SCIENTIFIC REPORT

Novel biosensors and smart tools for ultrasensitive detection of olive oils adulteration, PN-III-P4-ID-PCE-2020-0923

January 2022-Decembrer 2022

In 2022, the necessary activities in the implementation plan of the project from Stage 2 were carried out: **Detection of target molecules and development of new sensitive materials; Development and characterization of biosensors; Intelligent model development and their application in data analysis; System integration validation; Dissemination of results; Project management.**

Scientific description

Activity 2.1 Chromatographic analysis of markers. Development of new sensitive materials. Synthesis of Conductive Polymers and Molecularly Imprinted Polymers

Chromatographic and spectrometric analysis of phenolic markers

Most of the samples of olive oils (extra virgin olive oils from different producers, obtained by different technologies, from different varieties of olives, from different countries but also olive cake oils) and vegetable seed oils (corn, sunflower, flax, hemp, rape etc.) analyzed this year by chromatography, spectrometry and biosensors were commercial oils. Olive oils provided by Prof. Dr. Maria Lisa Clodoveo from the Carlos Moro University of Bari were also analyzed.

The present research aimed at a first stage to develop a targeted method for the quantification of compounds specific to vegetable oils and, in particular, olive oil, namely tyrosol, hydroxytyrosol, trigonelline, oleuropein, verbacoside, oleic acid and oleanolic acid by UHPLC -MS/MS (FullMS-vDIA), which was later used to quantify the respective compounds in vegetable oil samples in order to discriminate them using principal components analysis – PCA and cluster analysis – HCA. Also, the determination of minor phenolic compounds in oils was carried out by UHPLC-MS/MS, using the analysis method developed in step 1. HRMS data processing was carried out with the help of Compound Discoverer v. 2.0 software (Thermo Scientific, USA) using a working template for metabolomics, making it possible to identify other classes of compounds by reporting to databases including Chemspider, MzCloud, etc.

The determination of specific phenolic compounds (tyrosol, hydroxytyrosol, oleuropein), terpenic compounds (olivic and oleanolic acids) and trigonelline in vegetable oils by UHPLC-MS/MS involves three important steps, namely: (i) sampling and extraction of oil samples (solid phase extraction - SPE); (ii) data acquisition in FullMS-vDIA mode, which was performed using the Q Exactive Focus high-resolution mass spectrometer (Thermo Scientific, USA) coupled to a UHPLC Dionex high-performance liquid chromatograph for analyte separation; (iii) the processing and identification of data which was carried out with the help of the Xcalibur software. Also, by processing the spectral data with Compound Discoverer v. 2.0 software (Thermo Scientific, USA), using a working template for metabolomics, it was also possible to identify other classes of compounds by reporting to databases, including Chemspider , MzCloud, MassBank, etc. The vegetable oils investigated in this study are: extra virgin olive oil (EVOO), olive oils from local producers in Italy (MS*), commercially available olive oils (MS), but also other types of commercially available vegetable oils, such as: walnut oil (N), grape seed oil (STR), pumpkin oil (D), linseed oil (Fin), soybean oil (So), sesame oil (SU), palm oil (Palm), hemp oil (Cp), coconut oil (Co), sunflower oil (FL).

Isolation of the compounds of interest from the investigated vegetable oils was achieved by SPE extraction using SPE cartridges and a vacuum elution system (Vaccum Manifold, Varian). Three types of cartridges were used to optimize the extraction, namely: Strata NH2 cartridges (55 µm, 70 Å), 500 mg/6 mL, Strata Silica cartridges (55 µm, 70 Å), 500 mg/6 mL, and cartridges HyperSep silica 500 mg/6 mL, recovery studies performed showing better

performance (75-98% recovery rate) for Strata Silica (55 µm, 70 Å), 500 mg/6 mL cartridges that were further used for the extraction of the majority of phenolic compounds from vegetable oils. The SPE extraction protocol consists of: conditioning the SPE cartridges with 6 mL methanol and 6 mL hexane, followed by the addition of the sample solution (2.5 g oil in 6 mL hexane) and its penetration into the cartridge; washing the cartridges with 3x3 mL hexane and eluting the compounds from the cartridge with 10 mL methanol. The resulting sample solution is concentrated to dryness in a stream of nitrogen, after which the extract is taken up again with 1 mL methanol:ultrapure water = 80:20 solution, filtered through a 0.45 µm filter membrane and subjected to analysis. The schematic representation of the extraction step of the major phenolic compounds from vegetable oils using the SPE protocol is shown in Figure 1.

Figure 1. Schematic representation of the extraction of major phenolic compounds from vegetable oils prior to analysis by UHPLC-MS/MS

For UHPLC-MS/MS detection, the following categories of operational parameters were optimized and subsequently set: HESI ionization parameters, chromatographic separation parameters (mobile phase composition, gradient, flow rate), MS operating parameters (Full acquisition scan followed by fragmentation). The separation of the analytes was carried out on an analytical Kinetex C18 column (100 × 2.1 mm, 1.7 µm), at 30 °C, under the action of a gradient of mobile phases: HPLC water with 0.1% formic acid and HPLC methanol with 0.1% formic acid. The mass spectrometer was run in H-ESI (negative) ionization mode at an applied voltage of 3.0 kV. The nitrogen flow rate was 48 L/min, and the auxiliary flow was 11 L/minute at a temperature of 413°C, capillary temperature of 320°C. Data were acquired in Full MS – vDIA mode with simultaneous recording of precursor and resulting MS/MS fragments. Mass spectral data were recorded in the scan range of m/z 100–1500, with full scan resolution at 70,000, and MS/MS acquisition resolution at 35,000. The step normalized collision energy (NCE) was set to 35 eV. Data processing was carried out using Xcalibur software, and data quantification was carried out using the external standard method, in a linearity range of 25-1000 ng/mL. The coefficient of linearity ranged from 0.990 to 0.995, while the detection limit of the methods was calculated based on a signal to noise ratio of 3:1. To evaluate the performance of the analysis method, the investigation of the matrix effect was pursued by enriching the oil samples with known concentrations of the standard at a concentration level of 100 ng/mL, followed by the analysis of the resulting samples and the estimation of the recovery percentage, the results obtained being between 75-98%.

The identification of the compounds was carried out based on the comparison of the retention times with those of the reference substances and by the identification of the molecular ion and fragments resulting from fragmentation in the negative mode (Table 1 and Figure 2). For example, compounds such as: elenolic acid, including hydroxylated derivatives and aldehyde forms, oleacin and hydroxylated derivative, derived from oleuropein, oleocanthal, ligstroside, elenaic acid, were identified in extra virgin olive oil (Figure 2).

Figure 2. Identification of specific compounds in extra virgin olive oil by UHPLC–MS/MS, negative ionization and data processing with Compound Discoverer software

Table 1. Identification of phenolic compounds in vegetable oils by UHPLC-MS/MS

Compound	Retention	Molecular	Molecular	m/z	Mass fragments	
Trig						
Hyd						
Tyrc						
Vert						
Oleu						5
Olea						9,
Manitic acid	10.00	C30H48O4	472.3552	471.3479	471.3470, 472.3513	

The content of specific major phenolic compounds in the analyzed oils varies according to the oiling time, as follows: (i) in the original olive oils, the major quantified compounds are trigonelline with values between 0.834-22.514 mg/kg and hydroxytyrosol, with values between 0.008-24.582 mg/kg, while tyrosol, verbacoside and oleuropein showed lower values, respectively n.d.-2.553 mg/kg, 0.314-0.758 mg/kg and n.d.-0.243 mg/kg; (ii) higher amounts of trigonelline (5,774-34,062 mg/kg) and tyrosol (n.d.-4,363 mg/kg) were identified

in EVOO compared to original olive oils, while the content of hydroxytyrosol, verbacoside and oleuropein is smaller; (iii) higher amounts of trigonelline (2,074-26,985 mg/kg) and tyrosol (n.d.-10,386 mg/kg) were identified in commercial olive oils compared to original olive oils, while the content of verbacoside and oleuropein it is alike; (iv) in the analyzed vegetable oils, among the majority compounds are trigonelline (n.d.-65.129 mg/kg), with higher concentrations in grape seed oil and rapeseed oil and tyrosol (n.d.-12.758 mg/kg), with higher concentrations high in walnut, flax and hemp oils. Regarding the analyzed terpenic compounds, higher amounts of oleic acid and oleanolic acid correspond to commercial olive oils, with values between 0.241-18.733 mg/kg for oleic acid and 0.075-16.074 mg/kg for oleanolic acid (Table 2).

Table 2. Content of specific analyzed compounds in vegetable oils

Type	mg/kg	Trig	Htv	Tv	Verb	Oleur	MA	OA
								.d.
								446
Con								375
								074
Or								333
								559
								594
								147

The quantitative data resulting from the investigation of the minor phenolic compounds in the analyzed oils indicated that the main phenolic acids are hydroxybenzoic (HyB), dihydroxybenzoic (DHyB), p-coumaric (Ap-Coum), ferulic (AF), ellagic (Ael), cinnamic (Acin), but also flavonoids such as apigenin (Apg) and pinocembrin (PinoC), their content varying according to the type of vegetable oil (Table 3). As can be seen, higher concentrations of dihydroxybenzoic acid correspond to olive oils (n.d.-0.118 mg/kg), while hydroxybenzoic acid was quantified in higher amounts in vegetable oils (n.d.-0.381 mg/kg), with values higher for nut oil and pumpkin seed oil. p-coumaric acid was quantified in higher amounts in commercial olive oils (n.d.-0.806 mg/kg) and virgin olive oils (0.008-0.708 mg/kg), while higher amounts of ferulic acid (n.d.- 1.007 mg/kg) and ellagic acid (0.015-0.731 mg/kg) correspond to the other types of vegetable oils investigated. Cinnamic acid is predominant in olive oils, with values ranging from 0.023-4.832 mg/kg in original olive oils and n.d.-5.076 in commercial olive oils. Also, the content of apigenin and pinocembrin is higher in olive oils and extra virgin olive oils compared to the other types of vegetable oils.

Table 3. The content of analyzed phenolic compounds in vegetable oils

Type	mg/kg	DHyB	HyB	Ap-Coum	AF	Ael	Acin	Apg	PinoC
Veg									
E									
Origin									
Co									

Principal component analysis (PCA) was performed for exploratory data analysis of the content of tyrosol, hydroxytyrosol, trigonelline, oleuropiein, verbacoside, oleic acid, and oleanolic acid. The distribution of vegetable oils in the PC1-PC2 scores graph is shown in Figure 3, where a clear discrimination of olive oils and extra virgin olive oils, original and commercially available, is observed, compared to the rest of the oils. The results indicate that the specific markers of olive oils are: trigonelline, verbacoside, hydroxytyrosol, oleic and oleanolic acids, but also oleuropein and tyrosol, their distribution being, mainly, to olive oils and less to the other types of oils.

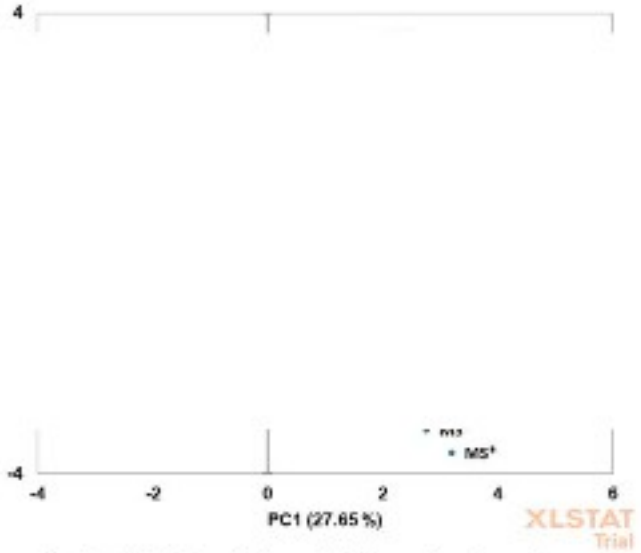


Figure 3. Scores plot of PCA of vegetable oils based on the profile of specific phenolic compounds

In order to understand the role of the determined compounds as quality indicators of olive oils and extra virgin olive oils, HCA-cluster analysis was also used to group the oils according to botanical origin. Figure 4 shows a hierarchical dendrogram for the grouping of oils based on the content of the 7 specific markers studied. The grouping of oils into three main clusters is observed, namely: cluster 1 which corresponds to grape seed oil, cluster 2 which groups most olive oils, EVOO, but also other types of vegetable oils such as rice, walnut, sesame and rapeseed oil and cluster 3 in which mainly vegetable oils are grouped, but also olive oils (figure 4).

Figure 4. HCA analysis of vegetable oils based on the content of tyrosol, hydroxytyrosol, trigonelline, oleuropein, verbacoside and oleic and oleanolic acids

Development of new sensitive materials

A series of new biosensors have been developed for the analysis of biomarkers in olive oil samples. For the construction of the biosensors, screen-printed carbon-based electrodes (SPCE), zinc tin oxide (ITO), gold (Au) and platinum (Pt) were used which were modified with different nanomaterials, peptides and enzymes. The nanomaterials used were carbon nanotubes, carbon nanofibers, mesoporous carbon, and graphene as such or functionalized

with different types of hydrophilic groups: -OH, >C=O, -COOH, -COONH2, etc. For example, manganese phthalocyanine, cobalt phthalocyanine, Prussian Blue or peptides were used as electron exchange mediators. A wide range of enzymes from the oxidase class were also used, for example tyrosinase, laccase, peroxidase, cholesterol oxidase.

For example, in one study, the first step for constructing enzyme biosensors was to modify carbon screen-printed electrodes (SPE/C) with single-layer carbon nanotubes. This procedure was carried out by dispersing a volume of 10 µL of a SWCNT suspension prepared in advance on the active surface of the supporting electrode. To prepare the SWCNT suspension, 10 mg of single layer carbon nanotube powder was dispersed in a solvent mixture consisting of dimethylformamide and ultrapure water in a 1:1 ratio. For optimal dispersion, the suspension was ultrasonicated for 30 minutes.

The optimal suspension volume (10 µL) was added to the surface of the SPE/C electrodes using an Eppendorf micropipette in two successive steps. After each step, the electrodes were dried in a desiccator at a temperature of 25 °C for 2 hours.

The second stage consisted in the construction of two types of enzymatic biosensors by modifying SPE/SWCNT with laccase and tyrosinase, respectively. The enzyme solutions were added onto the SWCNT/SPE surface by the casting technique, followed by cross-linking using glutaraldehyde vapors. After adding 5 µL of enzyme solution, the electrodes were kept in the desiccator for one hour for drying. Another 5 µL was added in the same way. Exposure to glutaraldehyde vapor was for 1 minute for each electrode. The cross-linking ensures a favorable electrical connection of the SWCNT with the enzyme. However, a longer exposure time could decrease the enzyme activity due to changes in the three-dimensional structure of the heteroprotein. After immobilization, the biosensors were stored at 4 °C until use, a maximum of 72 hours. Figure 5 shows the stages of the preparation process of laccase and tyrosinase-based biosensors and the schemes of the reactions that take place between glutaraldehyde and enzymes.

Figure 5. Procesul de preparare a biosenzorilor pe bază de tirosinază și respectiv lacază imobilizate pe electrozi serigrafiați de carbon modificați cu nanotuburi de carbon

Several steps were taken to prepare the modified GPH-MnPc/SPE sensor. Initially, an amount of 10 µL of 10^{-5} M manganese phthalocyanine solution in chloroform was added, by the drop-and-dry technique, sequentially, with drying breaks, on the surface of the screen-printed electrode modified with graphene (GPH). Drying was carried out at room temperature for 30 minutes. The addition of the manganese phthalocyanine solution was done using an Eppendorf micropipette. For the preparation of the MnPc-Tyr/SPE biosensor, GPH-MnPc/SPE was used as support. Using the drop-and-dry technique, a volume of 10 µL was added, sequentially, in two steps (5 µL in each step), with a 3-hour drying break between the two additions. Enzyme cross-linking was achieved by holding the sensor over a container with 5 mL of 2% glutaraldehyde for 1 minute. Glutaraldehyde vapor ensured the

immobilization of tyrosinase on the electrode surface by cross-linking. The biosensors were stored at 4 0C until use.

The characterization of the deposited sensitive materials (of the active surface of the biosensors) was performed by scanning electron microscopy and atomic force microscopy. Electrochemical methods (cyclic voltammetry (CV), differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS)) and spectrometric methods (FTIR) were also used. Some of the obtained results are presented below. The following figure shows the SEM images of some biosensors made, GPH-MnPc-Tyr/SPE (Figure 6) and OMC-Lac (Figure 7).

Figure 6. SEM image of the surface of the GPH-MnPc-Tyr/SPE biosensor

Figure 7. SEM image of the OMC-Lac biosensor surface

Also, a series of AFM images of the sensitive element of some biosensors are presented in the next figure (Figure 8).

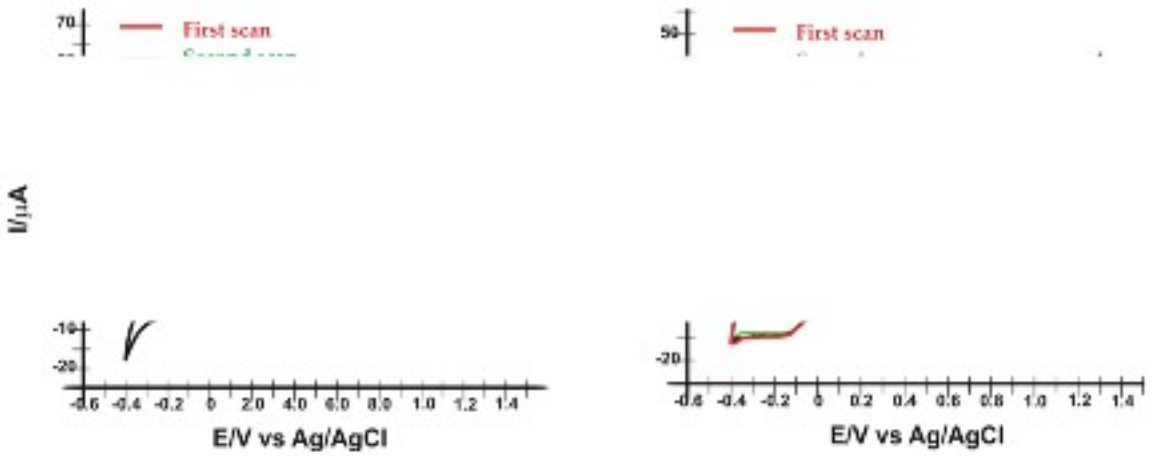
a) AFM image of a sensing layer containing tyrosinase and manganese phthalocyanine

b) AFM image of a sensitive -laccase layer deposited on mesoporous carbon

Figure 8. AFM images of the sensing element

After the construction of the biosensors, the characterization of the modified surfaces was carried out by several methods. CV was the first method by means of which the immobilization of enzymes on the supporting electrode was sought.

In Figure 9 we can see the oxidation of tyrosinase at the potential Epa=0.433 V (Ipa= 5.970 μA) and the reduction of the enzyme at Epc=-0.171 V (Ipc=14.739 μA). The redox process is quasi-reversible. No significant differences in the electrochemical behavior of tyrosinase are observed in successive scans. In the case of laccase, the oxidation peak appears only in the first scan at a potential Epa= 0.914 V (Ipa= 13.913 μA), which is irreversible. On the second and third scan, oxidation and reduction peaks are no longer evident, the signal being stable.



(a) (b)

Figure 9. Cyclic voltammograms recorded in 10-1 M phosphate buffer solution (PBS) with the SPE/SWCNT/Lac biosensor at pH 5.0 (a) and SPE/SWCNT/Tyr at pH 7.0 (b).

Analyzing Figure 9, it can be stated that the enzymes were immobilized on the surface of the supporting electrodes, observing their electrochemical behavior in both situations, but SPE/SWCNT/Tyr stands out through a more stable signal and more obvious peaks.

In the next step, the two biosensors were analyzed by means of EIS. In a three-electrode system, EIS analysis is performed by fixing an applied voltage, and the resulting Nyquist plot (typically used for resistive system analysis), shows the produced solution resistance (Rs), charge transfer resistance (Rct), and impedance Warburg (W).

The electrochemical processes that take place at the interface between SPE/SWCNT/Lac, respectively SPE/SWCNT/Tyr and the electrolyte chosen for analysis (in the present case K3[Fe(CN)6]/K4[Fe(CN)6] 10^{-3} M and KCl 10^{-1} M in molar ratio 1:1) are transposed in the form of an equivalent circuit involving resistors, capacitors and inductors. The Randles equivalent circuit shows solution resistance (Rs), electrode surface double layer capacitance (CdI), charge transfer resistance (Rct) and Warburg resistance (Zw) in a simplified manner, which is why it is most commonly used. According to Figure 10, the Nyquist diagrams obtained in this study (in the form of a semicircle) correspond to a simulated Randles circuit, also shown in the figure.

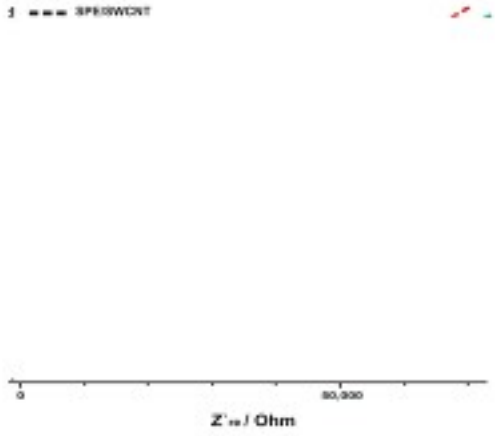


Figure 10. Nyquist plots of EIS for SPE/SWCNT (black line), SPE/SWCNT/Tyr (red line), SPE-SWCNT-Lac (green line) in 10-1 M and 10-3 M KCl solution [Fe(CN) 6]3−/4− in the frequency range from 0.01 Hz to 10 kHz, amplitude 10 mV. Inset figure: The equivalent circuit used to fit the electrochemical impedance spectra.

Another method by which enzyme immobilization was studied is the FTIR technique. The FTIR spectra of SPE/SWCNT, SPE/SWCNT/Lac and SPE/SWCNT/Tyr (Figure 10) were recorded in attenuated total reflectance (ATR) mode in the range 4000–500 cm^{-1} with a resolution of 4 cm^{-1}.

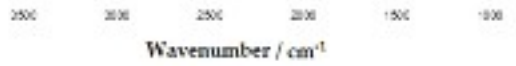


Figure 10. FTIR spectrum of SPE/SWCNT (green line), SPE/SWCNT/Tyr (blue line) and SPE/SWCNT/Lac (red line).

The spectra of the two biosensors highlight the stretching vibrations of the C=N groups at approximately 1644 cm-1 (amide I, protein-specific band), which indicates the interaction of glutaraldehyde with the nitrogen atoms of the ezime.

Synthesis of conducting polymers and molecularly imprinted polymers

In order to make new electrochemical sensors, electroconductive organic polymers were deposited using monomers derived from thiophene (3,4-ethylenedioxythiophene (EDOT), hydroxymethyl-EDOT) and pyrrole in the presence of different dopant anions (potassium hexacyanoferrate (II), perchlorate of lithium, sodium sulfate, Prussian Blue, etc.), some of them with a dual role, both doping agents and electron exchange mediators, an important process during electrochemical detection. Thus, innovative electrochemical sensors based on conductive polymers doped with different anions were obtained that can successfully detect markers in olive oil. In Table 4, the manufacturing method of the sensors is presented centrally.

Table 4. Experimental conditions for obtaining conducting polymers

Monomer		Doping agent	Electropolimerization	Electrolyte solutions used in
EDOT				
HMEDOT				
Pyrrole				

Polymer layers electrosynthesized by chronoamperometry in the presence of different doping agents were characterized by SEM and electrochemical. Examples of these characterizations are presented in Figure 11.

SEM image of the PEDOT layer doped with sulfate ions	SEM image of the PPy layer doped with sulfate ions

Figure 11. SEM images of sensitive layers of conducting polymers

The electrochemical responses of sensors based on conducting polymers varied depending on the nature of the polymer and the nature of the dopant. Thus, Figure 12 shows the responses of sensors based on PPY/FCN and PEDOT/FCN immersed in 0.1 M KCl solution.

Redox peaks related to the conductive polymer and in the case of the ferrocyanide dopant (FCN) and peaks due to it are observed.

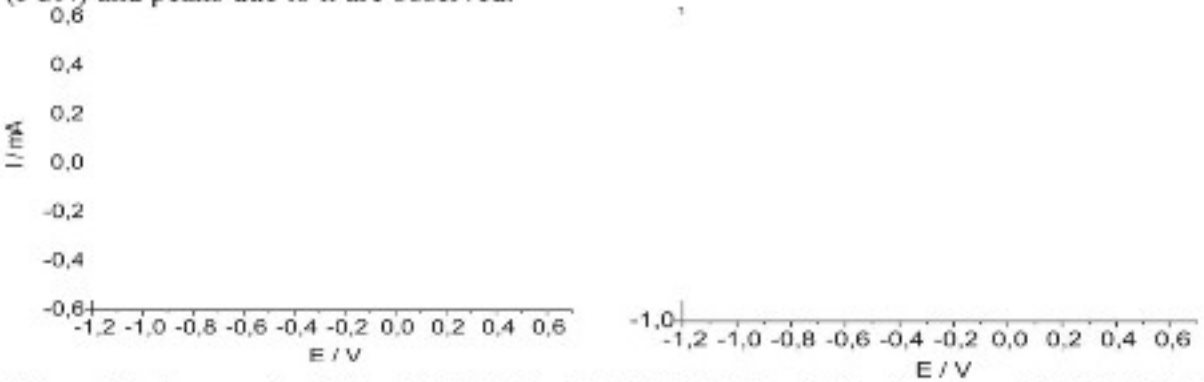


Figure 12. Sensor signals in PPY/FCN and PEDOT/FCN solution immersed in 0.1 M KCl solution during voltammetric signal stabilization process.

The obtained sensors can be used as such to obtain the chemical fingerprint of olive oils or they can be transformed into biosensors by immobilizing some enzymes, with a biocatalytic role in the determination of markers in olive oil. Biosensors with good sensitivity and selectivity for olive oil biomarkers were thus obtained.

Figure 13 shows the cyclic voltammograms of the PPY/FCN/Tyr biosensor when it is immersed in tyrosol solution of variable concentration and the corresponding calibration curve

Figure 13. Cyclic voltammograms of PPY/FCN/Tyr immersed in tyrosol solution of varying concentration; b) Calibration curve

The main biosensors based on conducting polymers, the enzymes used, the target compounds and the limits of detection (LOD) are presented in Table 5.

Table 5. Biosensors based on conducting polymers

						M
PE						
PE						
PE						
PE						
PP						
PP						
PP						

For the synthesis of molecularly imprinted polymers, acrylic acid and methacrylic acid were used as monomers, and oleuropein, tyrosol, hydroxytyrosol, verbascoside and trigonelline as target molecules. The procedure is similar for the two monomers and the crosslinking agent and initiator were ethylene glycol dimethyl acrylate and benzoyl peroxide for all cases. Next, the process carried out to obtain sensors of this type is presented.

0.02 g of acrylic acid (monomer) and 0.02 g of trigonelline (template molecule) were dissolved in 20 mL of ethanol and the mixture was ultrasonicated for 30 min. In another beaker, 400 µL of ethylene glycol dimethyl acrylate (crosslinker) and 0.0050 g of benzoyl peroxide (initiator) were dissolved in 5 mL of ethanol and the solution was left to stand for 30 min. The two solutions are mixed and then shaken vigorously for 1 hour at 25 °C. Afterwards, the mixture is heated to 50 °C for 90 min to complete the polymerization process. The molecular polymer-trigonelline adduct obtained in solid form is repeatedly washed with ethanol until the trigonelline template molecule is completely removed from the adduct. The molecularly imprinted molecular polymer is dried and stored cold in the absence of light.

The molecularly imprinted polymer thus obtained is dispersed in ethanol by ultrasonication and then deposited on the surface of screen-printed carbon electrodes, thus obtaining selective molecularly imprinted polymer-based sensors for the detection of markers in olive oil.

The sensors thus obtained were characterized by AFM and in Figure 14 the surfaces of sensors (a) C/polyacrylate/Verbascozide and (b) C/polymethacrylate/Trigonelline are shown. The homogeneity of the distribution of active centers on the surface of the sensors can be highlighted.

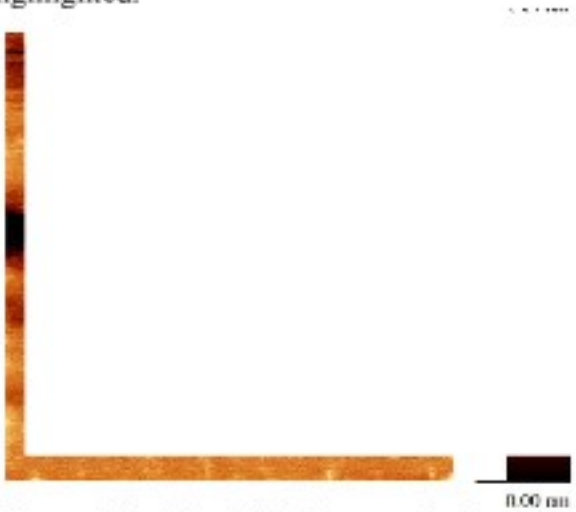


Figure 14. (a) AFM image of the C/polyacrylate/ Verbascozide sensor

Figure 14. (b AFM image of the C/polymethacrylate/Trigonelline

Figure 15 shows the responses of the C/polyacrylate/Hydroxytyrosol sensor to increasing amounts of hydroxytyrosol recorded by DPV and the calibration curve. The sensor has adequate selectivity and sensitivity for the determination of hydroxytyrosol in complex samples.

Figure 15. Sensor responses in hydroxytyrosol solution in the 50-300 nM range and calibration curve

Table 6 shows the main sensors based on molecularly imprinted polymers developed in this project and their detection limits.

Table 6. Sensors based on molecularly imprinted polymers

Sensor	LOD/nM	Sensor	LOD/nM
C/pol			
C/pol			
C/pol			

Through this method, molecularly imprinted polymer-based sensors with biomarkers from olive oil were obtained, which can be successfully used for the detection of additions of other oils in olive oil. The best results were obtained with trigonelline.

The results obtained in Activity 2.1 are very good and promising, the specific activities proposed for the second year of implementation have been achieved 100% and the general and specific objectives of this stage have been fully met.

Activity 2.2 Development of a multi-sensor platform. Characterization of biosensors in the presence of markers. Development of biosensor networks for the analysis of markers in olive oil

Development of a multi-sensor platform

In the project, a modular multisensor system based on electrochemical biosensors was developed for the determination of markers in olive oils and vegetable oil additives in extra virgin olive oil.

Characterization of biosensors in the presence of markers

For the analysis of pure or falsified olive oils using electrochemical biosensors, several procedures have been carried out to extract the polar fraction: extraction with methanol-water, extraction in HCl solution, emulsions with Triton x-100, eutectic solvents. The biosensors prepared and characterized morphologically, spectrometrically and electrochemically in the previous steps were used for the detection of some biomarkers in olive oil.

Next, as an example, the studies carried out for the electrochemical detection of oleuropein with two biosensors based on tyrosinase and laccase are presented. In the first step, the electrochemical behavior of oleuropein was studied using the two newly constructed biosensors, SPE/SWCNT/Lac and SPE/SWCNT/Tyr, by means of CV and square wave voltammetry (SWV). For each biosensor, a 10-4 M solution of oleuropein was prepared, with pH 5.0 (for Lac) and pH 7.0 (for Tyr).

CV was applied in the potential range from -0.4 to +1.3 V at a scan rate of 0.1 V/s. In Figure 16 (a) we can see the oxidation-reduction process of oleuropein for SPE/SWCNT/Lac and for SPE/SWCNT/Tyr recorded by CV. In both situations, well-defined peaks corresponding to the oxidation and reduction of oleuropein are observed, with close current intensities, but at

visibly different potentials, which suggests different electrochemical performances of the two biosensors.

SWV was applied after prior optimization of the parameters: f (frequency) = 15 Hz; Esw (applied impulse) = 90 mV, Es (potential rise) = 2 mV. Figure 16 (b) shows the square wave voltammograms recorded with the same biosensor in 10^{-4} M oleuropein solution (10-1 M PBS electrolyte).

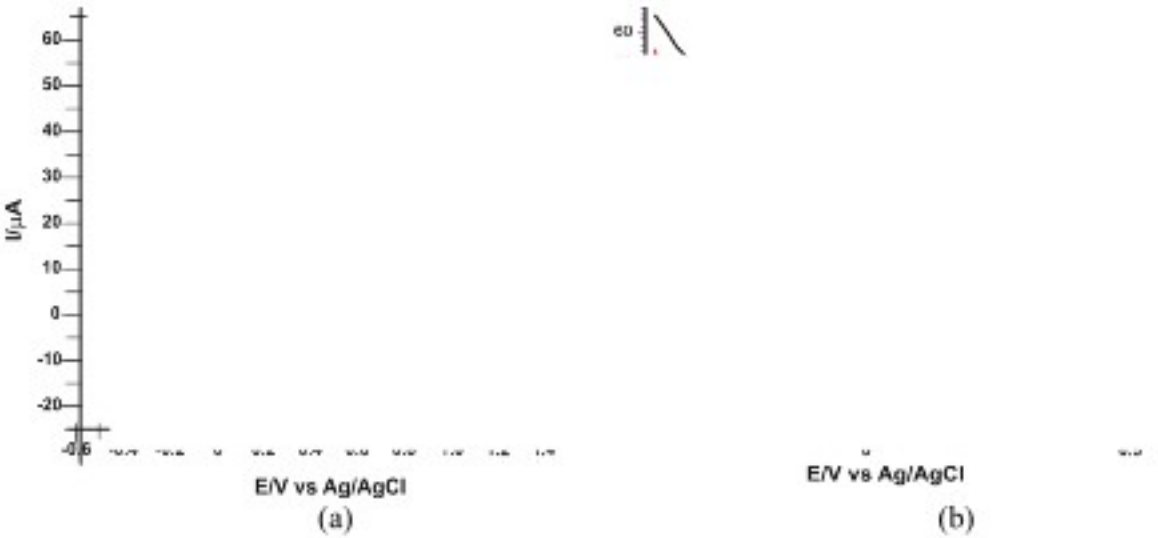


Figure 16. (a) Cyclic voltammograms of SPE/SWCNT (green line), SPE/SWCNT/Tyr (black line) and SPE/SWCNT-Lac (red line) in 10^{-1} M PBS (pH 7.0) containing 10^{-4} M OLEU. (b) Square-wave voltammograms of SPE/SWCNT/Tyr (black line) and SPE/SWCNT-Lac (red line) in 10−1 M PBS (pH 7.0) containing 10^{-4} M OLEU

To create the calibration curve, the square wave voltammograms of the two biosensors were recorded in oleuropein solutions in the concentration range 0.01 μM – 28.62 μM.

Linear regression equations were used to calculate the limits of detection (LOD = 3σ / m, where σ is the standard deviation and m the slope of the calibration curve) and the limits of quantification (LOQ = 10σ / s) and the results obtained are shown in Table 7.

Table 7. Linear dependence equation, R^2, LOD and LOQ for the two biosensors

SPE/				
SPE/SWCNT/Tyr				

SPE/SWCNT/Tyr is notable for lower detection and quantification limits, thus confirming the very good electrocatalytic properties of this biosensor.

The main biosensors realized in this project implementation year, target compounds and detection limits are presented in Table 8.

Table 8. Biosensors based on nanomaterials and enzymes

Nar de a			
Nar mul cart			
Nar mul ami			

Ca
Na per car
Ox
Ca
Na de
Na
Ca
Ox
Gr
Na per
Ca
Ca

Most of the biosensors made so far have proven to be suitable for building the biosensor network for the determination of adulteration of olive oils. Eight biosensors, with the best characteristics and different selectivities, were chosen to build the network of biosensors to determine the adulteration of olive oils: PEDOT/FCN/Tyrosinase, PPY/FCN/Tyrosinase, C/polyacrylate/Trigonelline, Carbon nanofibers/nanoparticles of gold/Cholesterol dehydrogenase, Single-wall carbon nanotubes, functionalized with carboxyl groups /Lacase, Carbon /Cobalt phthalocyanine/Lacase, Graphene oxide /Lacase, C/polymethacrylate/Oleuropein.

Development of biosensor networks for the analysis of markers in olive oil

For the design of the biosensor network that includes the biosensors with the best analytical performance and cross-selectivity for the simultaneous or sequential analysis of olive oil samples, several constructive variants were considered. After a thorough analysis, it was decided to use a system that includes 8 different biosensors, each equipped with a counter electrode and a reference electrode. By means of a multiplexer each three-electrode system is sequentially activated and the electrochemical signals with each biosensor are recorded.

Array with 8 different biosensors

After all the signals are recorded they are processed to determine the concentration of the biomarkers and the purity of the analyzed samples.

The specific activities provided for in activity A2.2 have been fully achieved, and the degree of achievement of the objectives is 100% for the second year of project implementation.

Activity 2.3 Developing intelligent models and applying them to data analysis

In this activity, intelligent models were developed by applying multivariate data analysis methods from different types of measurements made for samples of olive oils (extra virgin or from olive cakes), other vegetable oils such as sunflower oil, corn oil, canola oil, coconut oil, sesame oil, palm oil and mixtures thereof. The oil samples were analyzed individually and as mixtures of extra virgin olive oil and other oils in different proportions to train the systems and to determine the detection limits under laboratory conditions. Depending on the complexity of the analysis, 3, 4 or 6 replicates of the measurements were made to increase the significance and robustness of the developed models.

The models were built using several methods including principal component analysis (PCA), discriminant analysis solved by partial least squares (PLS-DA), soft and independent modeling of class analogy (SIMCA) and regressions by partial least squares (PLS1 and PLS2).

In the third year of implementation, when the number of analyzed samples will be sufficiently large, classification models based on artificial neural networks (ANN) and machine learning (ML) will be developed.

In A2.3, the specific activities were completed in full (100%). The main data analysis methods were selected and optimized, methods that will be finalized until the end of the activity and the creation of the final report for this activity.

Activity 2.4 Validation of the multi-parametric system in the analysis of real samples

To validate the results obtained with the biosensor system coupled with multivariate data analyses, the data obtained were analyzed for discrimination, classification and establishing correlations when using different vegetable oils or extra virgin olive oils classified according to: manufacturing technology, the variety of olives used, the country of origin, etc. The differences between extra virgin olive oils and olive cake oils were also studied. In addition, the performance of the multi-parametric system in classifying samples of extra virgin olive oils in which different concentrations of seed oils were added was determined.

Next, a series of results regarding the discrimination or classification of the analyzed oil samples and the correlation between the data obtained with the biosensors and their antioxidant properties are presented.

The discrimination of vegetable seed oil samples: linseed (USI), hemp (USC), pumpkin (USD), sesame (USS), rapeseed (UR), grape (GSO), rice (SOR), soybean (US) is presented in Figure 17. It can be seen that the biosensor system is capable of differentiating oils according to their biological origin.

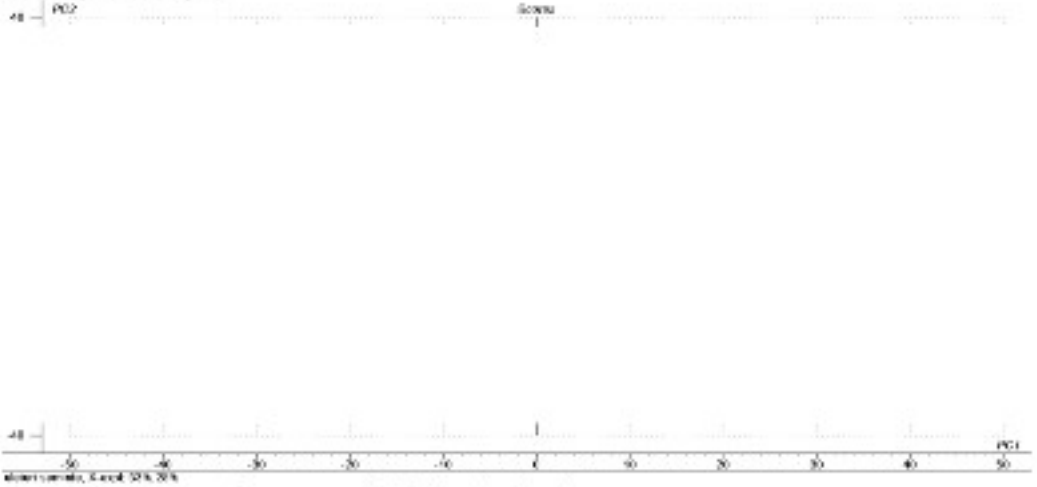


Figure 17. PCA scores plot on seed oil discrimination

Analysis of data from voltammetric measurements of blends of extra virgin olive oil and corn oil led to the results shown in Figure 18 and Figure 19.

Figure 18. PCA scores plot on the differentiation of virgin olive oil samples and corn samples mixed in different proportions (from 0% to 50%).

Figure 19. Scores graph of PCA sample 1 -> c% adulterant = 0%, sample 2 -> c% adulterant = 0.5%, sample 3 -> c% adulterant = 1% (the adulterant is corn oil).

It can be appreciated that the pure corn oil (sample 1 in Figure 18) is discriminated from the samples containing extra virgin olive oil. Also, oils adulterated with corn oil are differentiated even if the adulterant concentration is 0.5% (Figure 19).

Another study consisted in the differentiation and classification of some oil samples according to their type, biological origin, production technology and according to the country of origin. The results are presented in the form of three-dimensional scores graphs (Figure 20), differentiated by EVOO or POO - (Figure 20A), by type of oil – POO, EVOO and sunflower oil (Figure 20B), by production technology – biological EVOO, Unfiltered EVOO, classic EVOO and organic EVOO (Figure 20C) and by country of origin – Tunisia, Greece and Italy (Figure 20D). Figure 20 shows the PLS-DA scores graphs in which a separation of the groups formed on the basis of different classification criteria is observed.

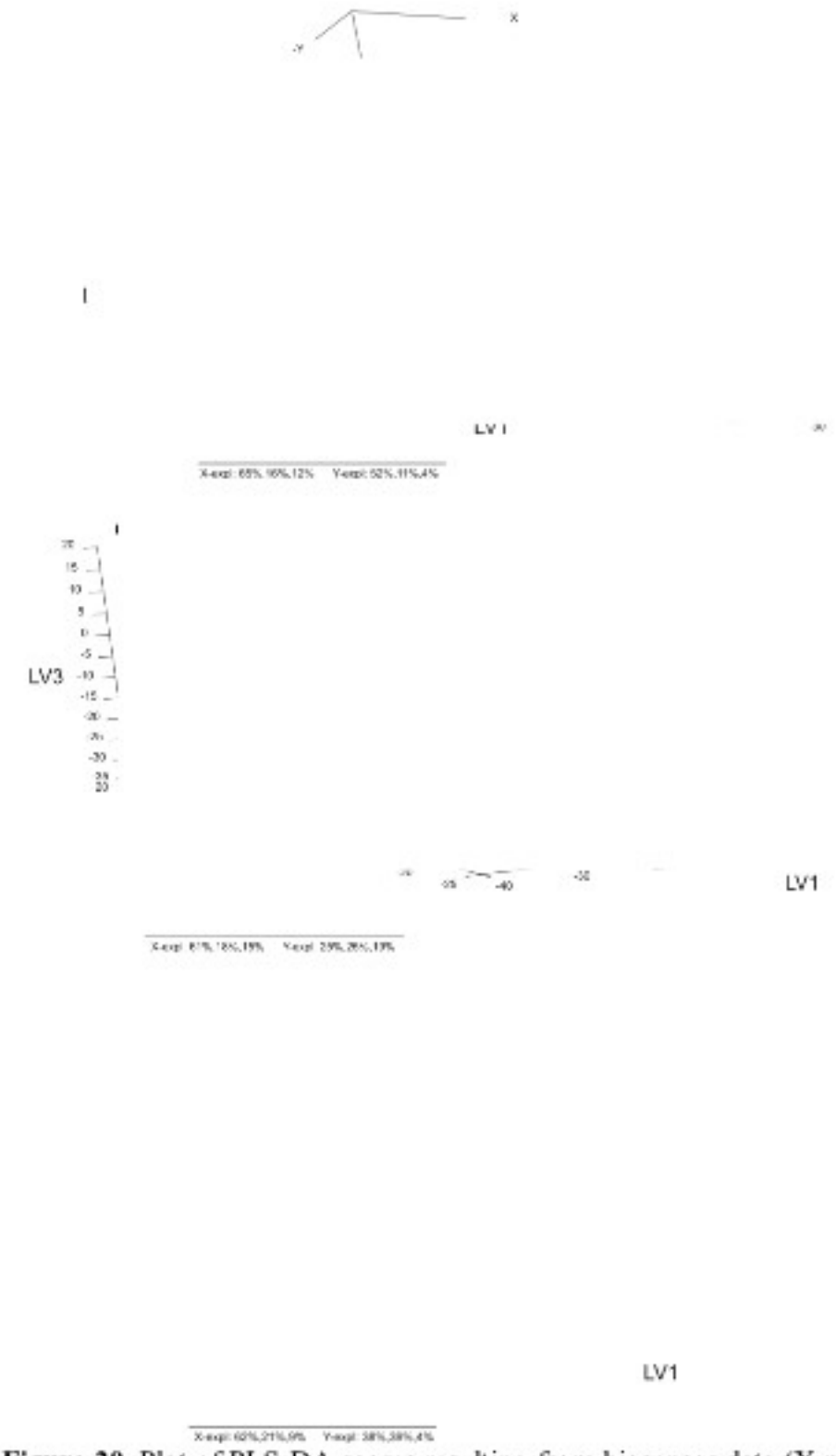


Figure 20. Plot of PLS-DA scores resulting from biosensor data (X matrix) corresponding to different oil classes (Y matrix): (A) EVOO versus POO; (B) POO versus EVOO versus sunflower oil; (C) organic EVOO versus unfiltered EVOO versus classic EVOO versus organic EVOO; (D) Tunisia versus Greece versus Italy.

The quantitative data of all PLS-DA models show in all cases correlation coefficients greater than 0.9 and small root mean square errors in both calibration and validation.

PLS1 was used as a prediction technique to correlate the biosensor data with antioxidant activity quantified by percentage inhibition capacity of DPPH and galvinoxyl. The results of the PLS1 regression models are shown as the dependence of the predicted results on the measured antioxidant activity results (Figure 21).

Figure 21. Plot of percent inhibition predicted from biosensor data vs. measured percent inhibition obtained from spectrophotometric methods for the galvinoxyl assay.

All six replicate measurements corresponding to each oil sample were included in the regression models. The first number in the sample coding (1 to 25) represents the oil sample and the second represents the replicates (1 to 6).

From these results it can be concluded that electrochemical data obtained with biosensors can be successfully used to classify oils based on different criteria such as oil type, production technology or country of origin and to determine oil adulteration.

Within Activity 2.4, the activities provided for in this year of implementation were completed in full (100%). The multi-sensor system has been validated on a range of oils and this process will be completed at the end of the project implementation period as set out in the work plan.

Activity 2.5 Participation in conferences, publication in ISI journals, patent application

For this project, we periodically updated the web page, including the relevant information regarding the research activities carried out within the project and the dissemination activity. The website address of the project is: www.busdoa.ugal.ro

The following results were disseminated:

Articles in Clarivate Analytics journals:

1. Bounegru, A.V.; Apetrei, C. Studies on the Detection of Oleuropein from Extra Virgin Olive Oils Using Enzymatic Biosensors. International Journal of Molecular Sciences **2022**, 23, 12569. IF 6,208. https://doi.org/10.3390/ijms232012569

2. Munteanu, I.G.; Grădinaru, V.R.; Apetrei, C. Sensitive Detection of Rosmarinic Acid Using Peptide-Modified Graphene Oxide Screen-Printed Carbon Electrode. Nanomaterials **2022**, 12, 3292. IF 5,719. https://doi.org/10.3390/nano12193292

3. Dăscălescu, D.; Apetrei, C. Development of a Novel Electrochemical Biosensor Based on Organized Mesoporous Carbon and Laccase for the Detection of Serotonin in Food Supplements. Chemosensors **2022**, 10, 365. IF 4,229. https://doi.org/10.3390/chemosensors10090365

4. Bounegru, A.V.; Apetrei, C. Sensitive Detection of Hydroxytyrosol in Extra Virgin Olive Oils with a Novel Biosensor Based on Single-Walled Carbon Nanotubes and Tyrosinase. International Journal of Molecular Sciences **2022**, 23, 9132. IF 6,208. https://doi.org/10.3390/ijms23169132

5. Munteanu, I.G.; Apetrei, C. Assessment of the Antioxidant Activity of Catechin in Nutraceuticals: Comparison between a Newly Developed Electrochemical Method and Spectrophotometric Methods. International Journal of Molecular Sciences **2022**, 23, 8110. IF 6,208. https://doi.org/10.3390/ijms23158110

6. Bounegru, A.V.; Apetrei, C. Simultaneous Determination of Caffeic Acid and Ferulic Acid Using a Carbon Nanofiber-Based Screen-Printed Sensor. Sensors **2022**, 22, 4689. IF 3,847. https://doi.org/10.3390/s22134689

Participation in international conferences

1. Alexandra Virginia Bounegru, Irina Georgiana Munteanu, Constantin Apetrei. Development of an electroanalytical method for detecting adulteration of extra virgin olive oils. SCDS-UDJG 2022 The Tenth Edition, Galaţi, 9-10 iunie 2022. Invited oral presentation, abstract published in Book of abstracts, p. 39.
2. Alexandra Virginia Bounegru, Constantin Apetrei. Sensors and Biosensors for Evaluating Olive Oil Quality. SCDS-UDJG 2022 The Tenth Edition, Galaţi, 9-10 iunie 2022. Oral presentation, abstract published in Book of abstracts Book of abstracts, p. 161.
3. Andreea Loredana Comănescu, Constantin Apetrei. Discrimination of vegetable oils by using spectrometric data and chemometrics methods. SCDS-UDJG 2022 The Tenth Edition, Galaţi, 9-10 iunie 2022. Poster, abstract published in Book of abstracts Book of abstracts, p. 241.
4. Andrei Geman, C. Apetrei. Determination of the addition of maize oil to extra virgin olive oil by FTIR method coupled with multivariate data analysis methods. SCDS-UDJG 2022 The Tenth Edition, Galaţi, 9-10 iunie 2022. Poster, abstract published in Book of abstracts Book of abstracts, p. 242.
5. Constantin APETREI. Spectrometric and electroanalytical methods for the determination of virgin olive oils adulteration. International Summer School - FOOD SAFETY AND HEALTHY LIVING -FSHL – 2022, 5-8 Septembrie 2022, Braşov – Romania, oral presentation, abstract published in Book of abstracts Book of abstracts, p. 142.
6. Constantin APETREI, Elisabeta-Irina GEANĂ, Irina Mirela APETREI. Electroanalytical method coupled with chemometry for detection of virgin olive oil adulteration. The 6th International Conference New Trends on Sensing - Monitoring – Telediagnosis for Life Sciences NT-SMT-LS 2022 Braşov, Septembrie 8-10, 2022. Oral presentation, abstract published in Book of abstracts Book of abstracts, p. 43.
7. Alexandra Virginia BOUNEGRU, Constantin APETREI. Electrochemical determination of hydroxytyrosol in extravirgin olive oils using screen-printed electrodes modified with single wall carbon nanotubes and tyrosinase The 6th International Conference New Trends on Sensing - Monitoring – Telediagnosis for Life Sciences NT-SMT-LS 2022 Braşov, Septembrie 8-10, 2022. Poster, abstract published in Book of abstracts Book of abstracts, p. 62.
8. Irina-Georgiana Bulgaru (Munteanu), Constantin Apetrei. Comparative study on the antioxidant activity of extra virgin olive oil samples using a newly developed electrochemical method and DPPH spectrophotometric assay. The 6th International Conference New Trends on Sensing - Monitoring – Telediagnosis for Life Sciences NT-SMT-LS 2022 Braşov, Septembrie 8-10, 2022. Poster, abstract published in Book of abstracts Book of abstracts, p. 81.
9. Constantin Apetrei, Andreea Loredana Comănescu, Andrei Daniel Geman, Irina Georgiana Munteanu, Alexandra Virginia Bounegru, Irina Mirela Apetrei, Elisabeta Irina Geană. Electrochemical (bio)sensor array coupled with multivariate data analysis for the assessment of virgin olive oil quality. "Priorities of Chemistry for a Sustainable Development", PRIOCHEM XVIII, Bucureşti, 26-28 Octombrie 2022. Invited oral presentation, abstract published in Book of abstracts Book of abstracts, p. 11.
10. Elisabeta-Irina Geana, Corina Teodora Ciucure, C. Apetrei. Authentication and detection of adulteration of extra virgin olive oil based on the composition of phenolic compounds. "Priorities of Chemistry for a Sustainable Development", PRIOCHEM XVIII, Bucureşti, 26-28 Octombrie 2022. Oral presentation, abstract published in Book of abstracts Book of abstracts, p. 63.

Participation in national conferences

1. Alexandra Virginia Bounegru, Constantin Apetrei. Noi biosenzori enzimatici pentru determinarea electrochimică a oleuropeinei din uleiuri de măsline extravirgine. Oral presentation. A XXXVI-a CONFERINŢĂ NAŢIONALĂ DE CHIMIE –CNChim-2022 Călimăneşti – Căciulata. Oral presentation, abstract published in Book of abstracts, p. 28.

Stage 2 results were disseminated through 6 oral presentations and 4 posters at various international conferences, 1 oral presentation at a national conference, and the publication of 6 articles in first-tier Clarivate Analytics scientific journals (5 from the Q1 quartile and 1 from the Q2 quartile). The dissemination activity planned for this year was carried out in full, 100%.

Activity 2.6 Acquisition activities. Elaboration of scientific reports

Acquisition activities were carried out in good conditions this year without delays. The annual scientific report for 2022 was prepared in accordance with the Contractor's requirements and is displayed on the project's web page, in Romanian and English.

The procurement activity ensured the full implementation of all activities foreseen in the second year of project implementation.

In the second year of implementation of the BUSDOA project, all the activities provided for in the implementation plan were carried out, the planned objectives were met 100% and valuable scientific results were obtained, a large part of which was disseminated through publication in scientific journals of prestige and also presented at international conferences.

Summary of progress: deliverables achieved, result indicators, dissemination of results

#	**Planned deliverables/indicators**	#	**Deliverables/indicators done in stage 2**	**Degree of achievement Stage 2**	**Degree of achievement Stage 1 and 2**
1	Chromatographic analysis of markers		Yes	100%	100%
2	Development of new sensitive materials		Yes	100%	100%
3	Synthesis of conducting polymers and molecularly imprinted polymers		Yes	100%	100%
4	Development of a multi-sensor platform		Yes	100%	100%
5	Characterization of biosensors in the presence of markers		Yes	100%	100%
6	Development of biosensor networks for the analysis of markers in olive oil		Yes	100%	100%
7	Developing intelligent models and applying them to data analysis		Yes	100%	50%*
8	Validation of the multi-parametric system in the analysis of real samples		Yes	100%	30%**
9	Attending conferences, publication in ISI journals, patent application (for the entire duration of the project)	6 6 1	6 11 0	100% 100% 0%	100% 100% 0%***
10	Acquisition activities			100%	100%
11	Elaboration of scientific reports Report for stage 2 Executive summary for stage 1 and 2	 1 1	 Yes Yes	 100% 100%	 100% 100%

* The deliverable will be completed in stage 3, when activities are planned for 6 months
* The deliverable will be completed in stage 3, when activities are planned for 12 months
*** The patent application will be submitted in stage 3, when activities are foreseen for 12 months

Project Manager,
Apetrei Constantin