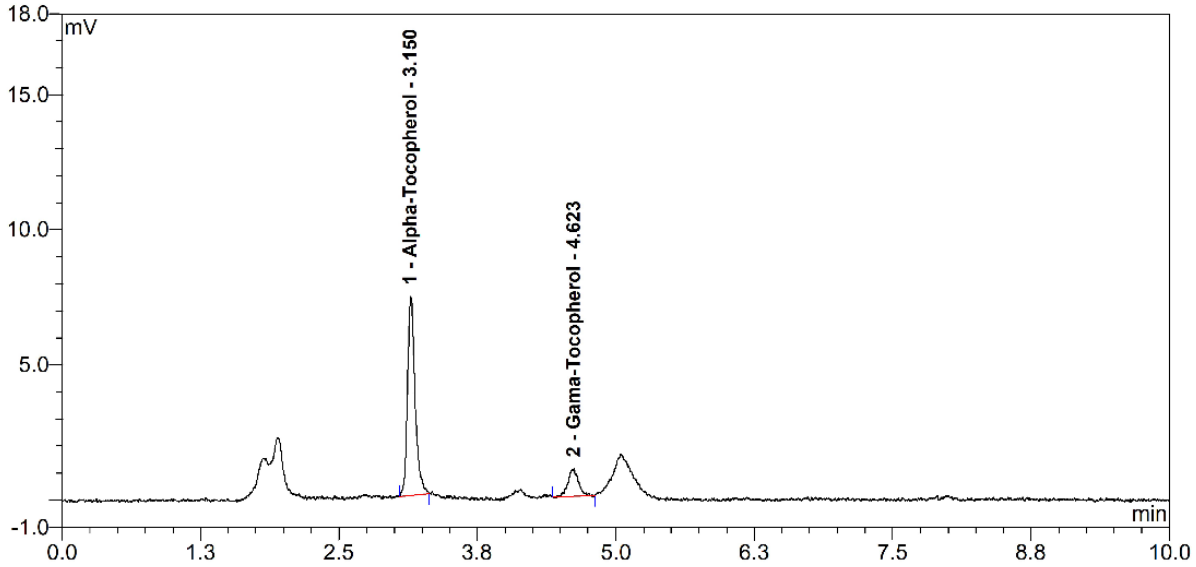

Figure A27 EVOO#1 diluted with 20% w/w rice bran oil

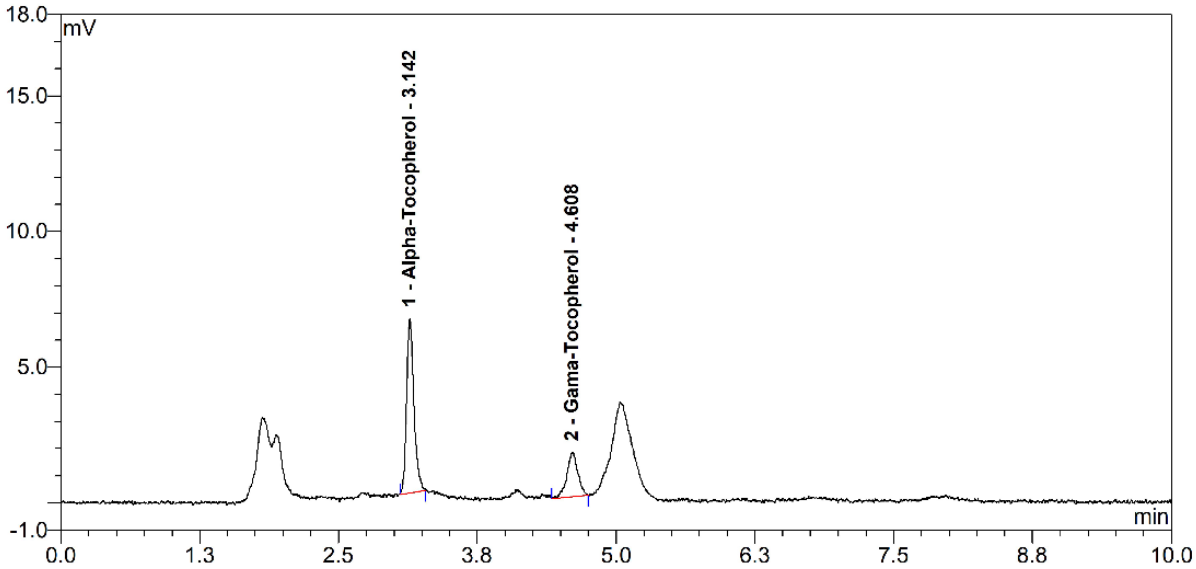

Figure A28 EVOO#1 diluted with 40% w/w rice bran oil

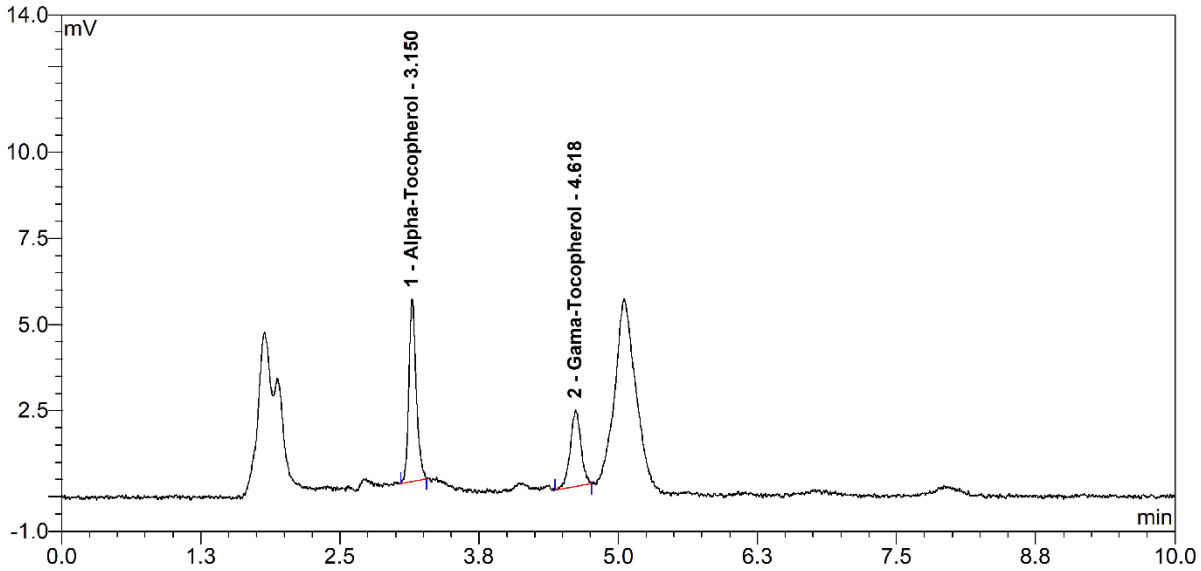

Figure A29 EVOO#1 diluted with 60% w/w rice bran oil

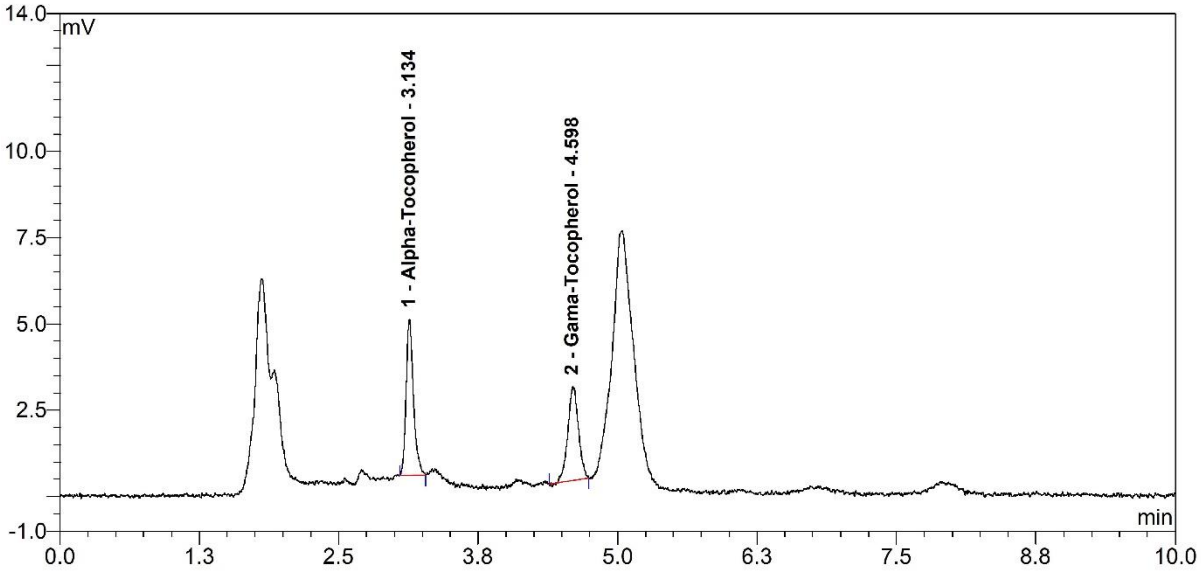

Figure A30 EVOO#1 diluted with 80% w/w rice bran oil

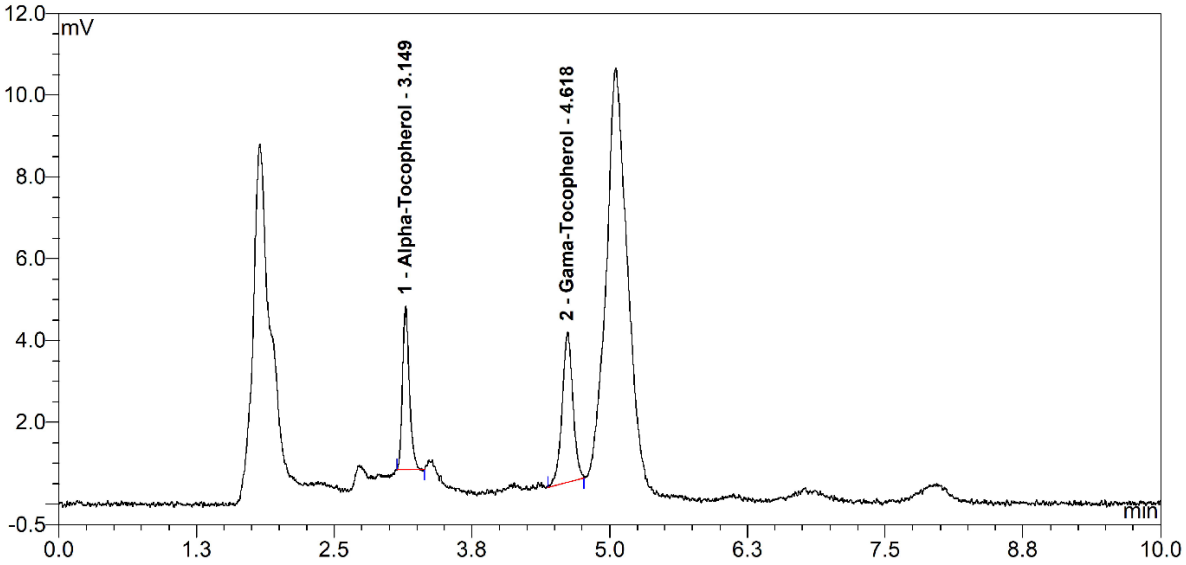

Figure A31 pure rice bran oil

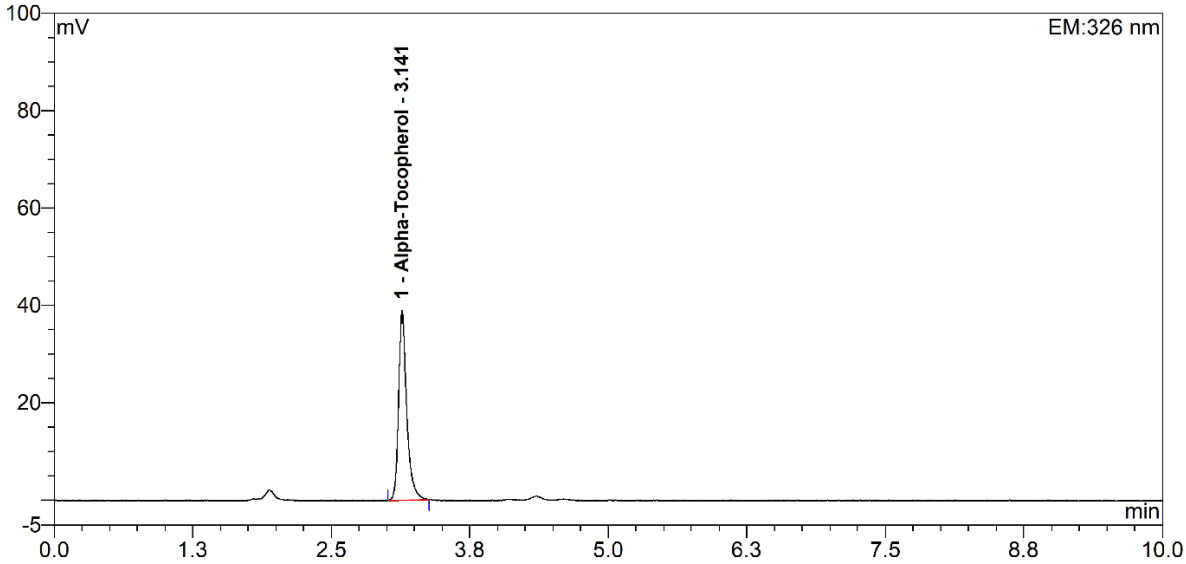

Figure A32 pure sunflower oil

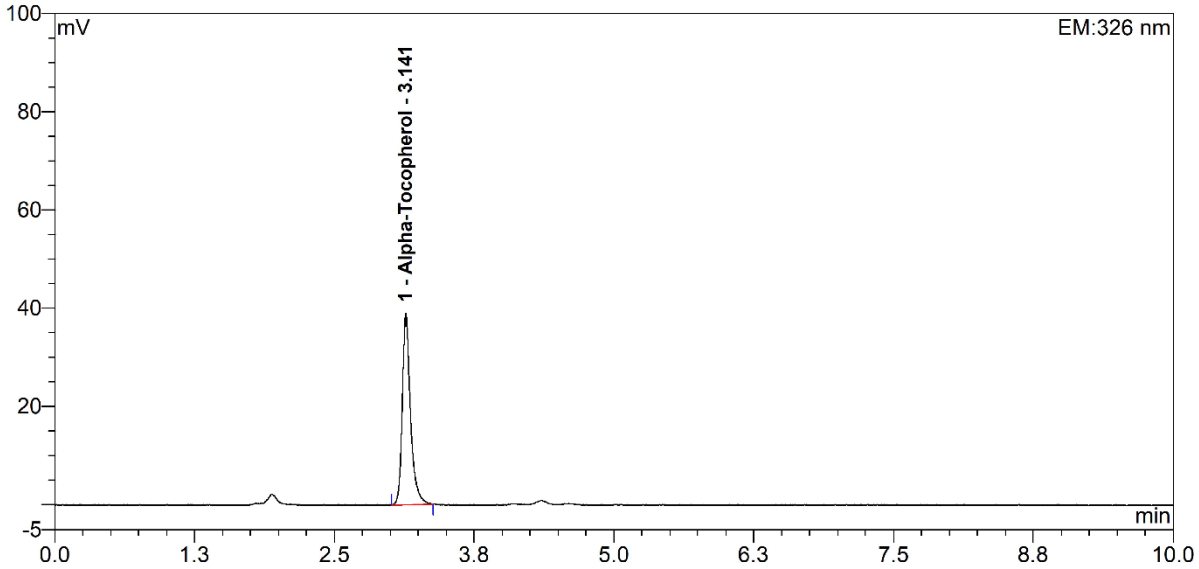

Figure A33 EVOO#1 diluted with 10% w/w sunflower oil

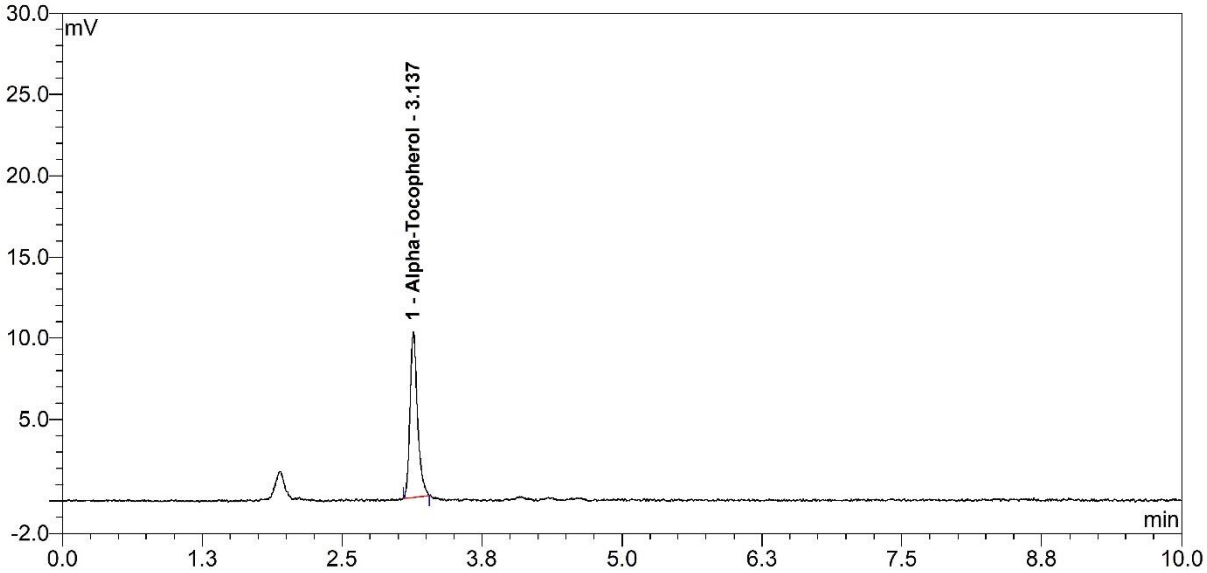

Figure A34 EVOO#2 diluted with 10% sunflower oil

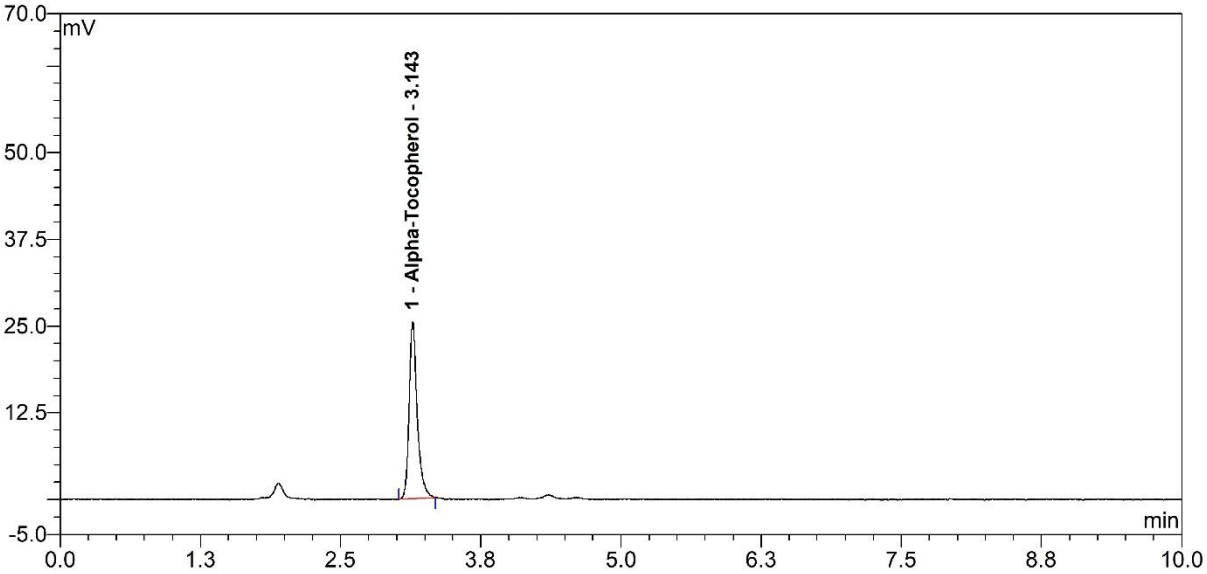

Figure A35 EVOO#1 diluted with 80% w/w sunflower oil

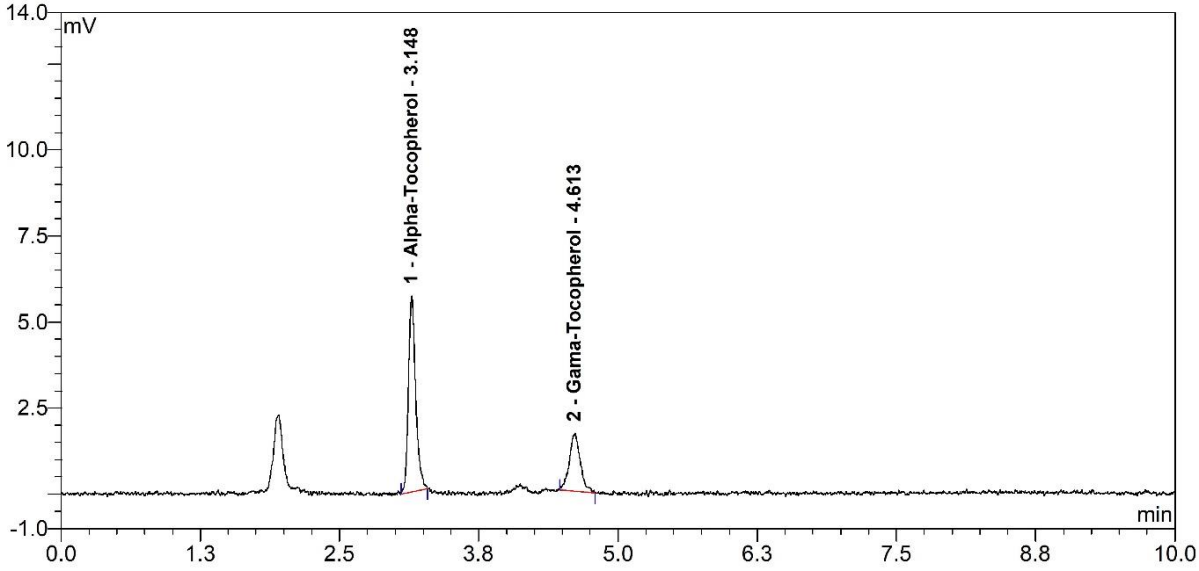

Figure A36 EVOO#1 diluted with 10% w/w canola oil

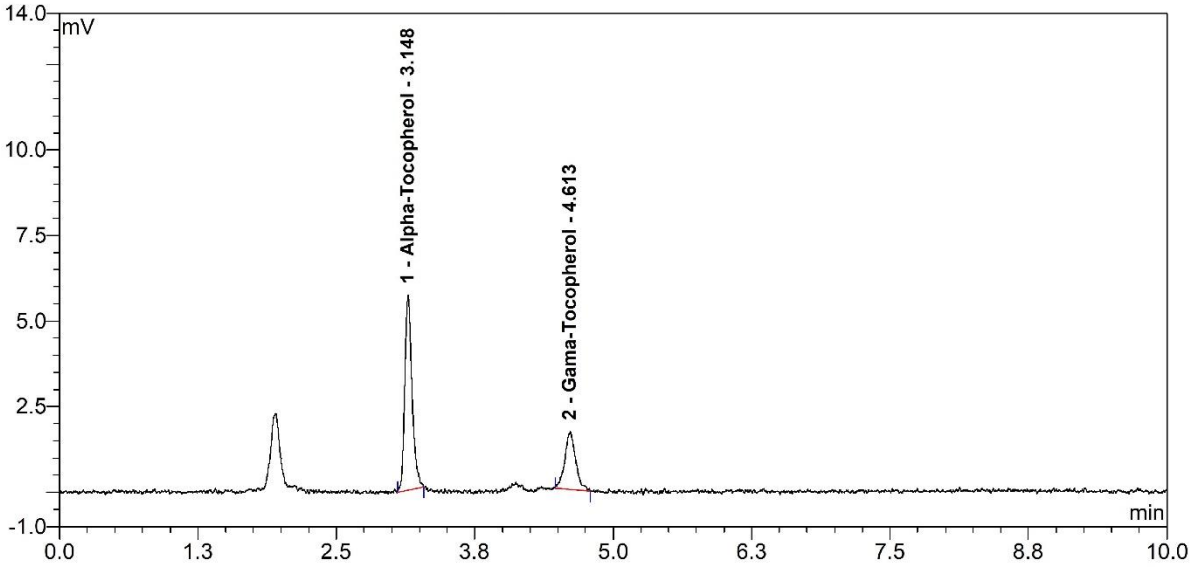

Figure A37 EVOO#2 diluted with 10% w/w canola oil

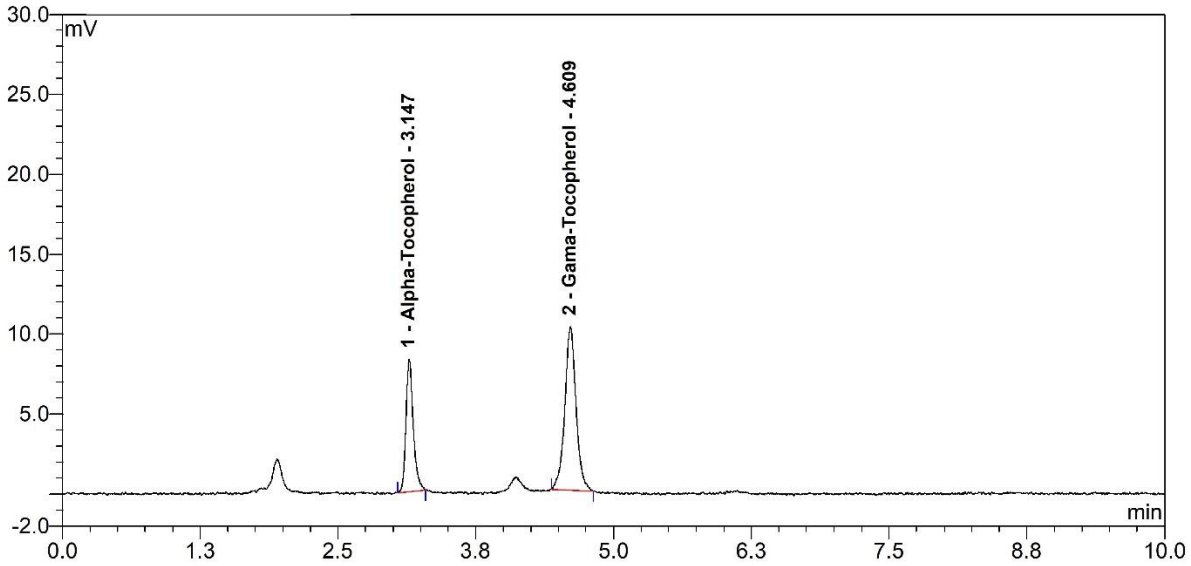

Figure A38 EVOO#1 diluted with 20% w/w canola

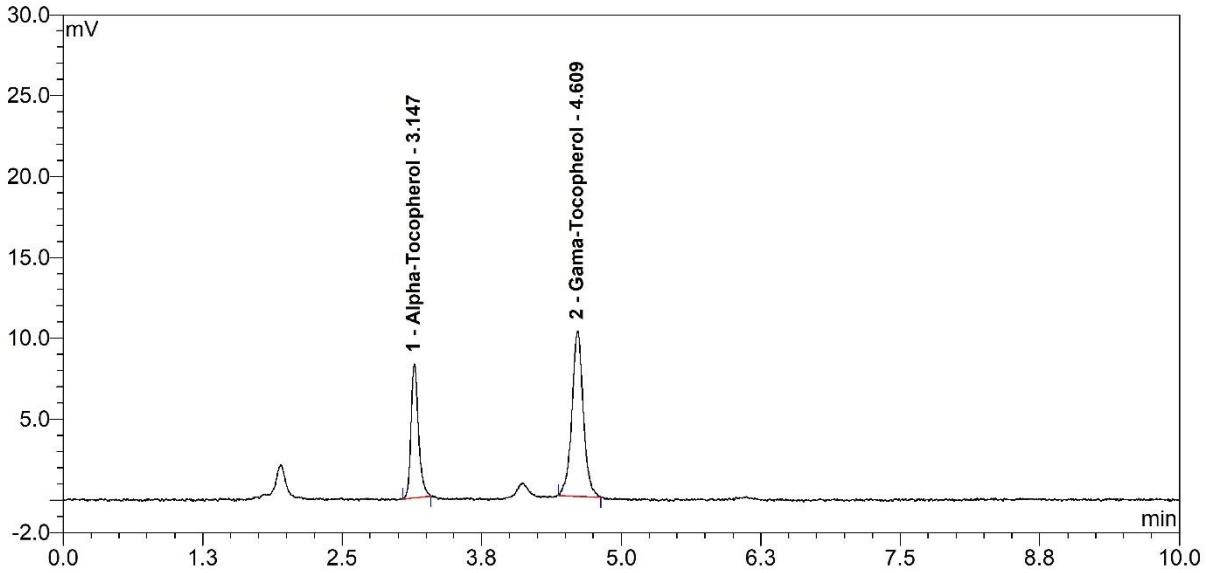

Figure A39 EVOO#1 diluted with 40% w/w canola

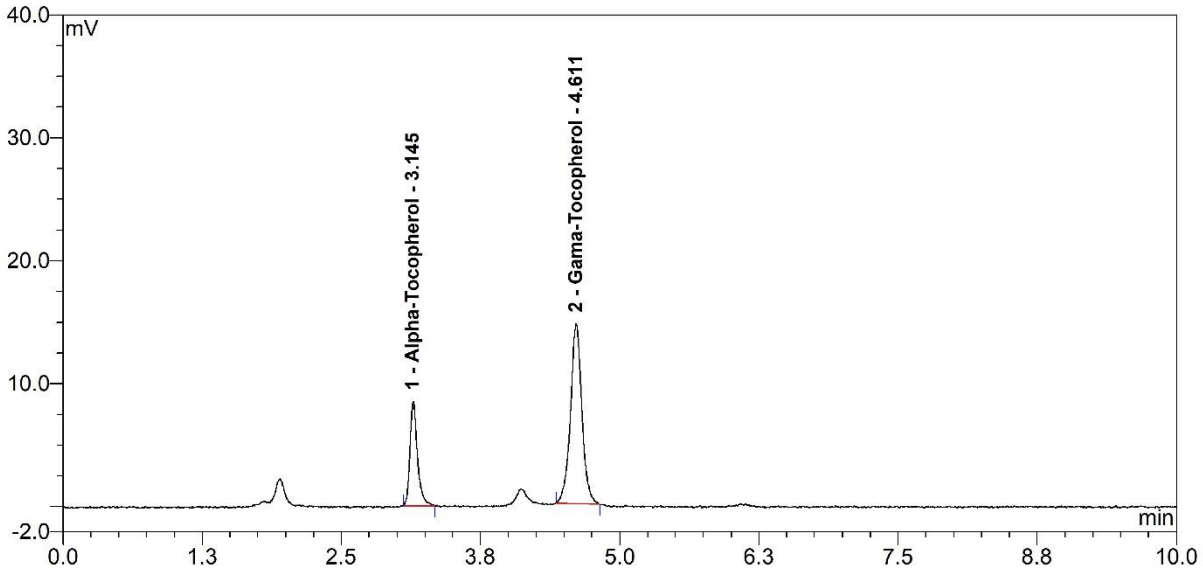

Figure A40 EVOO#1 diluted with 60% w/w canola

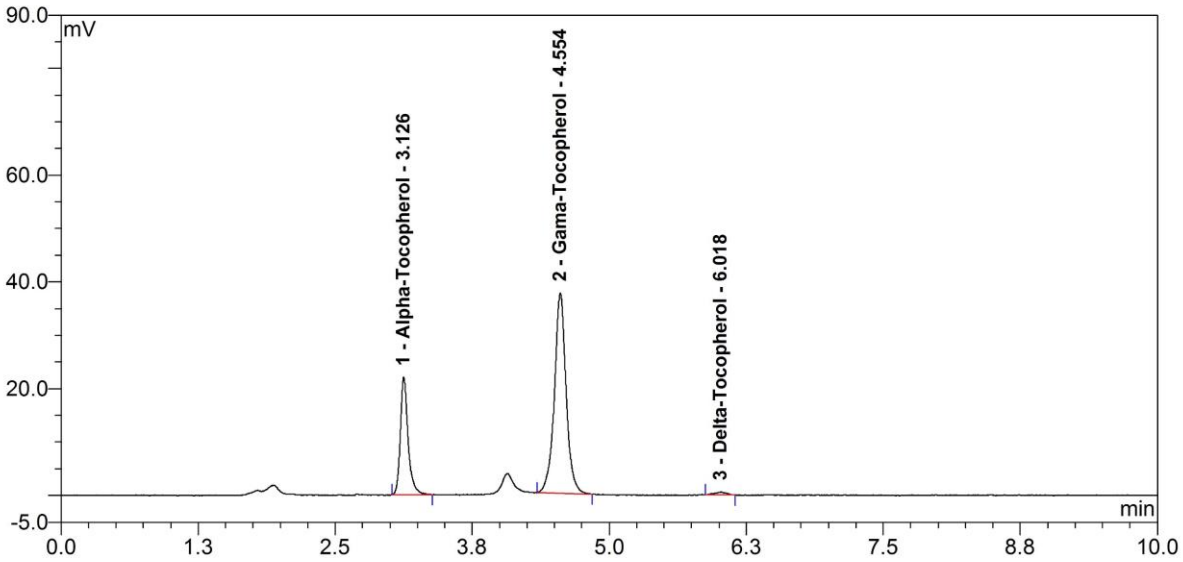

Figure A41 EVOO#1 diluted with 80% w/w canola