Project co-financed by the European Regional Development Fund

ARISTOIL

"Reinforcement of Mediterranean olive oil sector competitiveness through development and application of innovative production and quality control methodologies related to olive oil health protecting properties"

PRIORITY AXIS 1: Promoting Mediterranean innovation capacities to develop smart and sustainable growth

OBJECTIVE: 1.1 To increase transnational activity of innovative clusters and networks of key sectors of the MED area

Project website: http://aristoil.interreg-med.eu/

DELIVERABLE Number: 3.1.1
Title of DELIVERABLE: Guide for production and quality control of olive oil with increased health protecting properties

ACTIVITY n. 3.1: Development of methodology for monitoring olive oil quality

WP n. 3: STUDYING

PARTNER IN CHARGE: National and Kapodistrian University of Athens, Department of Pharmacognosy and Natural products Chemistry (UoA)

PARTNERS INVOLVED: 1. National and Kapodistrian University of Athens, Department of Pharmacognosy and Natural products Chemistry (UoA),
2. University of Cordoba, Department of Analytical Chemistry (UCO),
3. ARISTOLEO (ARI)
4. Free Municipal Consortium of Ragusa (Ragusa Consortium)

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Contents

Guide for production and quality control of olive oil with increased health protecting properties

General description**:** A guide including information about which factors (e.g variety, harvest time, irrigation, olive mill operation parameters) should be taken in account and how the quality control should be done has been created. The guide includes a report with the finally proposed methodologies for monitoring of olive oil quality in relation to its health-protecting properties and a guide with advantages and disadvantages of each method.

Methods optimization

The WP leader (UOA) had the responsibility to isolate and purify appropriate amounts of the ingredients of olive oil that should be used as standards for the calibration of the three analytical methods. More specifically during the first three to six months of the activities in WP3, UOA made available to UCO, UNIST and ARI the following compounds:

1. Oleocanthal
2. Oleacein
3. Oleuropein aglycon (monoaldehyde closed form)
4. Ligstroside aglycon (monoaldlehyde closed form)
5. Oleuropein aglycon (dialdehyde and enolic open forms) = Oleuropeindials+Oleomissional
6. Ligstroside aglycon (dialdehyde and enolic open forms) = Ligstrodials + Oleokoronal

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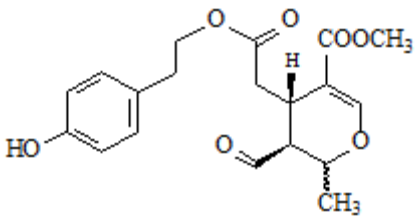
<td> 
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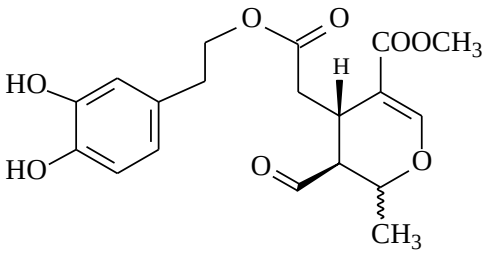
<td> 
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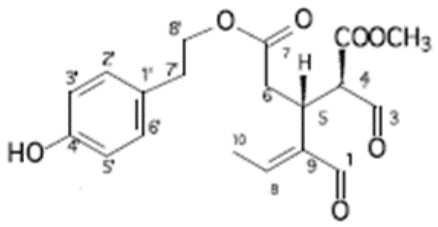
<td> 
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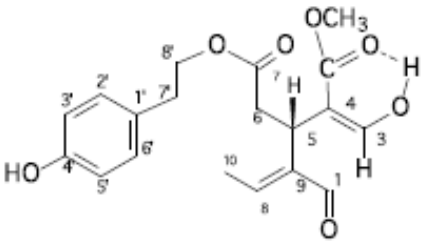
<td> 
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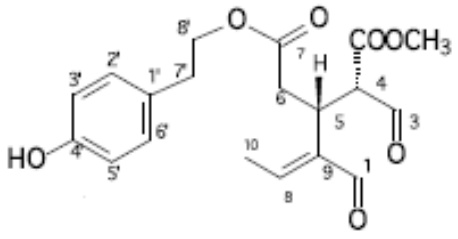
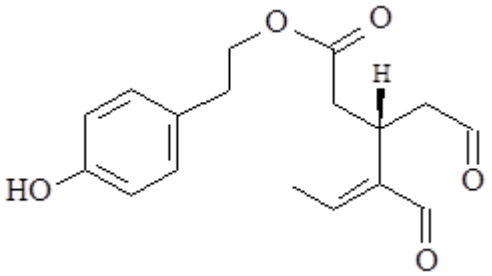
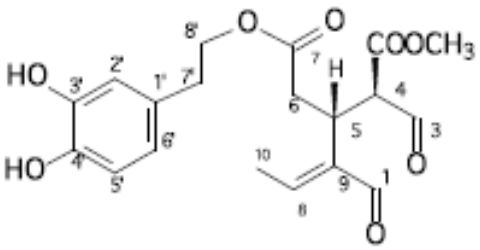
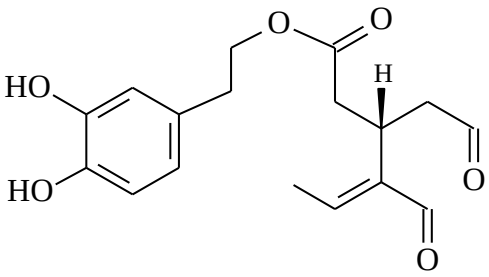
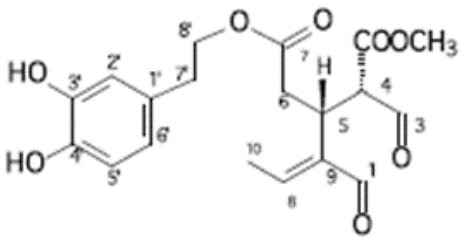
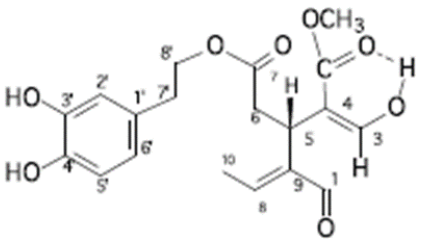
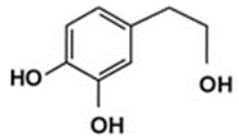
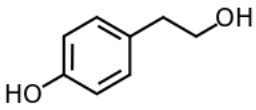
 **Ligstrodial 5S,4S**	 **Oleocanthal**
 **Oleuropeindial 5S,4R**	 **Oleacein**
 **Oleuropeindial 5S,4S**	 **Oleomissional**

All the above ingredients are not commercially available and constitute >95% of the phenolic content of olive oil that is related with the EU 432/2012 regulation about the health claim.

The above list was accomplished by commercially available standards of tyrosol and hydroxytyrosol which are found in small quantities in fresh oils but in higher amounts in old oils.



Hydroxytyrosol



Tyrosol

During the isolation procedure, **one new phenolic ingredient** (tyrosol derivative) was isolated and characterized and its name was given as **oleocanthalic acid.**

All the above standards were used to optimize the existing methodologies for olive oil analysis that had been previously developed by UOA, UCO and ARI.

More specifically, the existing methodologies of analysis by NMR, LC-MS/MS, colorimetry were optimized by each corresponding partner UOA, UCO, ARI to increase accuracy, precision, repeatability, sensitivity and time of analysis and reduction of artifacts formation and cost.

UOA optimized the NMR method at two magnetic fields (600 MHz and mainly 400 MHz) for all above mentioned standards targeting mainly to increase accuracy and reduce the problem of overlapping peaks. Alternative peaks were also investigated as candidates for quantitation in the case where the samples present overlapping.

UCO optimized the LC/MS/MS method and expanded it to include all available standards. Special care was taken to reduce the problem of artifacts formation.

ARI tested the specificity of the previously developed method in order to guarantee that the obtained measurement is associated only with the target compounds. The colorimetric aristoleo method was proven that it measures only the levels of oleocanthal and oleacein and is an alternative cheap way of measurement. There was a strong need to check that the method is selective for the two compounds and that there is no interaction with other similar compounds, included in the above mentioned standards list. Parameters that were investigated during the optimization phase included temperature, time, wavelength of measurement as well as reagents concentration and the need or not for centrifugal separation at the last step before the optical measurement.

It should be emphasized that in order to obtain homogeneous results the three measurement methods were calibrated using common chemical standards ingredients of olive oil. The purity of all standards provided by UOA was checked using a second commercially available internal standard (syringaldehyde). Every batch of standards that was used during the optimization phase was controlled by the second internal standard and any possible differences in purity was adjusted by the ratio to the internal standard.

Methods cross-validation

After the optimization procedure was completed (**June 2017**), the optimized methods were submitted to cross-validation using common real olive oil samples, in the form of a ring test. More specifically a small number of samples (10) were analyzed by UOA and then sent to UCO, ARI, UNIST for analysis. UOA used the NMR method, UCO the LC-MS/MS, ARI the colorimetric and UNIST used both the LC-MS/MS and the colorimetric method. The target of the ring test was that all partners will obtain the same results for the same samples in the limit of +/- 10%.

During the first ring test, big differences were observed because of the high temperature during the transportation of the samples. The high temperature led to significant decomposition of the samples and made necessary the repetition of the ring test in October 2017 with fresh olive oil samples that had been produced in the same month. During the steering committee meeting in Ragusa (October 2017) an expansion of the 3.1 activity was asked until the end of December 2017 to overcome this unexpected problem.

At the end of the development and optimization phase (12/2017), the results were evaluated and a guide for selection of appropriate method depending on the required applications has been prepared.

In parallel with the optimization procedure, a number of samples from all participating countries was analyzed with the existing methods (before the end of optimization). A number of samples coming from the autumn 2016 season and corresponding to the 10% of the samples that were analyzed from the autumn 2017 season was studied. The role of the part of the study was to collect preliminary data from the 2016 season that would help us to compare with the 2017 season and to identify the initial factors (e.g variety, harvest time, irrigation, olive mill operation parameters) that should be taken in account and how they influence the quality control.

Guide for production of olive oil with increased health protecting properties, including information about which factors (e.g variety, harvest time, irrigation, olive mill operation parameters) should be taken in account

Study of the cultivar influence on the phenolic profile of monovarietal virgin olive oils performed by the University of Cordoba

The University of Cordoba made an extensive study of the cultivar influence on the phenolic profile of monovarietal virgin olive oils.

This study was carried out to evaluate the influence of the cultivar on the phenolic profile of virgin olive oil. This research was carried out by setting the same agronomical and technological conditions for all cultivars in order to associate the phenolic variability to the cultivar influence.

The main conclusion of this study, was the remarkable variability found for nine phenolic compounds in the largest set of monovarietal VOOs analyzed to date. Genotype was the main factor contributing to this variability for all phenolic compounds with a percentage of total variance between 83% and 97%. The secoiridoid derivatives were the most abundant phenols of all monovarietal VOOs evaluated in this study. Various previously undistinguished olive cultivars were revealed to be very rich, interesting cultivars for certain phenolic compounds.

Multivariate analysis allowed detection of four groups of cultivars (G1, G2, G3 and G4) via their phenolic profile. G1 was characterized by a high concentration of oleuropein and ligstroside aglycon isomers and G2 by a high concentration of oleocanthal and oleacein; G3 was rich in two flavonoids (apigenin and luteolin). The last group, G4, included cultivars for VOOs that did not stand out in terms of the monitored phenols. The differences in the phenolic profiles of VOOs pertaining to G1 and G2 groups allowed detection of two independent pathways in the metabolism of oleuropein and ligstroside, through the involvement of demethylesterases and β-glucosidases.

Study of the variety, harvest time and olive mill operation parameters performed by the University of Athens

The University of Athens analyzed 300 samples from Greece, Italy, Croatia, Cyprus from the autumn 2016 period and made a first identification of the factors the can influence the phenolic content. The samples were collected from UNIST for Croatia, ANETEL for Cyprus, SVIMED for Italy and Region of Peloponnese for Greece.

The first conclusion is that specific varieties can lead to very high phenolic content while others were most often give low phenolic oils.

The most interesting sample was obtained from Cyprus, from the Kalamon variety harvested on September 2016. This specific sample presented oleocanthal level at 2645 mg/Kg which is the highest that has ever been recorded until now.

We also identified that extremely early harvest (e.g beginning of September) at least for some varieties (e.g Halkidikis, Koroneiki, Athinolia, Kalamon) can lead to very high phenolic content. On the contrary too late harvest (e.g. February) in most varieties was leading to low phenolic content. An exception was Lianolia from Corfu that presented relatively high oleocanthal content even until March or even April 2017.

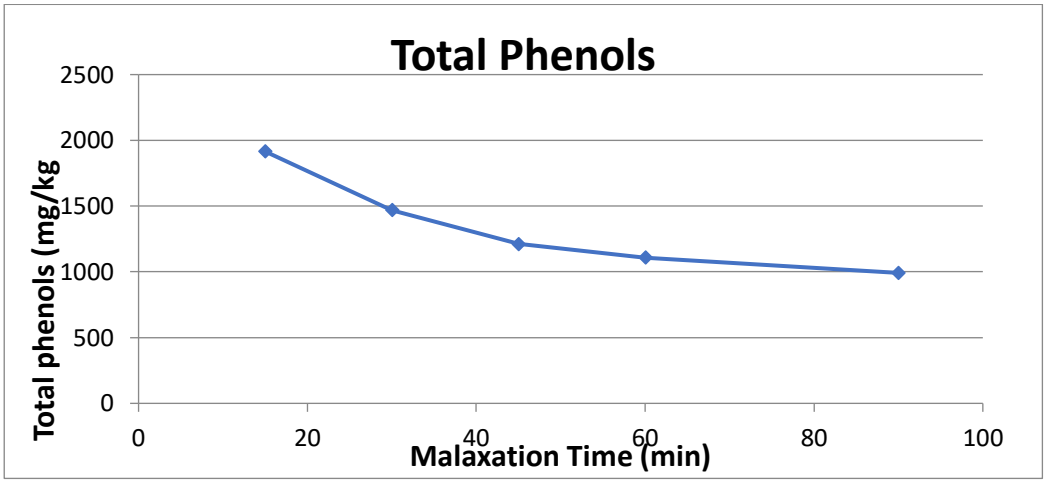
We also identified that olive oil samples coming from non-irrigated orchards had bigger chance to give high phenolic oils. Interestingly olive oil produced before and after rainfall presented differences up to 200 mg/Kg of phenols with the levels being reduced after the rainfall.

The most critical observation was that specific olive mill parameters and especially the time of malaxation could have a tremendous impact on the phenolic content and the phenolic profile as well as the addition or not of water during the malaxation or during the centrifugation.

The initial conclusions from the first period of study are the following:

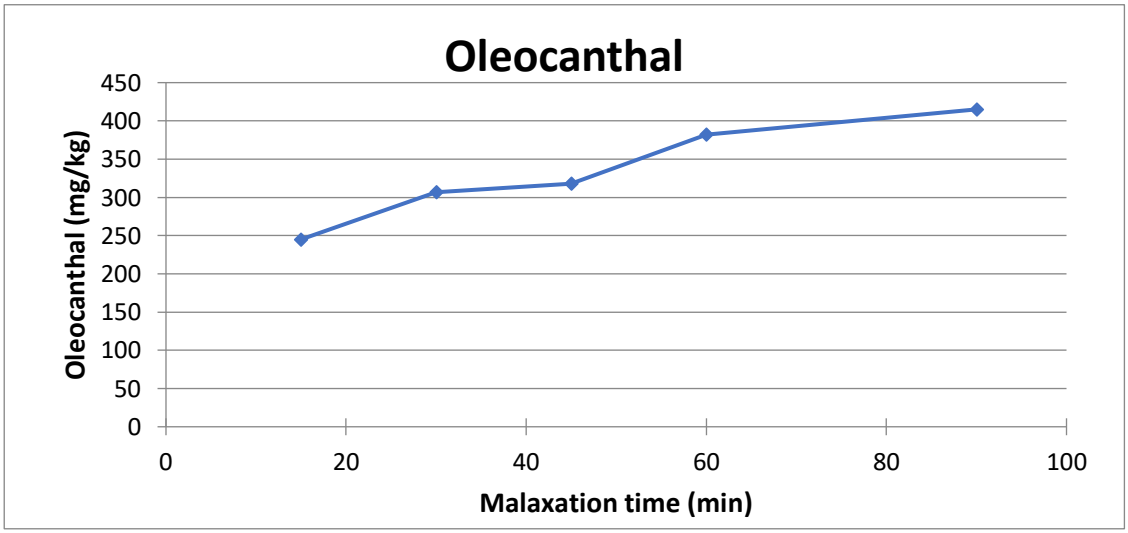
1) The increase of malaxation time, independently of the malaxation temperature leads to lower concentration of total phenolics

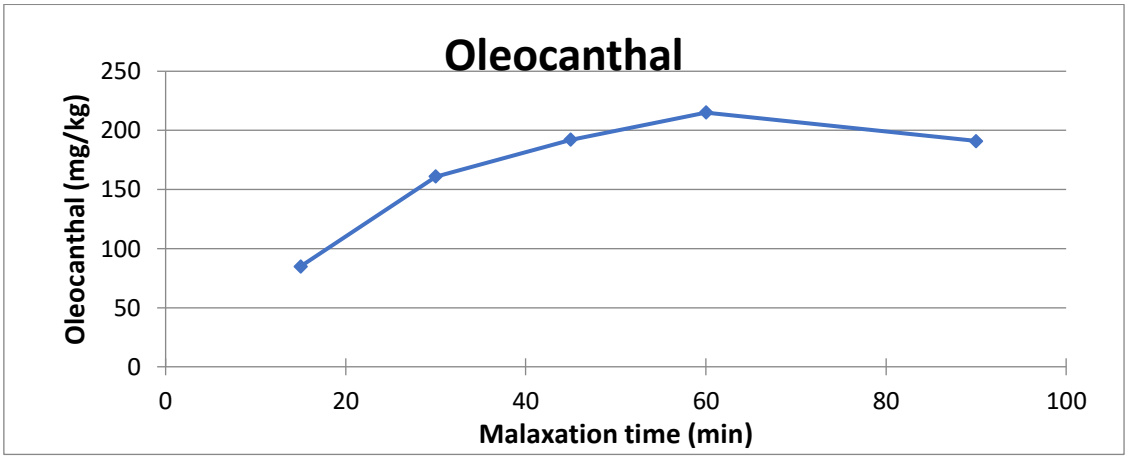
Following is an examples of Koroneiki variety from Lakonia malaxed at 28 C



2) In most cases the oleocanthal content increases with malaxation time

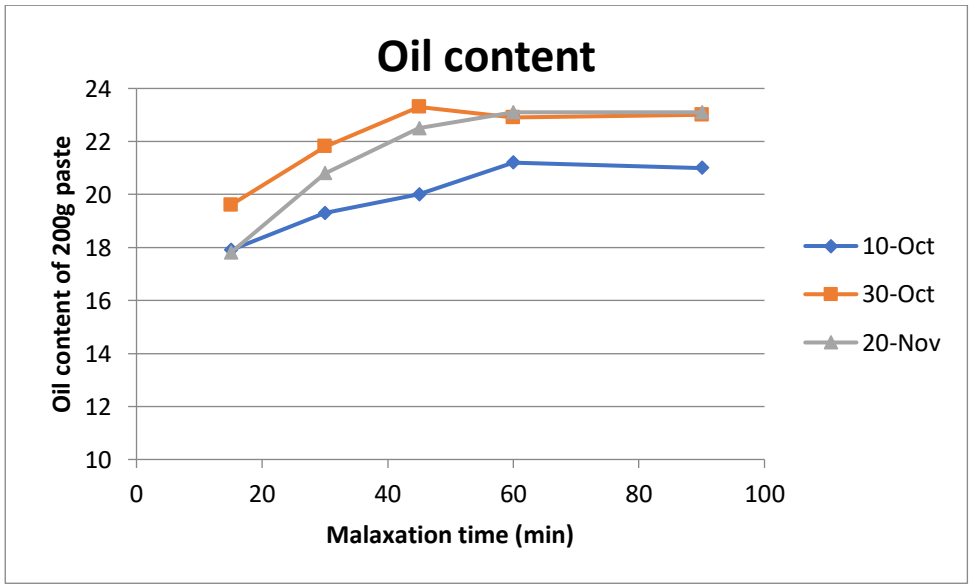
Following are two examples from Koroneiki variety



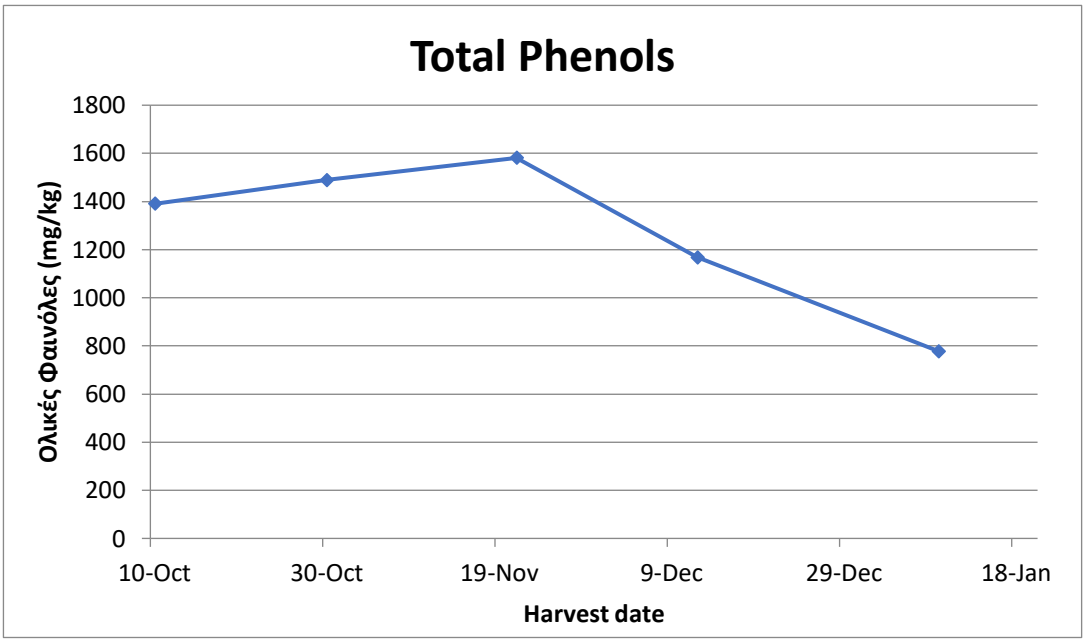


3) The olive oil yield increases with increase of malaxation time by 10 or up to 20% (for low temperatures of malaxation

4) The olive oil yield increases up to 20% depending on the maturity for a period from early October to late November



5) The increase of maturity index leads to decrease of total phenols. For Koroneiki variety there is a one-month period with stable high content after which there is a sudden drop.



6) We also observed that even in the same orchard, olives of the same variety harvested in the same day and produced in the same mill and the same conditions can lead to oils with very different phenolic content. This observation led to the identification of another critical parameter which the type of soil and especially the porosity of the soil and its ability to absorb rain water.

More detailed results will occur after the completion of activity 3.2

Guide for quality control of olive oil with increased health protecting properties

The guide includes a report with the finally proposed methodologies for monitoring of olive oil quality in relation to its health-protecting properties and a guide with advantages and disadvantages of each method.

Report with the finally proposed methodologies for monitoring of olive oil quality in relation to its health-protecting properties

ANALYTICAL METHOD FOR QUANTITATIVE ANALYSIS OF PHENOLIC COMPOUNDS IN OLIVE OIL BY NMR

This is the analytical method developed by the University of Athens for quantitative analysis of phenolic compounds in olive oil. The method is based on a simple sample preparation involving a liquid-liquid extraction with acetonitrile and subsequent analysis Nuclear Magnetic Resonance NMR. The method includes the determination of eight phenolic compounds, namely: oleuropein aglycon (four isomers), ligstroside aglycon (four isomers), oleacein and oleocanthal.

Detailed description of all the optimization trials are included as annex.

The **final procedure** that is proposed based on NMR methodology is the following:

- 5 g of filtered olive oil are weighed directly to Falcons of 50 ml.
- The olive oil is mixed with 20 ml cyclohexane, using a 20 ml graduated glass pipette.
- The mixture is homogenized manually for 1 minute.
- 25 ml of acetonitrile are added by using a 20 ml graduated glass pipette
- The mixture is homogenized again manually for 1 minute
- The mixture is then centrifuged at 4000 rpm for 5 minutes,
- A part of the acetonitrile phase (25 ml) is collected with a 25 ml graduated glass pipette and mixed with 1.0 mL of a syringaldehyde (4-hydroxy-3, 5-dimethoxybenzaldehyde) solution (0.5 mg/mL) in acetonitrile.

- The mixture is placed in a 100 ml round bottom flask and evaporated under reduced pressure using a rotary evaporator.
- The evaporated sample is placed in a dessicator for 10 minutes to completely remove from the sample all residual solvents
- The residue of the above procedure is dissolved in $CDCl_3$ (750 µL) and an accurately measured volume of the solution (550 µL) is transferred to a 5 mm NMR tube.

NMR Spectral Analysis

1H NMR spectra are recorded at 400 MHz (Bruker DRX400), 500 or 600 MHz. Typically, 32 scans are collected into 32K data points over a spectral width of 0–14 ppm with a relaxation delay of 1 s and an acquisition time of 1.7 s. Prior to Fourier transformation (FT) an exponential weighing factor corresponding to a line broadening of 0.3 Hz is applied. The spectra were phased corrected and integrated using MestRenova

Integration procedure at 400 MHz

At a first step, a baseline correction procedure is performed using multipoint selection.

At a second step, the peak of internal standard at 9.81 ppm is integrated and the integration value is set to 1

For Oleocanthal: the peaks at 9.62 ppm and 9.22 ppm are integrated. The integration value at 9.62 ppm (i1) is multiplied by 1.2 giving i2. i2 should give the same integration value as the peak at 9.22 ppm (i3). If not, the lowest between the two values (i2 and i3) is used for quantitation.

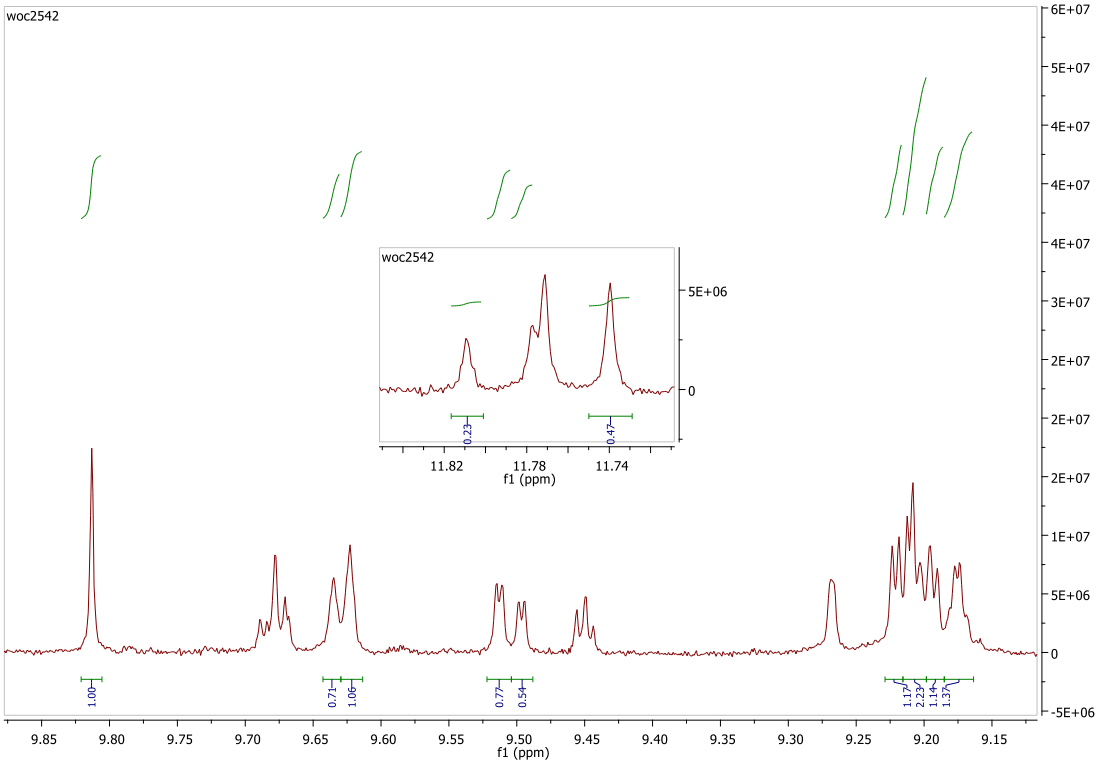
For Oleacein: the peaks at 9.64 ppm and 9.19 ppm are integrated. The integration value at 9.64 ppm (i4) is multiplied by 1.2 giving i5. i5 should give the same integration value as the peak at 9.19 ppm (i6). If not, the lowest between the two values (i5 and i6) is used for quantitation.

For oleuropein aglycon monoaldehyde form: The peak at 9.51 ppm is integrated (i7)

For ligstroside aglycon monoaldehyde form: The peak at 9.50 ppm is integrated (i8)

For oleuropein aglycon dialdehyde forms and oleomissional: the peaks at 11.81 ppm and the multiple peak at 9.16-9.18 ppm are integrated. The integration value at 11.81 ppm (i9) is multiplied by 4 giving i10. i10 should give the same integration value as the peak at 9.16-9.18 ppm (i11). If not, the lowest between the two values (i10 and i11) is used for quantitation. The two isomers of oleuropein aglycon dialdehyde forms and oleomissional are quantitated all together since they are in an equilibrium.

For ligstroside aglycon dialdehyde forms and oleokoronal: the peaks at 11.74 ppm and the multiple peak at 9.200-9.215ppm are integrated. The integration value at 11.74 ppm (i12) is multiplied by 4 giving i13. i13 should give the same integration value as the peak at 9.200-9.215 ppm (i14). If not, the lowest between the two values (i13 and i14) is used for quantitation. The two isomers of ligstroside aglycon dialdehyde forms and oleokoronal are quantitated all together since they are in an equilibrium.


woc2542
6E+07
5E+07
4E+07
4E+07
4E+07
3E+07
2E+07
2E+07
2E+07
1E+07
5E+06
0
-5E+06
1.00
0.71
1.06
0.77
0.54
1.17
2.23
1.14
1.37
9.85
9.80
9.75
9.70
9.65
9.60
9.55
9.50
9.45
9.40
9.35
9.30
9.25
9.20
9.15
f1 (ppm)
woc2542
5E+06
0
0.23
0.47
11.82
11.78
11.74
f1 (ppm)

Quantitation equations:

- For Oleocanthal:

C (mg/Kg of olive oil) = 164,77*(i2 or i3) + 16,48

- For Oleacein:

C (mg/Kg of olive oil) = 170,91*(i5 or i6) + 15,97

- For oleuropein aglycon monoaldehyde form

C (mg/Kg of olive oil) = 243,5 * i7 +4,58

- For ligstroside aglycon monoaldehyde form

C (mg/Kg of olive oil) = 232,7 * i8 +4,3

- For oleuropein aglycon dialdehyde forms (oleuropeindials + oleomissional)

C (mg/Kg of olive oil) = 250,73 * (i10 or i11) +5,43

- For ligstroside aglycon dialdehyde forms (ligstrodials + oleokoronal)

C (mg/Kg of olive oil) = 232,7 * (i13 or i14) +4,3

ANALYTICAL METHOD FOR QUANTITATIVE ANALYSIS OF PHENOLIC COMPOUNDS IN OLIVE OIL BY LC-MS/MS

This is the analytical method developed by the University of Cordoba for quantitative analysis of phenolic compounds in olive oil. The method is based on a simple sample preparation involving a liquid-liquid extraction with a hydroalcoholic extractant and subsequent analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The method includes the determination of eight phenolic compounds, namely: hydroxytyrosol, tyrosol, oleuropein aglycon (two isomers), ligstroside aglycon (two isomers), oleacein and oleocanthal. Due to the different concentration levels of these phenolic compounds

in olive oil, three injections per sample at three dilution factors are required. Next, the experimental protocol as well as the reagents, materials and instrumentation used for the analysis are described.

Reagents and standards

The solvents used for the analysis of phenols in olive oil were mass spectrometry (MS) grade methanol (MeOH) and *n*-hexane, both from Scharlab (Barcelona, Spain). MS-grade formic acid, also from Scharlab, was used as an ionization agent in the chromatographic mobile phases. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare both the aqueous mobile phase and the hydroalcoholic mixture used as extractant.

The evaluated phenols were hydroxytyrosol, tyrosol, oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycon (3,4-DHPEA-EA) and, ligstroside aglycon (p-HPEA-EA). The aglycon forms of oleuropein and ligstroside were discriminated according to their structures. Thus, it was possible to discriminate between the aldehyde open forms of oleuropein aglycon (AOleAgly, the sum of stereoisomers) and the monoaldehyde closed form of the oleuropein aglycon (MAOleAgly). By analogy, it was possible to discriminate between the aldehyde open forms of ligstroside aglycon (ALigAgly, the sum of stereoisomers) and the monoaldehyde closed form of ligstroside aglycon (MALigAgly). Standards for hydroxytyrosol and tyrosol were purchased from Extrasynthese (Genay, France). Oleacein, oleocanthal, and the aldehydic open forms of oleuropein aglycon and ligstroside aglycon were provided by Prof. Magiatis of the University of Athens (Greece). The monoaldehyde forms were quantified using the corresponding standards for the aldehyde open forms. Standard solutions of non-secoiridoid phenols were prepared in methanol (1 mg/mL), while secoiridoids were prepared at the same concentration in pure acetonitrile to preserve their stability and avoid undesired conversion to acetal and hemiacetal derivatives.

Sample preparation for analysis of phenolic compounds

Phenolic compounds were isolated by liquid-liquid extraction following previously optimized protocols (Verónica Sánchez de Medina, Priego-Capote, & Luque de Castro, 2015). For this purpose, 2 g of VOO are mixed with 2 mL *n*-hexane; then, 2 mL of 60:40 (v/v) methanol-water are added and shaken for 2 min, and the hydroalcoholic phase is separated by centrifugation. The extraction is repeated to enhance the extraction efficiency (V. Sánchez de Medina et al., 2017). The resulting phenolic extracts are analyzed by LC–QqQ MS/MS with three different dilution factors (1:2, 1:50 and 1:200 v/v) to encompass the concentration variability.

LC-MS/MS analysis of phenolic compounds

Analyses are performed by reversed-phase liquid chromatography followed by electrospray ionization (ESI) in negative mode and tandem mass spectrometry (MS/MS) detection. Five µL of extract are injected in triplicate into the LC system for chromatographic separation of the target compounds using a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 µm particle size) from Varian (Walnut Creek, CA, USA). The column compartment is kept at 30 ˚C. Mobile phase A is 0.1% formic acid in water, while phase B is 0.1% formic acid in MeOH. The gradient program, at a 0.4 mL/min constant flow rate, is as follows: initially, 50% phase A and 50% phase B are maintained for 0.5 min; from 0.5 to 2 min, mobile phase A is from 50 to 20%; and from min 2 to 4, mobile phase A is from 20 to 0%. This last composition is maintained for 1 min. After each analysis, the column is equilibrated for 5 min to the initial conditions.

The entire eluate is electrosprayed and monitored by MS/MS in Multiple Reaction Monitoring (MRM) mode for selective transitions from precursor to product ions for each analyte. The MRM parameters for the analysis of target phenols are listed in **Table 1**. The flow rate and temperature of the drying gas (N_2) are 10 L/min and 300 ^{0}C, respectively. The nebulizer pressure is 50 psi, and the capillary voltage is 3000 V. The dwell time is set at 200 µs.

Quantitation of the target compounds and statistical analysis

Absolute quantitative analysis is performed by calibration curves obtained using fresh refined oil (sunflower, olive, maize) spiked with the target phenols. The absence of quantifiable levels of phenols in the refined oil is checked by direct analysis with the developed method. Eight phenolic concentrations from 0.1 ng/mL to 5 µg/mL are injected in triplicate to obtain the calibration curves. The concentration of phenols in the monovarietal VOOs is determined with these models, using three replicates per sample.

Table 1. Multiple Reaction Monitoring (MRM) parameters for quantitative analysis of phenolic compounds by LC–MS/MS.

Phenol		Retention time (min)	Q1 voltage (V)	Precursor ion (*m/z*)	Collision energy (eV)	Quantitative transition (*m/z*)	Product ion confirmation (*m/z*)
Hydroxytyrosol		2.1	110	153.1	10	153-123	108
3.4-DHPEA-EDA (Oleacein)		4.3	110	319.1	12	319-59	139
3.4-DHPEA-EA	AOleAgly	4.6	110	377	12	377-275	307
	MAOleAgly	5.9	110	377	12	377-275	307
p-HPEA-EDA (Oleocanthal)		5.4	110	303.1	12	303-59	137
p-HPEA-EA	ALigAgly	5.5	110	361.1	12	361-291	101
	MALigAgly	6.2	110	361.1	12	361-291	101
Luteolin		6.3	170	285	35	285-133	175
Apigenin		6.6	170	269	35	269-117	151

AOleAgly – Aldehydic open forms of Oleuropein Aglycon; **MAOleAgly** – Monoaldehydic closed form of Oleuropein Aglycon.

ALigAgly – Aldehydic open forms of Ligstroside Aglycon; **MALigAgly** – Monoaldehydic closed form of Ligstroside Aglycon.

Certificate of analysis emitted for producers

Córdoba 16 June, 2017

Producer: XXXXXXXXXXXX

Samples: 1

Cultivar: Picual

Collection date: 15/11/17

Analytical method: Liquid–liquid extraction of phenolic compounds and analysis by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) in SRM mode.

Quantitation method: Absolute quantitation based on calibration models prepared with pure standard solutions of the analyzed phenols.

Compound	Concentration (mg/kg)
Hydroxytyrosol	0,9
Tyrosol	0,0
Oleacein	109
Oleocanthal	252
Oleuropein aglycon (open aldehydic forms)	634
Oleuropein aglycon (close monoaldehydic form)	32,1
Ligstroside aglycon (open aldehydic forms)	472
Ligstroside aglycon (closed monoaldehydic form)	485
Apigenin	1,0
Luteolin	2,4

Total content of hydroxytyrosol derivatives: 777 mg/kg

Total content of tyrosol derivatives: 1209 mg/kg

Total content in phenolic compounds included in the EFSA Health Claim: 1986,1 mg/kg

Total content of analyzed compounds: 1989,5 mg/kg

Comments:

The daily intake of 20 g of the analyzed olive oil provides **39,7 mg** of hydroxytyrosol, tyrosol and derivatives, an amount higher than that stated by the European Regulation 432/2012 (5 mg of daily intake) based on the EFSA Health Claim. Therefore, the intake of this olive oil according to the suggested amount provides the health benefits described in the Health Claim, with special emphasis on the protection of blood lipids against oxidation.

F. Priego-Capote

ARISTOMETRO

The final method of colorimetric optical measurement of the sum of oleocanthal and oleacein (D1 ratio) is the following:

- 7,5 ml of olive oil is placed in the ARISTOLEO 12 ml vial

- 1,5ml of reagent (p-hydroxyanthranilic acid) which is dissolved in acetic acid is added
- We agitate the sample for 30sec making sure that it becomes homogeneous and we wait for 30sec
- 1,5ml of distilled water is added and the vial is vigorously agitated for 30 sec
- We wait for 30min, we put the sample in the ARISTOMETRO and the concentration of the sum of oleocanthal and oleacein is automatically calculated.

Figure 1: ARISTOMETRO

The method of ARISTOLEO was validated using a zero phenolic sample of olive oil in which we added specific amounts of oleacein and oleocanthal, obtaining the following concentrations 0 , 50 , 150 , 250 , 500

, 750, 1000, 1250 and 1500 mg/kg . After the waiting time, the phases were separated and we measured the absorption in the aqueous phase using a laboratory spectrophotometer and ARISTOMETRO.

C(mg/kg)	A(639nm) using spectrophotometer	ARISTOMETRO C(mg/kg)	
0	0	0	47680
50	32	57	43980
150	94	156	37483
250	157	241	32527
500	313	527	20419
750	470	760	13547
1000	626	1020	9002
1250	783	1260	6001
1500	940	1510	4029

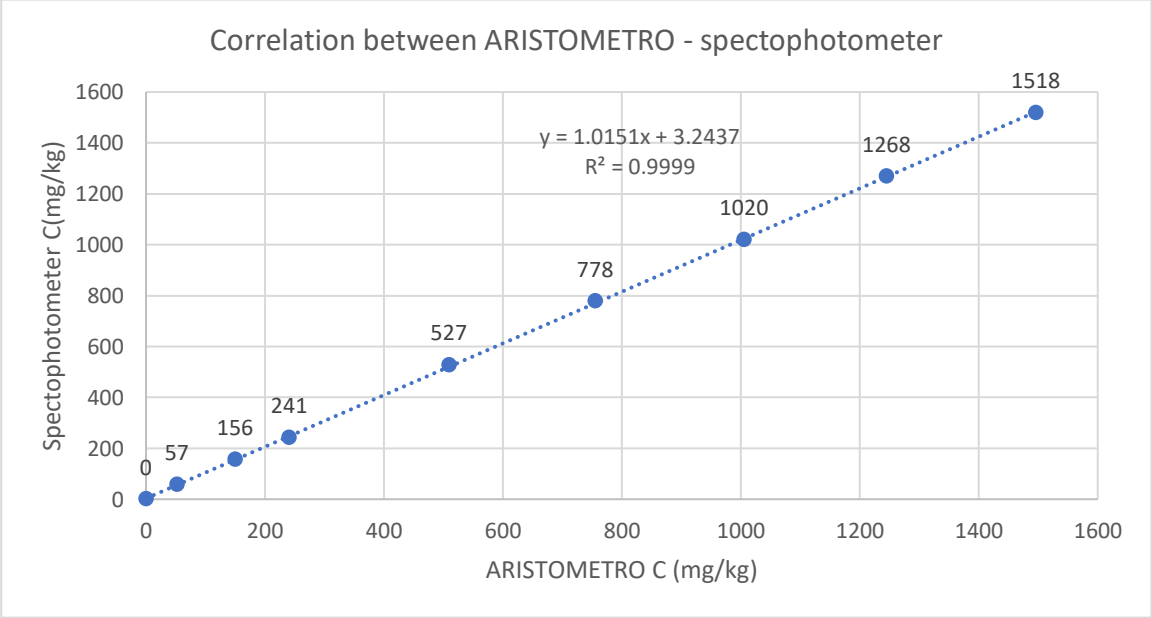


Diagram 1: Correlation of the sum of oleocanthal and oleacein using ARISTOMETRO and spectrophotometer

Oleocanthal and oleacein was added in specific concentration in olive oil , the optical measurement of the D1 ratio was applied and the absorptions was measured using ARISTOMETRO .

C(mg/kg) oleocanthal and oleacein	ARISTOMETRO C(mg/kg)
50	60
150	149
200	211
300	317
400	414
450	470
600	609
750	765
900	910
1050	1063
1200	1221
1350	1363
1500	1554

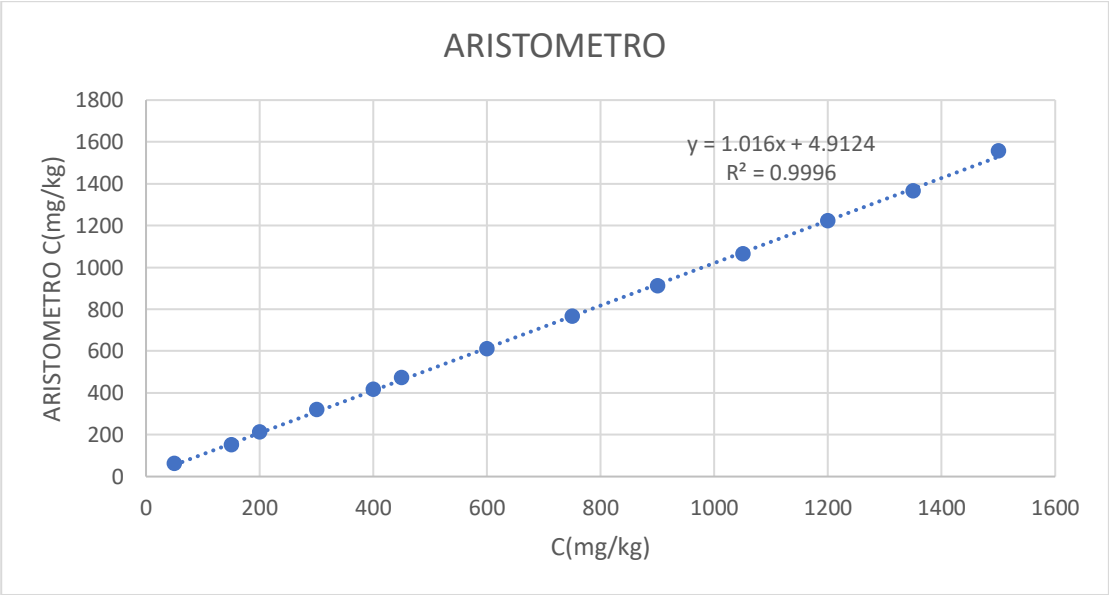


Diagram 2: Calibration curve of the sum of oleocanthal and oleacein using ARISTOMETRO

Advantages and disadvantages of each method

NMR

Advantages

Very cheap consumables

Short time of analysis ~1 min

No need for calibration with purified standards

Disadvantages:

Expensive

Needs special equipment and trained personnel

Is not currently able to measure the concentration of free hydroxytyrosol

LC-MS/MS

Advantages:

Can measure low concentrations of free hydroxytyrosol, tyrosol and also other ingredients like flavonoids and lignans

Disadvantages:

Expensive

Needs special equipment and trained personnel

Needs calibration with purified standards

ARISTOMETRO

Advantages:

Very cheap

No need for special training

Portable. The olive oil samples can be measured in the olive mill

Disadvantages:

-Can measure only the levels of oleocanthal and oleacein. If their sum is >250 mg/Kg then it is enough to certify that the oil can have a health claim. If the sum is <250 then it is necessary to perform one of the two other methods and measure all the phenolic ingredients in order to measure the total phenolics.

-Cannot measure separately the levels of oleocnanthal and oleacein but only their sum