

# Analysis of minor components in olive oil

Michael Murkovic<sup>a,\*</sup>, Sonja Lechner<sup>a</sup>, Ariane Pietzka<sup>a</sup>,  
Michael Bratacos<sup>b</sup>, Evangellos Katzogiannos<sup>b</sup>

<sup>a</sup> *Department of Food Chemistry and Technology, Graz University of Technology,  
Petersgasse 12/2, Graz 8010, Austria*

<sup>b</sup> *Department of Food Technology, Faculty of Food Technology and Nutrition,  
Technological Educational Institute of Athens Agiou Spyridonos Street, Egaleo, Athens 122 10, Greece*

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## Abstract

Virgin olive oil is well known for its high content of phenolic substances that are thought to have health-promoting properties. These substances also contribute to the distinctive taste of the oil. In this study, tyrosol, vanillic acid, luteolin, and apigenin were identified and quantified by liquid chromatography mass spectrometry (LC-MS). In the seven samples analysed, tyrosol, the most abundant, was in the range of 1.4–29 mg/kg, vanillic acid was in the range of 0.67–4.0 mg/kg, luteolin was in the range of 0.22–7.0 mg/kg, and apigenin was in the range of 0.68–1.6 mg/kg. It was also shown that in olive oil, squalene can be analysed by using a refractive index detector. In the samples analysed, squalene occurred in the range of 3.9–9.6 g/l.

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## 1. Introduction

The world production of olive oil is ca. 2.5 million tons, with Spain, Italy, Greece, and Maghreb countries major producers. Abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid ranges from 56% to 84% of total fatty acids, while linoleic acid, the major essential fatty acid and the most abundant polyunsaturated acid in our diet, is present in

\* Corresponding author. Tel.: +43-3168736495; fax: +43-3168736971.

E-mail address: [michael.murkovic@tugraz.at](mailto:michael.murkovic@tugraz.at) (M. Murkovic).

concentrations between 3% and 21% [1]. In addition to triglycerides and free fatty acids, olive oil contains a variety of nonsaponifiable compounds that add up to 1–2% of the oil and are important for its stability and unique flavour and taste.

It has been postulated that the components in olive oil in the Mediterranean diet, a diet which is largely vegetarian in nature, can contribute to the lower incidence of coronary heart disease and prostate and colon cancers. The Mediterranean diet includes the consumption of large amounts of olive oil, which contains high amounts of phenolic substances. The major phenolic compounds in olive oil are oleuropein, hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) and tyrosol (2-(4-hydroxyphenyl)ethanol). The phenolic compounds present in olive oil are strong antioxidants and radical scavengers. Typically, hydroxytyrosol is a superior antioxidant and radical scavenger to oleuropein and tyrosol [2].

It was shown by Owen et al. [3] that the health-promoting properties are a result of the unique profile of the phenolic fraction, along with high intakes of squalene and the monounsaturated fatty acid, oleic acid.

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane) is a terpenoid hydrocarbon occurring in high concentrations (60–75%) in the unsaponifiable fraction of olive oil.

The main focus of this study are the minor components such as polyphenols and squalene. In this study, the phenols [tyrosol, vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), *p*-coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid), ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid), luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one), apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one), oleuropein] as well as squalene were analysed. The analytes found in the samples are shown in Fig. 1.

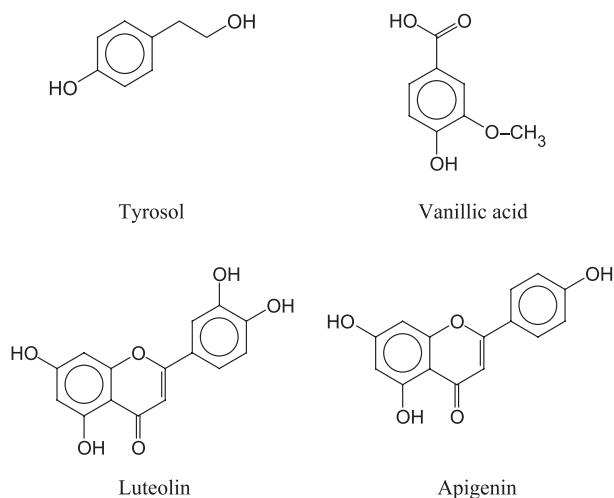


Fig. 1. Chemical structures of substances found in the olive oil extract analysed by HPLC-MS.

## 2. Materials and methods

The olives were grown in Greece and harvested in 2001. The oil was pressed in a small-scale laboratory press.

The polyphenols and other chemicals were bought from Merck (Darmstadt, Germany). Squalene was purchased from Sigma (St. Louis, MO, USA). Methanol, glacial acetic acid, acetonitrile, and acetone were of LiChrosolv quality from Merck. Water was prepared with Simplicity 185 (Millipore, Molsheim, France).

## 3. Analysis of squalene

### 3.1. Sample preparation

One hundred milligrams of the olive oil was diluted with the eluent to 2 ml, centrifuged, and used for high-performance liquid chromatography (HPLC) analysis.

### 3.2. HPLC analysis

Twenty-five microliters of the diluted oil was injected onto a reversed-phase column. The HPLC equipment used was an HP 1100 (Agilent, Waldbronn, Germany) equipped with an isocratic pump and a refractive index detector. For injection, a manual injector (Rheodyne) was used. The analytes were separated on a LiChrospher 100 (5  $\mu$ m, 250  $\times$  4 mm; Merck) and a precolumn LiChroCART 4-4 (LiChrospher 100; 5  $\mu$ m). The column was eluted with a mixture of 70% acetone and 30% acetonitrile (vol/vol). The flow rate was 1.0 ml/min. Squalene was identified by comparing the retention time with an authentic substance and comparing the mass spectrometry (MS) spectra of selected samples. For quantification, a standard addition method was used. All standard solutions were prepared daily from a stock solution, which was stored in the dark at  $-20^{\circ}\text{C}$ . The mean and standard deviation were calculated from data of triplicate analysis.

## 4. Analysis of polyphenols

### 4.1. Sample preparation

Five hundred milligrams of the olive oil was extracted with 500  $\mu$ l of methanol in 2-ml Eppendorf reaction tubes. After vigorous shaking, the vials were centrifuged at 13,000 rpm for 5 min. The upper methanolic phase was used for HPLC analysis.

### 4.2. HPLC analysis

Five microliters of the methanolic extract was injected onto a reversed-phase column. The HPLC equipment used was an HP 1100 (Agilent) equipped with a quaternary pump and a diode array (HP 1100) and mass selective detector (MSD; Agilent). For injection an

autosampler (HP 1100; Agilent) was used. The analytes were separated on a LiChrospher 100 (5  $\mu\text{m}$ , 125  $\times$  4 mm; Merck) and a precolumn LiChroCART 4-4 (LiChrospher 100; 5  $\mu\text{m}$ ). Separation was achieved by elution gradient using an initial composition of 90% water (pH adjusted to 3.1 with 0.2% acetic acid) and 10% methanol. The concentration of methanol was increased to 30% in 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40% in 10 min, maintained for 5 min, increased to 50% in 5 min, and maintained for another 5 min. Finally, the methanol percentage was increased to 60%, 70%, and 100% in 5-min periods. A flux of 1 ml/min and a temperature of 35 °C were also used (adopted from Ref. [4]). Ionisation was achieved by an electrospray. The parameters were set for the drying gas flow to 10 l/min, drying gas pressure to 40 psig, and drying gas temperature to 350 °C. To transfer the ions into the capillary, a voltage of 3500 V was used. The fragmentor voltage was set to 70 V.

For mass selective detection, the positively charged ions were analysed. For tyrosol, the ion with a molecular mass of 121 ( $\text{MH}^+ - \text{H}_2\text{O}$ ), vanillic acid 169 ( $\text{MH}^+$ ), luteolin 287 ( $\text{MH}^+$ ), and apigenin 270 ( $\text{MH}^+$ ) were selected. In the case of tyrosol, a fragment was used for quantification, which had a higher intensity than the molecular ion.

## 5. Results and discussion

The analysis of phenolic substances by using mass selective detection after liquid chromatographic separation showed that from the tested substances, only tyrosol, vanillic acid, luteolin, and apigenin were found. The other substances (*p*-coumaric acid, ferulic acid, and oleuropein) normally found in olive oil could not be identified in the samples investigated samples [5]. Elenolic acids and its derivatives were also not found. Hydroxytyrosol, which is a commonly found substance in olive oil, was not found using mass selective detection. Figs. 2 and 3 show chromatograms of an olive oil sample with the standards used for identification. Since the amount of oil was very limited, the original method of sample preparation published by Brenes et al. [4] was modified. The original method used 14 g of olive oil, which was extracted with a mixture of methanol (80%) and water (20%) with a further step of defatting the extract with hexane. In the method presented, here only a small amount of oil (500 mg) was used, which was extracted with an equal amount of methanol. Using mass selective detection, this crude extract could be used for analysis. Using commercially available olive oil of extravirgin quality, both extraction methods were compared, giving similar results (data not shown). The limit of quantification was 6.3  $\mu\text{g/ml}$  for squalene, 0.4  $\mu\text{g/ml}$  for tyrosol, 0.2  $\mu\text{g/ml}$  for vanillic acid, 0.2  $\mu\text{g/ml}$  for luteolin, and 0.2  $\mu\text{g/ml}$  for apigenin.

Tyrosol was the most abundant substance in the analysed samples. The concentration ranged from 1.4 to 29 mg/kg. The other phenolic substances (vanillic acid, luteolin, and apigenin) occurred in a lower range from 0.68 to 7.0 mg/kg. Oleuropein, which normally occurs in the range of nondetectable to 11 mg/kg in the oil [6], was not present in our samples (Fig. 2).

Squalene was analysed using the refractive index detector. For a positive identification of squalene and for validation of the method, MS detection was used. However, the method presented here shows the possibility of analysing substances that have no

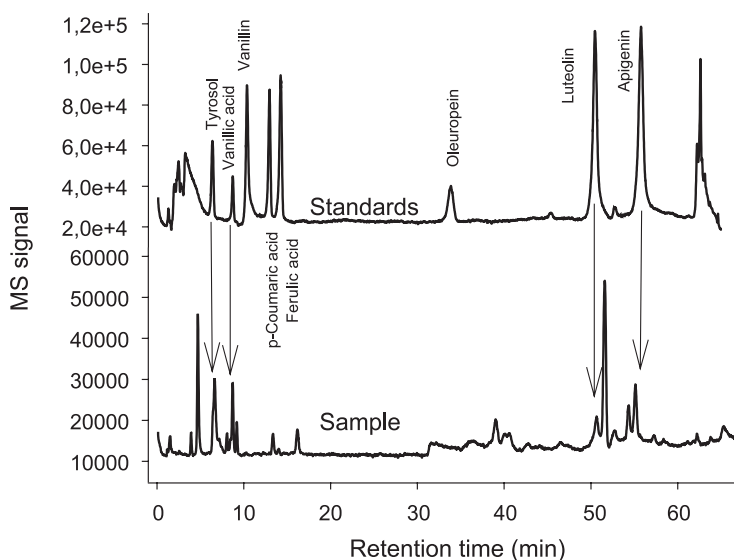


Fig. 2. Chromatogram of an olive oil sample. The identified substances are marked with arrows.

satisfactory UV absorption. The method used was originally developed for analysis of triglycerides. For this, the oil was diluted with the eluent (a mixture of 70% acetonitrile and 30% acetone). As can be seen in Fig. 2, squalene elutes as a distinct peak in the chromatogram. The triglycerides elute later, which is not shown in the chromatogram. For analysis of squalene, coupled methods of HPLC and gas chromatography (GC) have been published [7].

The squalene concentration is in the range of 5.1–9.6 g/l (Table 1). Squalene is known to occur in concentrations between 0.8 and 12 g/kg in virgin olive oil [8]. The samples

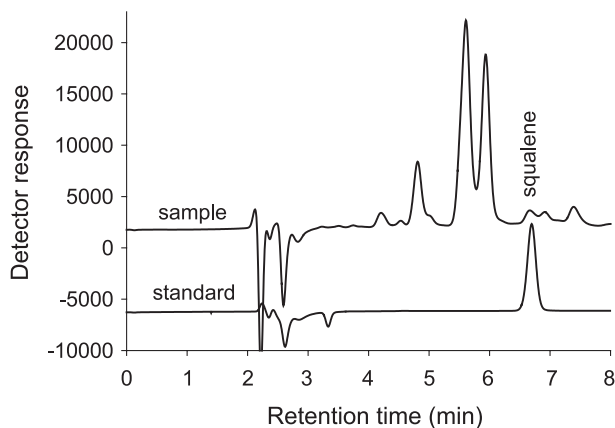


Fig. 3. Chromatogram of an olive oil sample for determination of squalene.

Table 1

Concentrations of phenolic substances (mg/kg) and squalene (g/l) in olive oil with standard deviation ( $n=3$ )

Analyte	Analysed oil sample							
	<i>m/z</i>	1	2	3	4	5	6	7
Tyrosol	121	5.5 ± 0.46	11 ± 0.90	3.5 ± 0.12	1.4 ± 0.14	2.0 ± 0.06	2.2 ± 0.22	29 ± 1.74
Vanillic acid	169	0.72 ± 0.07	1.7 ± 0.08	2.4 ± 0.08	4.0 ± 0.08	0.91 ± 0.02	3.0 ± 0.06	0.67 ± 0.09
Luteolin	287	1.9 ± 0.08	0.22 ± 0.01	4.4 ± 0.12	7.0 ± 0.49	3.5 ± 0.21	1.1 ± 0.08	4.1 ± 0.08
Apigenin	271	1.2 ± 0.04	0.68 ± 0.02	1.1 ± 0.03	1.2 ± 0.07	1.5 ± 0.11	1.5 ± 0.02	1.6 ± 0.07
Squalene	—	5.1 ± 0.08	7.5 ± 0.03	5.8 ± 0.28	6.5 ± 0.13	9.6 ± 0.13	7.8 ± 0.12	3.9 ± 0.07

analysed for this study were in the same range and it could be shown that HPLC with refractive index detector can be used for the determination of squalene in olive oil (Fig. 3).

## References

- [1] Tiscornia E, Forina M, Evangelisti F. Chemical composition of olive oil and its variations induced by refining. *Riv Ital Sostanze Grasse* 1982;59:519–56.
- [2] Tuck KL, Hayball PJ. Major phenolic compounds in olive oil: metabolism and health effects. *J Nutr Biochem* 2002;13:636–44.
- [3] Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalter B, et al. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol* 2000;1:107–12.
- [4] Brenes M, Garcia A, Garcia P, Rios JJ, Garrido A. Phenolic compounds in Spanish olive oils. *J Agric Food Chem* 1999;47:3535–40.
- [5] Visioli F, Galli C. Olive oil phenols and their potential effects on human health. *J Agric Food Chem* 1998;46:4292–6.
- [6] Perri E, Raffaelli A, Sindona G. Quantitation of oleuropein in virgin olive oil by ion spray mass spectrometry—selected reaction monitoring. *J Agric Food Chem* 1999;47:4156–60.
- [7] Moreda W, Pérez-Camino MC, Cert A. Gas and liquid chromatography of hydrocarbons in edible vegetable oils. *J Chromatogr, A* 2001;936:159–71.
- [8] Cert A, Moreda W, Pérez-Camino MC. Chromatographic analysis of minor constituents in vegetable oils. *J Chromatogr, A* 2000;881:131–48.