Project co-financed by the European Regional Development Fund

Kalamata, 21/10/2019

To the technical committee of the International Olive Council

Dear Sir/Madam,

The steering committee of the Interreg Med ARISTOIL project is pleased to present you the outcome of the collaborative study between the National and Kapodistrian University of Athens, Department of Pharmacognosy and Natural products Chemistry (UoA) and the University of Cordoba, Department of Analytical Chemistry (UCO) regarding the development of two analytical methodologies for the quantitative measurement of the phenolic ingredients of olive oil required by the EU 432/2012 health claim regulation. Both methods have been validated for their precision and accuracy and give comparable results. The validity of the two methods has been checked through the analysis of the same samples in the two participating laboratories.

The two methods have been used by the two Universities in the analysis of about 6,500 olive oil samples from Spain, Greece, Italy, Croatia and Cyprus during the last three years and all the obtained data have been used for statistical evaluation of the olive oil quality in the five participating countries.

The ARISTOIL project represents about 3,000 producers who would like to express their support toward the adoption of new methods by IOC for the certification of health claim of olive oil.

We would like to propose you to accept for evaluation the two methods and consider them as a reliable and acceptable way to measure the phenolic content of olive oil for the certification of the EU health claim. If the evaluation by the experts of IOC is successful, we would like to propose you to permit their use as optional reference methods. We are aware of the high cost of the used equipment and we can understand that both methods cannot become obligatory but the complexity of the problem makes necessary their use at least as long as the legislation of the health claim remains as it is now.

In the following page we present the methods that have been developed, optimized and validated and we also present an example of comparison between the results obtained by the two methods.

We remain at your disposal for any further information regarding the developed methodologies.

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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 PI 1.b

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/

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Olive Oil Extraction and Sample Preparation for NMR Analysis

Following, the analytical protocol is described in detail.

- Falcon tubes 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 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.

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.

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.

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.

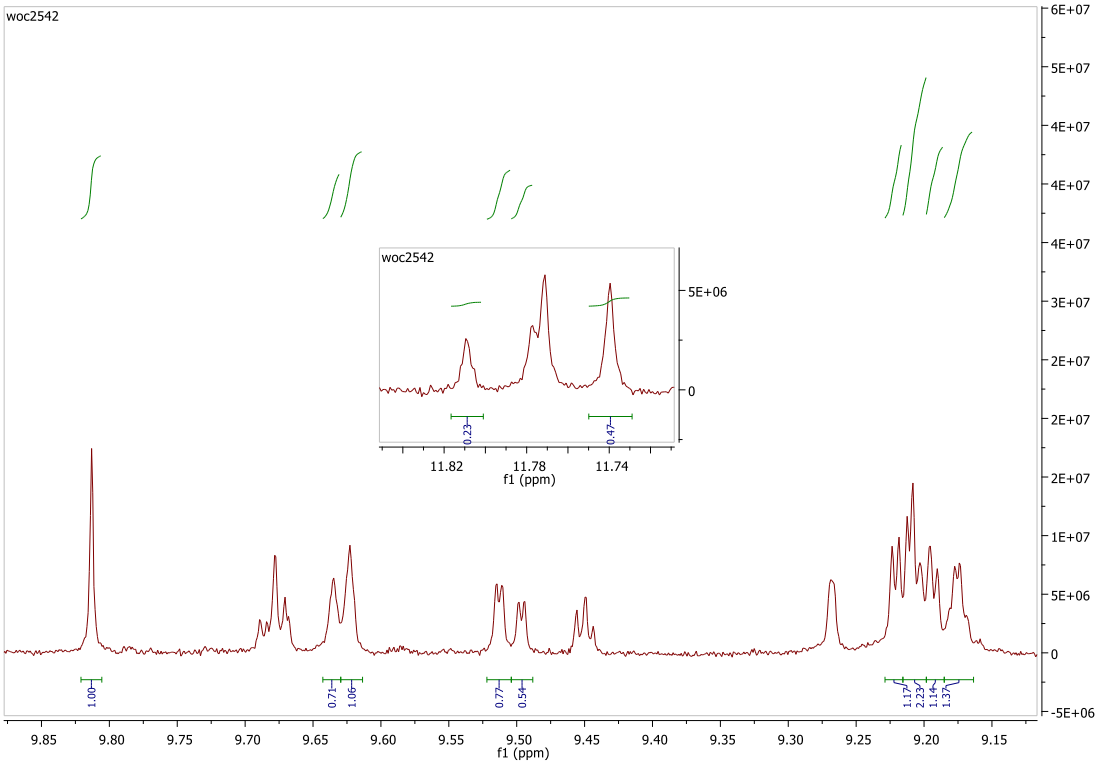
For Oleacein: the peaks at 9.64 ppm and 9.19 ppm are integrated. The integration value at 9.64 ppm (i4) is multiplied by 1.2 giving i5. i5 should give the same integration value as the peak at 9.19 ppm (i6). If not, the lowest between the two values (i5 and i6) is used for quantitation.

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.



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

The sum of the concentrations of the above compounds represents the total content of olive oil regarding the hydroxytyrosol and tyrosol derivatives required by the EU 432/2012 health claim.

Analysis 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.

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.

The exchanged real olive oil samples showed the following results in Table 2:

Table 2.

Code	Hydroxy tyrosol	Oleacein	Oleo canthal	Oleuropein Aglycon (mono aldehyde)	Oleuropein Aglycon (di aldehyde)	Ligstroside aglycon (mono Aldehyde)	Ligstroside aglycon (di Aldehyde)	Total Phenols
1	2,7	418,9	81,1	333,0	37,2	177,1	186,7	1236,7
2	2,2	398,1	82,8	325,8	40,1	180,5	197,9	1227,3
3	1,3	250,3	79,2	84,1	22,1	45,1	29,4	511,4
4	1,2	115,3	37,6	258,8	173,6	137,4	485,1	1209,0
5	0,6	103,9	49,8	162,3	115,5	86,8	288,6	807,6

6	0,4	45,8	31,1	83,9	66,1	44,9	116,7	388,9
7	0,6	48,6	10,3	8,4	0,0	1,4	0,0	69,3
10	0,6	52,1	18,4	29,9	9,8	12,4	15,3	138,5

Comparison of the results obtained by the two methods

Real olive oil samples from Spain were transferred to UOA. The samples were initially analyzed in the certification Center in Spain and randomly selected among hundreds of different olive oil samples that had been previously analyzed in Spain. The target of the exchange of samples between the centers was to check the agreement between the results.

In parallel UOA prepared a batch of purified standards including:

- Oleocanthal
- Oleacein
- Oleuropein aglycon (monoaldehyde form)
- Ligstroside aglycon (monoaldehyde form)
- Oleuropein aglycons (Oleomissional and diadehyde forms)
- Ligsroside aglycons (Oleokoronal and dialdehyde forms)

All the fresh standards were prepared in the UOA in acetonitrile solution at concentration 1.0 mg/ml with high accuracy after measurement of purity by NMR.

The standards were transferred to UOC and were used for the construction of calibration curves.

All the exchanged olive oil samples were analyzed by UOA and UCO according to the procedures described in the previous pages.

The comparison between the results obtained in the two certification centers showed in all cases differences lower than 10% for each analyte.