SCIENTIFIC REPORT

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

January 2021-December 2021

This year, 2021, the activities included in the project implementation plan of Stage 1 were carried out: Stage 1 - **Detection of target molecules and development of new sensitive materials Development and characterization of biosensors Development of intelligent models and their application in data analysis Dissemination of results Management project.**

Stage summary

In this year of the project implementation a series of the research were carried out: (1) chromatographic characterization of markers in olive and other vegetable oils by spectroscopic (UV-Vis and FT-IR) and chromatographic (UHPLC-MS / MS) methods); (2) novel sensitive materials have been developed for the development of novel biosensors, based on conductive polymers and molecularly printed polymers; (3) the development of a multi-sensor platform necessary for the analysis of olive oil adulteration, for which biosensors were studied and characterized in the presence of phenolic markers, optimizing the experimental conditions and measurement parameters so that maximum electroanalytical performance is selected. Thus, the first biosensors that will be integrated in biosensor arrays for the analysis of markers in olive oil and the determination of adulteration with different vegetable oils; (4) for the analysis of experimental data, intelligent models of multivariate data analysis have been developed and applied for the analysis of physico-chemical, chromatographic, spectrometric, spectroscopic and electrochemical data for the purpose of discrimination, classification of oil samples according to authenticity and the determination of the limits from which the biosensor system is able to detect the addition of other oils to the olive oil. Some of the results were presented at prestigious international conferences or published in ISI journals. A number of important data are being processed for the writing of a patent application, which is an important deliverable of this project, but also of other articles in ISI journals.

Task 1.1 Chromatographic analysis of markers; Development of newly sensing materials; Synthesis of conductive polymers and molecularly imprinted polymers.

Chromatographic and spectroscopic analysis of phenolic markers

Some of the olive oil samples used in spectrophotometric and chromatographic analysis, and also in studies of design and optimization of novel biosensors were provided by Prof. Dr. Maria Lisa Clodoveo from Carlos Moro University of Bari and consisted of samples produced by small farmers in the Puglia region and also from samples obtained in the laboratory of the University of Bari through a new extraction process involving the use of ultrasound. In total 17 samples of olive oil were used. Some of the samples are shown in Figure 1.

Figure 1. Image of samples of olive oils of Italian origin analyzed in the project

Other samples of olive oils from different producers, from different varieties of olives as well as other vegetable oils were purchased from the project (36 samples).
Olive oil samples were analyzed by IR spectroscopy, UV spectrophotometry and Vis spectrophotometry. The FTIR spectra obtained will be used as chemical fingerprints of authentic olive oils being considered standards in studies to determine the falsification of oils with other vegetable oils.
The UV spectra obtained were used as chemical fingerprints belonging to standard samples but also for evaluating parameters characteristic of olive oils such as absorbance values at 225 nm, 232 nm, 270 nm, 266 nm, 274 nm.
Visible spectra were used as input data for calculating color parameters in the CIELab system, which are useful in discrimination and classification studies (L *, a *, b *, C, H, S).
To determine the compounds with antioxidant properties present in the oil samples, the Folin-Ciocalteu method was used to determine the total content of polyphenolic compounds.
Other ways to assess antioxidant activity were free radical scavenging methods. DPPH, ABTS and galvinoxyl free radical scavenging methods were used.
These results will be used in the validation stages of biosensors, in order to assess the concordance between the responses of biosensors and other types of physico-chemical analyzes, but also the differences between pure and falsified samples with other vegetable oils.
For the studies in laboratory conditions we made mixtures of different oils in different proportions in order to be able to determine the differences that appear and what the sensitivity limits of the methods are. Thus, more than 50 mixtures of olive oils were analyzed in which other vegetable oils from levels ranging from 0.1% to 50% were added.
Vegetable oils characterized by HPLC-MS / MS are: extra virgin olive oil (MS), walnut oil (N), grape seed oil (STR), pumpkin oil (D), flaxseed oil (I), soybean oil (SO), sesame oil (SU), hemp oil (C), poppy seed oil (M), sunflower oil (FL), corn oil (P). An experiment was also performed on the controlled adulteration of extra virgin olive oil with different percentages of corn oil (0.5-50%).
The extraction of the polar fraction from the investigated vegetable oils was carried out according to the protocol of the International Olive Council (COI / T.20 / DocNo 29, Nov. 2009). To do this, place 2 g of oil in an extraction tube, add 1 mL of internal standard (0.015 mg / mL syringic acid prepared in a mixture of methanol / water 80/20 (v / v)) and then mix with a vortex for 30 sec. Add 5 mL of extraction solution (methanol / water 80/20 (v / v)), vortex again for 1 minute, then the resulting mixture is subjected to ultrasonic extraction in the ultrasonic bath for 15 minutes, at room temperature. The sample is then centrifuged at 5,000 rpm for 25 minutes. An aliquot of the supernatant phase is filtered through a 1 ml plastic syringe using nylon syringe filters (0.45 μm) before injection into the HPLC system. The schematic representation of the extraction stage of phenolic compounds from the polar (unsaponifiable) fraction of vegetable oils is shown in Figure 2.

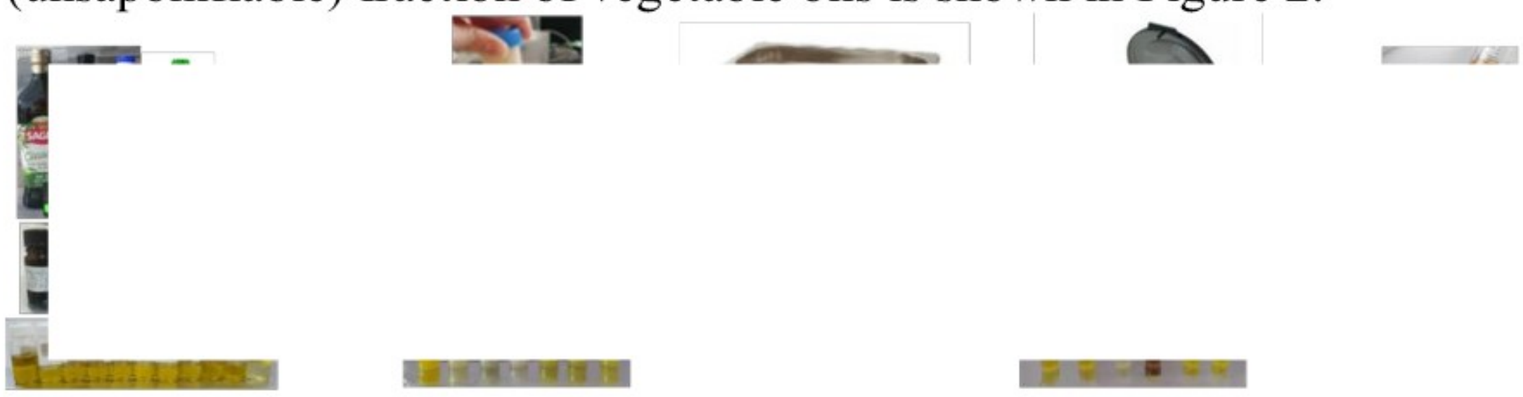


Figure 2. Schematic representation of the extraction of phenolic compounds from vegetable oils before analysis by UHPLC-MS / MS

The determination of the polyphenol content was performed by UHPLC-MS / MS with ESI ionization, using a high resolution mass spectrometer Q Exactive™ Focus Hybrid Quadrupole - OrbiTrap (ThermoFisher Scientific) equipped with HESI, coupled to a high performance liquid chromatograph UltiM 3000 UHPLC (ThermoFisher Scientific). Chromatographic

separation was performed on a Kinetex® C18 column (100 × 2.1 mm, particle diameter 1.7 μm), at a temperature of 30 °C. Mobile phase: A - water with 0.1% formic acid and B - methanol with 0.1% formic acid, elution in gradient at a flow rate between 0.3 and 0.4 mL / min. The mass spectrum was recorded in the negative ionization mode in a range between 100-800 m / z, at a resolution of 70,000. Nitrogen was used as collision gas and auxiliary gas at a flow rate of 11 and 48 arbitrary units, respectively. The applied voltage was 2.5 kV and the capillary temperature was 320 °C. The energy of the collision cell ranged from 30 eV to 60 eV. The data was purchased and processed using the Xcalibur software package (Version 4.1). Calibration was performed in the concentration range 0 - 1000 μg / L for each of the phenolic acids and flavonoids, by serial dilution with methanol of the standard mixture of concentration 10 mg / L. The phenolic compounds determined by this method are: apigenin, galangin, kaempferol, izorhamnetin, chrysin, pinocembrin, gallic acid, abscisic acid, p-coumaric acid, syringic acid, caffeic acid, chlorogenic acid, ferulic acid, ellagic acid, vanillic acid, acid p-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, t-cinnamic acid, (+) - catechin and (-) - epicatechin.

The identification of quantified phenolic compounds in vegetable oils was based on the comparison of retention times with those of the reference substances and by the identification of the molecular ion and the fragments resulting from negative ionization (Table 2). The identification of the main phenolic acids and flavonoids in extra virgin olive oil is shown in Figure 3. In the absence of standards for the major phenolic compounds in olive oil, the identification of other compounds in the extract was based on the search for the deprotonated molecule, [M – H] - and resulting fragments, but also by comparison with the specific literature. Thus, in addition to the quantified compounds, other phenolic compounds such as tyrosol, oleocanthal (p-HPEA-EDA), luteolin, but also oleuropein mono-aldehyde aglycone (3,4-DHPEA-EA) were identified in the extract resulting from EVOO (Figure 3).

Table 2. Identification of phenolic compounds in vegetable oils by UHPLC-MS/MS

#	Compound	Retention time [min]	m/z $[M-H]^-$	Mass fragments	Linearity (μg/L), R^2
Phenolic acids					
1	A				7
2	A				1
3	A				6
4	A				
5	A				2
6	A				6
7	A				6
8	A				9
9	A				6
10	A				1
11	A				5
Flavonoids					
12	(-				3
13	(-				9
14	Q				8
15	N				7
16	H				8
17	R				5
18	K				6
19	Is				7
20	A				7

21	F					97
22	(					99
23	(					89
24	F					33
Stilbenes						
25	t					88

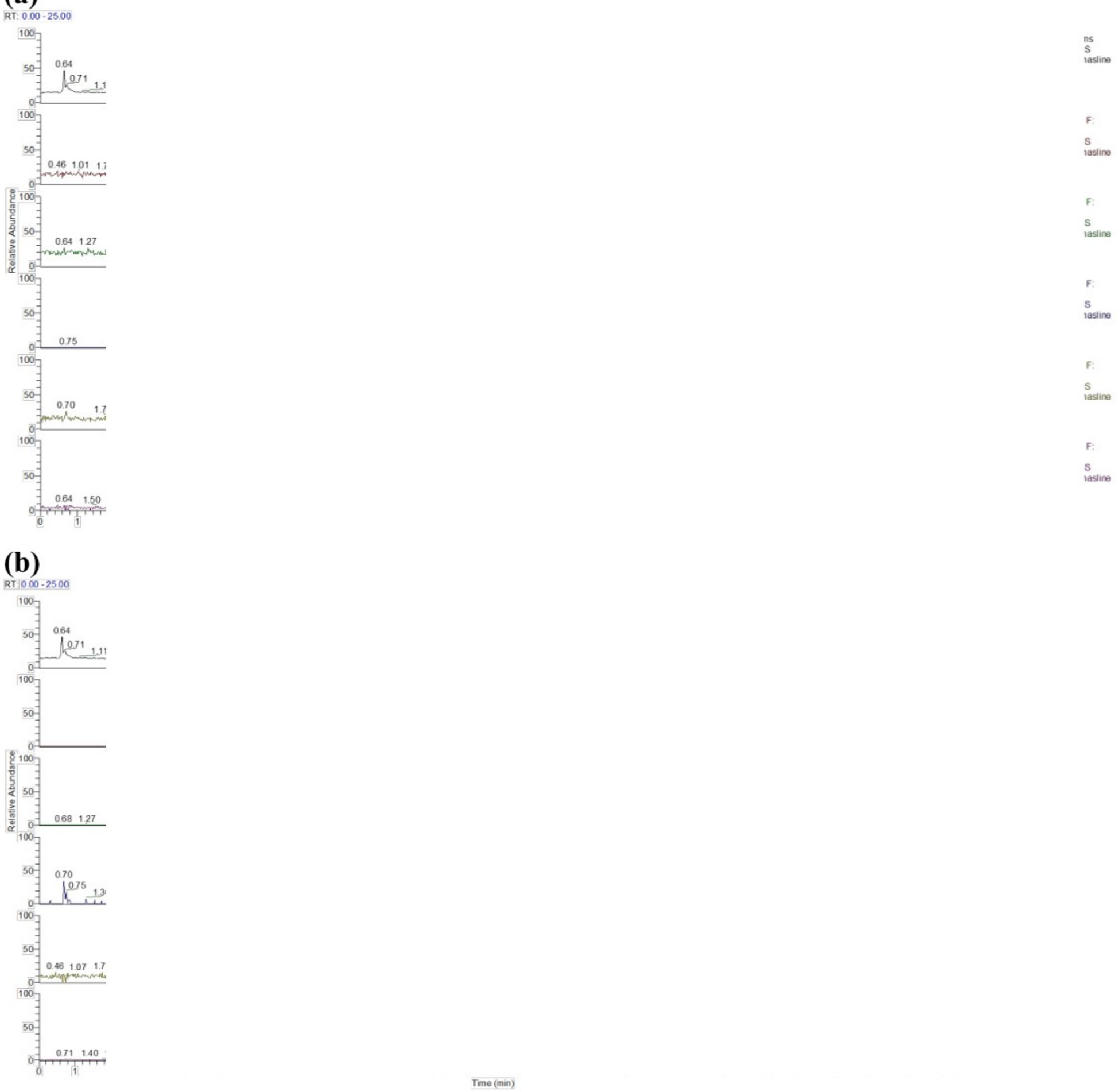


Figure 3. Identification of phenolic compounds in the liquid extract of extra virgin olive oil (EVOO) by UHPLC – MS / MS, negative ionization: (a) phenolic acids, (b) fllavonoids

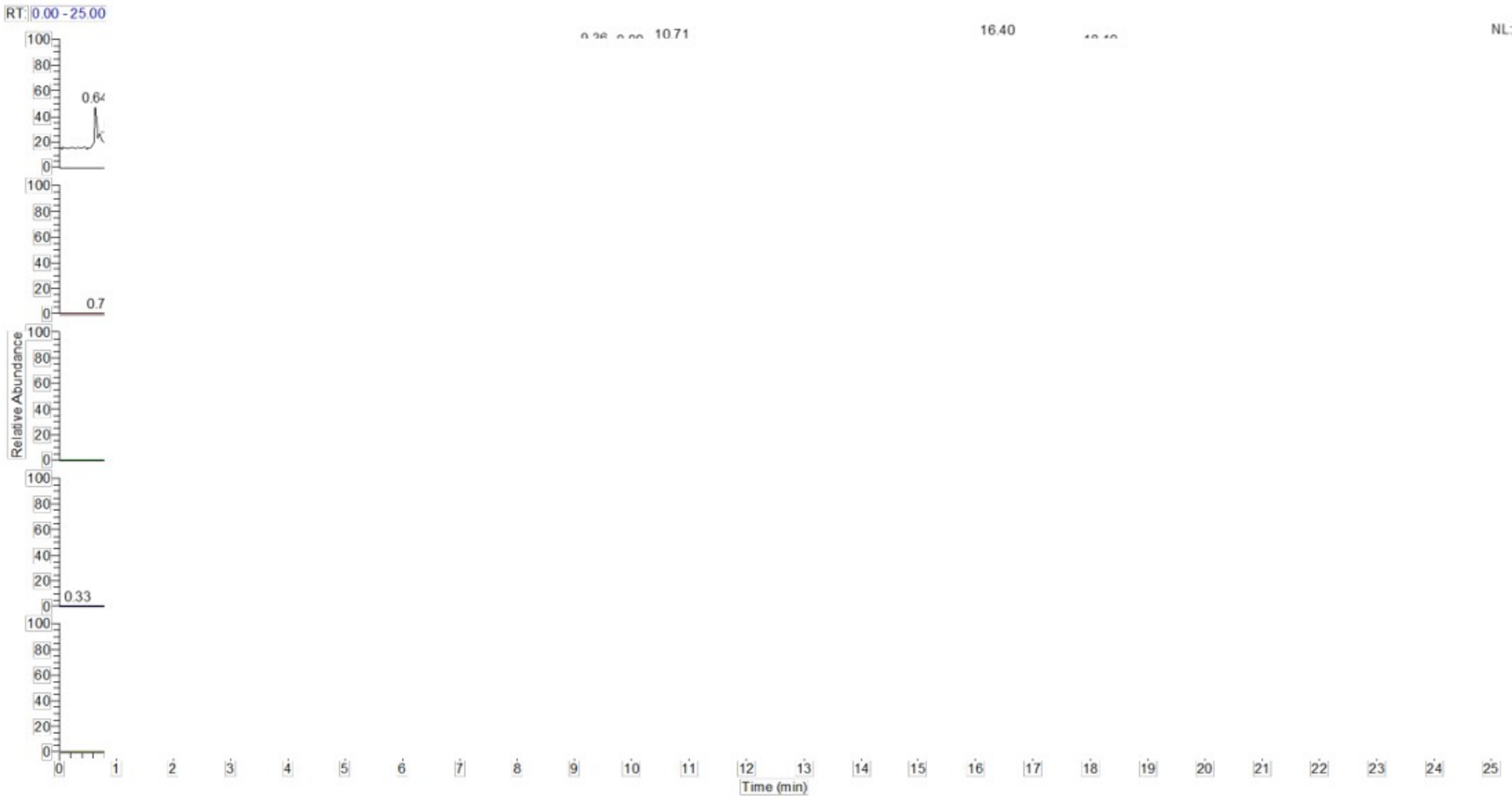


Figure 4. Identification of representative phenolic compounds in the liquid extract of extra virgin olive oil (EVOO) by UHPLC – MS / MS, negative ionization

Quantitative data from the investigation of phenolic compounds in vegetable oils indicated that the main phenolic acids identified are p-coumaric, ferulic, ellagic, abscisic and cinnamic acids, their content varying depending on the type of vegetable oil (Figure 5). As can be seen, walnut oil (N) and extra virgin olive oil (MS) have a higher content of ellagic acid (2,439 and 1,195 mg / kg, respectively), while sesame seed oil has a higher content of high ferulic acid (2.730 mg / kg) compared to other types of vegetable oils. EVOO oil has a cinnamic acid content of 2.242 mg / kg (average value), a much higher value compared to other oils (n.d. - 0.359 mg / kg), thus being a representative phenolic marker for EVOO. Among the quantified flavonoids, pinostrobin, apigenin, quercetin and isorhamnetin are the majority, with values between nd. - 5.085 mg / kg for pinostrobin, 0.011 - 1.651 mg / kg for apigenin, nd. - 1,048 mg / kg for quercetin and 0.003 - 0.506 mg / kg for isorhamnetin respectively..

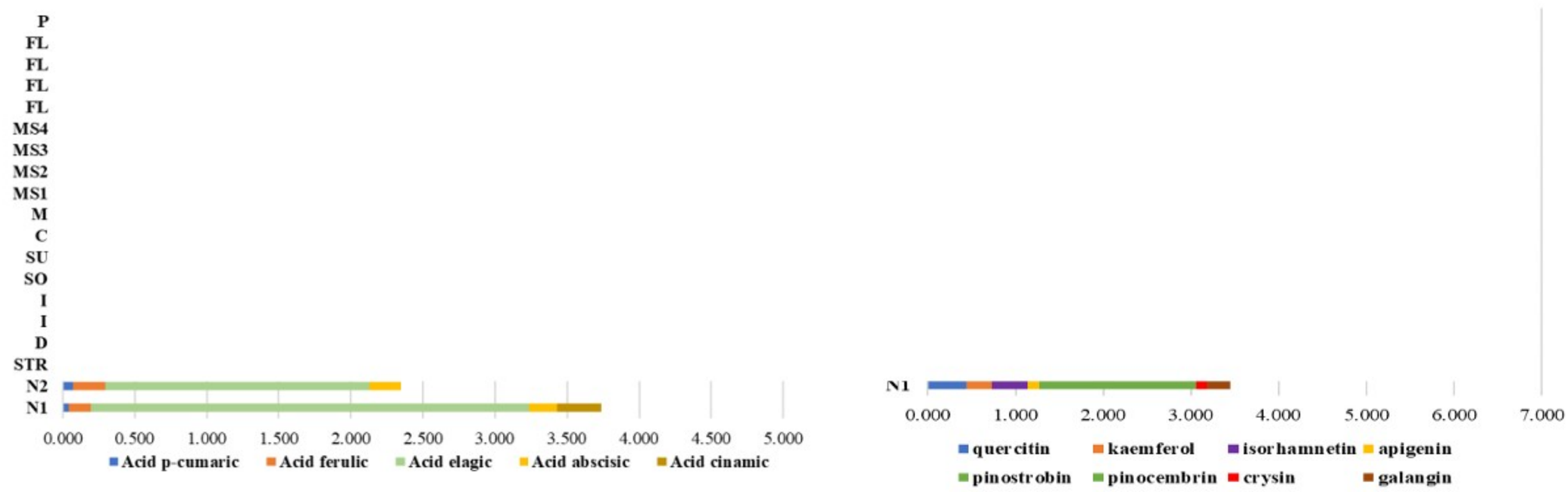


Figure 5. UHPLC-MS / MS profile of phenolic compounds (phenolic acids and flavonoids) in vegetable oils

For exploratory analysis of quantitative polyphenolic data, principal component analysis (PCA) was performed. The first two main components (PC 1 and PC 2) with 53.64% of the total variations were extracted for analysis. The distribution of vegetable oils in the PC1-PC2 score graph is shown in Figure 5, where a clear discrimination of extra virgin olive oil and walnut oil is observed compared to the rest of the oils.

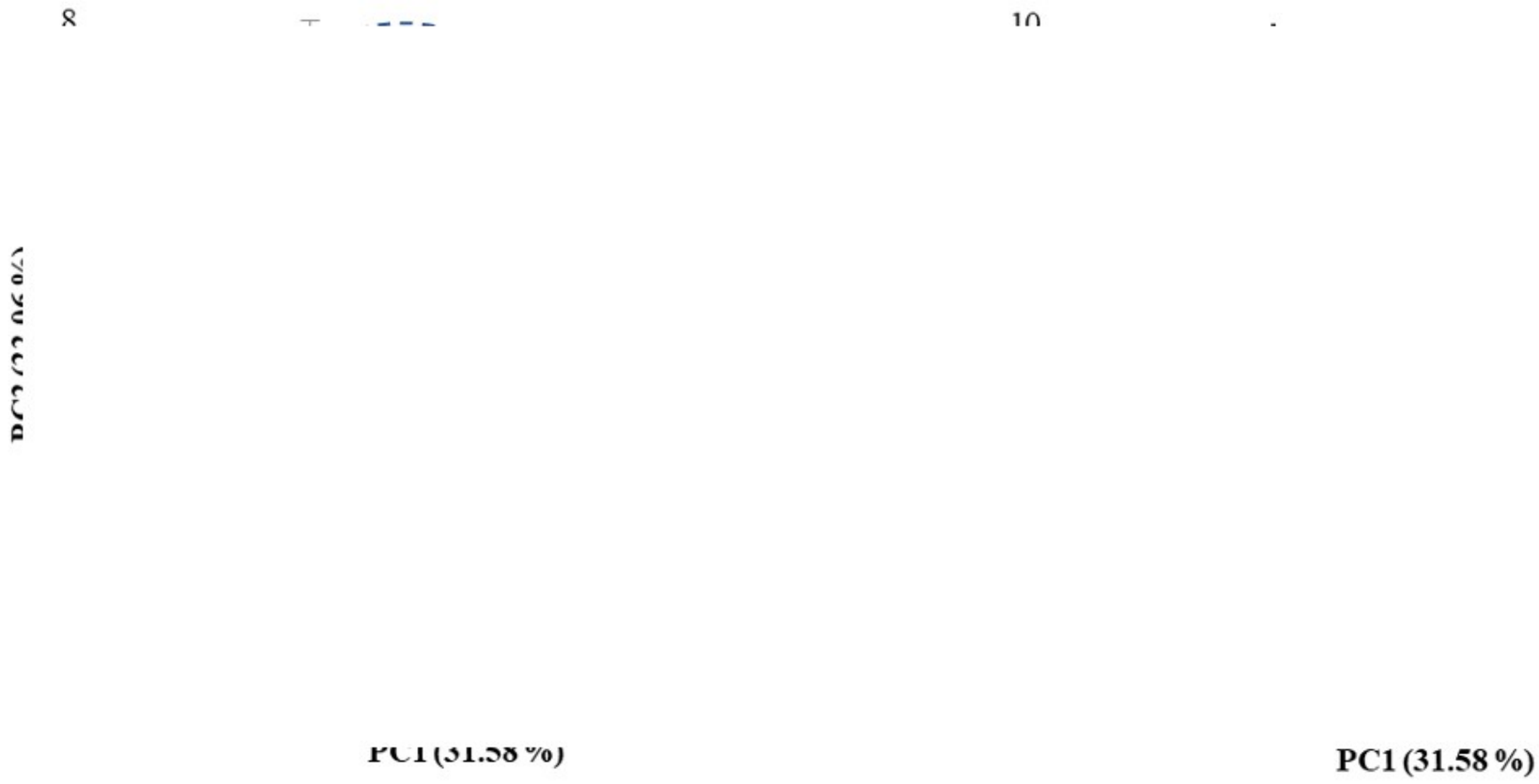


Figure 6. PCA of vegetable oils based on the profile of phenolic compounds (MS - extra virgin olive oil, N - walnut oil, grape seed oil (STR), pumpkin oil (D), flaxseed oil (I), soybean (SO), sesame oil (SU), hemp oil (C), poppy seed oil (M), sunflower oil (FL), corn oil (P)

The results indicate that the phenolic markers specific to extra virgin olive oil are coumaric and cinnamic acids, apigenin, quercetin, isorhamnetin, pinocembrin and (+) - catechin, while the phenolic markers specific to walnut oil are p-hydroxybenzoic, chlorogenic and abscisic acids, but also flavonoids such as galangin, rutin, kaempferil, hesperidin.

Hierarchical Cluster Analysis (HCA) based on quantitative data on phenolic compounds in vegetable oils, grouped the investigated oils into four clusters, namely: cluster C1, which groups most of the investigated olive oils; cluster C2, which includes soybean, hemp, flax, grape seed and pumpkin oils; cluster C3 grouping walnut oils and a sample of extra virgin olive oil from Bulgaria and cluster C4 grouping sunflower, sesame and corn oils (Figure 7). As can be seen, based on the minor phenolic compounds, there is a very clear differentiation of extra virgin olive oil from other vegetable oils, being grouped in a well-defined cluster. Vegetable oils obtained from soybeans and seeds (hemp, poppy, flax, grape seeds) have a similar profile of phenolic compounds, being different from that of vegetable oils obtained from sesame, sunflower and corn.

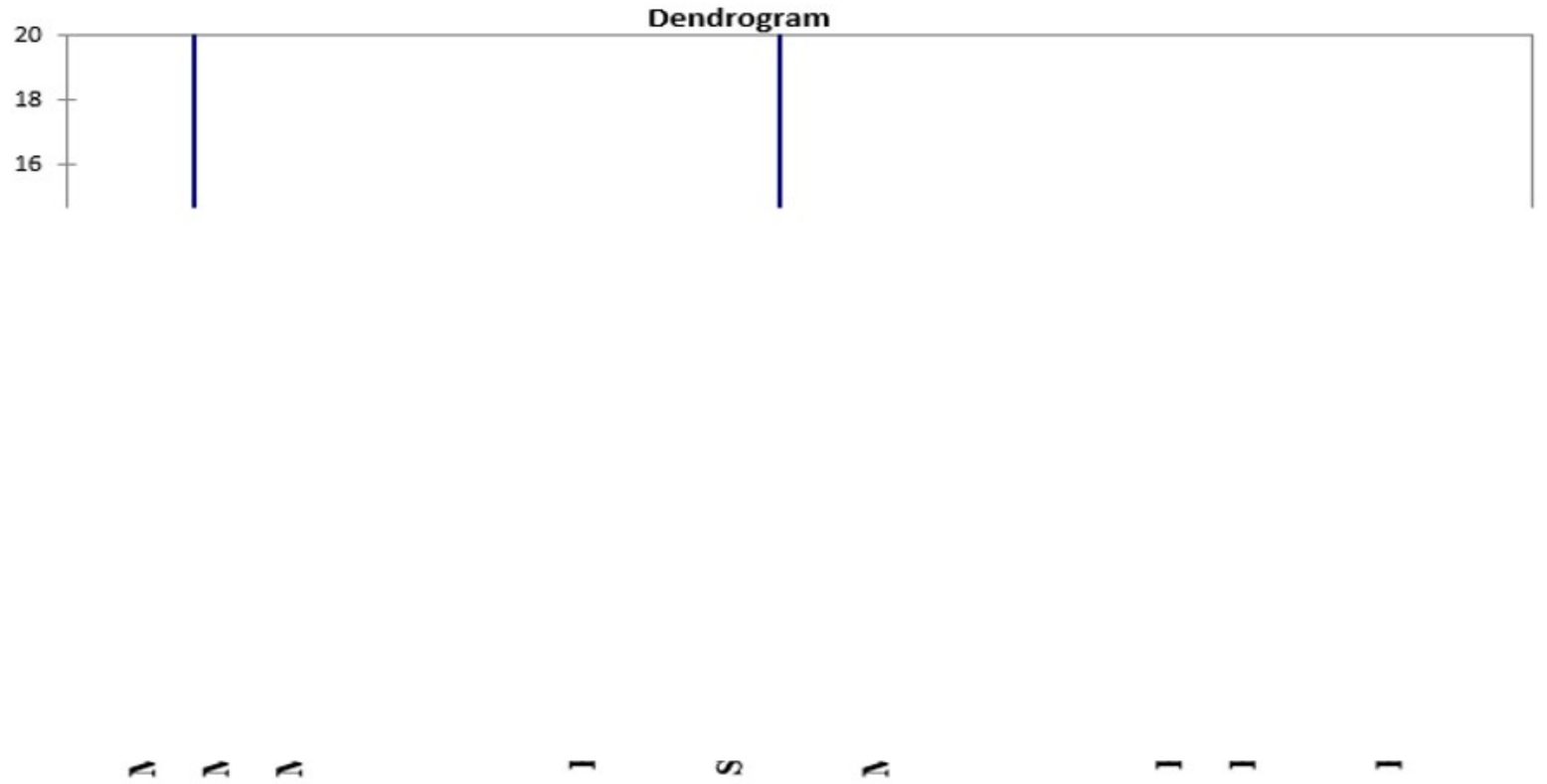


Figure 7. HCA analysis of vegetable oils based on phenolic compounds

Concluding the data on the profile of phenolic compounds in various vegetable oils, it can be stated that extra virgin olive oil has a special composition of phenolic compounds, being similar to that of walnut oils, but also with sunflower and corn oils. This indicates that

sunflower and corn oils may be potential adulterants of extra virgin olive oil (EVOO), by adding them, in different percentages, to EVOO.

Following the PCA analysis of the quantitative data on phenolic compounds in the oil samples resulting from the mixing of EVOO oil with different percentages of corn oil (A2 - 0.5%, A3 - 1%, A4 - 2.25%, A5 - 3 %, A6 - 5%, A7 - 7.5%, A8 - 10%, A9 - 19.5%, A10 - 50%) there is a distinction between counterfeit EVOO oils and maize oil, especially adulterated EVOO oils, which contain more than 3% corn oil (A5 - A10), being located on the left side of the PC1 axis. Adulterated EVOO oils with lower percentages of corn oil (0.5%, 1% and 2.5% - A2, A3 and A4) are grouped on the right side of the PC1 axis, along with the non-adulterated extra virgin olive oil. The main phenolic markers underlying the differentiation of pure and adulterated EVOO oils are ellagic and caffeic acids, lighthouse and flavonoids such as galangin, chrysin and pinocembrin (Figure 8).

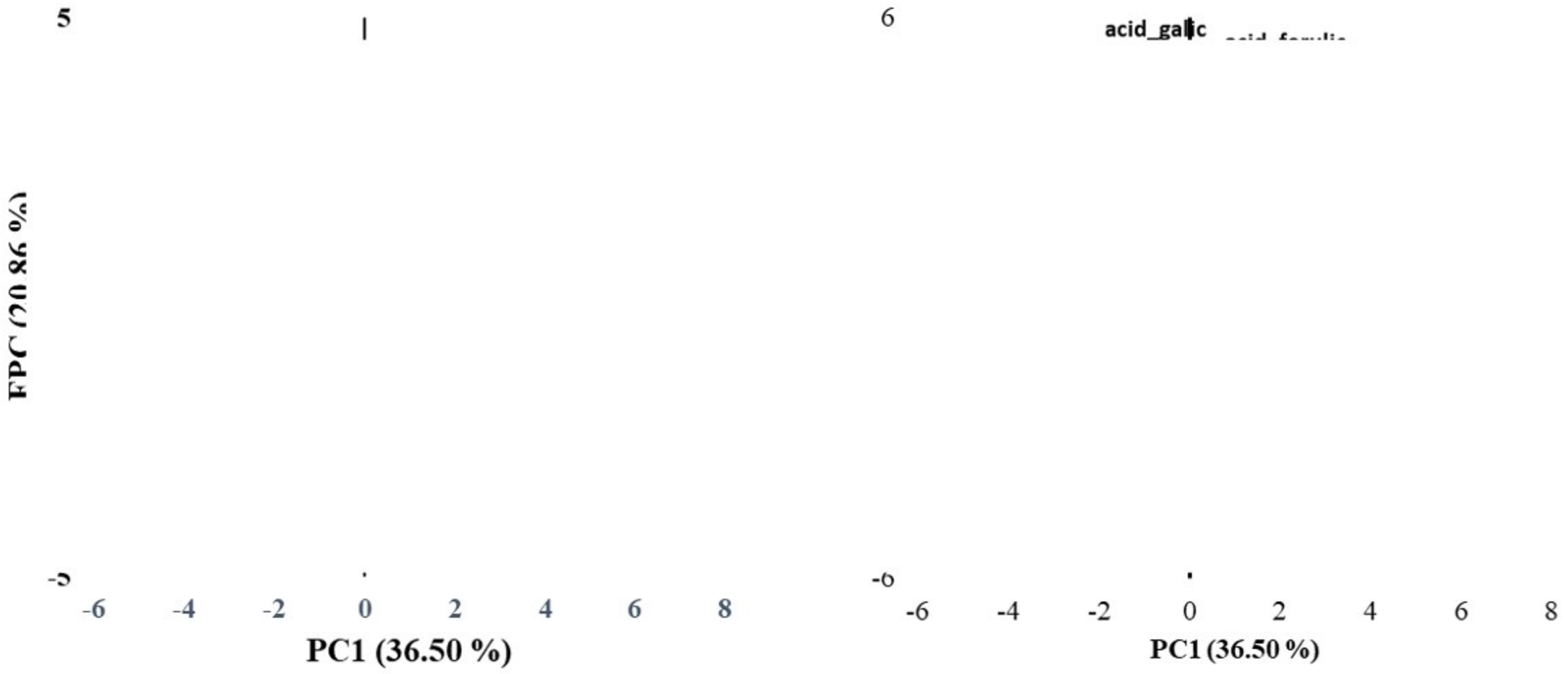
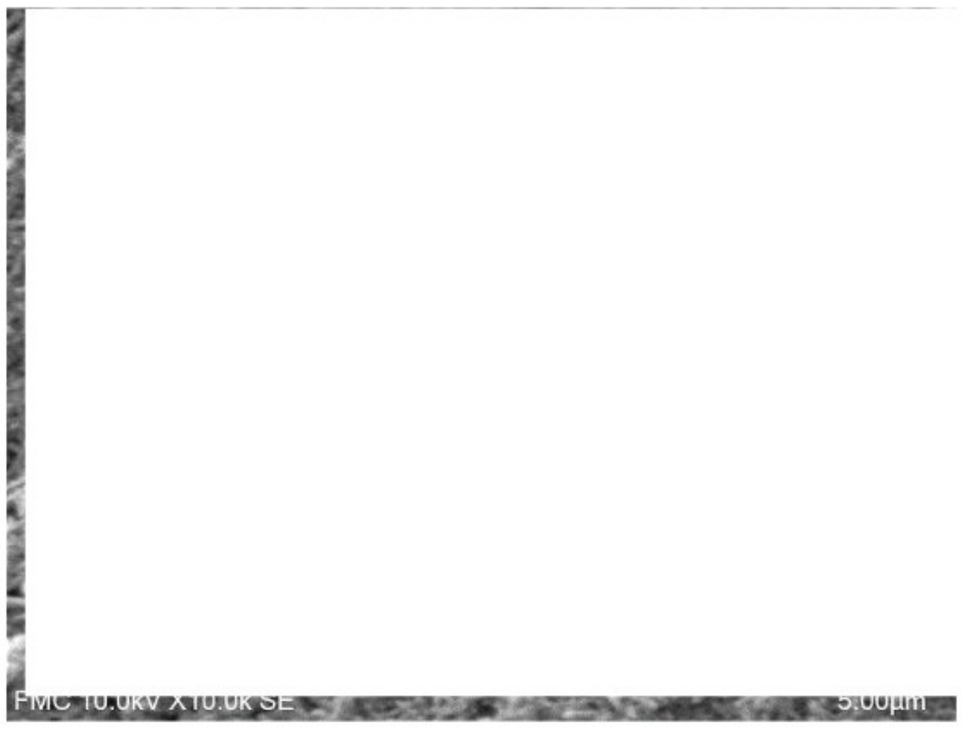


Figure 8. PCA of adulterated extra virgin olive oils with different percentages of corn oil

Therefore, based on the experiment of controlled adulteration of EVOO with different percentages of corn oil, it can be concluded that the profile of the phenolic compounds investigated allowed the differentiation of counterfeit EVOO oils with more than 3% corn oil.

Development of newly sensitive materials

Carbon screen-printed electrodes (SPCE), tin and zinc oxide (ITO), gold (Au) and platinum (Pt) were used to develop novel biosensors for the analysis of biomarkers in olive oil samples. These screen-printed electrodes have been used as a support for immobilizing nanomaterials to increase the sensitivity and selectivity of electrochemical sensors. These sensors have also been used for enzyme modification to increase sensitivity and selectivity. The nanomaterials used were carbon nanotubes functionalized with carboxyl or amido groups in order to favor the interactions between the enzyme and the immobilization matrix, without the need for the crosslinking step. Carbon nanofibers and hydroxyl-functionalized graphene were also used. The characterization of the sensitive materials was performed by scanning electron microscopy and atomic force microscopy. Some of the results obtained are presented below (Figure 9 and Figure 10).



a) SEM image of screen-printed carbon electrode modified with carbon nanofibers

b) SEM image of screen-printed carbon electrode modified with carbon nanotubes and tyrosinase

c) SEM image of screen-printed carbon electrode modified with core-shell ZnS/CdSe quantum dots

d) SEM image of screen-printed carbon electrode modified with graphen and gold nanoparticles

Figure 9. SEM images of the sensitive element

a) AFM image of a sensitive layer containing cobalt phthalocyanine deposited on a mica support

b) AFM image of a sensitive layer containing cobalt phthalocyanine and lacase deposited on a mica support

Figure 10. AFM images of the sensitive element

Synthesis of conductive polymers and molecularly imprinted polymers

In order to develop novel electrochemical sensors, the deposition of electrically conductive organic polymers was performed using monomers derived from thiophene (3,4-

ethylenedioxythiophene (EDOT), hydroxymethyl-EDOT) and in the presence of various doping anions (potassium hexacyanoferrate (II), perchlorate lithium, postaxium perchlorate, Prussian Blue), some of which have a dual role, both doping agents and mediators of electron exchange, a process that takes place during electrochemical detection.
Therefore, innovative electrochemical sensors based on conductive polymers doped with different anions (some of them with electrocatalytic properties or electron exchange mediators) have been obtained that can successfully detect markers in olive oil. A number of functionalized commercial monomers (pyrrole, acrylic acid, methacrylic acid), doping agents and target molecules (oleuropein, tyrosol, hydroxytyrosol, verbacoside) were used for the synthesis of molecularly printed polymers. After synthesis, the template molecules were removed by treatment, with the appropriate solvents releasing the active sites.
Electrochemical sensors based on molecularly printed polymers with biomarkers from olive oil were obtained, which can be used successfully to detect the addition of other oils. Particular attention has been paid to trigoneline, which is found in many vegetable oils but is not found in olive oil. Molecularly imprinted polymers with trigonelin were made and used successfully in the determination of oils added to olive oils.

The results obtained in Activity 1.2 are very good and promising, the specific activities proposed for this year have been achieved 100% and the objectives for this stage have been fully met.

Task 1.2 Development of a multi-sensor platform Characterization of biosensors towards markers Development of multi-biosensing arrays for the analysis of olive oil markers

Development of a multi-sensor platform
In the project, a series of activities were carried out for the development of a multisensor modular system, which would include several measurement possibilities using different detection techniques: amperometry, voltammetry, potentiometry and conductometry. Also, the way of simultaneous integration and measurement with colorimetric biosensors using UV or Vis spectrometry was studied.
The possibility to perform the measurements simultaneously or successively, which must be the design of the electrochemical cell, what amount of sample is required to perform the measurements, was evaluated.
The preliminary conclusions were that the use of only electrochemical methods is more feasible. It will be studied whether optical biosensors have an important role in determining the markings in olive oils, which would justify the more complicated experimental design and the use of two equipment for control, measurement and acquisition of experimental data.
Characterization of biosensors towards markers
For the analysis of pure or adulterated olive oils using electrochemical biosensors, several processes were performed for the extraction of the polar fraction: extraction with methanol-water, extraction in HCl solution, emulsions with Triton x-100, eutectic solvents.
The biosensors prepared and morphologically characterized in the previous stages were used for the detection of biomarkers in olive oil.
The following are examples of studies performed for the electrochemical detection of hydroxytyrosol with 3 new biosensors based on lutetium phthalocyanine and tyrosinase, peroxidase or laccase.
The role of the electron exchange mediator in the electrochemical detection of hydroxytyrosol was highlighted by recording cyclic voltammograms in a 10^{-4} M hydroxytyrosol and 10^{-1} M KCl solution, shown in Figure 11.

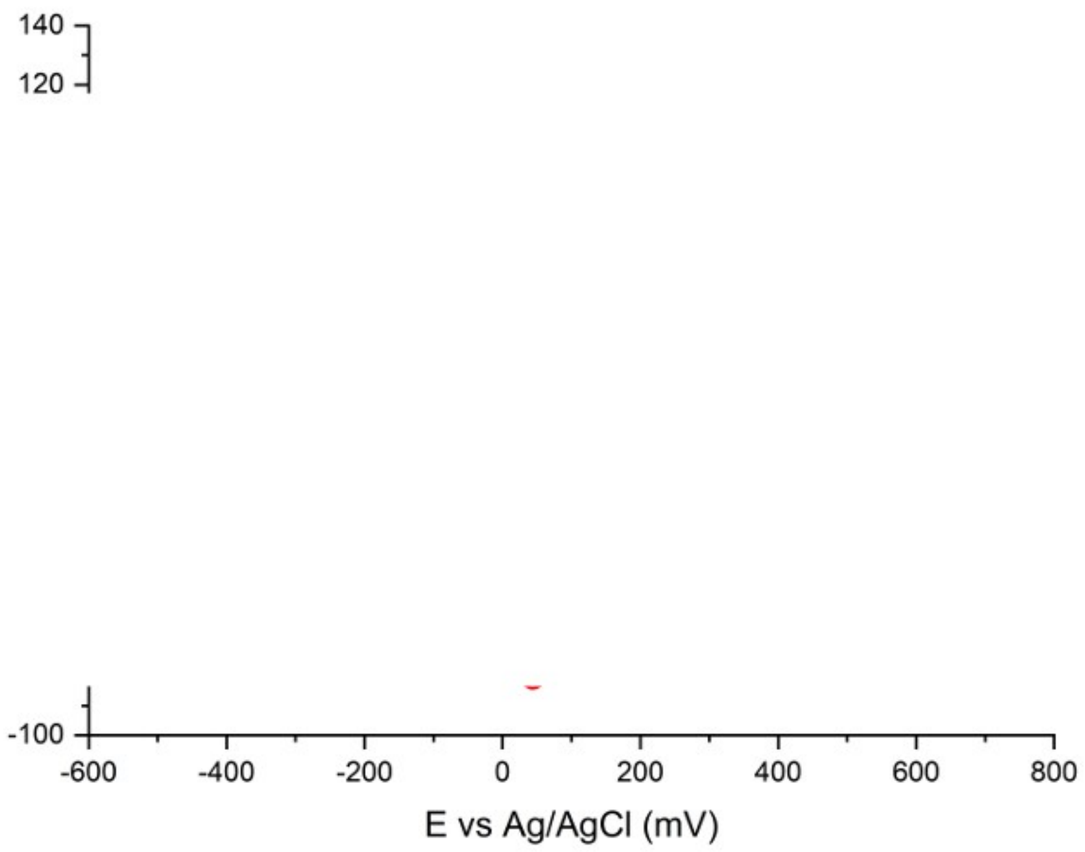
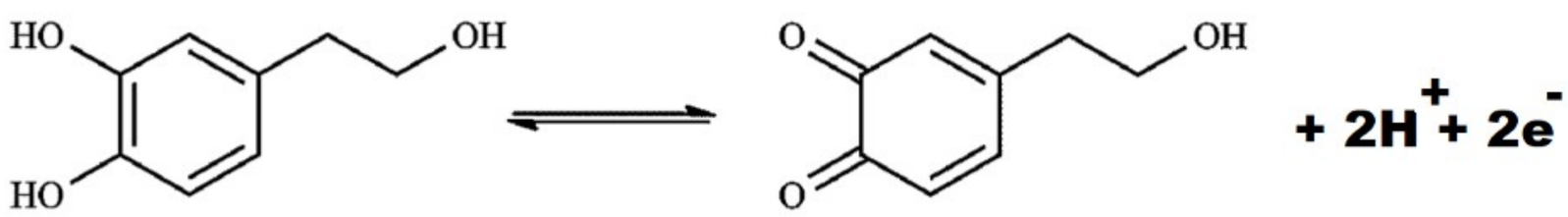


Figure 11. Cyclic voltammograms of biosensors in 10^{-4} M hydroxytyrosol and 10^{-1} M KCl solution

It is obvious that the sensitivity of the biosensor increases greatly due to the mediating effect of $LuPc_2$, which facilitates the transfer of electrons between the redox reaction of hydroxytyrosol and the sensitive surface of the biosensor. The mechanism of the quasi-reversible redox reaction of hydroxytyrosol is shown in the following scheme and involves the exchange of 2 electrons and two protons.



For the most sensitive determination of hydroxytyrosol, the experimental conditions, the pH of the solution to be analyzed, the buffer solution and the ionic strength, temperature, etc. were optimized.

To determine the sensitivity of the biosensor, the calibration curve was performed by recording cyclic voltammograms in hydroxytyrosol solution of different concentrations, in the range of 2-200 μM. The cyclic voltammograms obtained are shown in Figure 12.

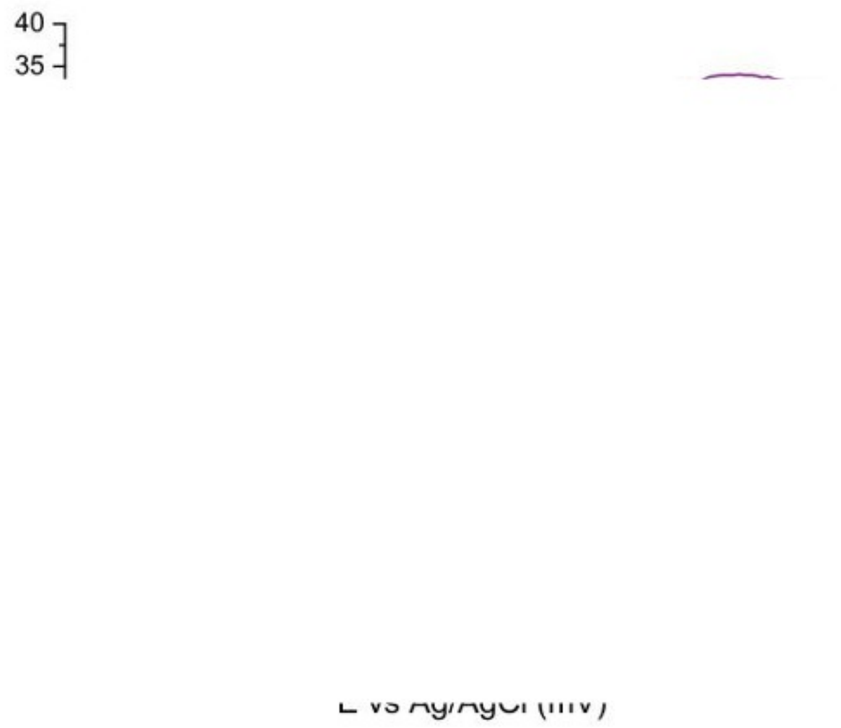


Figure 12. Cyclic voltammograms of tyrosinase-based biosensor in hydroxytyrosol solutions of various concentrations

From the graphical representation of the anodic and cathodic peak currents, respectively, depending on the analyte concentration, good linearities were obtained, with correlation coefficients higher than 0.9. (Figures 13 and 14).

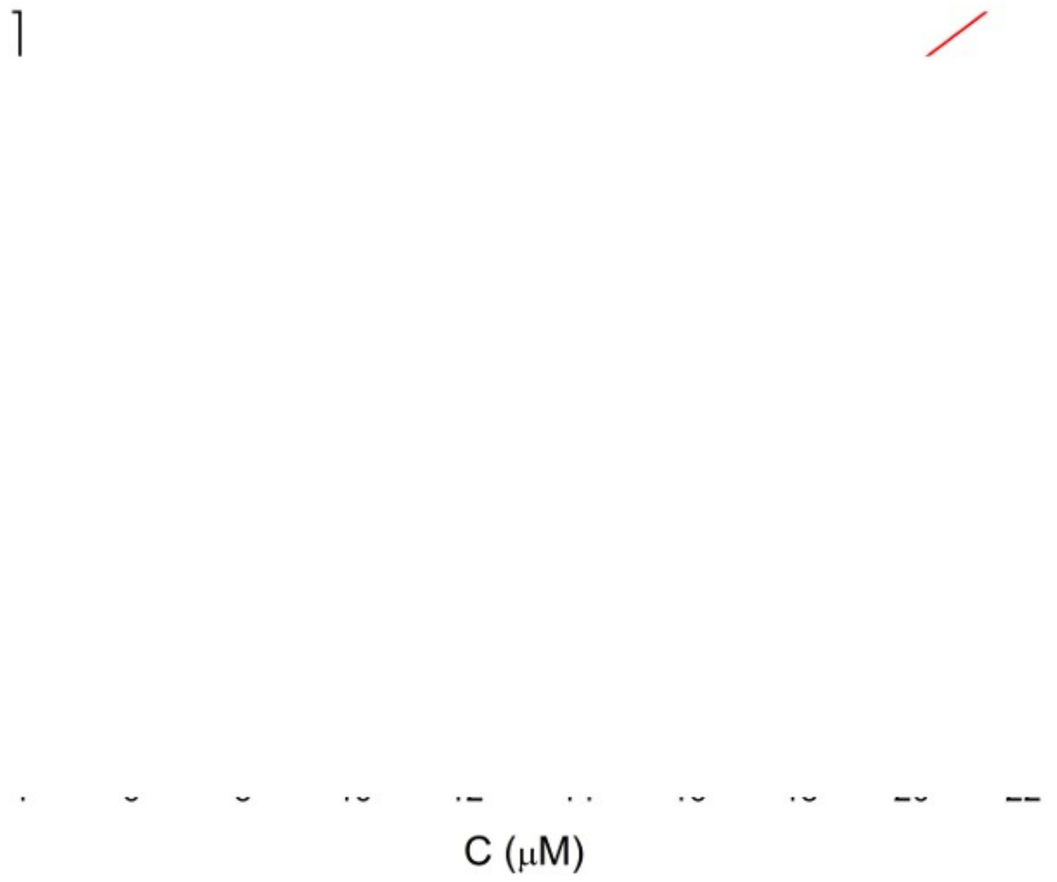


Figure 13. Linear calibration corresponding to the anodic peak for hydroxytyrosol

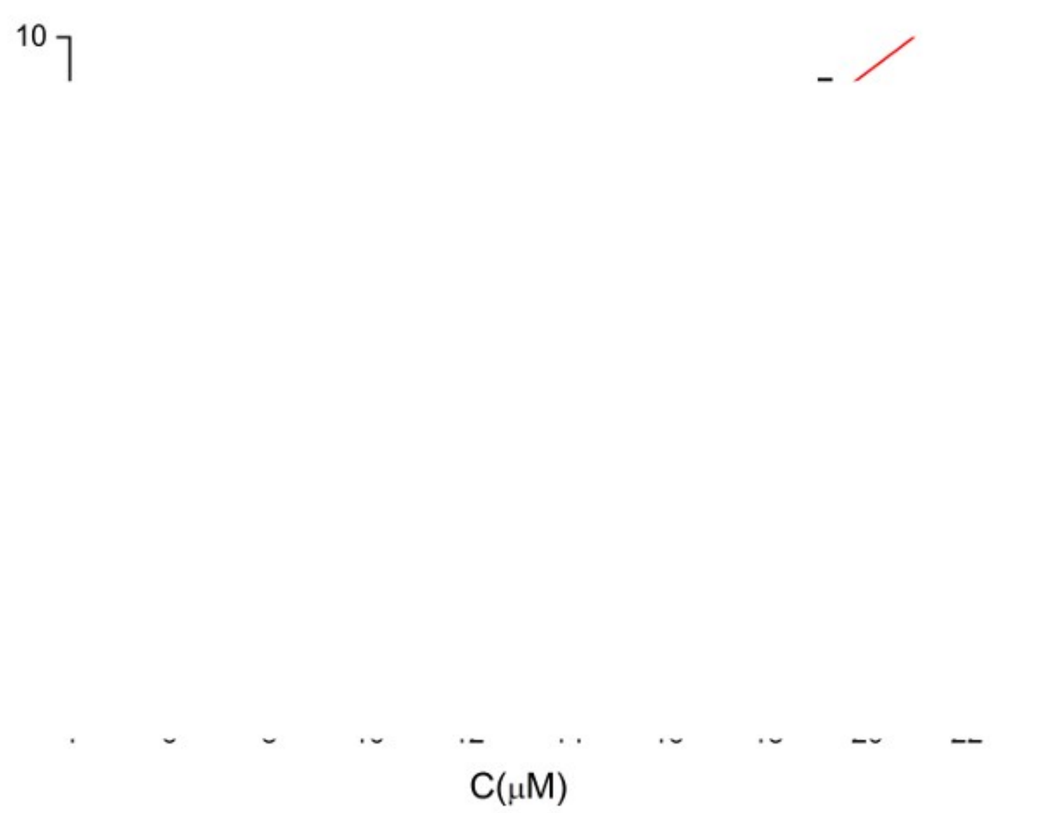


Figure 14. Linear calibration corresponding to the cathodic peak for hydroxytyrosol

Good values of the detection limit were obtained for both peaks, 1.36 μM for the cathodic process and 2.38 μM for the anodic peak.

It was determined that the kinetics of the cathodic process, which corresponds to the reduction of the ortho-quinonic derivative of hydroxytyrosol formed in the enzymatic reaction, is of the Michaelis-Menten type.

$$I = \frac{I_{máx} \cdot [S]}{K_M^{app} + [S]}$$

Apparent Michaelis Menten constant and maximum reaction rate were calculated from the Lineweaver-Burk equation:

$$\frac{1}{I} = \frac{K_M^{app}}{I_{máx}} \frac{1}{[S]} + \frac{1}{I_{máx}}$$

using the calibration data (figure 15).

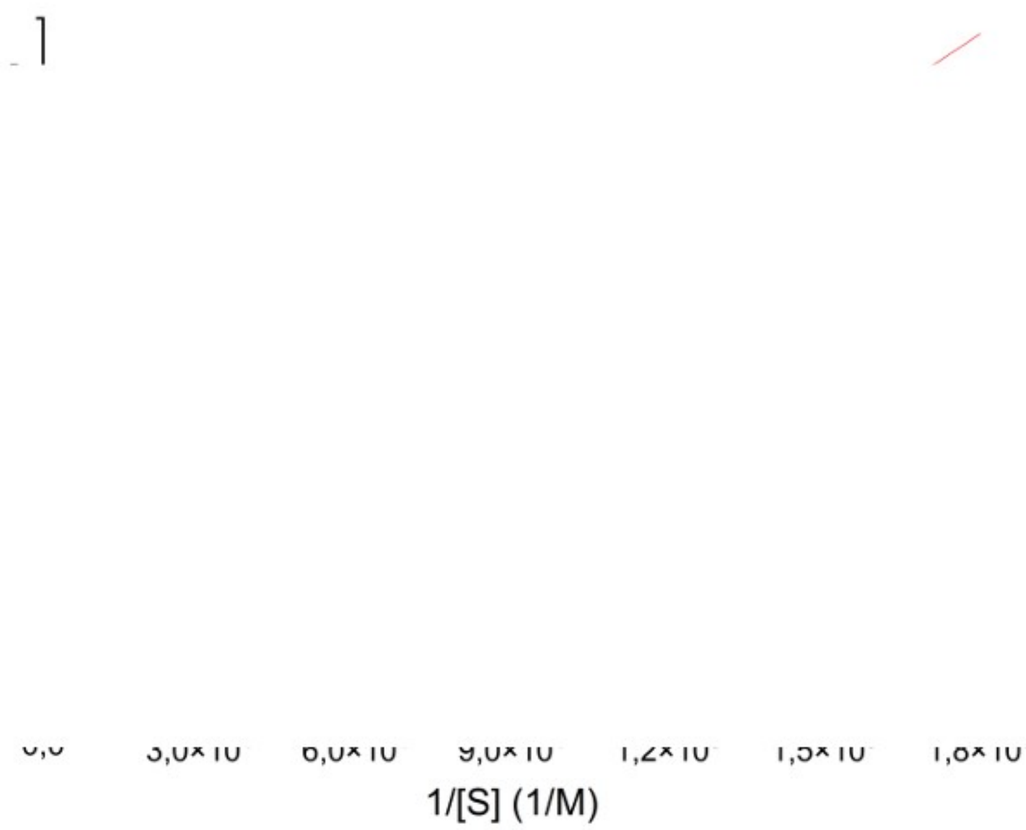


Figure 15. Graphical representation of the Lineweaver-Burk equation corresponding to the anodic peak

The results obtained, K_M (66.77µM) and I_{max} (24.22 µA) demonstrate that the enzyme immobilized in the biosensor retains its activity, has a good affinity for the substrate and therefore the biosensor has a very good sensitivity.
The results obtained for the three biosensors in this study are presented in Table 3.

Table 3. Sensitivity data of biosensors and enzymatic kinetics parameters

Enzyme	**Oxidation peak**		**Reduction peak**		**Michaelis-Menten parameters**	
	E (V)	LOD(µM)	E (V)	LOD(µM)	K_M (µM)	I_{max} (µA)
Tyr						
Perc						
Lac						

The biosensor with the best analytical performance was used to determine tyrosol and caffeic acid in samples of refined, virgin and extra virgin olive oil. The process for extracting the phenolic compounds is shown in the diagram below:

Centrifugare

electrochimica

In optimal conditions, 5 mL of hexane standards or oil samples (1 or 0.150 g depending on the oil) diluted to 5 mL with hexane were placed in test tubes. Then, 100 µL of 1 M aqueous HCl solution was added and the mixture was stirred for 2 minutes using a vortex stirrer. The phases were then separated by centrifugation for 10 minutes at 4000 rpm. The upper organic phase was carefully removed with a pipette, and the remaining aqueous acid phase (i.e., 40 µL) was extracted with a syringe for final analysis by cyclic voltammetry using the phthalocyanine biosensor based on lutetium and laccase. The results obtained are presented in Table 4.

Table 4. Determination of caffeic acid and tyrosol in olive oils.

	Acid cafeic	Tirosol
Sample		
ROO		
VOO		
EVOO		

[a] The standard deviation of three replicate analyzes.

[b] The coefficient of variation is shown in parentheses.

Phthalocyanine-based biosensors and enzymes have been shown to be suitable for building the array of biosensors for adulterated olive oils. The biosensors that will constitute the array of biosensors for determining the falsification of oils will be completed in the second year of implementation, when the work stage is completed and the deliverable provided in the general implementation plan of the project will be achieved.

Development of multi-biosensing arrays for the analysis of olive oil markers

In order to achieve the array of biosensors and the integration of biosensors with the best analytical performance, a series of experimental designs have been developed that can allow simultaneous or sequential analysis in the same sample to be analyzed with higher or lower sample amounts. An example is shown in the following scheme.

Array with 8 different biosensors

Electrochemical cell

Multiplexer for sequential measurements

The reference and auxiliary electrodes will be integrated in the same device or will be external electrodes. This will be decided based on the experimental setup.
The activity carried out was laborious, the objectives proposed at this stage were fully met.

The specific activities provided in activity A1.2 have been carried out in full, and the degree of fulfillment of the objectives is 100% for this year of implementation of the project.

Task 1.3 Smart tools models development and applications in data analysis

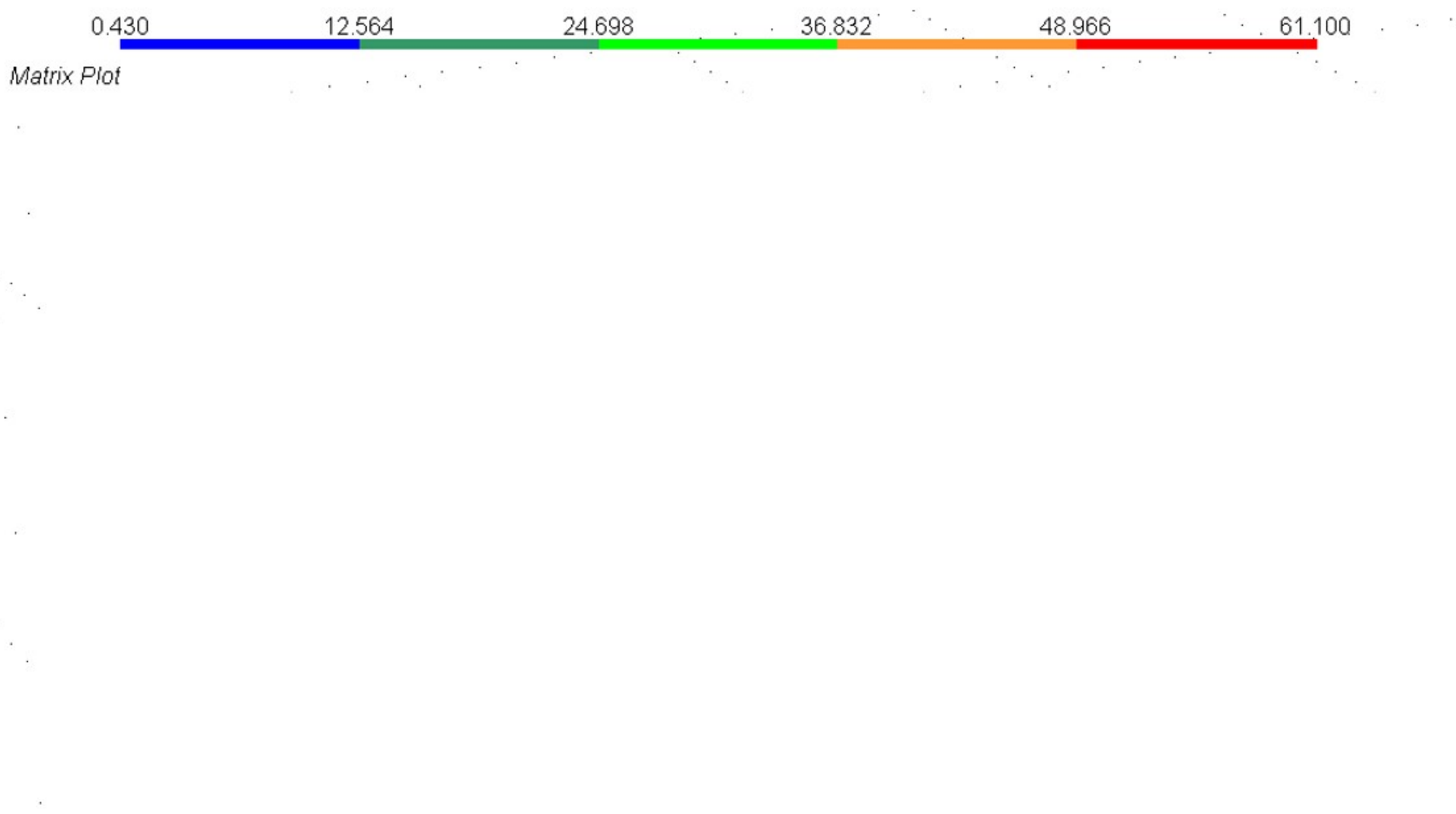
In this activity were applied various methods of analysis of multivariate data from different types of measurements made for samples of olive oils (extra virgin or olive cakes - pomace, chance), other vegetable oils such as sunflower oil, corn oil, rapeseed oil, coconut oil, sesame oil, palm oil and mixtures thereof. Mixtures of extra virgin olive oil and other oils in various proportions were prepared to drive the systems and to determine the limits of detection under controlled conditions.
Regardless of the nature of the experimental data (UV or Vis spectra, FTIR spectra, chromatograms, cyclic voltammograms, square wave voltammograms, etc.), two strategies were approached.
The first method of analysis consisted in the analysis of experimental data, obtained as such with the help of programs that control the equipment. In this case, the experimental data was exported in formats compatible with Excel, Origin, or Mathlab (.xls, .txt, .ascii, .dat). Then the data corresponding to the determined variable and characteristic for the analyzed sample (absorbance, intensity, current) were arranged in the form of matrices of the type presented in Table 5.

Table 5. The structure of the input matrix for the application of multivariate data analysis methods

		Variable 1	Variable 2	…	Variable n
Sam					
Sam					
…	….				

Sam

This approach is feasible with laboratory equipment and some problems were encountered when the matrices had to be transposed for the data to have the structure shown in Table 5. The problem could be solved using Matlab or The Unscrambler programs, which have a number large enough rows and columns to transpose the input matrix.
Another method of performing the input data matrix for multivariate analyzes involved data pre-processing steps. Several methods have been used such as:
• selection of relevant variables using genetic algorithms, implemented in Matlab
• calculation of color coordinates in the case of Vis spectra
• applying the Kernel method to reduce the voltammetric data to a small number (for example 10) by calculating the integrals for in different potential domains
Input data were initially analyzed by various exploratory methods such as Principal Component Analysis and Linear Factor Analysis.
The following is an example of a study to see the effectiveness of the input data pre-processing method. Six biosensors based on lacase, tyrosinase and peroxidase were used and with mediators ferocene, Prussian Blue and manganese phthalocyanine and two groups of oils were analyzed, some pure and some counterfeit.
Figures 16, 17 and 18 show graphically the initial input matrix and preprocessed by the Kernel method or genetic algorithms.



pca84

Figure 16. Graphic presentation of the raw data matrix

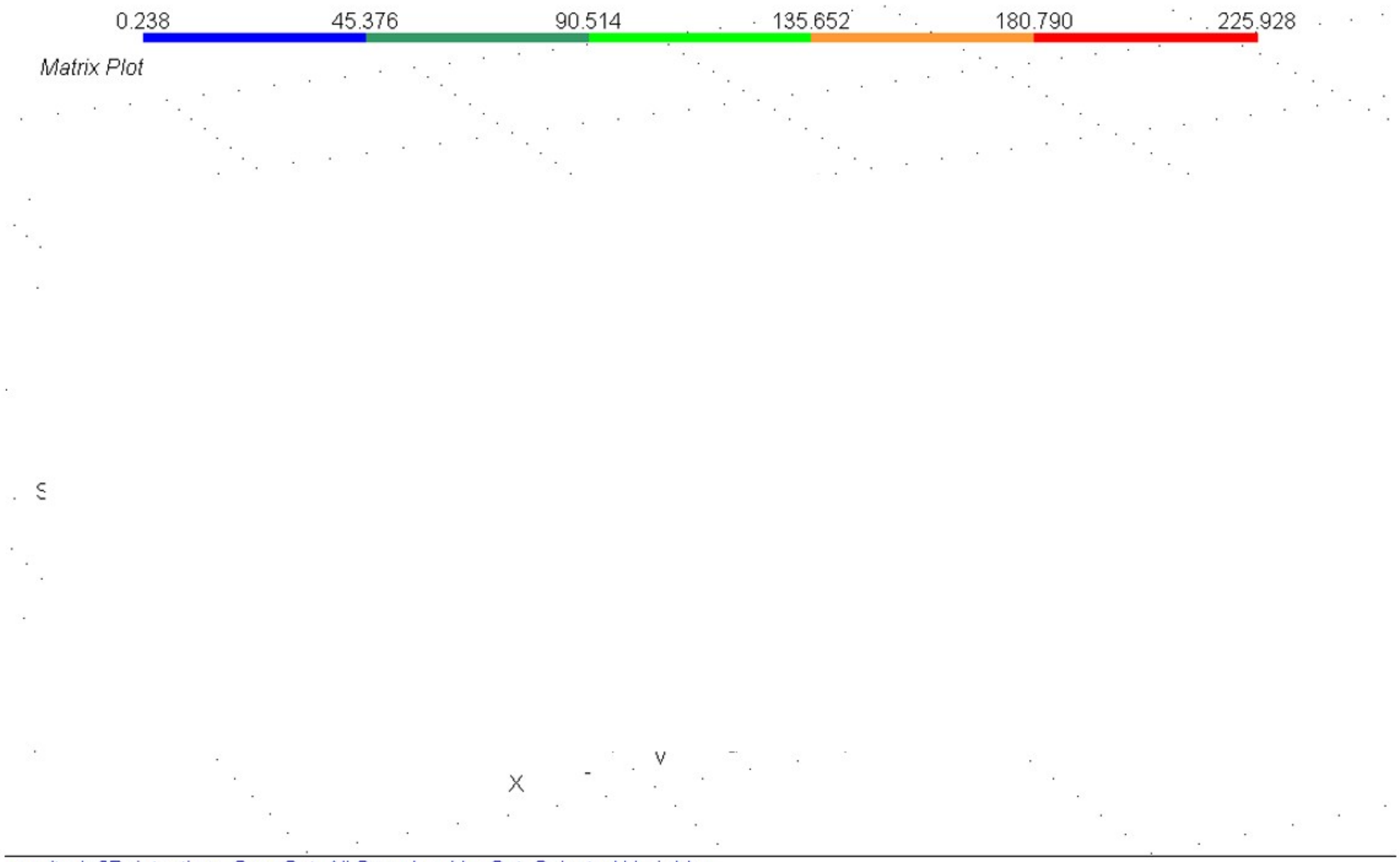


Figure 17. Graphical presentation of the data matrix processed by the Kernel method

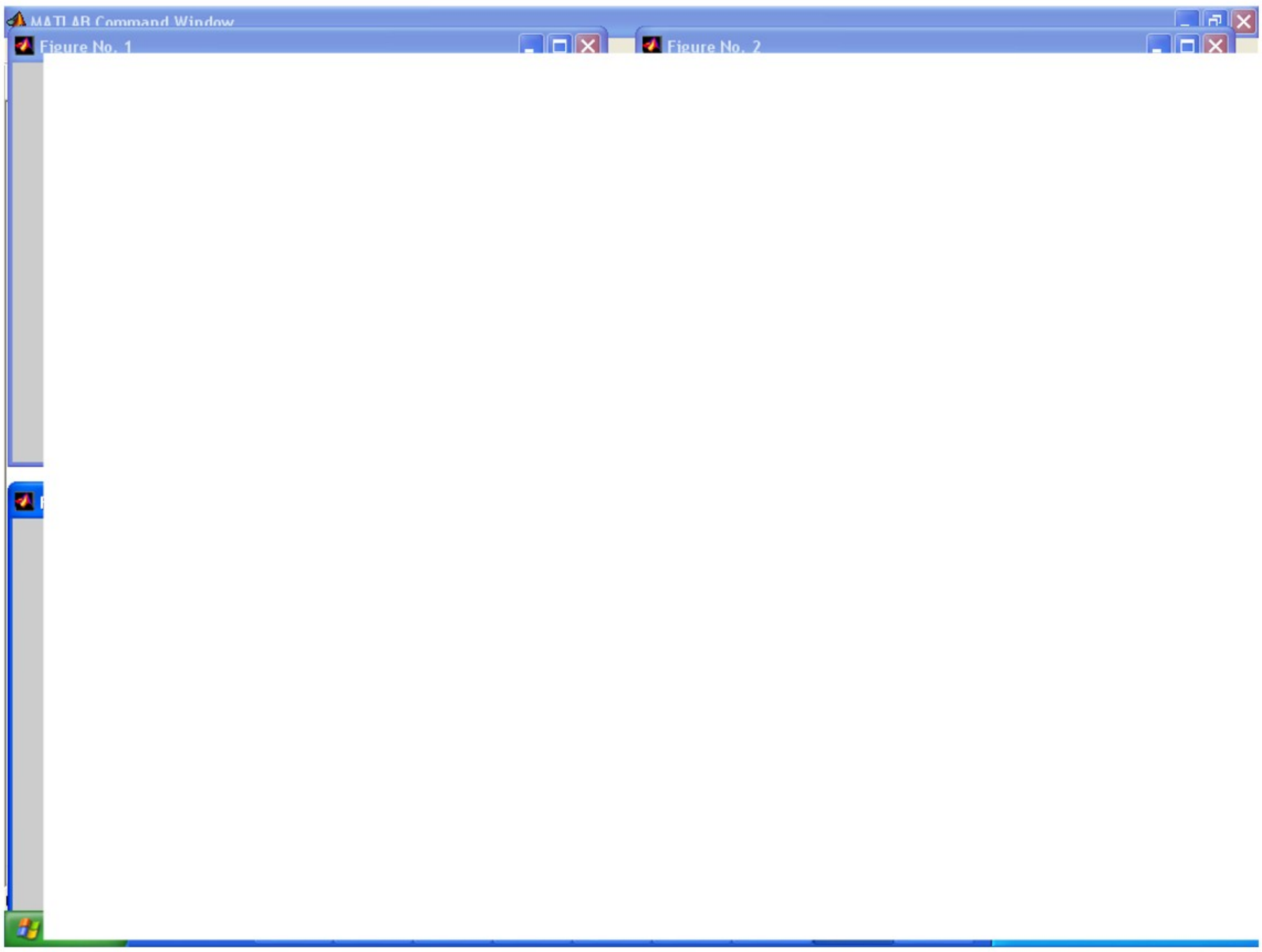


Figure 18. Application of genetic algorithms for the selection of relevant variables for the classification of the samples to be analyzed

The results obtained by applying the Analysis of the principal components to the original and pre-processed data are presented in the form of graph scores (obtained in Matlab or The

Unscrambler) in Figures 19, 20 and 21. The two types of samples are 1 - adulterated oils and 2 - pure oils.

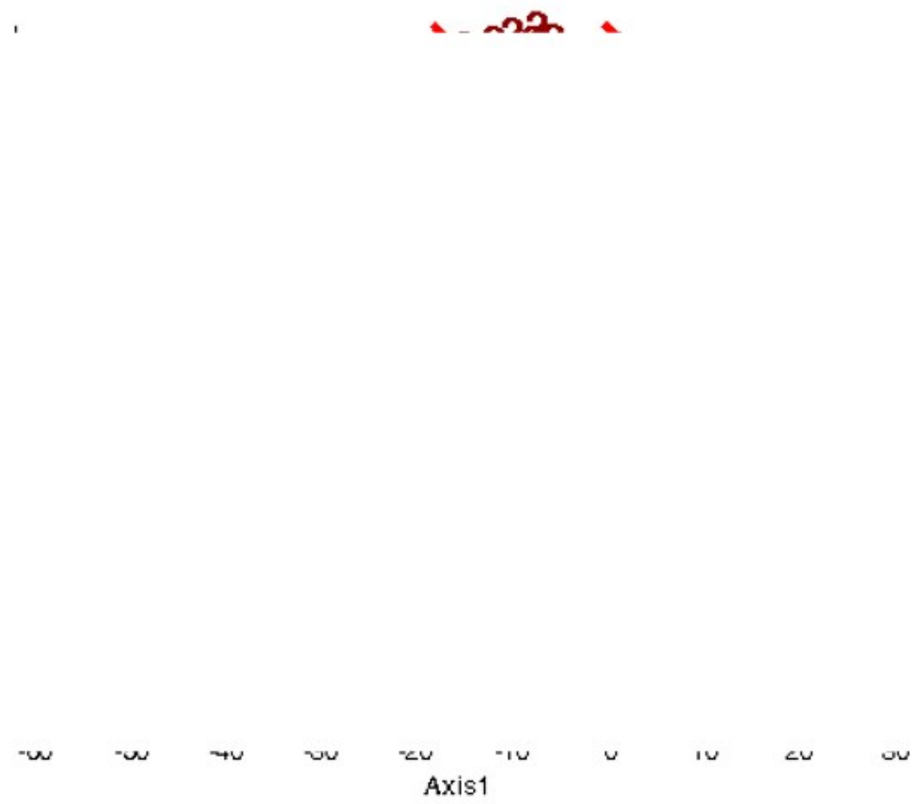


Figure 19. Scores plot of the PCA using the entire square wave voltammograms data

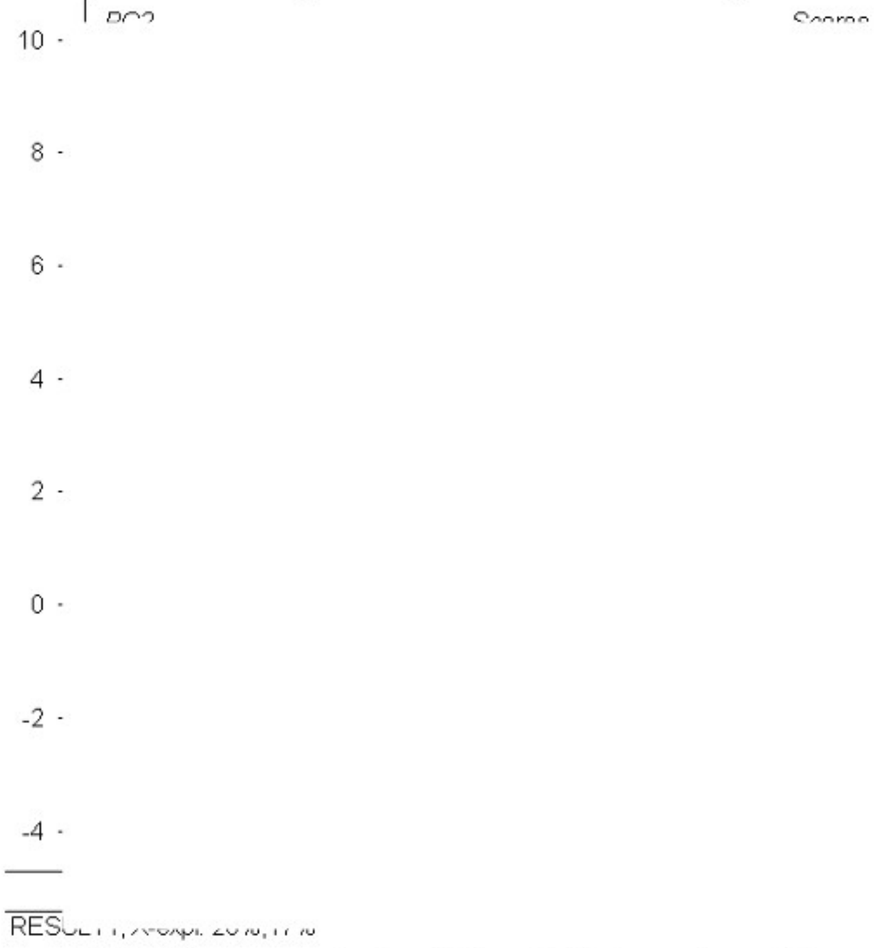


Figure 20. Scores plot of the PCA using Kernel method for the pre-treatment of the voltammetric data

Figure 21. Scores plot of the PCA using 160 representative variables resulting from the application of genetic algorithms

It is observed that the best results are obtained when genetic algorithms are used to select representative variables.
For the classification of samples and the construction of conformity models, the Discriminant Analysis solved by the least partial squares method (PLS-DA) and SIMCA (Soft independent modeling of class analogies) were used. Classification models based on artificial neural networks have also been developed. One such study included the analysis of three types of olive oils classified according to their polyphenol content. The PLS-DA results are shown in Figure 22 and Table 6.

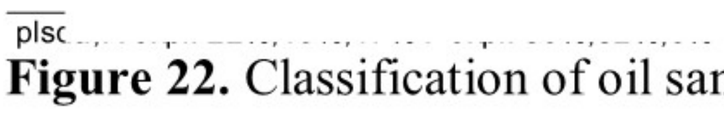


Figure 22. Classification of oil samples according to the content of polyphenolic compounds

Table 6. Quantitative data resulted from PLS-DA

Oil type	Calibration		Validation	
	S			
Oil P	C			
Oil A	C			
Oil H	C			

It is observed that a very good classification of olive oils is obtained according to the polyphenolic content with correlation coefficients higher than 0.95 both in calibration and validation and very low RMSEC and RMSEP errors.
Another area of research involved combining different types of data, spectral, electrochemical and chromatographic, in order to discriminate and classify samples of olive oil. It was assessed whether the fusion of experimental data increases the classification capacity of the system. In general, increasing the amount of information increases the classification power of the analytical system.
To establish correlations between different types of data, which can be used to quantify (predict) antioxidant properties (in the form of total polyphenol content or free radical scavenging capacity) but also the concentration of representative biomarkers for olive oils.
The predicted values were compared with those obtained experimentally (figure 23).

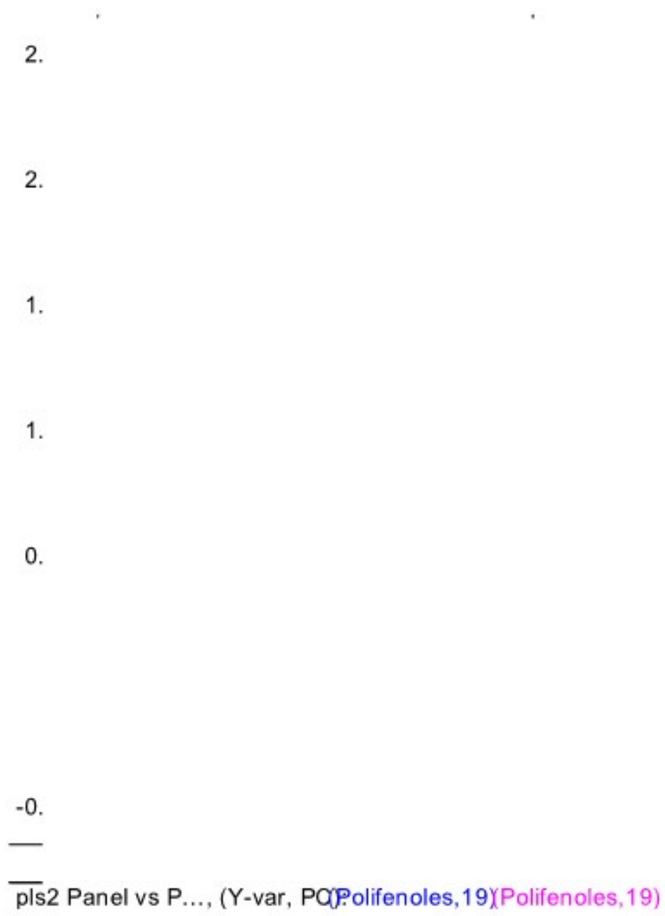


Figure 23. The correlation graph between the estimated value of the total polyphenols content with the biosensors and the value determined by the Folin-Ciocalteu method

Very good correlations were obtained between the quantitative values determined with the electrochemical biosensors and those obtained by standard analysis techniques.

In activity A1.3, the specific activities were carried out in full (100%), thus selecting the optimal methods of analysis that will be completed until the full completion of the activity and the achievement of the final deliverable for this activity.

Task 1.4 - Participation to conferences, publication in peer review journals, applications for patents

For this project we have created a web page that is regularly updated and contains relevant information on the progress in the implementation of the project. The address is: www.busdoa.ugal.ro
Some of the results obtained by the team members during the reported period were published in scientific journals in the red band (Q1 quartile of the field) or were presented at international conferences. These include results from most of the specific activities carried out in this year of project implementation, the other part of the results being being drafted for publication in ISI journals and a patent application.

ISI papers:
1. Munteanu, I.G.; Apetrei, C. Analytical Methods Used in Determining Antioxidant Activity: A Review. International Journal of Molecular Sciences 2021, 22, 3380. IF 5,923. https://doi.org/10.3390/ijms22073380
2. Bounegru AV, Apetrei C. Laccase and Tyrosinase Biosensors Used in the Determination of Hydroxycinnamic Acids. International Journal of Molecular Sciences 2021; 22(9):4811. IF 5,923.
https://doi.org/10.3390/ijms22094811
3. Bounegru, A.V.; Apetrei, C. Evaluation of Olive Oil Quality with Electrochemical Sensors and Biosensors: A Review. International Journal of Molecular Sciences, 22, 12708. IF 5,923.
https://doi.org/10.3390/ijms222312708

Participation in international conferences

1. Constantin Apetrei, Alexandra Virginia Bounegru, Irina Georgiana Munteanu, Irina Mirela Apetrei. Electrochemical sensors and biosensors based on polypyrrole for detection of phenolic compounds in olive oils. Scientific Conference of Doctoral Schools CDS-UDJG 2021, The Ninth Edition, Galați, 10th and 11th of June 2021, Oral, Abstract published in Abstract Book p. 117
2. C. Apetrei, I.M. Apetrei. Detection of Olive Oil Adulteration Using Electrochemical Sensors and Biosensors. XXVIth International Symposium on Bioelectrochemistry and Bioenergetics, Online, 9-13 May, 2021, Cluj-Napoca, Romania. Abstract published in Abstract Book, S2-O-15, page 97, Oral presentation.
3. Alexandra Virginia Meresescu (Bounegru), Constantin Apetrei. Development of novel biosensor for the detection of p-coumaric acid in phenolic extracts from virgin olive oils. Biosensors 2021, The 31st Anniversary World Congress on Biosensors, 26-29 July 2021, poster, P1.008
4. C. Apetrei, A. V. Bounegru, I.G. Munteanu, I.M. Apetrei. Development of a sensitive method for the voltammetric detection of phenolic compounds in extra virgin olive oils. CSAC2021: 1st International Electronic Conference on Chemical Sensors and Analytical Chemistry, session Electrochemical Devices and Sensors, 1st–15th July 2021, poster, sciforum-046141

The results of stage 1 were disseminated through oral presentations at 4 prestigious international conferences and the publication of 3 ISI articles in top-level scientific journals in the red zone (Q1). The dissemination activity planned for this year has been 100% completed.

Task 1.5 - Acquisition activities Elaboration of scientific reports

The procurement activities were carried out in good conditions, although there were problems with the supply of materials due to the delay in international deliveries. The annual scientific report has been prepared in accordance with the Contractor's requirements and is posted on the project's website in Romanian and English.
The procurement activity included all the processes, from offers, specifications, evaluation of technical offers, to the reception of materials, equipment, chemicals, oil samples, etc. provided in the project implementation plan and which ensured the implementation of the activities provided in the first year of implementation of this project.
The scientific activity report for 2021 was prepared by the implementation team under the conditions stipulated by UEFISCDI. The report, in Romanian and English, can also be found on the project website.

In this year of implementation of the project, all the activities provided in the implementation plan were carried out, the planned objectives were 100% fulfilled and valuable results were obtained, partially disseminated in scientific journals and presented at international conferences.

Project manager,
Apetrei Constantin