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: 3.3.1
Title of DELIVERABLE: Certification Centre Operational e-guide

ACTIVITY n. 3.3: Operational Instrument

WP n. 3: STUDYING

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Status:

Draft □	*Final* ×	*Version n. 01*

Distribution:

Confidential ×	*Public* □	

Date: 31 December 2017

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Contents

Certification's centre e-Guide

The following protocol must be applied for the operation of the Certification Center:

1. Sample recording

Olive oil samples receipt and entry must take place in a separate room from the analytical laboratory. Olive oil samples are acquired in glass, iron or plastic bottles, of 250ml content. This requires a large and stable surface for the initial storage until the samples proceed to analysis. Three forms signed by the producer must accompany all samples. The producer's form where the producer records his contact information, then the olive grove form where the producer refers his grove information, and last the sample form where the producer describes details about his olive oil and the production procedures.

There is always, at least one person, who has as an exclusive responsibility the management of the samples before analysis. The first thing that we do when we receive a sample is to create an entry in the system for the producer. The entry gives a unique code to the producer. Then, in the producer registry, we record all contact information and details about the company and the brand names used by the producer.

After this, we create an olive grove entry that corresponds to each producer. Producers may own more than one grove, so we create entries for every grove of the producer. In the grove section, we write down every information related to the orchard. We record the coordinates and the geographic position of the grove. Moreover, we entry details about the geology of the grove, soil analysis and any possible sprinkling. Finally, we state every variety included in this orchard and the tree population of every variety.

Finally, we process to create a unique entry for every sample of olive oil. In this step it is very important to correspond the correct sample to the grove where it comes from, for producers who have many orchards. In this section, we record every information

available about the olive oil sample. We state the grove from where the olive was produced, and the variety of the specific sample. We report the date and time of olive gathering and olive oil production, and the storage means as well. We, also, state the name and the type of the olive oil mill and the malaxation conditions.

2. Storage

100 ml of each olive oil sample are filtered through corrugated paper filter (60 g/cm^2). The filtered olive oil is stored in dark bottles of 100 ml, hermetically sealed in the freezer. The code number for each sample is written on the bottle and the lid. This code is used during the whole analytical procedure. The technician who analyzes the samples knows only this code without any data for the sample.

3. NMR Analysis

3.1 Olive Oil Extraction and Sample Preparation

Following, the analytical protocol is described in detail.

- Falcons of 50 ml resistant to cyclohexane were obtained by Labcon (CT1155). The code number is written both on the body and on the lid of each falcon.
- 5 g of filtered olive oil are weighed directly to the corresponding falcon. The analytical scale used is a KERN ABJ120-4NM with accuracy of 0.0001 g.
- The olive oil is mixed with 20 ml cyclohexane (HPLC grade), by using a 20 ml graduated glass pipette (accuracy 100 µl). The mixture is homogenized manually for 1 minute.
- 25 ml of acetonitrile are added by using a 20 ml graduated glass pipette (accuracy 100 µl) and the mixture is homogenized again manually for 1 minute
- The mixture is then centrifuged at 4000 rpm for 5 minutes, in a Thermo Electron Corporation-Multifuge 3S/D-37520 Osterode centrifuge. Before centrifugation falcon lids must be unscrewed to avoid breakage.
- 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 internal standard is added by automatic pipette NICHIRYO NICHIPET EX after it has been left to come to room temperature
- The mixture is placed in a 100 ml round bottom flask and evaporated under reduced pressure using a rotary evaporator (R-114 Buchi). The temperature of the waterbath must not exceed 40 ° C.
- The evaporated sample is placed in a dessicator for 10 minutes to completely remove from the sample humidity and residual solvents
- The residue of the above procedure was dissolved in $CDCl_3$ (CAS No: 865-49-6) (750 µL) and an accurately measured volume of the solution (550 µL) was transferred to a 5 mm NMR tube.

3.2. NMR Spectral Analysis

1H NMR spectra were recorded at 400 MHz (Bruker DRX400). Typically, 50 scans were collected into 32K data points over a spectral width of 0–16 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 was applied. The spectra were phased corrected and integrated using MestRenova.

3.2.1 Internal Standard (IS) preparation

IS solution is prepared in acetonitrile at a concentration of 0.5 mg/mL and kept in a refrigerator. Syringaldehyde (98% purity) was obtained by Fluorochem (Cas No: 394-31-0). 50 mg of syringaldehyde are weighed into 5 ml vial by analytical scale (KERN ABJ120-4NM). The substance dissolves in acetonitrile (HPLC grade), is transferred exhaustively into a 100 ml volumetric flask and 100 ml acetonitrile are added. Finally, the concentration of each IS solution is verified by calculating the absorption in 302 nm.

3.3 Analysis Certificate for producers – example

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Athens, 21/12/2017
Cert.Num: 1718-C00352

CERTIFICATE OF ANALYSIS

Analysis Date: 13/12/2017

Owner: THEODORAKOPOULOS ALEXANDROS

Origin: PLATANOS PULIA MESSINIA GREECE

Production Date: 20/11/2017

Chemical Analysis

Oleocanthal	172	mg/Kg
Oleacein	127	mg/Kg
Oleocanthal + Oleacein (index D1)	298	mg/Kg
Ligstroside aglycon (monoaldehyde form)	51	mg/Kg
Oleuropein aglycon (monoaldehyde form)	72	mg/Kg
Ligstroside aglycon (dialdehyde form)	116	mg/Kg
Oleuropein aglycon (dialdehyde form)	45	mg/Kg
Total tyrosol derivatives	339	mg/Kg
Total hydroxytyrosol derivatives	244	mg/Kg
Total phenols analyzed	583	mg/Kg

Comments :

The levels of oleocanthal and oleacein are higher than the avarage values (135 and 105 mg/Kg respectively) of the sample included in the international study performed at the University of California, Davis

The daily consumption of 20 g of the analyzed olive oil provides 11.7 mg of hydroxytyrosol, tyrosol or their derivatives (>5 mg) and consequently the oil belongs to the category of oils that protect the blood lipids from oxidative stress according to the Regulation 432/2012 of the European Union.

It should be noted that oleocanthal and oleacein present important biological activity and they have benn related with anti-inflammatory, antioxidant, cardioprotective and neuroprotective activity.

The chemical analysis was performed according to the method published in J.Agric. Food Chem., 2012, 60 (47) , pp 11696-11703, J.Agric. Food Chem., 2014 62 (3) , 600-607 and OLIVAE, 2015, 122, 22-33.

*Oleomissional+Oleuropeindial **Ligstrodial+Oleokoronal

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4. Certification with LC-MS/MS

Sample preparation for analysis of phenolic compounds

Phenolic compounds were isolated by liquid-liquid extraction. 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. 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.

4.1. 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.

4.2. Analysis Certificate for producers - example

Córdoba 16 June, 2017

Producer: XXXXXXXXXXX

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.