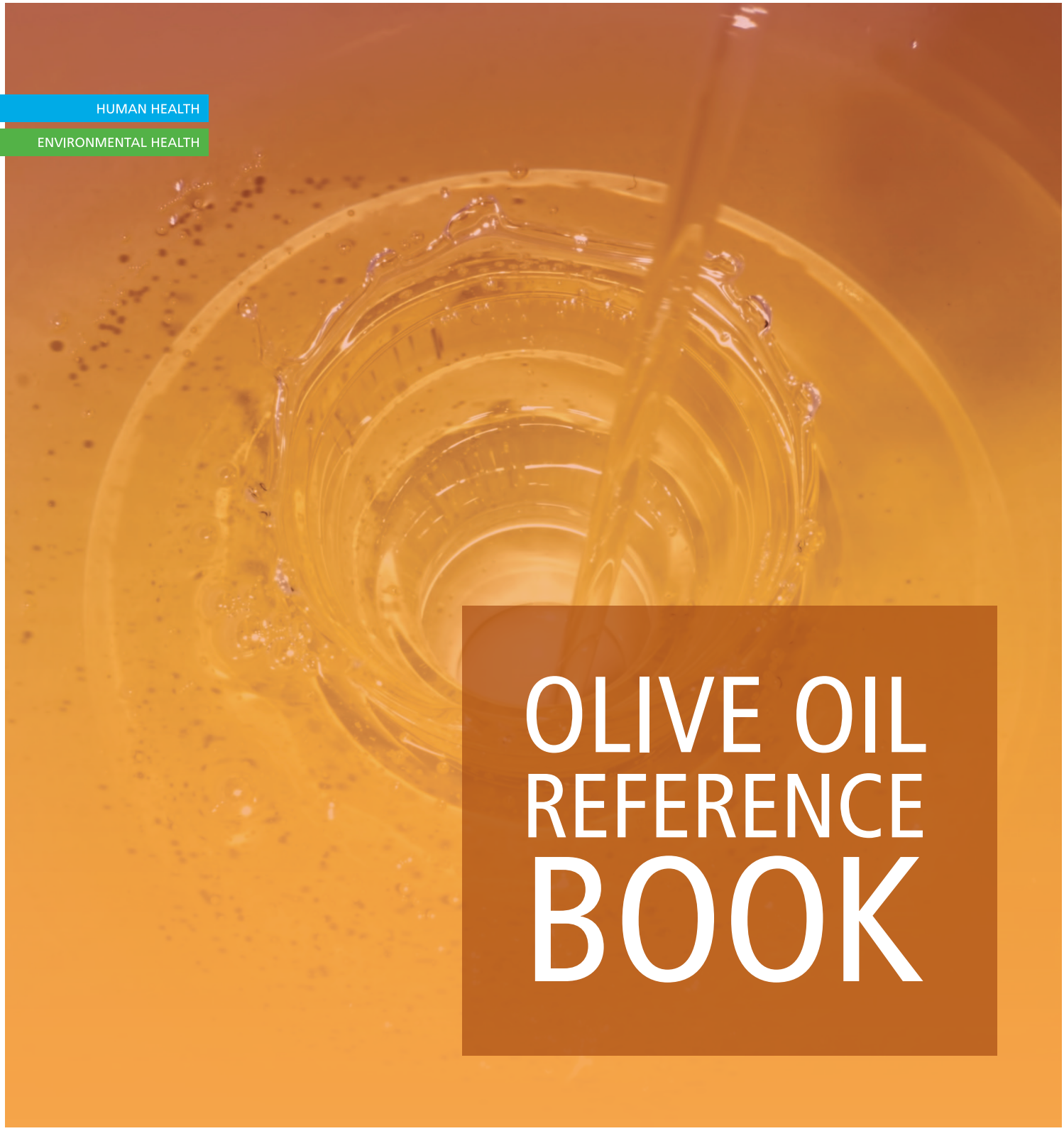
HUMAN HEALTH

ENVIRONMENTAL HEALTH



# OLIVE OIL REFERENCE BOOK

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# OLIVE OIL

## Olive oil: an introduction

Olive oil is the product of transformation obtained from the olive fruit (Olea europaea). Its recorded origins date back to the twenty-fourth century B.C, when the cultivation of this plant began to spread throughout the Mediterranean. In the case of extra virgin olive oil, it has been prized through the ages for possessing unique nutritional, medicinal and cosmetic properties, and even ceremonial value. As a result, it is one of the most expensive -- and most adulterated -- food products in history. While the creation and consumption of olive oil remain concentrated within the Mediterranean region, the global export of extra virgin olive oil is continuously growing. That poses a series of challenges raised by the worry of intentional adulteration for financial profit or unintentional environmental contaminants, such as pesticides.

How real are those concerns? In 1981, more than 600 people died because of adulterated olive oil containing rapeseed oil and aniline. Researchers at the University of California released the findings of a now famous 2010 study showing more than two-thirds of the extra virgin olive oil sold in California (and likely across the US) is neither extra virgin nor in some cases even olive oil, as the real oil is often adulterated with cheaper, more available oils to benefit fruadsters at the expense of human health. Today, extra virgin olive oil requires testing to ensure its authenticity, purity, and safety.

### The Rise of Regulation

Depending on olive processing and qualitative characteristics of the finished product, olive oil is classified into different product classes.

*Table 1.* Product classes of olive oils.

| Mechanical extraction - (virgin oils) | Chemical and physical extraction - (refined oils) |
|---|---|
| Extra virgin olive oil | Refined olive oil |
| Virgin olive oil | Olive oil (mix of virgin + refined oils) |
| Lampante olive oil | Crude olive-residue oil |
| | Refined olive-residue oil |
| | Olive residue oil |

For the classification of the product within the different classes, global regulatory bodies establish a number of chemical parameters, in conjunction with organoleptic analysis (panel test), in order to determine which class a specific olive oil belongs. As already noted, high market value, of true extra virgin olive oil has led to numerous adulteration and fraudulent schemes that undermine the quality attributes and sometimes even the safety of olive oil.

Therefore, the quality parameters covered by the EU law guarantees the authenticity of the oil first, and its quality in the broadest sense, which depends on the quality of olives, milling technologies and mode of preservation.

## The Chemistry of Olive Oil

From a chemical point of view, olive oil can be divided into two fractions, depending on their behavior in presence of a strong alkaline solution (NaOH or KOH) and heating, i.e. saponifiable and unsaponifiable fractions:

- **Saponifiable** (98% to 99% of total weight), composed of substances able to form soaps in the conditions mentioned; this fraction include free fatty acids or esterified fatty acids with glycerol to form triglycerides, diglycerides and monoglycerides, containing for 75% to 85% unsaturated fatty acids (mainly oleic and linoleic acids) and for 15% to 25% saturated fatty acids (palmitic and stearic acids).

- **Unsaponifiable** (1-2%), formed by micro-components that do not form soaps in the conditions mentioned; even where present in small quantities, this fraction is very important from a nutritional and analytical point of view to check the authenticity of the oil and its stability; it contains mostly sterols, fat-soluble vitamins, waxes, aliphatic alcohols, aromatic compounds and antioxidants.

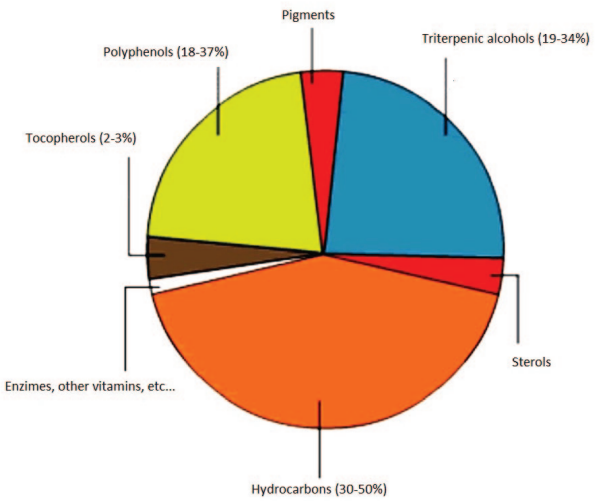


*Figure 2.* Chemical composition of Unsaponifiable fractions of olive oil.

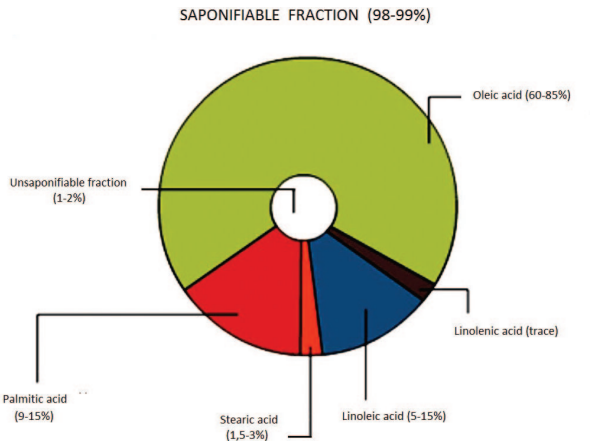


*Figure 1.* Chemical composition of Unsaponifiable and Saponifiable fractions of olive oil.

Regulations require analytical parameters and threshold values for individual product classes as listed in (Table 1), together with the scores to be obtained in the organoleptic evaluation carried out by means of a panel test (for virgin oils). The physiochemical parameters established by regulations are as follows:

1. **Free acidity**: It is an indirect measure, carried out by a simple acid-base titration of free fatty acid content in oil. It is expressed as a percentage of oleic acid and since these molecules are produced by triglycerides hydrolysis, their content increases if oil and/or olives preservation was not appropriately managed.

2. **Peroxide number**: It is a measure of the oxidative state of the oil, performed by titration with iodine and thiosulphate and expressed as mg of O2 absorbed by oil. The oxidation involves the formation of peroxides, which affect the stability of the product promoting hydrolytic rancidity. High values of this parameter are indicative of an improper olive manipulation or preservation management.

3. **K (UV-Vis)**: It measures the oil absorption in the UV to highlight the addition of refined oils, since during the refining process double bonds present in the polyunsaturated fatty acids change their position and form triens and diens conjugates (resulting in an increasing in the A270 and the A232 nm respectively); the K value is rather a relative measure of the absorbance peak at 270 against the entire UV spectrum. High values of these three parameters are indicative of an addition of refined oil to virgin or extra virgin oils.

4. **Acid composition** (Fatty Acid Methyl Esters, FAME): Through GC-FID technique it is possible to determine the relative abundance of fatty acids in oil after triglyceride hydrolysis and subsequent saponification in basic medium (to obtain the corresponding methyl esters). The presence of fatty acids normally absent in the olive (e.g. behenic acid, arachidic acid, etc…) is indicative of adulteration of the product with oils obtained from different seeds.

5. **Total Sterols, Erythrodiol and Uvaol**: It recovers the unsaponifiable fraction and separates the sterol fraction by preparative chromatography. It should then be performed a silanization reaction to analyze the sterolic composition by means of GC-FID. The sterol profile is species-specific, so adulterations of the product with other oils are easily detectable. The erythrodiol and uvaol content is higher in olive-residue oils due to the chemical extraction by solvent and a high content of these two compounds is indicative of adulteration caused by pomace oils or oils from different species.

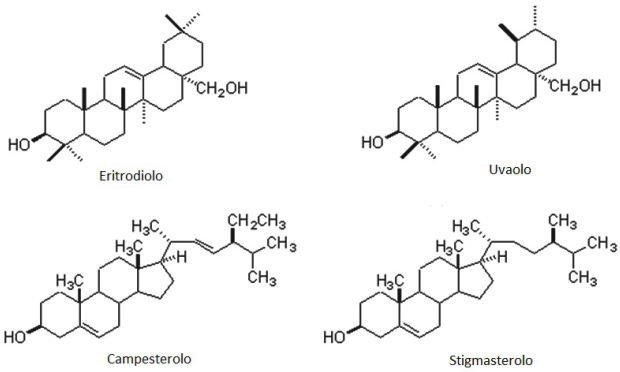


*Figure 3.* Structure of sterol characteristics in olive oil.

6. **Trans isomers of fatty acids**: The normal configuration of double bonds in unsaturated fatty acids is the cis. The processes of bleaching and deodorization modify the configuration forming trans isomers that can be quantified by GC-FID on a suitable capillary column.

7. **ΔECN42**: This method is based on the separation and quantification of the various triglycerides present in oil. The triglycerides composition is a function of primary fatty acid composition of oil; thus the expected composition of triglycerides can be calculated starting from the fatty acid composition (GC-FID of FAMEs).

The expected value is compared with the value measured by HPLC-RI for some of the triglyceride classes (in particular those with equivalent number of carbons equal to 42, such as the tri-linolein). Differences between expected and measured concentration values allows identity of adulterations with other oils having a different triglycerides composition.

8. **3.5-Stigmastadiene**: This compound is formed during the refining processes from sterols. Its determination in virgin olive oil by means of preparative chromatography and subsequent analysis by GC-FID allows detecting the presence of refined oils, even in very low concentrations.

9. **2-glyceryl monopalmitate**: The biosynthesis of triglycerides occurs through site-specific enzymes, which starting from free fatty acids and glycerol form the triglyceride. In virgin olive oils only about 2% of palmitic acid present is bonded on position 2, while in oil artificially esterified the bonding with glycerol occurs in a random manner and significantly increase this percentage. The concentration of 2-glyceryl monopalmitate is determined after site-specific enzymatic digestion of triglycerides (which eliminates fatty acids in position 1 and 3) and subsequent GC-FID analysis of monoglycerides.

10. **FAMEs/FAEEs** (methyl and ethyl esters of fatty acids): The presence of FAMEs and FAEEs in oil is indicative of an occurred reaction of free fatty acids with methanol (formed by degradation of cell walls) or ethanol (formed during fermentation processes). Their presence indicates an incorrect manipulation of olives or a slight oil deodorization (to eliminate off-odors produced by microorganisms). Their content can be measured without carrying out any oil saponification and using appropriate preparative chromatography, performed prior to the analysis by GC.

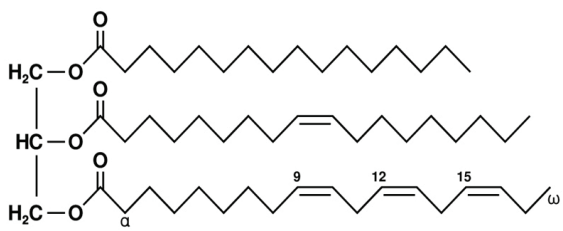


*Figure 4.* Triglyceride consisting of Palmitic acid, Oleic acid and Linolenic acid.

11. **Waxes**: The wax content is higher in olive-residue oil, since these compounds are found mainly in olive stone and their extraction is increased using organic solvents. Waxes are simultaneously determined by GC-FID analysis of FAMEs and FAEEs and an excessive concentration of these compounds indicates an adulteration with pomace oils. Waxes are also an important parameter in discriminating crude olive-residue oil from lampante olive oil, combining their concentration with aliphatic alcohols content.

12. **Aliphatic alcohols**: The measure of total aliphatic alcohols, in combination with those of waxes, uvaol and erythrodiol allow distinguishing crude olive-residue oil from lampante olive oil. These molecules are mainly extracted in oils obtained by the use of solvents (such as the case of pomace oil).

*Table 2.* Examples of some fatty acids (in bold are those most common in olive oil).

| Abbreviation | Double Bonds | Common Name | Chemical Formula |
|---|---|---|---|
| C 14:0 | 0 | Myristic acid | $CH_3 - (CH_2)_{12} - COOH$ |
| **C 16:0** | **0** | **Palmitic acid** | **$CH_3 - (CH_2)_{14} - COOH$** |
| **C 18:0** | **0** | **Stearic acid** | **$CH_3 - (CH_2)_{16} - COOH$** |
| C 20:0 | 0 | Arachid acid | $CH_3 - (CH_2)_{18} - COOH$ |
| C 22:0 | 0 | Behenic acid | $CH_3 - (CH_2)_{20} - COOH$ |
| C 16:1 | 1 | Palmitoleic acid | $CH_3 - (CH_2)_7 - CH = CH - (CH_2)_5 - COOH$ |
| **C 18:1** | **1** | **Oleic acid** | **$CH_3 - (CH_2)_7 - CH = CH - (CH_2)_7 - COOH$** |
| **C 18:2** | **2** | **Linoleic acid** | **$CH_3 - (CH_2)_4 - CH = CH - CH_2 - CH= CH - (CH_2)_7 - COOH$** |
| C 18:3 | 3 | Linolenic acid | $CH_3 - CH_2 - CH = CH - CH_2 - CH= CH - CH_2 - CH = CH - (CH_2)_7 - COOH$ |

APPLICATION BRIEF

Gas Chromatography

# Analysis of FAMEs – FAEEs and quantification of waxes

## Introduction

The chemical and physical properties of these classes of compounds allow obtaining an effective separation in a single chromatographic run. The Fatty Acid Methyl Esters (FAMEs) and Fatty Acid Ethyl Esters (FAEEs) may be indicative of an incorrect handling/ processing of the raw material (olives) or deodorization processes in which the oil may be subjected.

The content in Waxes is normally higher in olive pomace as mainly contained in the stone and principally extracted with the use of solvents in the extraction process.

The extent of these two parameters allows differentiating the olive oil from the olive pomace oil or, in case of extra virgin olive oils, identifying oils obtained by blending with lampante or deodorized virgin oils.

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## Principle of the Method

The FAMEs and FAEEs are naturally formed by reaction of free fatty acids with methanol or ethanol within badly maintained olives or low quality oils obtained therefrom.

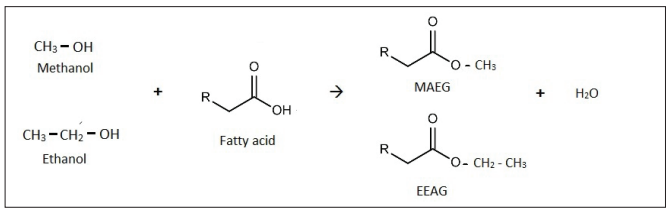



*Figure 1.* Esterification reaction.

The waxes contained in the olive oil are synthesized from the plant by esterification of fatty acids with long chain aliphatic alcohols. The aliphatic chains present all an even number of carbon atoms.

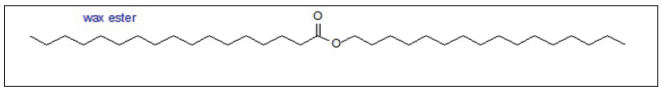



*Figure 2.* Wax.

*Table 1.* Instrumention.

| | |
|---|---|
| Gas Chromatograph: | Clarus 580 GC with PPC |
| Injector: | On-column or PSS Injector |
| Detector: | FID Flame Ionization Detector |
| Software: | TotalChrom® Computing and Instrumentation Management Software |
| Column: | Elite 5 HT, 15 m, 1 µm, 0.32 mm Part No. N932-6274 |

*Figure 3.* GC Clarus 580.

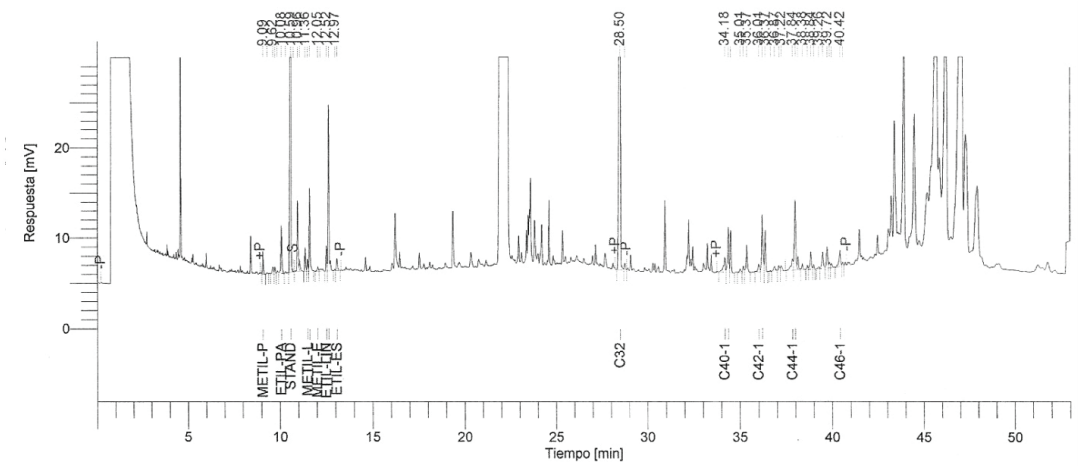
## Sample Preparation

It should be used two internal standards: Methyl heptadecanoate (for FAMEs and FAEEs) and Lauryl arachidate (for Waxes).

The fraction containing FAMEs, FAEEs and Waxes is obtained from the oil by passing it through a preconditioned packed silica gel column with n-hexane and then eluted with n-hexane/ethyl ether 99/1. The solvent in the collected fraction is eliminated with a Rotavapor and the residue re-suspended with 2-4 ml of heptane (A Solution).

*Table 2.* Analytical Method.

| | |
|---|---|
| Injection volume: | 1.5 µl of A Solution |
| Injector temperature: | 5 °C above the oven temperature |
| Heating Ramp: | 80 °C (held 1 min)<br>80 °C – 120 °C (30 °C/min)<br>120 °C – 340 °C (5 °C/min)<br>340 °C (held 10') |




| Waxes - Esters Report | | | | | |
|---|---|---|---|---|---|
| Time (min) | Area (uV*sec) | Area (%) | Waxes (Mg) | Esters (Mg) | Component Name |
| 9.087 | 7079.48 | 0.73 | 0.0000 | 4.3978 | Methyl Palmitate |
| 10.081 | 19021.01 | 1.96 | 0.0000 | 11.8159 | Ethyl Palmitate |
| 10.588 | 321954.85 | 33.18 | ------- | 0.0000 | Standard |
| 11.500 | 4045.93 | 0.42 | 0.0000 | 1.2567e-08 | Methyl Linoleate |
| 11.613 | 30948.55 | 3.19 | 0.0000 | 19.2254 | Methyl Oleate |
| 12.052 | 2242.47 | 0.23 | 0.0000 | 6.9652e-09 | Methyl Stearate |
| 12.519 | 9195.41 | 0.95 | 0.0000 | 2.8561e-08 | Ethyl Linoleate |
| 12.631 | 66769.61 | 6.88 | 0.0000 | 41.4776 | Ethyl Oleate |
| 13.082 | 5382.20 | 0.55 | 0.0000 | 1.6717e-08 | Ethyl Stearate |
| 28.496 | 374952.08 | 38.64 | ------- | 0.0000 | C32 |
| 34.381 | 33430.76 | 3.45 | 0.0000 | 0.0000 | C40 |
| 36.201 | 39511.24 | 4.07 | 0.0000 | 0.0000 | C44 |
| 37.993 | 45696.08 | 4.71 | 0.0000 | 0.0000 | C42 |
| 40.419 | 10142.63 | 1.05 | 0.0000 | 0.0000 | C46 |
| | 970372.03 | 100.00 | 68.6918 | 76.9168 | |

*Figure 4.* Analysis of FAMEs, FAEEs (first portion of the chromatogram) and Waxes (final part).

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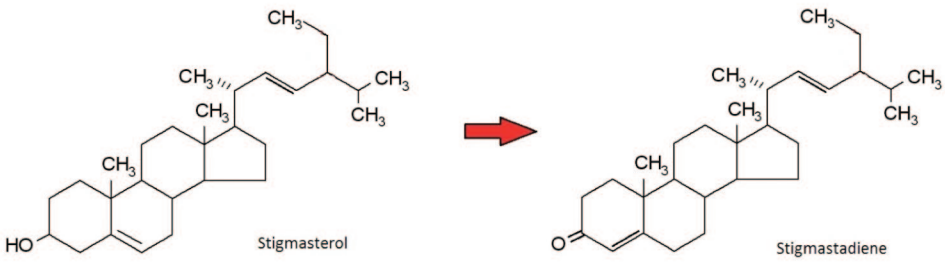
APPLICATION BRIEF

Gas Chromatography

# Determination of 3,5-Stigmastadiene Content

## Introduction

The stigmastadiene is a compound that is formed in small quantities starting from stigmasterol during the refining processes of oils carried out thermally or with the use of active carbons. In virgin olive oils, this compound is practically absent and therefore its determination allows to detect the presence of refined oils (olive, olive-residue, sunflower, palm, etc.) in virgin olive oils, even if they're added in small quantities.




## Principle of the Method

After isolation of the unsaponifiable fraction, the latter is fractioned by chromatography on preparative silica gel column to recover the fraction containing the steroids. The recovered fraction is subjected to derivatization and subsequently analyzed by GC-FID to quantify the 3,5-stigmastadiene. The method can be reliably applied to all vegetable oils, but its accuracy is good only if the stigmastadiene content lies between 0.01 and 4.0 mg/kg.

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## Sample Preparation

Start with 20 grams of oil, then add an appropriate amount of 3,5-cholestadiene (internal standard) and 75 mL of 10% KOH in ethanol. After the saponification is completed, the unsaponifiable fraction is recovered in hexane and passed on a silica gel packed column, eluting with 1 mL/min of hexane. The first fraction (about 25-30 mL) containing the saturated hydrocarbons is eliminated, while the next 40 mL fraction containing the steroidic-hydrocarbons is recovered and concentrated by rotavapor until a volume of 200 µL.

*Table 1.* Instrumentation.

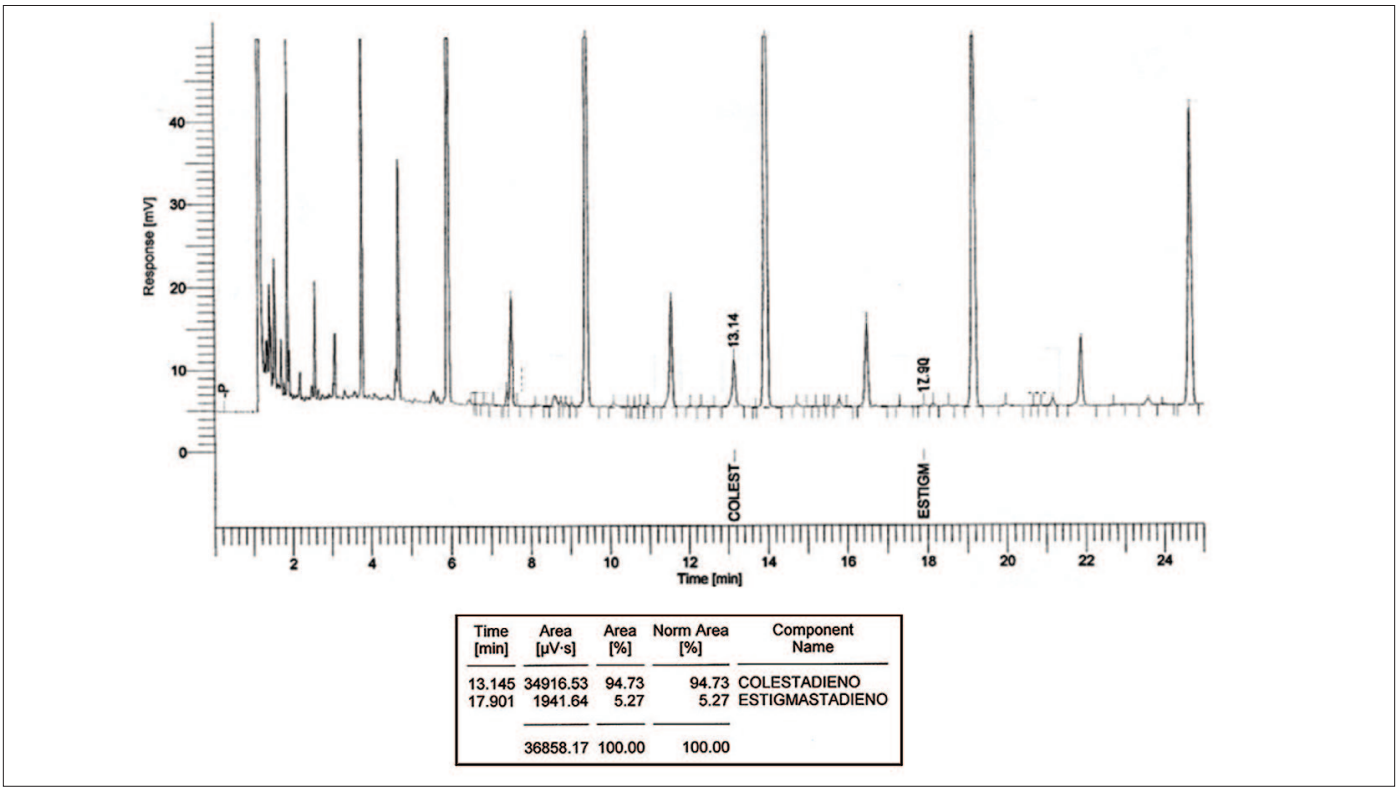
| | |
|---|---|
| Gas Chromatograph: | Clarus 580 GC with PPC |
| Injector: | Split/Splitless Injector |
| Detector: | FID Flame Ionization Detector |
| Software: | TotalChrom® Computing and Instrumentation Management Software |
| Columns: | Elite 5, 30 m; 0.25 µm, 0.25 mm Part No. N9316076 |

*Table 2.* Analytical Method.

| | |
|---|---|
| Injection Volume: | 1.0 µL |
| Injector Temperature: | 300 ˚C |
| Column Temperature: | 320 ˚C |
| Heating Ramp: | 235 ˚C (held 6 min)<br>2 ˚C/min up to 285 ˚C<br>285 ˚C (held 10 min) |
| Carrier Flow: | 40 psi (Split = 20 mL/min) |

## Chromatogram

The internal standard peak appears after about 13 min, whereas the experimental conditions employed present a retention time of 17.9 minutes for the 3,5-stigmastadiene. This compoound is often associated with small quantities of its isomer, but usually during the analysis, these two compounds originate a single chromatographic peak. If two peaks are obtained, the result should be given as the sum of the two areas and expressed, referring to the internal standard, as mg/kg.




| Time [min] | Area [µV·s] | Area [%] | Norm Area [%] | Component Name |
|---|---|---|---|---|
| 13.145 | 34916.53 | 94.73 | 94.73 | COLESTADIENO |
| 17.901 | 1941.64 | 5.27 | 5.27 | ESTIGMASTADIENO |
| | 36858.17 | 100.00 | 100.00 | |

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APPLICATION BRIEF

Gas Chromatography

# Total Sterols and Uvaol + Erythrodiol

## Introduction

The sterol composition is used as key quality and authenticity indicators that essentially fingerprint olive oils.

Erythrodiol (commonly understood as the set of erythrodiol and uvaol diols) is a constituent of the unsaponifiable fraction, characteristic of some types of fats. Its concentration is significantly higher in the olive oil resulting from chemical extraction, if compared to other oils that contain it (pressed olive oil, grapeseed oil). Therefore its determination can be used to detect the presence of refined oil in virgin olive oil.

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*Table 1.* Sterolic compounds detectable in olive oil.

| Peak | Identification | |
|---|---|---|
| 1 | cholesterol | Δ-5-cholesten-3β-ol |
| 2 | cholesterol | 5α-cholestan-3β-ol |
| 3 | brassicasterol | [24S]-24-methyl-Δ-5,22-cholestadien-3β-ol |
| 4 | 24-methylene-cholesterol | 24-methylene-Δ-5,24-cholesten-3β-ol |
| 5 | campesterol | [24R]-24-methyl-Δ-5-cholesten-3β-ol |
| 6 | campesterol | [24R]-24-methyl-cholestan-3β-ol |
| 7 | stigmasterol | [24R]-24-ethyl-Δ-5,22-cholastadien-3β-ol |
| 8 | Δ-7-campesterol | [24R]-24-methyl-Δ-7-cholesten-3β-ol |
| 9 | Δ-5,23-stigmastadienol | [24R,S]-24-ethyl-Δ-5,23-cholestadien-3β-ol |
| 10 | chlerosterol | [24S]-24-ethyl-Δ-5,25-cholastadien-3β-ol |
| 11 | β-sotosterol | [24R]-24-ethyl-Δ-5-cholestan-3β-ol |
| 12 | sitostanol | 24-ethyl-cholestan-3β-ol |
| 13 | Δ-5-avenasterol | [24Z]-24-ethylidene-5-cholesten-3β-ol |
| 14 | Δ-5,24-stigmastedienol | [24R,S]-ethyl-Δ-5,24-cholestadien-3β-ol |
| 15 | Δ-7-stigmastenol | [24R,S]-24-Ethyl-Δ-7,24-cholestadien-3β-ol |
| 16 | Δ-7-avenasterol | [24Z]-24-ethyliden-Δ-7-cholesten-3β-ol |

## Principle of the Method

The sterol determination is carried out by gas chromatography using FID as detector.

The analysis is not possible on untreated sample, but should be carried out on a purified extract of the unsaponifiable fraction obtained by two steps briefly summarized below.

*Table 2.* Instrumention.

| | |
|---|---|
| Gas Chromatograph: | Clarus 580 GC with PPC |
| Injector: | Split/Splitless Injector |
| Detector: | Flame Ionization Detector (FID) |
| Software: | TotalChrom® Computing and Instrumentation Management Software |
| Column: | Elite 5; 30 m, 0.25 µm, 0.25 mm Part No. N931-6076 |

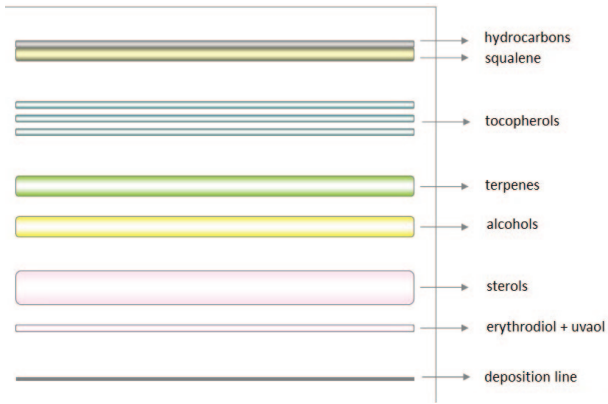
*Figure 1.* Clarus 580 GC.

## Sample Preparation

For the analysis of the oil, sample must be suitably prepared as summarized below:

1. The oil (with α-cholestanol added as internal standard) is subjected to saponification with 2 N KOH in ethanolic solution; the unsaponifiable matter is extracted with ethyl ether and recovered after phase separation.

2. The sterol fraction is separated from the extract by thin-layer chromatography (TLC), using basic silica gel plates and an eluent phase of hexane/ethyl ether 65/35 or benzene/acetone 95/5. The bands obtained are highlighted by spraying the plate with 2',7'-dichlorofluorescein; sterols are then recovered from the silica gel by scraping its corresponding and from the plate.




Since sterols are not volatile enough to be directly analyzed in gas chromatography, after recovery from TLC plate a to trimethylsilyl derivatization is required prior to the GC-FID analysis; the reaction occurs with (CH3)3Si-Cl trimethylsilyl chloride in pyridine:

**R-OH + (CH3)3Si-Cl ⟹ RO-Si(CH3)3**

*Table 3.* Analytical Method.

| | |
|---|---|
| Injection volume: | 1.0 µl |
| Injector temperature: | 290 °C |
| Column Temperature: | 300 °C |
| Heating Ramp: | 270 °C (held 30 min) |

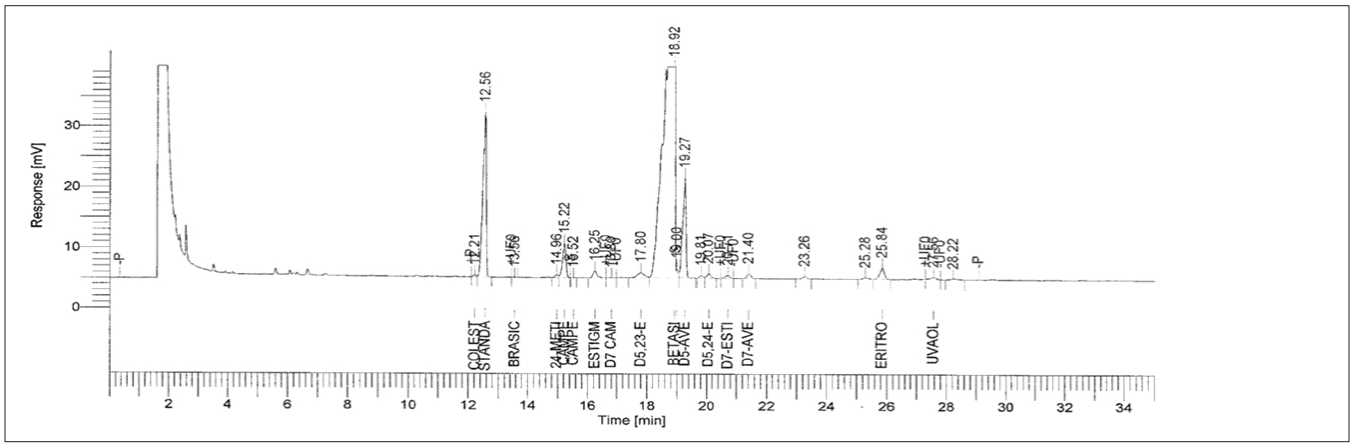



*Figure 2.* The chromatogram obtained, with the peaks of sterols indicated in Table 1 as well as those of Uvaol and Erythrodiol (in the last part of the chromatogram). The 12.56' peak is referred to the α-cholestanol internal standard.

*Table 4.* Percentage of individual sterols in the total sample (excluding erythrodiol and uvaol).

| Group Report for: ERI+UVA | | | | | | |
|---|---|---|---|---|---|---|
| Peak # | Time (min) | Area | Area (%) | BL | ISTD Amt. Ratio | Component Name |
| 1 | 12.214 | 2007.02 | 0.13 | BV | 1.8683 | Cholesterol |
| 3 | 13.557 | 257.41 | 0.02 | MM | 0.2396 | Brassicasterol |
| 4 | 14.963 | 3455.54 | 0.23 | BV | 3.2167 | 24-Methyl Cholesterol |
| 5 | 15.224 | 44296.74 | 2.96 | VB | 41.2350 | Campesterol |
| 6 | 15.520 | 234.40 | 0.02 | *BB | 0.2181 | Campestanol |
| 7 | 16.250 | 11737.39 | 0.78 | BB | 10.9261 | Stigmasterol |
| 8 | 16.800 | 489.80 | 0.03 | MM | 0.4559 | D7 Campesterol |
| | 19.000 | 1396687.88 | 93.27 | | 1300.1508 | Beta Sitosterol |
| 15 | 20.715 | 5067.99 | 0.34 | MM | 4.7177 | D7 Stigmasterol |
| 16 | 21.399 | 8247.20 | 0.55 | BB | 7.6772 | D7 Avenasterol |
| 19 | 25.843 | 20704.83 | 1.38 | VB | 19.2737 | Erythrodiol |
| 20 | 27.563 | 4219.17 | 0.28 | MM | 3.9276 | Uvaol |
| | | 1497405.36 | 100.00 | | 1393.9069 | |

$$\% \text{ of sterol x} = \frac{A_x}{\Sigma A} \cdot 100$$

$A_x$ = peak area for x;

$\Sigma A$ = total peak area for sterols.

*Table 5.* Percentage of Uvaol and Erythrodiol of the total sterols.

| Group Report for: Sterols | | | | | | |
|---|---|---|---|---|---|---|
| Peak # | Time (min) | Area | Area (%) | BL | ISTD Amt. Ratio | Component Name |
| 1 | 12.214 | 2007.02 | 0.14 | BV | 1.8683 | Cholesterol |
| 3 | 13.557 | 257.41 | 0.02 | MM | 0.2396 | Brassicasterol |
| 4 | 14.963 | 3455.54 | 0.23 | BV | 3.2167 | 24-Methyl Cholesterol |
| 5 | 15.224 | 44296.74 | 3.01 | VB | 41.2350 | Campesterol |
| 6 | 15.520 | 234.40 | 0.02 | *BB | 0.2182 | Campestanol |
| 7 | 16.250 | 11737.39 | 0.80 | BB | 10.9261 | Stigmasterol |
| 8 | 16.800 | 489.80 | 0.03 | MM | 0.4559 | D7 Campesterol |
| | 19.000 | 1396687.88 | 94.85 | | 1300.1508 | Beta Sitosterol |
| 15 | 20.715 | 5067.99 | 0.34 | MM | 4.7177 | D7 Stigmasterol |
| 16 | 21.399 | 8247.20 | 0.56 | BB | 7.6772 | D7 Avenasterol |
| | | 1472481.37 | 100.00 | | 1370.7056 | |

$$\text{Erythrodiol \%} = \frac{A_1 + A_2}{A_1 + A_2 + \Sigma A_{sterols}} \times 100$$

$A_1$ = peak area for erythrodiol

$A_2$ = peak area for uvaol

$\Sigma A_{sterols}$ = total peak area for sterols

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010212A_01

APPLICATION BRIEF

Gas Chromatography

# Determination of the Acidic Composition of Triglycerides and Trans Fatty Acids

## Introduction

The saponifiable fraction of olive oil is composed primarily of triglycerides, consisting of one molecule of glycerol on which are esterified up to three fatty acid chains (all with an even number of carbon atoms) saturated and unsaturated. Since the biosynthesis of fatty acids within the plant is variable depending on the species considered, the presence of fatty acids normally absent in the olive oil is indicative of adulteration of the product with oils obtained from different seeds. The percentage composition of the other fatty acids commonly present in olive oil (oleic, linoleic, palmitic, etc.) is also used for determining the ECN42, in the calculation of theoretical composition of triglycerides. The chromatographic separation on an appropriate capillary column allow to separate and quantify not only the normal (cis) fatty acid, but also their *trans*-isomers. These compounds are important markers of heat treatments (deodorization) or refining (e.g. activated carbons).

## Principle of the Method

Before proceeding to the determination of fatty acids by GC-FID, a glycerides hydrolysis should be performed to cleave the free fatty acids linked to glycerol. In this way, the fatty acids can be transformed (by a process of trans-esterification with methanol in basic conditions) in their respective methyl esters, which have a higher volatility and a lower polarity.

Methylated fatty acids can be injected into the column, separated and detected with FID. In this way it is possible to obtain a profile of the acidic composition of the oil in terms of fatty acid-derived methyl esters (FAMEs). The identification and quantification of individual fatty acids are carried out by comparing areas and retention times with those of the internal standard.

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## Sample Preparation

A possible sample preparation is described in the A Method (cold trans esterification) reported in the Annex X B of EC Regulation 2568/91 and can be summarized in the following steps:

1. Weigh 0.1 g of sample
2. Add 2 mL of heptane
3. Add 0.2 mL of 2 N methanolic KOH
4. Shake and recover the supernatant after stratification of the phases.

*Table 1.* Instrumentation.

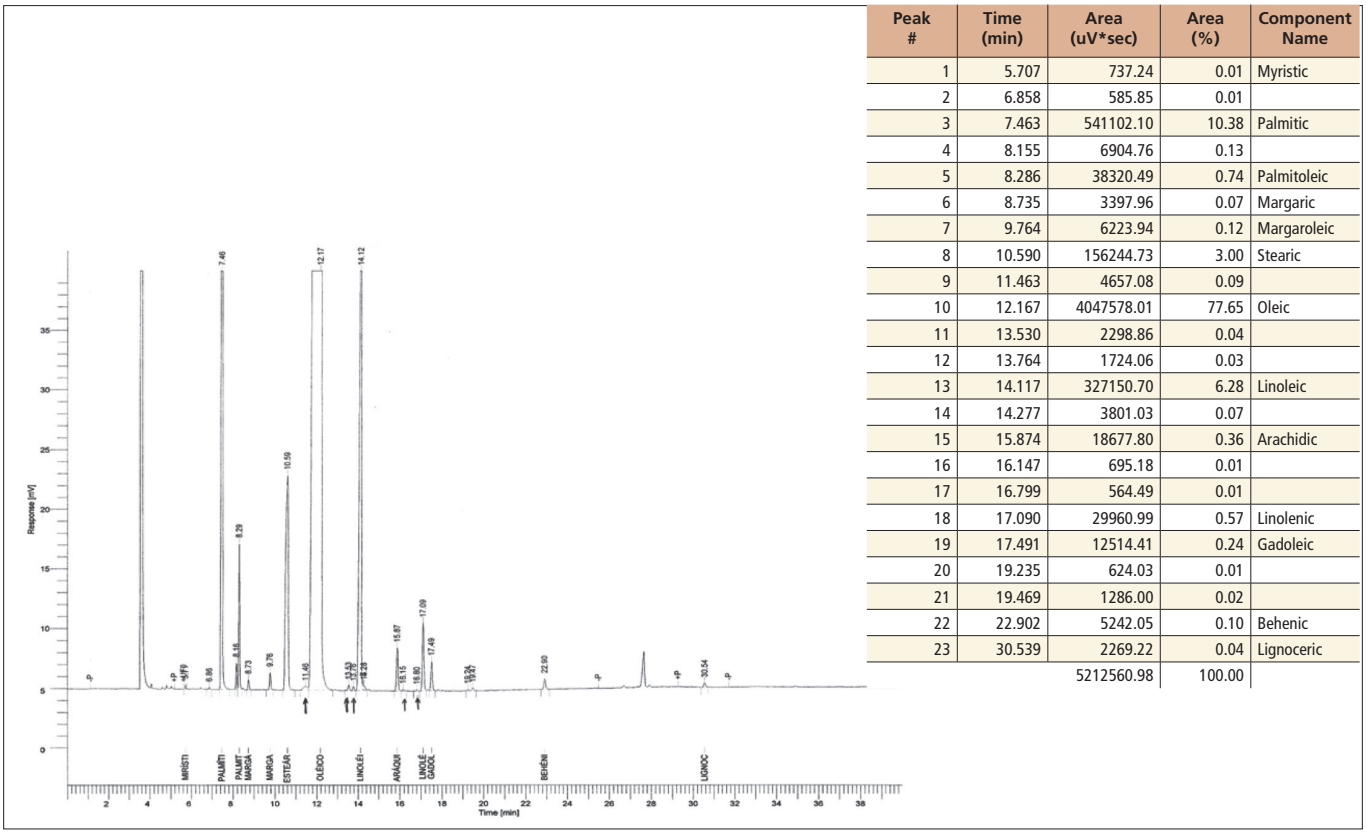
| | |
|---|---|
| Gas Chromatograph: | Clarus 580 GC with PPC |
| Injector: | Split/Splitless Injector |
| Detector: | FID Flame Ionization Detector |
| Software: | TotalChrom® Computing and Instrumentation Management Software |
| Columns: | Elite Column, 60 m; 0.25 µm, 0.25 mm Part No. N9316508 |

*Table 2.* Analytical Method.

| | |
|---|---|
| Injection Volume: | 1.0 µL |
| Injector Temperature: | 200 °C |
| Column Temperature: | 300 °C |
| Heating Ramp: | 170 °C (held 13 min)<br>1.5 °C/min up to 190 °C<br>190 °C (held 10 min) |

From the chromatogram analysis, it is possible to calculate the percentage composition of some Fatty Acids as required by the Regulation (Myristic, Linolenic, Arachidic, Eicosenoic, Behenic, and Lignoceric Acids). It is also possible to determine and express their concentration as a percentage of total fatty acids to calculate:

- The sum of TRANS isomers of Oleic Acid (C18:1);
- The sum of TRANS isomers of Linoleic and Linolenic Acids (C18:2 and C18:3).




| Peak # | Time (min) | Area (uV*sec) | Area (%) | Component Name |
|---|---|---|---|---|
| 1 | 5.707 | 737.24 | 0.01 | Myristic |
| 2 | 6.858 | 585.85 | 0.01 | |
| 3 | 7.463 | 541102.10 | 10.38 | Palmitic |
| 4 | 8.155 | 6904.76 | 0.13 | |
| 5 | 8.286 | 38320.49 | 0.74 | Palmitoleic |
| 6 | 8.735 | 3397.96 | 0.07 | Margaric |
| 7 | 9.764 | 6223.94 | 0.12 | Margaroleic |
| 8 | 10.590 | 156244.73 | 3.00 | Stearic |
| 9 | 11.463 | 4657.08 | 0.09 | |
| 10 | 12.167 | 4047578.01 | 77.65 | Oleic |
| 11 | 13.530 | 2298.86 | 0.04 | |
| 12 | 13.764 | 1724.06 | 0.03 | |
| 13 | 14.117 | 327150.70 | 6.28 | Linoleic |
| 14 | 14.277 | 3801.03 | 0.07 | |
| 15 | 15.874 | 18677.80 | 0.36 | Arachidic |
| 16 | 16.147 | 695.18 | 0.01 | |
| 17 | 16.799 | 564.49 | 0.01 | |
| 18 | 17.090 | 29960.99 | 0.57 | Linolenic |
| 19 | 17.491 | 12514.41 | 0.24 | Gadoleic |
| 20 | 19.235 | 624.03 | 0.01 | |
| 21 | 19.469 | 1286.00 | 0.02 | |
| 22 | 22.902 | 5242.05 | 0.10 | Behenic |
| 23 | 30.539 | 2269.22 | 0.04 | Lignoceric |
| | | 5212560.98 | 100.00 | |

*Figure 1.* Chromatograph.

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010206A_01

APPLICATION BRIEF

Liquid Chromatography

# Determination of ΔECN42 and Trilinolein Content

## Introduction

The composition of triglyceride is a function of elementary acidic composition of the oil. By means of suitable mathematical calculations, it is possible to obtain the expected (or theoretical) composition of triglycerides, starting from the acid composition (GC-FID of methyl esters). The trilinolein content, calculated by theoretical method and measured by HPLC-RI, allows calculating the ΔECN42, i.e. the percentage difference between these two values.

## Principle of the Method

Comparing the expected value with the measured one obtained by HPLC-RI for some of the classes of triglycerides, it is possible to identify adulteration of the oil with other oils having a higher content of linolenic acid (seed oils). For olive oil, this type of analysis is focused only on certain characteristic categories of triglycerides, i.e. those having an equivalent number of carbon atoms (ECN) equal to 42. The ECN is calculated by counting the carbon atoms which constitute fatty acids and subtracting 2 for each double bond contained in the chain. The possible triplets of fatty acids forming a molecule with ECN equal to 42 are listed in the Fig 1. Usually they originate only 3 peaks in HPLC.

The percentage difference between the theoretical content (calculated from the acidic composition assayed as described previously) and the actual content of triglycerides with ECN42 (measured by HPLC-RI) provides the ΔECN42 parameter. In case of adulteration of olive oils (naturally lacking in linolenic acid) with other oils rich in linolenic acid (seed oils), the ΔECN42 value increases because the determined trilinolein content is higher than that calculated by theoretical method from the acid composition.

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## Sample Preparation

For the quantitative determination of triglycerides, the sample must be suitably prepared for HPLC-RI analysis. 2.5 grams of oil are weighed and diluted in 20 mL of elution mixture 87:13 (v/v) of petroleum ether and ethyl ether. This compound is then purified on silica gel eluting 150 mL of eluent mixture, evaporated to dryness with a rotavac, and then weighed and re-suspended in acetone (up to a 5% concentration). The extract thus obtained can be injected into the HPLC-RI system for the separation and quantification of triglycerides. A faster preparation protocol that can be used for routine analysis of edible oil (when official method is not required) is based simply on the dilution of 0.5 g of filtered oil sample into 10 mL of proprionitrile and direct injected in HPLC system.

*Table 1.* Instrumentation.

| | |
|---|---|
| Liquid Chromatograph: | Flexar HPLC |
| Detector: | Flexar RI Detector |
| Software: | TotalChrom® Computing and Instrumentation Management Software |
| Columns: | Analytical C18 5 µm; length 250 mm x 4.6 mm; 110Å Carbon load 13%; end capped Part No. N9303514 |

*Table 2.* Analytical Method.

| | |
|---|---|
| Injection volume: | 20 µL |
| Column temperature: | room temperature |
| Solvents: | acetone (A):acetonitrile (B) |
| Gradient: | isocratic 50:50 |
| Flow: | 1.5 mL/min |

For the determination of triacylglycerols with ECN42, the first group of three peaks (See Fig.1 and Table 3) representing them should be well separated. The content of triglycerides ECN42 is expressed as percentage of total triglycerides present (up to ECN 54).

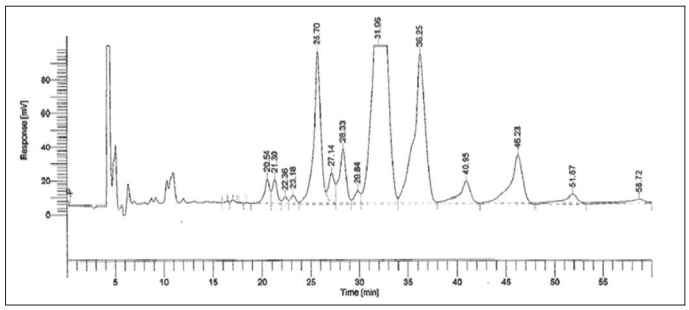


*Figure 1.* Chromatogram.

*Table 3.* Peak table.

| Peak # | Time (min) | Area (uV*sec) | Area (%) |
|---|---|---|---|
| 5 | 20.537 | 545274.77 | 1.38 |
| 6 | 21.298 | 503325.06 | 1.27 |
| 7 | 22.359 | 127740.75 | 0.32 |
| 8 | 23.184 | 187789.49 | 0.47 |
| 9 | 25.697 | 4523907.92 | 11.43 |
| 10 | 27.143 | 689213.50 | 1.74 |
| 11 | 28.331 | 1543488.30 | 3.90 |
| 12 | 29.838 | 312383.93 | 0.79 |
| 13 | 31.965 | 19457911.25 | 49.14 |
| 14 | 36.249 | 7235378.36 | 18.27 |
| 15 | 40.946 | 1075540.16 | 2.72 |
| 16 | 46.228 | 2521006.47 | 6.37 |
| 17 | 51.867 | 514188.93 | 1.30 |
| 18 | 58.722 | 265018.64 | 0.67 |
| | | 39596276.23 | 100.00 |

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APPLICATION BRIEF

Gas Chromatography

# Determination of 2-Glyceryl monopalmitate

## Introduction

The biosynthesis of triglycerides is a process mediated by the action of site-specific enzymes operating the esterification of glycerol with the fatty acid chains. The specificity in the action of these enzymes implies that, in the triglycerides of olive oils, only about 2% of glycerides linked in position 2 of glycerol are represented by palmitic acid. In the case of esterified oils artificially processed, the attack occurs in a nonspecific random manner resulting in a substantial increase of this percentage.

## Principle of the Method

The concentration of 2-glyceryl monopalmitate is determined after enzymatic digestion of triglycerides with pancreatic lipase (Fig. 1), which hydrolyzes only the ester bonds in positions 1 and 3, leaving intact the bond in position 2 of glycerol. The GC-FID analysis allows quantifying the percentage of 2-glyceryl-monopalmitate among all the 2-monoglycerides obtained from the digestion. The identification of the compounds takes place by comparison of retention times with those of the reference standard.

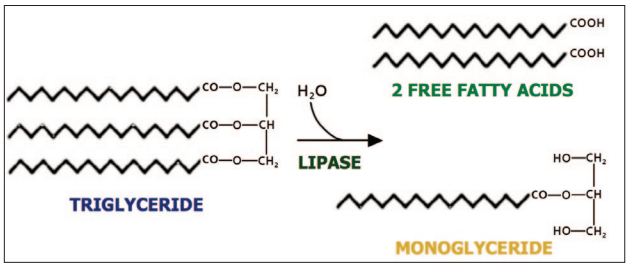


*Figure 1.* Triglycerides Enzymatic Digetion.

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## Sample Preparation

The preparation of the sample consists of several steps. The basic steps are summarized below:

- If the oil to be analyzed does not have a free acidity of more than 3%, it is possible to proceed with the sample preparation without performing any neutralization;
- 1 gram of oil is diluted in 10 mL of n-exane/diethyl ether 87/13, to be subsequently loaded (1 mL) on a 500 mg SPE cartridge. After this, an elution with 4 mL of n-hexane/ethyl ether 9/1 is performed and the eluate is dried in a nitrogen stream;
- The enzymatic digestion occurs by adding a suitable buffer and the pancreatic lipase, working at 40 °C under shaking;
- The glyceride fraction is recovered in 1 mL of ethyl ether by extraction and centrifugation of the lysate;
- 100 µL of supernatant are subjected to silanization to make the 2-monoglycerides volatile; then, after recovering the analytes in 5 mL of hexane, it is possible to perform the GC-FID analysis.

*Table 1.* Instrumentation

| | |
|---|---|
| Gas Chromatograph: | Clarus® 580 GC with PPC |
| Injector: | On-Column or PPS Injector |
| Detector: | Flame Ionization Detector (FID) |
| Software: | TotalChrom® Computing and Instrumentation Management Software |
| Columns: | Elite 5-HT (15 m x 0.32 mm I.D. x 1 µm film) Part No. N9326274 |

*Table 2.* Analytical method

| | |
|---|---|
| Injection volume: | 0.5 - 1.0 µL (on column) |
| Injector temperature: | < 68 °C (temperature of solvent evaporation) |
| Column temperature: | 350 °C |
| Heating Ramp: | 60 °C (1 min)<br>Up to 180 °C (15 °C/min)<br>Up to 340 °C (5 °C/min)<br>340 °C for 13 min |
| Flow: | 20 psi |

## Chromatogram

From the chromatogram (Fig. 2) it is possible to obtain the peak area relative to the 2-glyceryl monopalmitate and the result is expressed as a percentage area of the total of areas represented by monoglycerides in the sample.

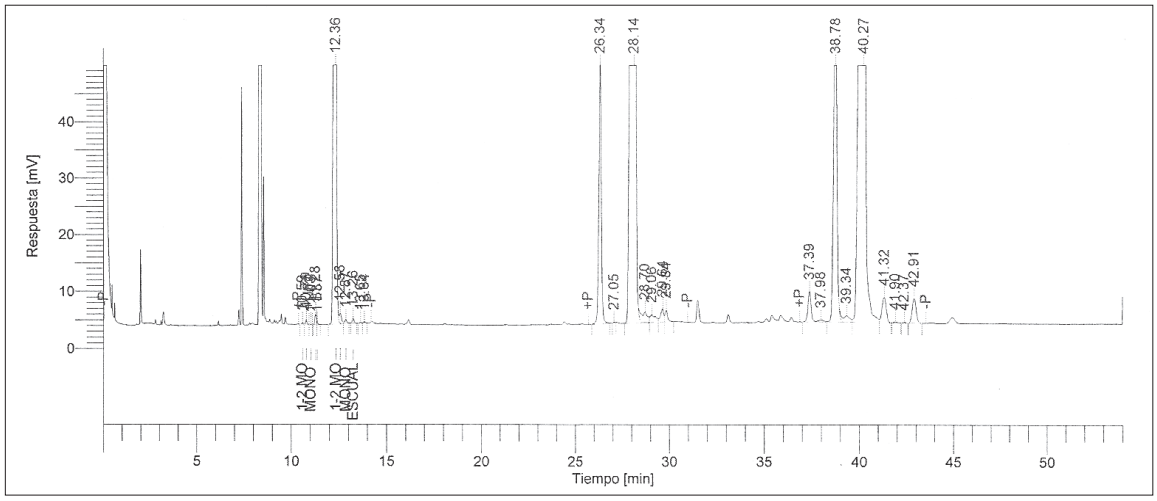


*Figure 2.* Chromatogram

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010203A_01

APPLICATION NOTE

UV/Vis Spectroscopy

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# Determination of Olive Oil Purity and Degree of Oxidation using the LAMBDA XLS

## Introduction

Olive oil is well established in the food industry. Demand continues to grow not only because of its distinct flavor, but also because of an increased awareness of its health benefits. In fact, the FDA allows producers of olive oil to place a health claim on their products because there is some scientific evidence to support a risk reduction of coronary heart disease by consuming a higher proportion of monounsaturated fat in one's diet. This is significant because olive oil is considerably rich in monounsaturated fats, most notably oleic acid. It is therefore of interest to producers to know the quality of the oil, its state of preservation, and changes brought about in it by technological processes.

The quality of the olive oil is studied by measuring the characteristics of the absorption bands between 200 and 300 nm. These are frequencies related to conjugated diene and triene systems. A low absorption in this region is indicative of a high-quality extra virgin olive oil, whereas adulterated/refined oils show a greater level of absorptions in this region.

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## Instrumentation

The PerkinElmer LAMBDA™ XLS UV/Vis spectrophotometer, shown in Figure 1, is a standalone, robust scanning spectrophotometer with no moving parts and a unique Xenon® Lamp Source (XLS) with a typical lifetime of five years.

The LAMBDA XLS is equipped with a large LCD screen making running methods and viewing data easier. Results can be printed, stored, or exported into Microsoft® Excel® for use on your personal computer.

## Method

Olive oil samples were diluted in iso-octane (2,2,4- trimethylpentane). All samples were measured in matched, synthetic fused silica cuvettes (10 mm is the recommended pathlength) running a solvent blank as a reference.

Absorption measurements for purity determination were made at 232, 266, 270 and 274 nm. K values were calculated according to the equation shown in Figure 2.

The purity of olive oil can be determined from three parameters:

- K232 absorbance at 232 nm
- K270 absorbance at 270 nm
- Delta K (Figure 3)

The LAMBDA XLS was used to collect UV data from four different label graded commercial olive oil samples.

## Results

The results collected using the LAMBDA XLS for the four different graded olive oils are shown in Table 1.

## Conclusion

The LAMBDA XLS is a reliable and cost effective system appropriate for keeping up with regulations around the standard method for measuring olive oil purity using a UV spectrophotometric technique.

*Table 1.* Measured and Permitted K Values of Commercial Olive Oils.

| | Measured K Values | | | | |
|---|---|---|---|---|---|
| Olive Oil Sample Type | K232 | K266 | K270 | K274 | Delta K |
| Extra Virgin - Sample 1 | 1.897 | 0.151 | 0.148 | 0.135 | 0.005 |
| Extra Virgin - Sample 2 | 1.717 | 0.201 | 0.189 | 0.173 | 0.002 |
| Virgin - Sample 3 | 1.436 | 0.240 | 0.248 | 0.223 | 0.016 |
| Olive Oil - Sample 4 | 3.000 | 0.640 | 0.832 | 0.458 | 0.283 |


*Figure 1.* LAMBDA XLS UV/Vis spectrophotometer.

$$K\lambda = \frac{Abs\lambda}{D \times L}$$

D = Dilution gr/L

L = cuvette pathlength

*Figure 2.* K equation for λ nm.

$$\text{Delta K} = K_{270} - \frac{K_{266} + K_{274}}{2}$$

*Figure 3.* Delta K equation.

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APPLICATION BRIEF

Gas Chromatography

# Determination of Aliphatic Alcohols

## Introduction

The aliphatic alcohols are contained in the unsaponifiable fraction of olive oil. These molecules have an even number of carbon atoms (from 20 to 30) mostly located inside the stone and are partially extracted by mechanical processes. Their content in the oil may be increased where extraction is performed with solvents (as in the case of refined oil) and therefore the concentration of these compounds, in combination with other parameters (erythrodiol, uvaol and wax content) allows to distinguish crude olive-residue oil from lampante oil.

## Principle of the Method

Oil is added with 1-eicosanol as internal standard prior to proceed to cold saponification with potassium hydroxide. The unsaponifiable fraction is then recovered with ethyl ether, and the fraction of the alcohol is subsequently separated by thin-layer chromatography. After recovery from TLC plate the alcohols are derivatized to trimethylsilyl ethers and analyzed by capillary gas chromatography.

## Sample Preparation

It starts from 5 grams of oil, to which is added an appropriate amount of 1-eicosanol (internal standard); the sample is then added with 50 mL of 2N KOH in ethanol and the saponification reaction is completed through stirring and water additions. Finally, the unsaponifiable fraction is extracted in ethyl ether. The fraction obtained is dried and resuspended in chloroform in order to reach a concentration equal to about 5% (m/v). 100 µL of the extract thus obtained are subjected to thin-layer chromatography using as mobile phase a 65:35

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(v/v) hexane/ethyl ether solution to separate the fraction of the aliphatic alcohols. After plate development with 2'7'-dichlorofluorescein, the alcohol band can be recovered from the plate to carry out the derivatization process with pyridine-hexamethyldisilazane-trimethylchlorosilane 9:3:1 (v/v/v) in order to obtain trimethylsilyl ethers, and then proceed to perform the gas chromatographic analysis on the same column used for the determination of sterols and erythrodiol.

*Table 1.* Instrumentation.

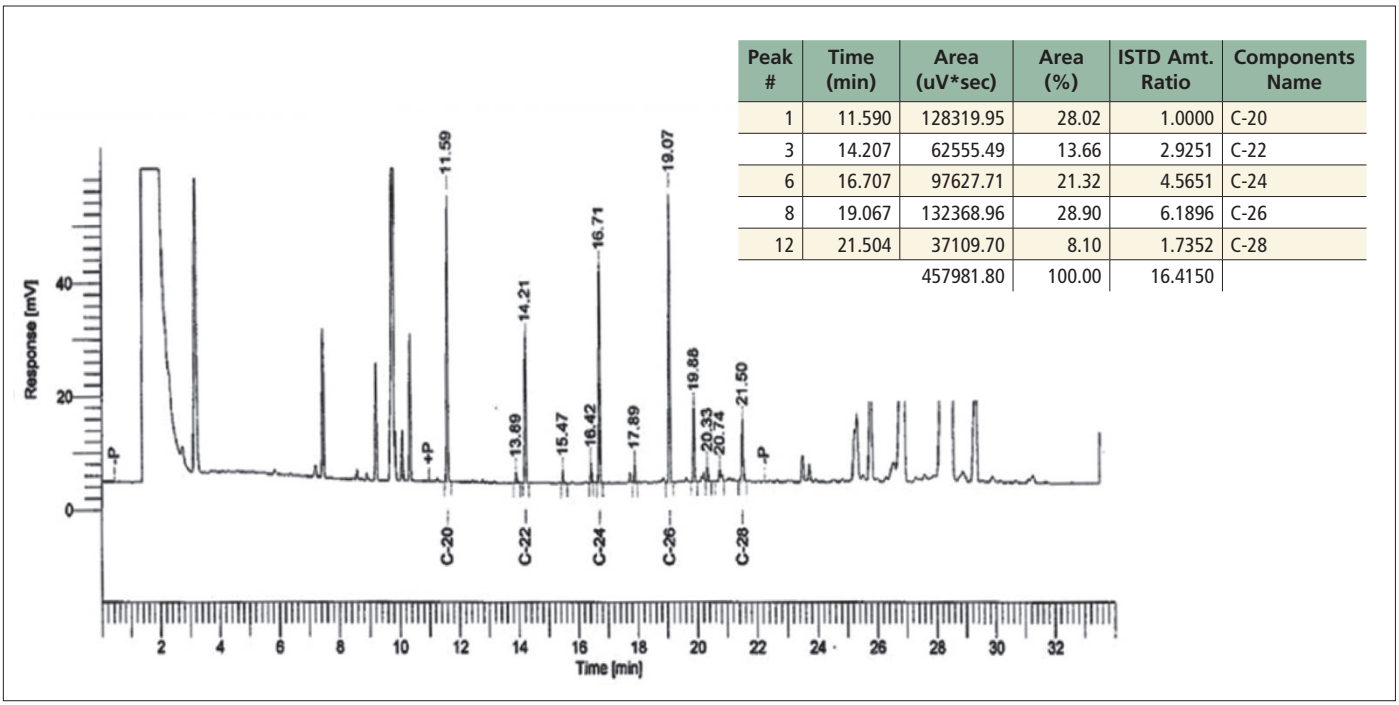
| | |
|---|---|
| Gas Chromatograph: | Clarus 580 GC with PPC |
| Injector: | Split/Splitless Injector |
| Detector: | FID Flame Ionization Detector |
| Software: | TotalChrom Computing and Instrumentation Management Software |
| Columns: | Elite 5; 30 m, 0.25 µm, 0.25 mm<br>Part No. N9316076 |

*Table 2.* Analytical Method.

| | |
|---|---|
| Injection Volume: | 1.0 µL |
| Column Temperature: | 290 °C |
| Column Temperature: | 300 °C |
| Heating Ramp: | 190 °C (held 2 min)<br>Up to 295 °C (6 °C/min)<br>295 °C (held 14 min) |

## Chromatogram

The chromatogram obtained by GC-FID is similar to that reported in the following figure. The internal standard peak (C 20) has a retention time of 11.59 minutes in the operating conditions applied. The content of total aliphatic alcohols is quantified as the sum of the C-22, C-24, C-26 and C-28 peaks, and expressed as equivalent mg of standard per kg of sample.




| Peak # | Time (min) | Area (uV*sec) | Area (%) | ISTD Amt. Ratio | Components Name |
|---|---|---|---|---|---|
| 1 | 11.590 | 128319.95 | 28.02 | 1.0000 | C-20 |
| 3 | 14.207 | 62555.49 | 13.66 | 2.9251 | C-22 |
| 6 | 16.707 | 97627.71 | 21.32 | 4.5651 | C-24 |
| 8 | 19.067 | 132368.96 | 28.90 | 6.1896 | C-26 |
| 12 | 21.504 | 37109.70 | 8.10 | 1.7352 | C-28 |
| | | 457981.80 | 100.00 | 16.4150 | |

*Figure 1.* Chromatogram.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APPLICATION BRIEF

Liquid Chromatography

# Group Separation of Total Sterols and Aliphatic Alcohols in Olive Oil by HPLC

## Introduction

Some official analytical methods (e.g. determination of total Sterols and Aliphatic Alcohols) within the sample preparation provide a separation phase performed through thin-layer chromatography with the purpose of separating, recovering and submitting to analysis the fractions of interest.

The thin-layer chromatography (TLC), while being recognized as a valid separation method, requires some skill by the operator (deposition of the sample, selection and recovery of the fractions) and can not be easily automated.

The separation of some fractions can be easily obtained using an HPLC system (e.g. the same used for the ΔECN42 parameter) equipped with a suitable column and an appropriate fraction collector.

## Principle of the Method

The fractions of aliphatic alcohols and that of Total Sterols contained in the unsaponifiable fraction are separated and collected automatically and then analyzed according to specific procedures.

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## Sample Preparation

The sample undergoes saponification process as previously described for aliphatic alcohols. The unsaponifiable fraction is recovered and diluted to 5% in n-hexane/ethyl ether 50/50, filtered and then injected in the HPLC system to separate and collect the different classes of compounds that compose the unsaponifiable fraction.

*Table 1.* Instrumentation.

| | |
|---|---|
| Liquid Chromatograph: | HPLC Flexar® |
| Injector: | Manual or Automatic Sample Injection System |
| Detector: | Refractive Index (RI) Detector |
| Sampler: | Fraction Collector Part No. N0911044 |
| Software: | TotalChrom Computing and Instrumentation Management Software |
| Columns: | Brownlee™ Analytical Silica Column Part No. N9303526 |

*Table 2.* Analytical Method.

| | |
|---|---|
| Injection Volume: | 200 µL |
| Column Temperature: | Room Temperature |
| Solvents: | n-hexane and Ethyl Ether |
| Gradient: | Isocratic 50:50 |
| Flow: | 1.2 mL/min |
| Detector: | Refraction Index |
| Column: | Analytical Silica 5 um; 250 mm x 4.6 mm Part No. N9303526 |

## Chromatogram

Applying the experimental conditions previously described (Tables 1 and 2) it is possible to collect two fractions (See Fig.1) that contain two of the major components of the unsaponifiable fraction:

- from 5 to 9.5 minutes the aliphatic alcohols can be recovered;
- from 9.5 to 25 minutes the sterols fraction, comprehensive of erytrodiol and uvaol can be recovered.

The other components of the unsaponifiable fraction are eluted in the first part of the chromatograms and are not separated with this protocol.

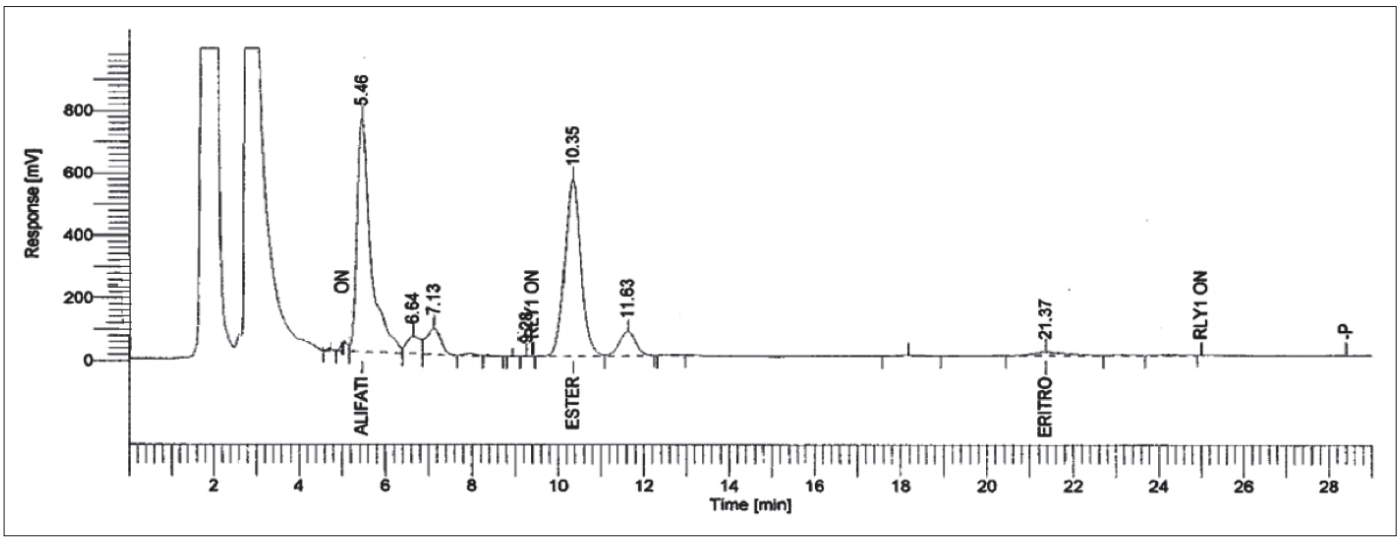


*Figure 1.* Chromatogram.

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APPLICATION NOTE

FT-IR Spectroscopy

**Authors:**

Ian Robertson

PerkinElmer, Inc.
Shelton, CT

# Advantages of Adulterant Screen for Detection of Olive Oil Adulteration by Attenuated Total Reflectance (ATR) FT-IR

## Introduction

Olive oil is an increasingly popular food product worldwide, with consumption in the U.S. alone having increased by about 50% in the last 10 years. Over three million tons annually of olive oil are produced worldwide, with approximately 75% of this being produced in Spain, Italy, and Greece. The U.S. now imports over 300,000 tons of olive oil annually.

Olive oil is considered to be healthy edible oil and is linked to the low incidence of heart disease associated with a Mediterranean diet. It is low in Saturated Fatty Acid (SFA) and Polyunsaturated fats (PUFA) but high in the healthier Monounsaturated fats (MUFA), known to lower cholesterol.

Extra Virgin Olive Oil (EVOO) is a premium product that can command a higher price than "standard" olive oils. This makes it highly susceptible to fraudulent activity. A report by the E.U. Committee on the Environment, Public Health, and Food Safety says olive oil is among the products most prone to food fraud. There were 267 oil adulteration incidents reported to the U.S. Pharmaceutical Food Fraud Database, with the vast majority occurring over the past three years.

Adulteration of EVOO with lower quality olive oils, or other lower cost edible oils, is frequently reported in the media. The most common adulterants include: hazelnut oil, sunflower oil, soybean oil, corn oil, rapeseed oil, and olive pomace oil. Fraudulent activities, such as dilution or even substitution with other lower cost oils containing additional chemicals, that enable the oil to appear to be of higher quality oil and pass routine screening tests are on the rise.

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This application note describes a fast, simple, low-cost solution to screen olive oils for adulteration.

## Materials and Methods

Mid-infrared spectroscopy is a well-established technique for the analysis of edible oil samples. The PerkinElmer Spectrum Two™ FT-IR, a high-performance compact FT-IR instrument utilizing the modern ATR sampling technique, offers fast and easy measurements of samples within the food industry. Diamond™ ATR accessories, such as the PerkinElmer Universal ATR (UATR), are extremely robust and allow the instrument to be used in the harshest of laboratories or even in remote environments. The Diamond ATR crystal requires only a very small volume of the sample to be tested and can easily be cleaned between samples, in situ, using laboratory tissue and a small amount of a suitable solvent, such as hexane for edible oils.

In this study the PerkinElmer Spectrum Two, equipped with a UATR sampling accessory, has been used to analyze a series of pure and adulterated olive oils and common adulterant spectra. A typical olive oil spectrum is shown in Figure 2. Spectra were recorded at 4 $cm^{-1}$ resolution with a scan time of one minute per sample.

The prominent features in the spectrum are the bands in the region of 2930 $cm^{-1}$ due to the –CH- stretch of the hydrocarbon chains and in the region of 1740 $cm^{-1}$ due to the carbonyl groups in the triglyceride.

## Discriminating Olive Oil from Other Edible Oil Types

The infrared spectra of different edible oils will be similar, only varying by the constituent chains on the triglyceride backbone, since their molecules contain the same chemical groups. However, there are small, observable differences between the different oil types. Figure 3 shows the ATR spectra of three different oil types: olive oil, sunflower oil, and rapeseed (canola) oil.

These spectral differences are significant enough to be able to develop a classification method for these different oils. There are a variety of ways to classify materials based on their infrared spectra. For this type of problem Soft Independent Modeling of Class Analogy (SIMCA), a Principal Components Analysis (PCA) based method, is a good approach to take. Building a SIMCA method requires the measurement of a variety of samples for each type of material you wish to classify. The calibration set of samples should cover all sources of variation normally encountered for that particular material, such as different sources, different batches, or different manufacturing processes. The method will build individual models to completely characterize each of the materials. Each material, in this case the individual oil types, generates its own cluster in this model that should be separated from the other clusters calculated for the other materials being classified. A SIMCA model has been generated for the three types of edible oils in this study. Figure 4 shows the SIMCA model with each oil having its individual cluster, clearly separated from those of the other materials.

*Figure 1.* The PerkinElmer Spectrum Two and UATR.

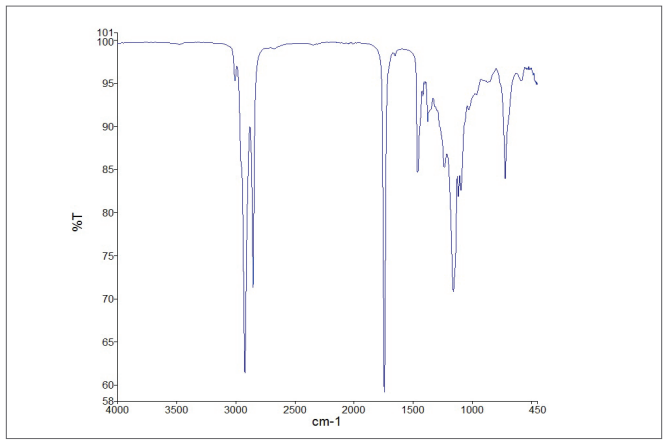


*Figure 2.* Diamond ATR spectrum of olive oil.

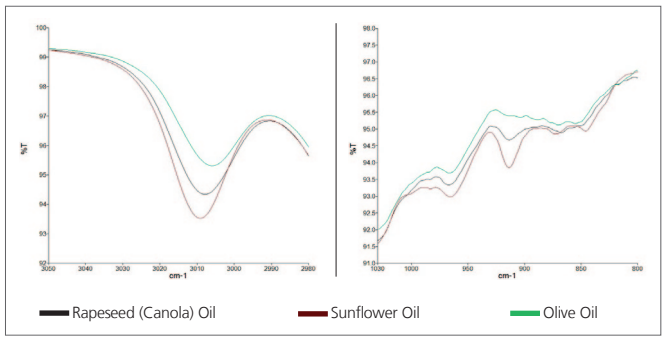


*Figure 3.* Spectral differences between olive oil, rapeseed oil and sunflower oil.

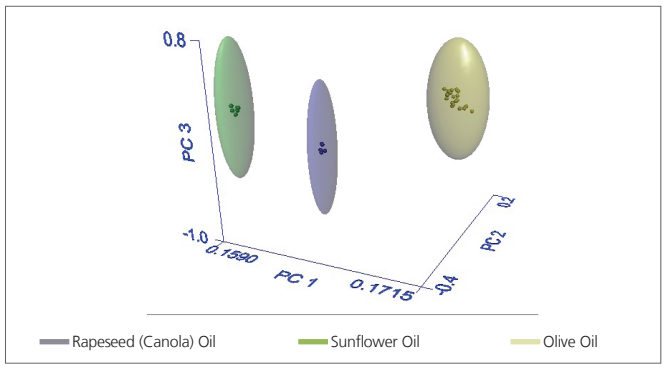


*Figure 4.* SIMCA model for three edible oil types. Olive oil, rapeseed oil, and sunflower oil.

Classifying a material consists of measuring the IR spectrum and using the SIMCA model to predict to which cluster the spectrum belongs. If the spectrum does not fall into one of the three classes of materials then it is likely to be a different material or contaminated/adulterated oil. Further data investigation would be required to determine the reason that the sample has failed the test.

## Quantifying Levels of Known Adulterants in Olive Oil

If the identity of the adulterant is known then it is possible to quantify the amount of adulterant present. This involves the preparation and measurement of the IR spectra of standard mixtures of the olive oil with the adulterant oil. The IR spectra for a series of standards are shown in Figure 5.

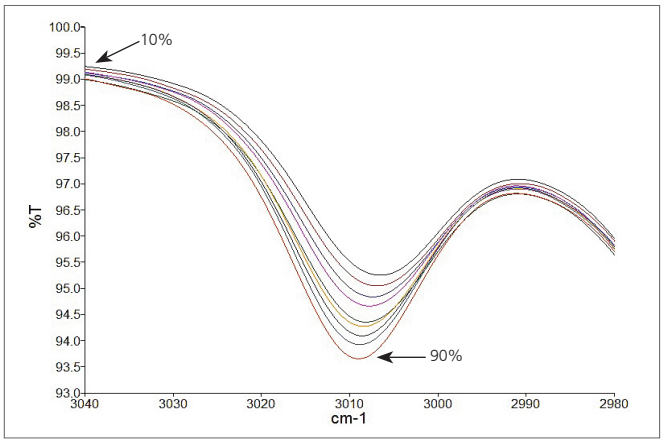


*Figure 5.* Standards from 10% - 90% Sunflower Oil.

Partial Least Squares (PLS1) Calibrations have been generated for mixtures of olive/sunflower oils and olive/rapeseed oils ranging from 0 to 100% olive oil. The calibrations are shown in Figure 6.

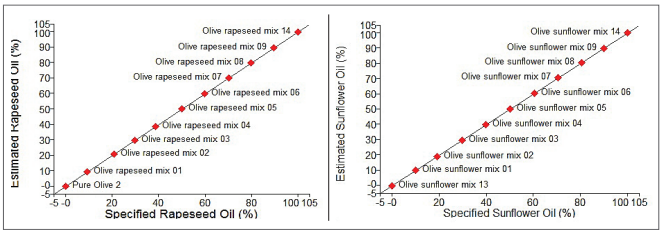


*Figure 6.* PLS1 Calibrations for Olive/Rapeseed and Olive/Sunflower oils.

An independent validation set of three samples were used to test the calibration model. The validation plot is shown in Figure 7.

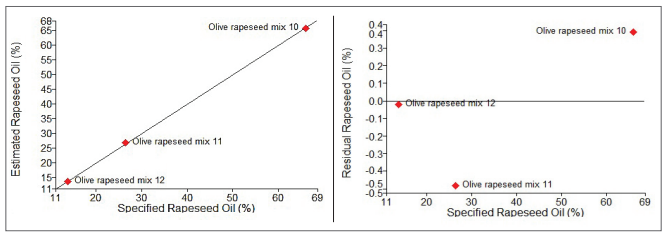


*Figure 7.* Independent validation samples for olive/rapeseed mixtures.

## Adulterant Screen™ Algorithm for Detecting "Known" and "New" Adulterants in Olive Oil

The two statistical approaches taken so far would allow for:
a.) checking that the material is the correct material (SIMCA) and
b.) quantifying the amount of a single, known adulterant (PLS).

An alternative approach is available using an Adulterant Screen Algorithm. The approach is simple:

1. Generate a library of unadulterated material samples spectra exactly as for SIMCA. This library should span as much as possible the natural variation of the material, due to differences between batches, suppliers or processing parameters, etc.
2. Generate spectra of adulterants of concern. These spectra should be of the pure adulterant material, not mixtures. (As new adulterant materials emerge these can easily be added to the adulterant library in the future.)

These two sets of spectra are registered in the software, and the method is ready to use.

In this study, a series of 24 olive oil spectra were measured from commercially purchased oils. These 24 spectra were used to generate a library of the unadulterated material. The objective of this study was to specifically look for adulteration with either sunflower or rapeseed oils. Single spectra of the two adulterants were measured and stored with the method. The Adulterant Screen method was tested using samples adulterated with known concentrations of the other oil types and also with pure olive oil. The results are shown in Table 1.

*Table 1.* Adulterant Screen results for a series of method validation standards.

| Sample Name | Adulterant | Level | Unidentified Components | Adulterant Screen Pass/Fail |
|---|---|---|---|---|
| Sunflower 18.66% Std | Sunflower Oil | 0.19208 | Probable | Fail |
| Sunflower 68.80% Std | Sunflower Oil | 0.69011 | Probable | Fail |
| Sunflower 38.10% Std | Sunflower Oil | 0.38183 | Probable | Fail |
| Sunflower 100.0% Std | Sunflower Oil | 1.00328 | Probable | Fail |
| Rapeseed 66.02% Std | Rapeseed Oil | 0.64944 | Probable | Fail |
| Rapeseed 26.41% Std | Rapeseed Oil | 0.26367 | Probable | Fail |
| Rapeseed 13.79% Std | Rapeseed Oil | 0.14083 | Probable | Fail |
| Rapeseed 100.0% Std | Rapeseed Oil | 0.99191 | Probable | Fail |
| Pure Olive Oil | No Adulterants | - | Unlikely | Pass |

In all cases, except the pure olive oil, the adulterated samples generated a "Fail" result indicating the presence of an adulterant. Not only does the Adulterant Screen algorithm correctly identify the adulterant, but it also gives an estimated level of that contaminant without the requirement for running quantitative calibration standards. The level of the contaminant is reported as the proportion of the total spectrum contribution arising from that component. The results table demonstrates the ability of this algorithm to classify like SIMCA and additionally provide approximate estimates of concentration of the adulterants without the need to generate extensive quantitative models.

When a sample spectrum is scanned, the algorithm first compares it to a PCA model generated from the reference materials. This model is then augmented with each of the adulterant spectra in turn. If including a given adulterant in the model greatly increases the fit of the sample spectrum, it is likely that the adulterant is actually present in the sample.

Figure 8 shows the residuals observed from the analysis of 13.79% rapeseed validation standard.

*Note: the spectral region from 2450-1850 $cm^{-1}$ (the region where the diamond absorptions due to the Diamond ATR are intense) was excluded from the method.*

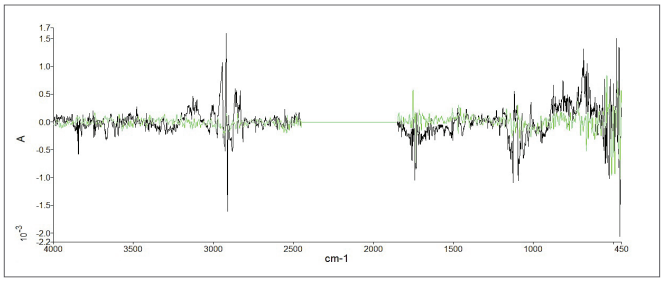


*Figure 8.* Spectral residuals before (black) and after (green) fitting adulterants.

In this case the residuals are significantly decreased by fitting the spectrum of the pure rapeseed oil indicating the presence of that adulterant in the sample.

## Summary

ATR-FT-IR on the Spectrum Two allows for a fast, easy, and low-cost method for screening olive oil samples for adulterants. The information required from the analysis will determine which will be the most appropriate data analysis method to use. Data has been demonstrated using three different approaches – SIMCA , PLS, and Adulterant Screen. These are summarized below:

SIMCA – Is the product what it says it is and does it fall within the expected variation within that class of material? If not, further data analysis will be required.

PLS - For known adulterants it is possible to generate complete quantitative calibrations by preparing suitable standard mixtures. This will give accurate quantitative results.

Adulterant Screen algorithm – Is the product what it says it is and has it been adulterated? If adulteration is likely then try to identify the adulterant from known adulterants and give a semi-quantitative measure of how much of the adulterant is present.

The Adulterant Screen algorithm offers significant benefits over the other two approaches:

### Faster method development

- The Adulterant Screen algorithm simply requires the collection of the spectra of the unadulterated material and the known adulterants.

### Simple upgrade of methods

- When new potential adulterants are identified they can simply be added to the library of adulterant spectra.

### Greater sensitivity than SIMCA

- Achieved by utilizing a library of spectra of potential adulterants.

Whichever statistical approach is utilized it can be deployed using a Spectrum Touch™ method, employing a simple user interface for the routine operator. Figure 9 is an example of the results screen for an adulterated sample.

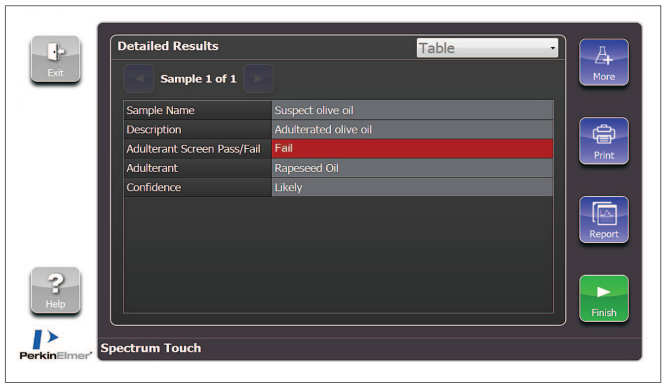


*Figure 9.* Spectrum Touch software showing result from Adulterant Screen.

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APPLICATION NOTE

Mass Spectrometry

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# Rapid Measurement of Olive Oil Adulteration with Soybean Oil with Minimal Sample Preparation Using DSA/TOF

## Introduction

Among edible oils, olive oil shows important and outstanding characteristics due to its differentiated sensorial qualities (taste and flavor) and higher nutritional value. It is an important oil that is high in nutritional value due to its high content of antioxidants (including vitamin E)[1]. Several health benefits, such as its ability to lower LDL cholesterol and its anti-inflammatory activity, associated with its consumption were initially observed among Mediterranean people[2,3]. Olive oil is one of the most adulterated food products of the world due to its relatively low production and higher prices as compared to vegetable and seed oils.

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Olive oil and other oils are composed mainly of triacylglycerols. These molecules are derived from the esterification of three fatty acid molecules with a glycerol molecule and these fatty acids determine the fatty acid composition of oils. Olive oil contains more oleic acid and less linoleic and linolenic acids than other vegetable and seed oils. Oleic acid is a monounsaturated fatty acid, whereas linoleic and linolenic acids are polyunsaturated fatty acids. The main fatty acids in olive oil are: oleic acid (65-85%), linoleic acid (4-15%), palmitic acid (7-16%) and linolenic acid (0-1.5%). The main fatty acids in soybean oil are: oleic acid (19-30%), linoleic acid (48-58%), palmitic acid (7-12%) and linolenic acid (5-9%)[4,5]. Therefore, the ratio of linoleic and linolenic acid to oleic acid in olive oil can be used as a way to detect its adulteration with soybean oil and other seed oils such as corn, safflower, sunflower and sesame oil, which have a higher content of linoleic and linolenic acids and lower amount of oleic acid in comparison to olive oil[6]. Using this strategy with the AxION® Direct Sample Analysis™ Time-of-Flight mass spectrometry system (DSA/TOF), we detected adulteration of olive oil with soybean oil.

The addition of vegetable and seed oils of low commercial and nutritional value to olive oil results in frequent problems for regulatory agencies, oil suppliers and consumers. A lot of scientific effort has been spent to develop rapid, reliable, cost effective analytical approach for measurement of adulteration of olive oils with other oils. In the past, methods employing gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) hyphenated to MS (HPLC/MS) have been implemented for this purpose[7,8,9,10]. These methods are time consuming, expensive and require extensive sample preparation, method development and derivatization. In this work, we demonstrated that the AxION Direct Sample Analysis (DSA™) system integrated with the AxION 2 Time-of-Flight (TOF) mass spectrometer can be used for rapid screening of adulteration of olive oil with soybean oil. The advantages of this method, compared to conventional techniques, are that no chromatography is required, the combination of direct sampling from the olive oil is done with minimal or no sample preparation and mass spectra results are generated in seconds.

## Experimental

Olive oil and soybean oil were purchased from a local supermarket. Both oils were diluted to 1% in iso-propanol with 10 mM ammonium acetate. After dilution, the oils were mixed in different proportions to simulate the adulteration of olive oil with soybean oil at different percentages of 5, 10, 25 and 50. All oils and their mixtures were measured with an AxION 2 DSA/TOF system with minimal sample preparation. Five µl of each sample was pipetted directly onto the stainless steel mesh of the AxION DSA system for ionization and analysis. The DSA/TOF experimental parameters were as follows: corona current of 5 µA and heater temperature of 350 °C. The AxION 2 TOF MS was run in negative ionization mode with flight voltage of 8000 V and capillary exit voltage of -120 V for the analysis. Mass spectra were acquired in a range of m/z 100-700 at an acquisition rate of 5 spectra/s. All samples were analyzed within 30 sec. To obtain excellent mass accuracy, the AxION 2 TOF instrument was calibrated before each analysis by infusing a calibrant solution into the DSA source at 10 µl/min.

## Results

Figure 1 and Figure 2 show the mass spectra for a 1% solution of olive oil and soybean oil in iso-propanol with 10 mm ammonium acetate in negative ion mode using DSA/TOF, respectively. The mass spectra shows that the fatty acids, oleic, linoleic and linolenic, are present in both oils, but their relative amount is different in the two oils. The data shows that the response ratio for linoleic acid to oleic acid (L/O) was 0.18 and 1.86 in olive oil and soybean oil, respectively. Also, the response ratio for linolenic acid to oleic acid (Ln/O) was 0.017 and 0.29 in olive oil and soybean oil, respectively. Therefore, the higher response ratio for linoleic and linolenic acid to oleic acid can be used to detect adulteration of olive oil with soybean vegetable oil using DSA/TOF. This is supported further by data in Figure 3 which shows that response ratio of linoleic and linolenic acid to oleic acid was higher roughly by a factor of 2 for olive oil adulterated with 10 % soybean vegetable oil in comparison to olive oil. Figures 4 and 5 show that the response ratio for linoleic acid and linolenic acid to oleic acid increased, with

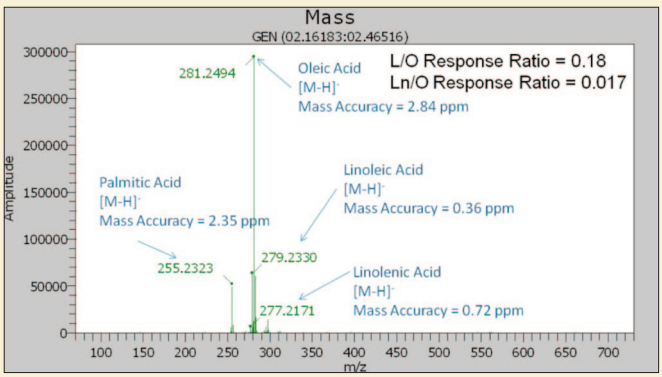


*Figure 1.* Mass spectra of olive oil diluted by a factor of 100 in negative mode using DSA/TOF.

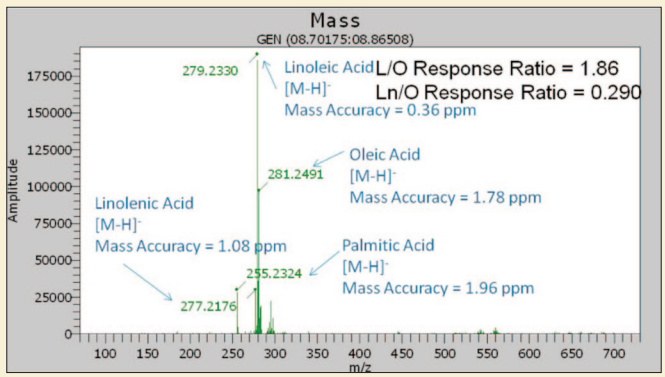


*Figure 2.* Mass spectra of soybean oil diluted by a factor of 100 in negative mode using DSA/TOF.

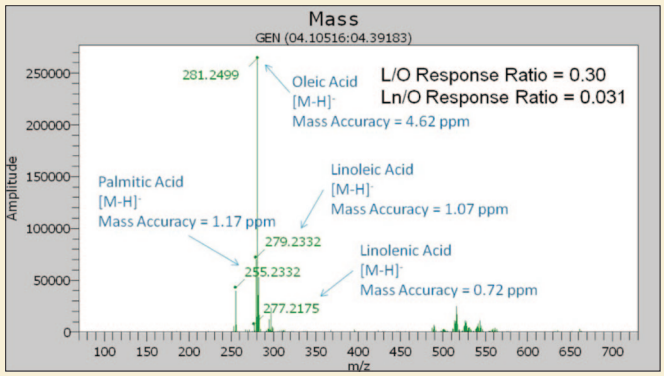


*Figure 3.* Mass spectra of olive oil adulterated with 10 % soybean oil diluted by a factor of 100 in negative mode using DSA/TOF.

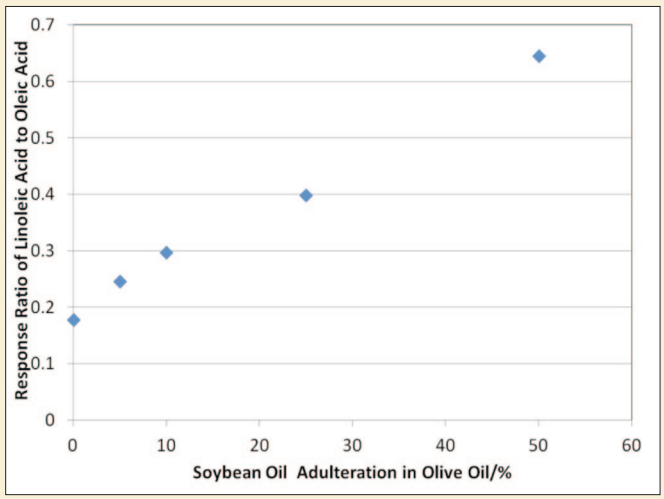


*Figure 4.* Effect of olive oil adulteration with different levels of soybean oil on response ratio of linoleic acid to oleic acid.

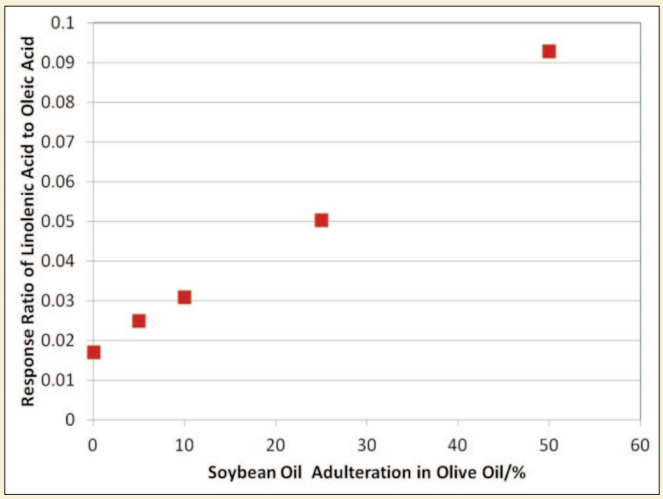


*Figure 5.* Effect of olive oil adulteration with different levels of soybean oil on response ratio of linolenic acid to oleic acid.

an increase in adulteration of olive oil with soybean oil from 5 to 50 %. This further confirmed that adulteration of olive oil with soybean oil can be detected by measuring the response ratio for linoelic and linolenic acid to oleic acid with DSA/TOF. All mass measurements showed good mass accuracy with an error of less than 5 ppm.

## Conclusion

This work shows the first work for rapid screening of adulteration of olive oil with soybean oil using DSA/TOF. The data showed that the higher response ratio for linoleic and linolenic acid to oleic acid in olive oil can be used to detect its adulteration with soybean oil. The mass accuracy of all measurements was less than 5 ppm with external calibration. All samples were screened with minimal sample preparation, in 30 sec per sample. In comparison to other established techniques such as LC/MS and GC/MS, DSA/TOF will improve laboratory productivity and decrease operating costs and analysis time.

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APPLICATION NOTE

Differential Scanning Calorimetry

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# Detecting the Adulteration of Extra Virgin Olive Oil by Controlled Cooling DSC

## Introduction

Food adulteration normally makes the news with cases like melamine in milk[1]. However, high-value products are often subjected to adulteration by lower-value materials and this can be difficult to detect. As a high-priced produce, a pint of extra virgin olive oil (EVOO) is close in cost to that of a half gallon of food-grade olive pomace oil. University of California at Davis has reported that the majority of the extra virgin olive oils sold in California fail the tests for the same (EVOO), using a variety of techniques (ultraviolet and visible spectroscopy [UV/Vis], gas chromatography [GC], liquid chromatography [LC]), and wet methods[2]. However, considering the way EVOO is made, one would expect a relationship to its thermal properties.

Differential scanning calorimetry (DSC) is commonly used to analyze foods in both quality control and research labs[3, 4]. DSC is often used to compare materials on heating, but cooling studies often give more information as materials can respond more thermodynamically under controlled cooling[5].

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## Experimental

### Materials

Initial samples of four commercial olive oils were obtained locally and then samples of high-grade EVOO were obtained directly from small producers. In addition, samples of freshly pressed mono and multi-varietal EVOO, along with refined and salvage oil with known processing histories, were also obtained. All samples were stored in a cool, dark room, when not used, under $N_2$ purge.

### Instrumental

4-8 mg samples of the various oils were pipetted into pre-weighted and matched aluminum DSC sample pans (PerkinElmer Part No. 02190041). These were then run on a PerkinElmer DSC 8500 under $N_2$ purge at 20 cc/min and cooled from room temperature to -60 °C at a rate of 5 °C per minute. A two-stage refrigerated cooler was used. Once at -60 °C, they were held there for three minutes to ensure complete cooling. Then, the samples were heated back to room temperature at 10 °C per minute. All samples were run in triplicate and the results averaged.

*Figure 1.* The DSC 8500 is a dual furnace power compensated design differential scanning calorimeter capable of very precise control on heating and cooling.

## Results

The commercial samples of olive oil show distinct thermal differences. Below, extra virgin (solid), refined (dashed), and pomace (dotted) olive oils are shown during the cooling run.

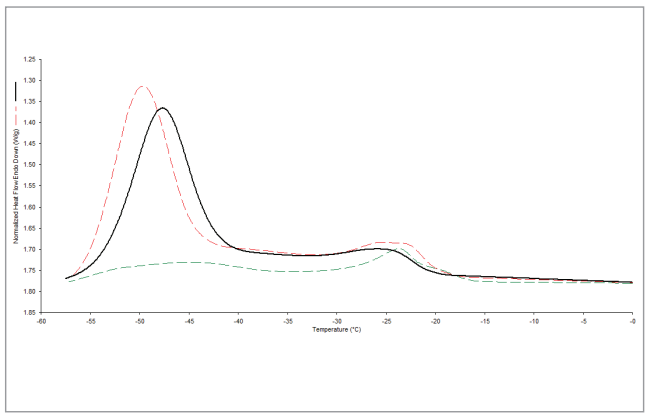

*Figure 2.* Grocery store grades of extra virgin, refined and pomace olive oils were run by controlled cooling in the DSC. Notice the distinctive fingerprints, particularly of the pomace oil, which lacks the low temperature peak.

As shown in Figure 3, we ran a series of EVOO samples that we were reasonably sure were truly extra virgin, as well as two received directly from a Texas-based producer who could assure this. While preliminary, the data shows some interesting features. First of all, the higher-temperature peak appears in the same temperature range as the pomace oil peak but is very small, even compared to the grocery store EVOO. This data suggests that the grocery store EVOO may be diluted with another oil.

Secondly, it appears that there are shifts in the peak shapes and temperatures with the varietal and origin of the oil. For example, note the difference in shape and peak position of the low temperature peak between the Spanish Arbeguina and the Spanish Arbosana. Origin appears to complicate, as seen in the Texas versus Spanish Arbeguina scans. Futher work would be needed to see if this holds, but based on previous work with nut oils[6], it seems likely.

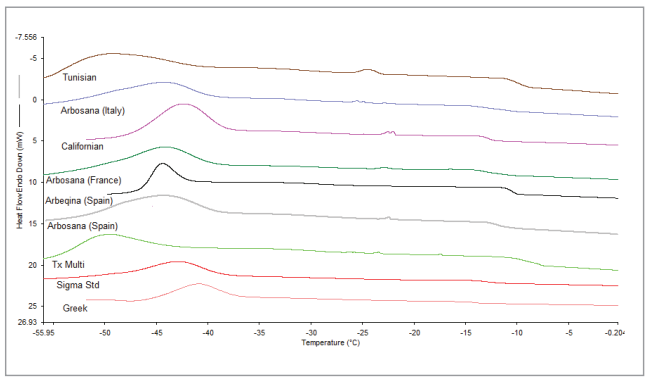


*Figure 3.* High-quality EVOO from small batch suppliers. The Texas EVOOs were of known origin. Note the lack of strong "pomace peak".

Characterizing these differences is often done by taking partial areas under the curve, as shown in Figure 4. This is shown for EVOO and a large high-temperature peak similar to that of the pomace oil was seen in all the grocery store samples in contrast to the truly EVOO samples in Figure 3.

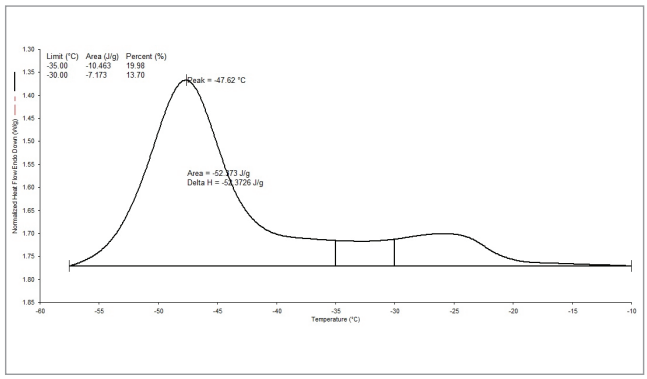


*Figure 4.* Multiple peak areas in a DSC scan analyzed by the partial areas technique. Only three partial areas are shown above for clarity.

With the "pomace peak" occurring in the -20 °C to -10 °C range and the major "EVOO peak", it appears likely that one could sort materials based on this approach. To test this, we created blends of EVOO and pomace oil in three amounts. The thermograms are overlayed in Figure 5. This data was used to construct a simple model from the partial area data shown above. Linear regression suggests we can estimate the addition of more than 7% olive oil-based adulterant to the olive oil. Based on this approach, we suspect the grocery store EVOO to have 12-15% adulterant or to be pressed at higher temperatures (see Figure 6). More exacting model techniques, such as those used in TIBCO Spotfire® software, are expected to give better results.

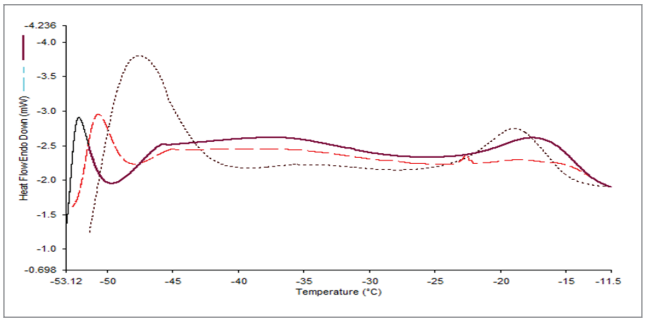


*Figure 5.* 25% EV (dashed), 50% EV (solid), and 75% EV (dotted) oils during the cooling run.

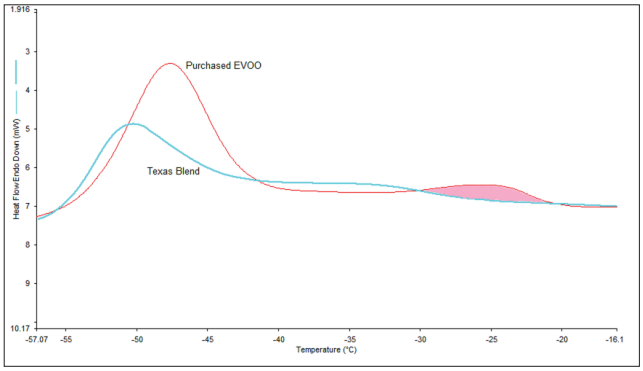


*Figure 6.* Overlay of the purchased EVOO and the Texas blend of known EVOO. The area of increased "pomace oil" is highlighted.

## Conclusion

Controlled cooling in the DSC represents a way to extract information from food products not normally accessible by other methods. Extra virgin olive oils have a distinct cooling profile that is different from lesser grades and apparently this profile is quite responsive to changes in composition. This gives a method for addressing adulterants as well as possibly identifying the varietal used to produce the oil. Futher work is planned on the effect of temperature and UV radiation.

## Acknowledgements

We thank Steve Beines of Texas Olive Ranch/Cowgirl Brands for useful discussions and olive oil samples of known history as well as the LAPOM group at University of North Texas for space and support,

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APPLICATION NOTE

Gas Chromatography/
Mass Spectrometry

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# The Determination of Low Levels of Benzene, Toluene, Ethylbenzene, Xylenes and Styrene in Olive Oil Using a TurboMatrix HS and a Clarus SQ 8 GC/MS

## Introduction

Levels of benzene, toluene, ethylbenzene, xylenes and styrene (BTEXS) are a concern in olive oil. These compounds find their way into olive trees and hence into the olives and olive oil mainly as a result of emissions from vehicles, bonfires, and paints into ambient air near the orchards.

Various methods have been developed to detect and quantify these compounds down to levels of 5 ng/g (5 ppb w/w). This application note describes an easy to perform method using PerkinElmer® Clarus® SQ 8 GC/MS with a TurboMatrix™ 110 headspace sampler to achieve detection limits below 0.5 ng/g.

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## Method

The experimental conditions for this analysis are given in Tables 1 to 4.

***Table 1.*** **GC Conditions.**

| Gas Chromatograph | Clarus 680 |
|---|---|
| Column | 30 m x 0.25 mm x 1.0 µm Elite-Wax |
| Oven | 35 °C for 1 min, then 10 °C/min to 130 °C |
| Injector | Programmable Split Splitless (PSS), 180 °C, Split OFF |
| Carrier Gas | Helium at 1.0 mL/min constant flow (7.2 psig initial pressure), HS Mode ON |

***Table 2.*** **HS Conditions.**

| Headspace System | TurboMatrix 110 HS Trap in standard HS mode (trap port capped). |
|---|---|
| Vial Equilibration | 90 °C for 20 minutes |
| Needle | 130 °C |
| Transfer Line | 140 °C, long, 0.150 mm i.d. fused silica (chosen to facilitate rapid conversion to HS trap operation for other applications) |
| Carrier Gas | Helium at 35 psig |
| Injection Time | 0.15 min |

***Table 3.*** **MS Conditions.**

| Mass Spectrometer | Clarus SQ 8 MS, Large Turbo Pump |
|---|---|
| Scan Range | 35 to 350 Daltons |
| Electron Energy | 70eV |
| Scan/Dwell Time | 0.1 s |
| Interscan/Interchannel Delay | 0.02 s |
| Source Temp | 200 °C |
| Inlet Line Temp | 200 °C |
| Multiplier | 1400V |

***Table 4.*** **Sample Details.**

| | |
|---|---|
| Sample | 10.00 ±0.01 g of olive oil weighed directly into vial |
| Vial | Standard 22-mL vial with aluminum crimped cap with PTFE lined silicon septum |

## Calibration solutions

1 mL of each BTEXS component was added to a 100-mL volumetric flask and diluted to volume with methanol. 1 mL of this stock solution was further diluted to 100 mL with methanol to produce the working solution used to fortify 'clean' olive oil for calibration purposes. The w/v concentration of each analyte in each of these two solutions is given in Table 5.

***Table 5.*** **BTEXS concentrations in calibration solutions.**

| Component | Stock Solution (µg/µL) | Working Solution (ng/µL) |
|---|---|---|
| Benzene | 8.77 | 87.7 |
| Toluene | 8.70 | 87.0 |
| Ethylbenzene | 8.67 | 86.7 |
| p-Xylene | 8.80 | 88.0 |
| m-Xylene | 8.64 | 86.4 |
| o-Xylene | 8.80 | 88.0 |
| Styrene | 9.06 | 90.6 |
| Methanol | Balance | Balance |

## Experimental

### Method Optimization

Figure 1 shows a total ion chromatogram (TIC) obtained from an empty vial into which 2 µL of the working mixture of the BTEXS components in methanol was injected and fully evaporated. The conditions given in Tables 1 to 3 were applied.

Excellent peak shape is apparent and a full baseline separation of all components has been achieved. Meta-xylene and para-xylene are easily separated on this highly polar chromatographic column. A solvent delay of 4.6 minutes eliminates the appearance of the methanol solvent peak in this chromatogram.

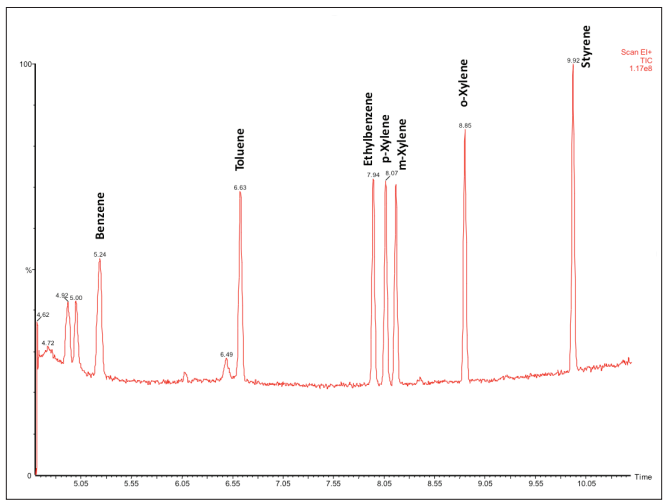


*Figure 1.* Chromatogram of 2 µL of working calibration solution added to an empty 22-mL HS vial.

Figure 2 shows a chromatogram (with the same scaling as Figure 1) run under the same analytical conditions of 2 µL working calibration mixture mixed into a 10 g sample of 'clean' olive oil. The analyte peaks are either close to the background noise level or are obscured by other components. The effective concentration of each analyte in the oil is approximately 17 ng/g (or ppb w/w). We need to see levels below 5 ng/g with this analysis and so it is clear that this will be a challenge with the method used to produce this chromatogram. The BTEXS compounds obviously have an affinity for the olive oil and so the partition coefficients are not favorable to the headspace phase – only a very small fraction of these will make it into the headspace.

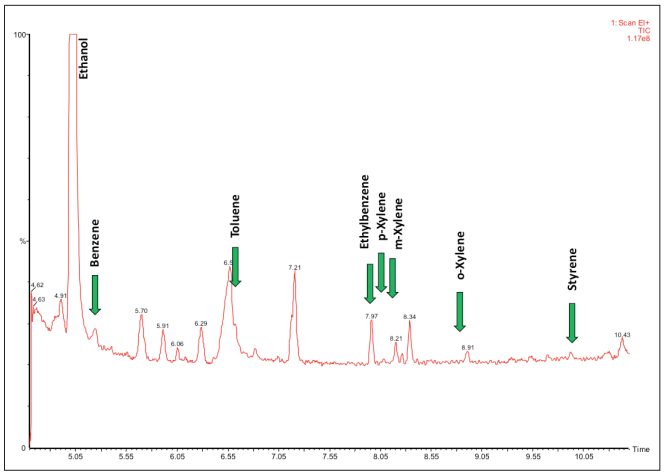


*Figure 2.* Chromatogram of 17 ng/g BTEXS in 10 g olive oil in a 22-mL HS vial with expected analyte retention times annotated.

By using the MS single-ion recording (SIR) mode of operation, the detector sensitivity and selectivity is significantly enhanced as shown in Figure 3. This chromatography was produced using the same chromatographic conditions as for Figure 2 but with the mixed single ion/full ion (SIFI) regime given in Figure 4 applied.

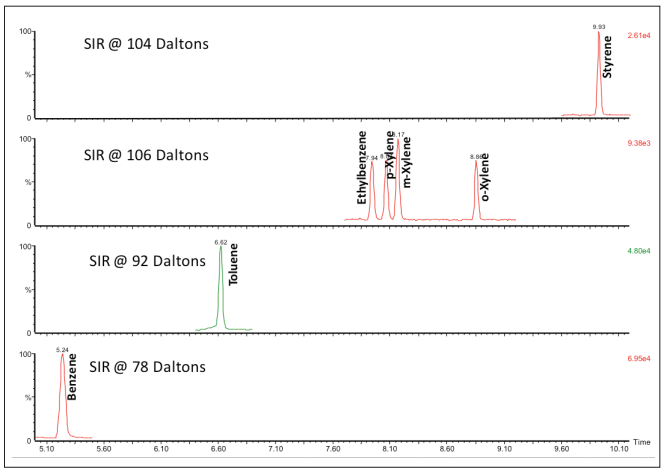


*Figure 3.* Chromatogram of 17 ng/g BTEXS in 10 g olive oil using SIFI settings given in Figure 4.

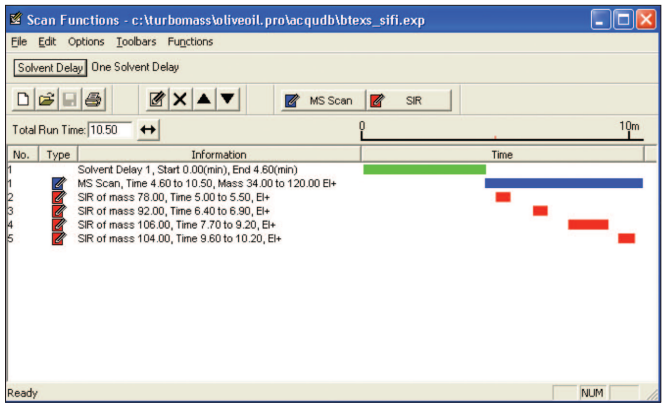


*Figure 4.* SIFI™ settings used to produce the chromatography shown in Figure 3.

## Linearity

A series of calibration mixtures was prepared by adding volumes of the working solution to clean olive oil as listed in Table 6. Note – this is often referred to as "method of standard addition".

***Table 6.* Calibration mixture preparation.**

| Olive Oil (g) | Working Solution (µL) | Nominal Concentration (ng/g) |
|---|---|---|
| 10.00 | 0 | 0 |
| 10.00 | 0.5 | 4.4 |
| 10.00 | 1.0 | 8.8 |
| 10.00 | 2.0 | 17.6 |
| 10.00 | 3.0 | 26.3 |
| 10.00 | 4.0 | 35.1 |
| 10.00 | 5.0 | 43.9 |
| 10.00 | 10.0 | 87.8 |

These mixtures were chromatographed using the conditions given in Tables 1 to 3. The analyte peak areas were obtained from the SIR traces. The clean olive oil was an off-the shelf product found to have low levels of BTEXS. The analyte peak areas found in this oil were subtracted from the calibration mixture responses, which were then used to prepare linear calibration profiles.

Figures 5 and 6 show calibration plots for the first and last eluting analytes, benzene and styrene, and Table 7 shows the least squares fit for each analyte. The linearity is excellent across this low concentration range especially for a complex sample matrix like olive oil.

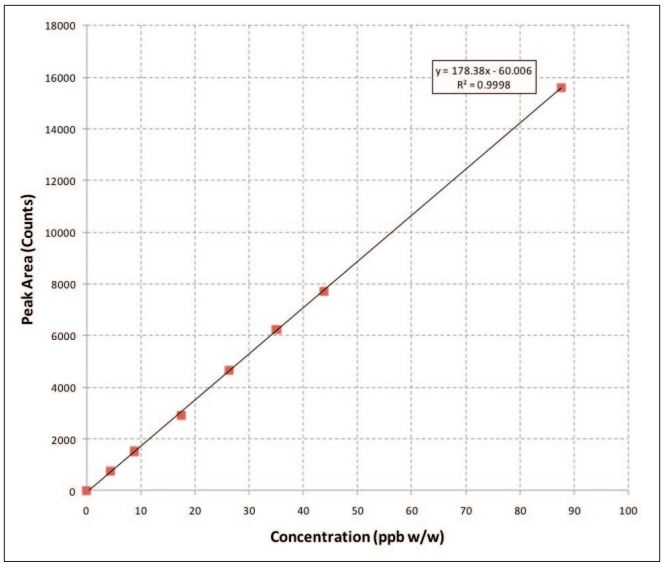


*Figure 5.* Calibration plot for benzene.

## Quantitative Precision

Ten samples of the clean olive oil were fortified with 5 µL of the working solution. Each was analyzed using the conditions given in Tables 1 to 3 and the amount of each analyte was determined using the calibration data from Table 7. The results are given in Table 8. An overall precision of 1.69 to 3.76% relative standard deviation is a very good result from this complex matrix.

## Detection Limits

Figure 7 shows chromatography of a low-level sample. The calculated signal to noise ratios were used to predict the analytical detection limits shown in Table 9 based on a 2:1 ratio. These limits are over an order of magnitude below that of the 5ng/g requirement.

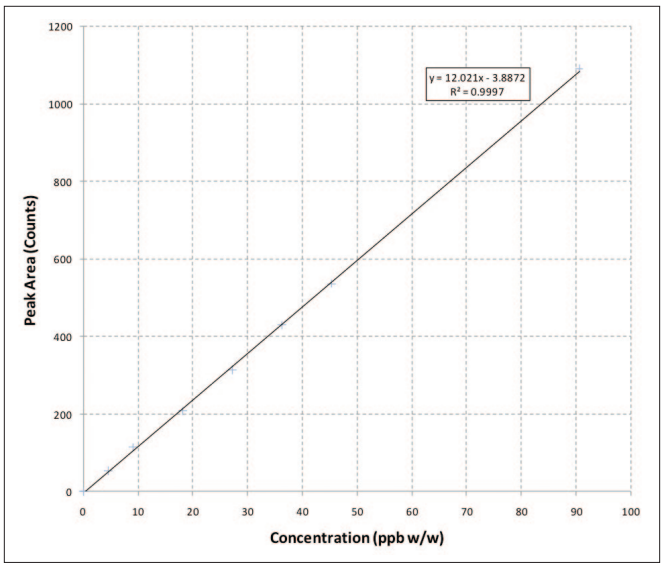


*Figure 6.* Calibration plot for styrene.

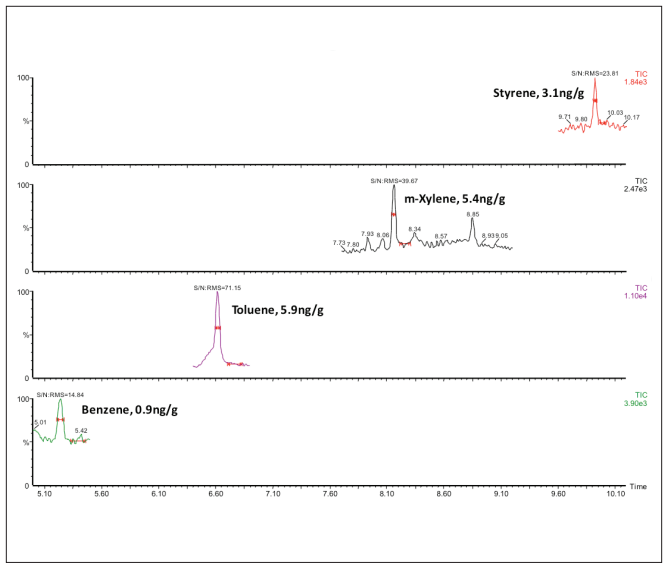


*Figure 7.* Chromatography of a sample containing low-levels of BTEXS with annotated signal to noise values.

***Table 7.* Least squares linear fit to calibration data.**

| Statistic | Benzene | Toluene | Ethylbenzene | p-Xylene | m-Xylene | o-Xylene | Styrene |
|---|---|---|---|---|---|---|---|
| Slope | 178.38 | 51.465 | 10.07 | 11.568 | 10.708 | 8.4239 | 12.021 |
| Intercept | -60.006 | -1.6527 | -5.6768 | -6.7959 | -1.1014 | -6.7186 | -3.8872 |
| $r^2$ | 0.9998 | 0.9986 | 0.9995 | 0.9997 | 0.9998 | 0.9995 | 0.9997 |

*Table 8.* **Quantitative precision.**

| Run # | Concentration in Spiked Sample (ng/g) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Benzene | Toluene | Ethylbenzene | p-Xylene | m-Xylene | o-Xylene | Styrene |
| 1 | 42.84 | 48.01 | 43.17 | 41.05 | 44.09 | 43.53 | 43.83 |
| 2 | 42.60 | 46.35 | 44.46 | 42.95 | 46.24 | 45.43 | 45.16 |
| 3 | 44.27 | 47.42 | 45.45 | 44.85 | 49.32 | 46.98 | 48.32 |
| 4 | 43.30 | 47.17 | 44.85 | 42.51 | 46.98 | 45.55 | 45.66 |
| 5 | 42.87 | 45.44 | 43.56 | 40.09 | 44.65 | 44.25 | 45.16 |
| 6 | 42.40 | 43.83 | 43.66 | 40.27 | 44.18 | 42.46 | 42.75 |
| 7 | 42.90 | 49.37 | 44.56 | 41.91 | 45.49 | 44.01 | 45.25 |
| 8 | 43.30 | 45.03 | 44.85 | 42.08 | 45.95 | 44.13 | 44.66 |
| 9 | 41.91 | 44.18 | 43.37 | 40.35 | 44.37 | 43.65 | 44.33 |
| 10 | 41.77 | 46.41 | 42.17 | 41.30 | 44.18 | 42.23 | 42.92 |
| Mean | 42.82 | 46.32 | 44.01 | 41.74 | 45.54 | 44.22 | 44.81 |
| RSD% | 1.69 | 3.76 | 2.25 | 3.51 | 3.66 | 3.26 | 3.53 |

*Table 9.* **Predicted limits of detection.**

| Compound | Predicted Limit of Detection (ng/g) |
|---|---|
| Benzene | 0.12 |
| Toluene | 0.16 |
| Ethylbenzene | 0.26* |
| p-Xylene | 0.26* |
| m-Xylene | 0.26 |
| o-Xylene | 0.26* |
| Styrene | 0.26 |

** Peaks too small to quantify and so are based on value for m-Xylene.*

## Sample Analysis

Seven different branded bottles of olive oil were purchased from a local supermarket and analyzed using this method. The results are given in Table 10. The determined concentrations are well within the range of this method.

*Table 10.* **Results from analysis of supermarket samples.**

| Sample Source(s) | Concentration in Sample (ng/g) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Benzene | Toluene | Ethylbenzene | p-Xylene | m-Xylene | o-Xylene | Styrene |
| California | 0.89 | 5.86 | 1.66 | 1.45 | 5.24 | 3.77 | 3.07 |
| Italy, Greece, Spain, Tunisia | 2.86 | 27.55 | 6.12 | 5.86 | 16.73 | 8.75 | 41.34 |
| Italy, Spain, Greece, Tunisia | 3.07 | 24.22 | 13.47 | 7.85 | 23.64 | 13.97 | 39.59 |
| Italy, Spain, Tunisia, Turkey, Argentina | 2.99 | 17.03 | 3.74 | 3.44 | 9.35 | 6.14 | 40.09 |
| Spain, Argentina | 2.43 | 34.99 | 7.22 | 7.42 | 18.97 | 10.65 | 126.11 |
| Italy, Spain, Greece, Tunisia, Morocco, Syria, Turkey | 4.09 | 35.71 | 19.13 | 17.10 | 59.31 | 28.10 | 61.05 |
| Italy, Greece, Spain, Tunisia | 1.25 | 2.79 | ND | 1.80 | 3.74 | 3.17 | 7.39 |

## Conclusions

This method uses the new Clarus SQ 8 GC/MS to great effect. Sample preparation is extremely easy – 10 g of olive oil is weighed into a standard headspace vial and then sealed with a crimped cap. The analysis is fully automated and takes just 10.5 minutes for the chromatography and an additional 3.5 minutes for cool-down and equilibration between analyses.

Sub-ppb levels are possible using standard headspace sampling of light aromatics in a complex natural oil matrix without the need for vapor pre-concentration (for example with an HS Trap). Excellent quantitative performance has been demonstrated and the system is easily able to see low concentrations of these compounds in olive oil bought from a local supermarket.

## PerkinElmer Accessories and Consumables for this application:

| Item Description | Part No. |
|---|---|
| Elite Wax | N9316485 |
| Injector Port Septa 6pk | N6101748 |
| Ferrules | 09920104 |
| H/S Vials/Caps/Septa | N9303992 |
| Marathon Filament | N6470012 |
| Ergo Crimper | N6621037 |

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APPLICATION NOTE

Near-Infrared Spectroscopy

**Authors:**

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# Quality Control of Olives by Near-Infrared Spectroscopy and AssureID Software

## Introduction

Olive oil is an increasingly popular food product worldwide, with global production exceeding 3.0 million tons in 2011 and showing steady annual growth. Despite these huge volumes, however, margins are relatively small in olive oil production so quick and easy analysis of oil quality is vital to maintain process efficiency. Rapid, reliable analysis can contribute to process and quality improvements in numerous ways. For example,

- Assessment of raw olive acceptability. If the olives have been collected from the ground rather than fresh from the tree, they may be of poor quality with high acidity and hence lower value.
- Measurement of water and oil content. These parameters determine the price of the olives, with those having a greater oil content commanding a higher price.
- Process optimization. After extracting the oil, the remaining pulp or by-product (called alperujo in Spain) should have only minimal oil content, typically around 2% or less. If the oil exceeds this level, a problem with the process is indicated.

This note describes how a PerkinElmer Frontier™ FT-NIR spectrometer and AssureID software have been used by an olive oil producer in Spain to improve productivity by implementing the above analyses within their routine production.

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## Materials and Methods

A PerkinElmer Frontier FT-NIR spectrometer equipped with an upwards-facing reflectance accessory and sample spinner (NIRA) was used for all measurements.

Olive samples were milled to a paste and placed in a glass petri dish before analysis. Spectra were collected between 10000 and 4000 $cm^{-1}$ at 16 $cm^{-1}$ resolution, with an accumulation time of 30 seconds per sample.

The olive samples were also analyzed for oil and water content following the customer's established laboratory procedures.

Some of the measured spectra are shown in Figure 1. Typically for NIR spectra, the absorption features are broad and overlapped, although several prominent features can be assigned either to water or to organic C–H modes in the oil.

## Assuring Olive Quality

SIMCA is a powerful chemometric method for sample classification that builds independent models for each sample class – in this case, fresh and old olives. New samples are tested against both models, and identified as belonging (or not) to one of the material classes. Compared to traditional methods of identification such as spectral correlation, SIMCA has a much greater ability to distinguish between relatively similar materials, even in the presence of natural variation – provided this is captured in the training set data used to build the models.

AssureID software was designed from the ground up to streamline the process of building SIMCA models, and breaks the procedure down into a series of straightforward, logical steps:

1. Define materials and acquire spectra of known references.
2. Optionally, configure algorithm parameters and spectral pre-processing such as baseline correction: the default settings are tailored to the instrument and sample type and in most cases will produce good results without modification.
3. Calibrate the method. The software automatically builds the models and determines the acceptance thresholds.
4. Review the classification results (for example, see Figure 2). Any issues with the data or performance of the method will be flagged by the troubleshooting engine, allowing corrective action to be taken.
5. The validated method is then deployed as a workflow within the dedicated Analyzer module of AssureID, allowing routine use of the method.

## Quantitative Modeling of Oil and Water Content

The oil and water contents of the olives are key parameters for quality, and both contribute to the NIR spectrum. The complex nature of NIR spectra often makes it impossible to develop quantitative models based on the absorbance at a single wavelength. However, multivariate (chemometric) methods such as partial least squares regression (PLS) still function in the presence of overlapping bands, and can allow models to be built.

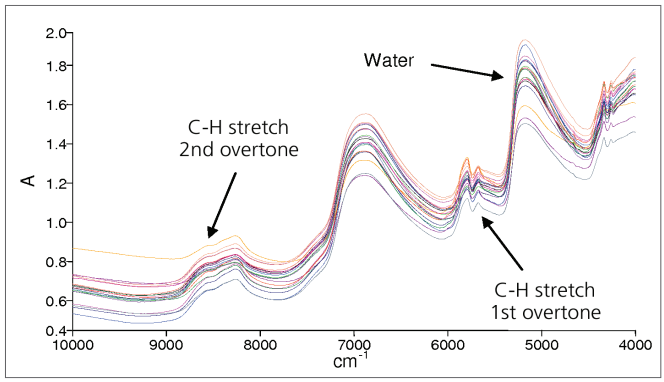


*Figure 1.* NIR spectra of some of the olive samples.

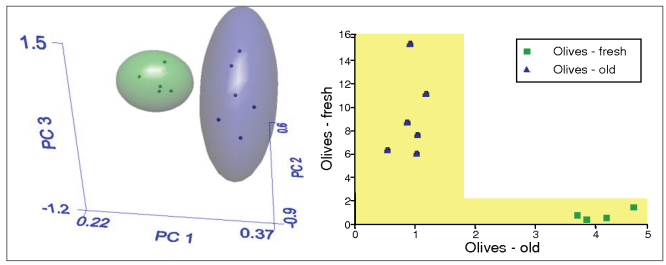


*Figure 2.* Overview PCA (left) and Cooman's (right) plots for the models to discriminate old and fresh olives. Each axis represents the residual distance against one model. A clear separation of points in the top-left and bottom-right corners, as seen here, indicates that the model is comfortably distinguishing the two types of olive.

The olive spectra and properties determined by chemical analysis were loaded into PerkinElmer Quant+ software. One third of the data were designated as a validation set to verify the performance of the model. The spectra were pre-processed with first-derivative baseline correction.

The calibration and validation results are summarized in Table 1 and Figure 3. The models use a modest number of latent variables and show good linearity and precision over the range of available samples. The standard errors of prediction (SEPs) were 1.5 % and 1.7 % for oil and water, respectively.

*Table 1.* Summary of results for the PLS modeling of oil and water in olives.

| Property | Oil (%) | Water (%) |
|---|---|---|
| Range | 14–41 | 34–61 |
| Mean | 25 | 46 |
| Standard deviation | 5 | 6 |
| No. of latent variables | 5 | 3 |
| Validation SEP | 1.5 | 1.7 |

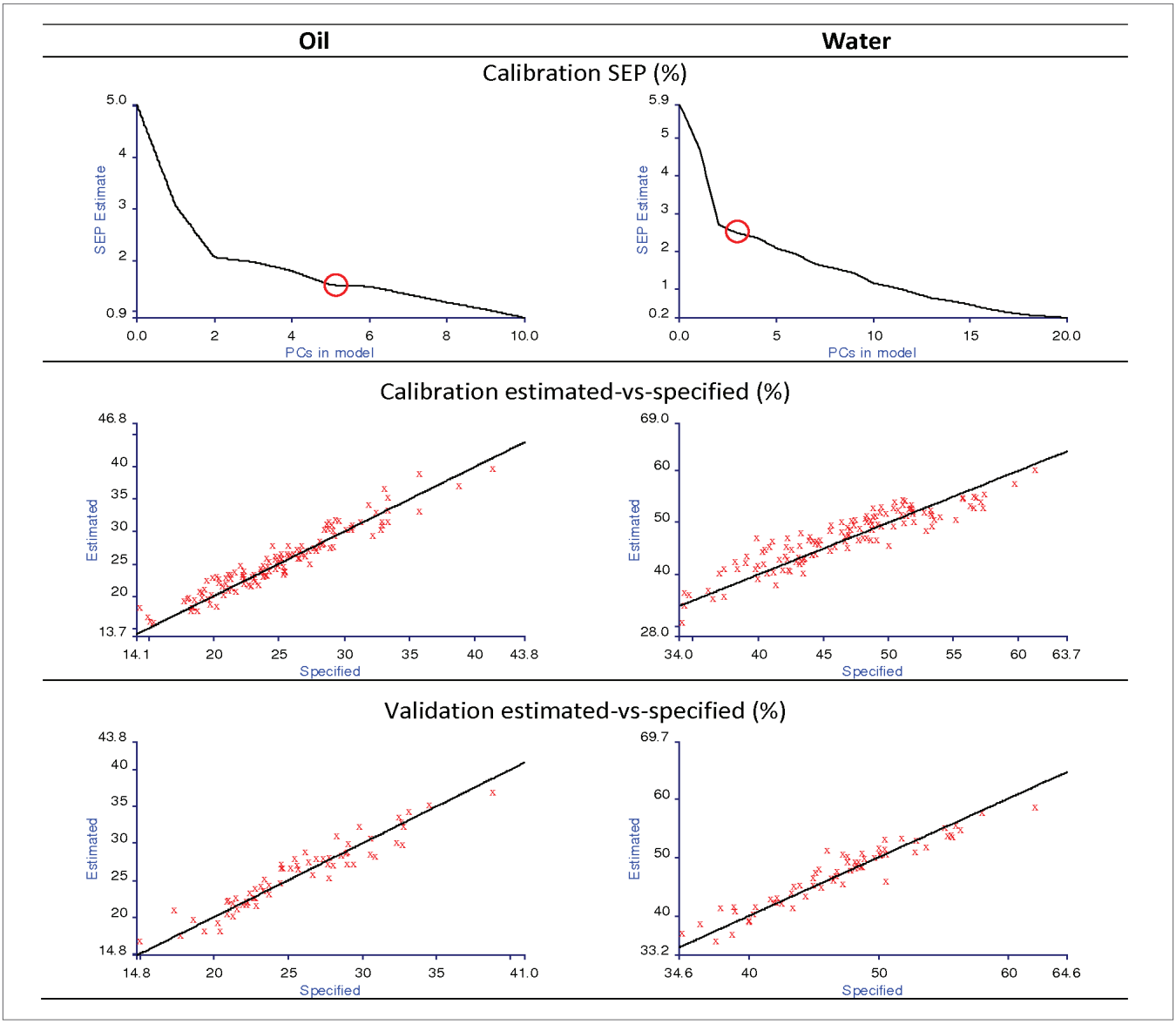


*Figure 3.* Calibration and validation results for oil (left column) and water (right column).

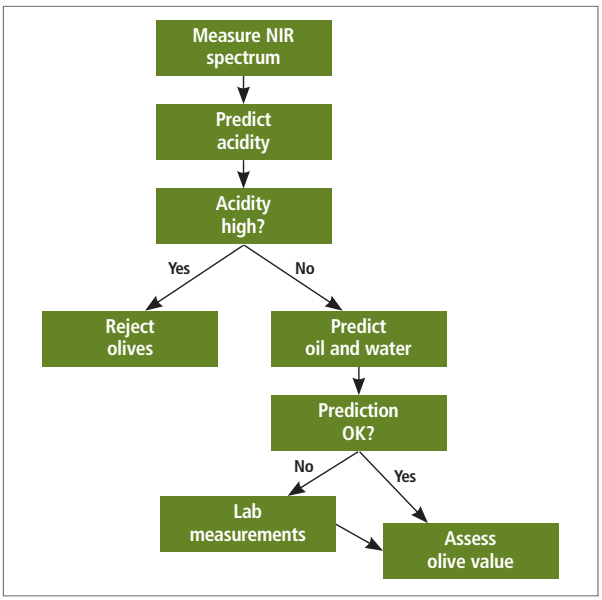


*Figure 4.* Flow chart for olive analysis by NIR spectroscopy with AssureID.

These quantitative models were also incorporated into the AssureID analysis. After checking the olives for quality, acceptable olives will be further analyzed for oil and water content (as shown in the flowchart in Figure 4).

While AssureID allows sophisticated analyses using both qualitative and quantitative chemometric methods, its design as separate method-building and analysis modules ensures that the end-user is presented with a simple interface, as shown in Figure 5.

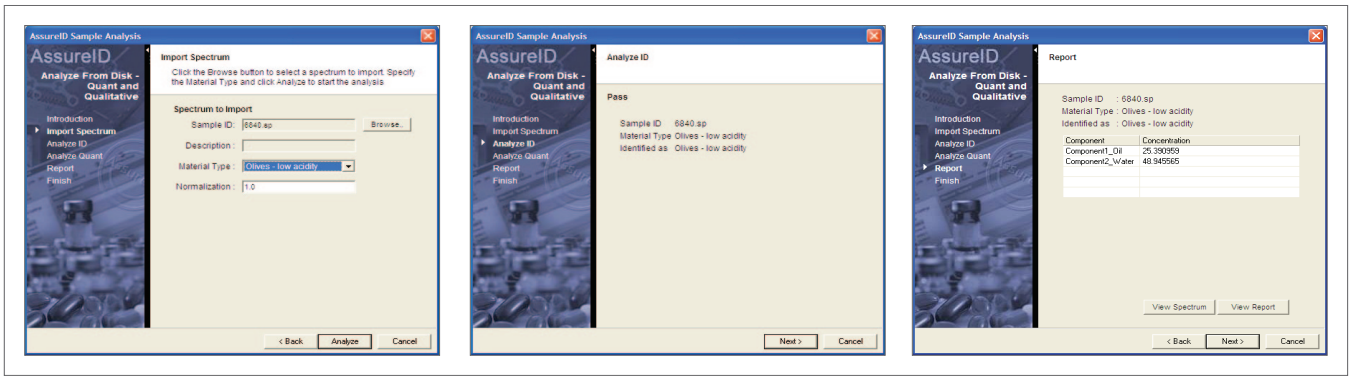


*Figure 5.* AssureID workflow for olive analysis.

## Conclusions

Increasing pressure on food and food ingredient manufacturers to increase efficiency while maintaining product quality has created a need for rapid and precise analysis of materials at all stages of the processing chain. Near-infrared spectroscopy provides rich information about physical and chemical properties of many food materials, and combined with chemometric techniques can provide unequalled speed and precision of analysis. In this note we have shown how the Frontier near-infrared spectrometer from PerkinElmer, in conjunction with AssureID software, is being used to perform three key analyses in olive processing: checking for excess acidity to reject poor-quality olives, measuring the oil and water content to assess olive value, and measuring the oil content in the alperujo by-product to verify extraction efficiency.

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