Rapid determination of hydrophilic phenols in olive oil by vortex-assisted reversed-phase dispersive liquid-liquid microextraction and screen-printed carbon electrodes

Elena Fernández, Lorena Vidal, Antonio Canals

www.elsevier.com/locate/talanta

PII: S0039-9140(17)31277-8
DOI: https://doi.org/10.1016/j.talanta.2017.12.075
Reference: TAL18211

To appear in: *Talanta*

Received date: 22 July 2017
Revised date: 19 December 2017
Accepted date: 22 December 2017

Cite this article as: Elena Fernández, Lorena Vidal and Antonio Canals, Rapid determination of hydrophilic phenols in olive oil by vortex-assisted reversed-phase dispersive liquid-liquid microextraction and screen-printed carbon electrodes, *Talanta,* https://doi.org/10.1016/j.talanta.2017.12.075

Rapid determination of hydrophilic phenols in olive oil by vortex-assisted reversed-phase dispersive liquid-liquid microextraction and screen-printed carbon electrodes

Elena Fernández, Lorena Vidal*, Antonio Canals

Departamento de Química Analítica, Nutrición y Bromatología e Instituto Universitario de Materiales, Universidad de Alicante, P.O. Box 99, E-03080 Alicante, Spain

*Corresponding author: Tel.: +34965909790 ext. 2232; fax: +34965903697. lorena.vidal@ua.es.

Abstract

A novel approach is presented to determine hydrophilic phenols in olive oil samples, employing vortex-assisted reversed-phase dispersive liquid-liquid microextraction (RP-DLLME) for sample preparation and screen-printed carbon electrodes for voltammetric analysis. The oxidation of oleuropein, hydroxytyrosol, caffeic acid, ferulic acid and tyrosol was investigated, being caffeic acid and tyrosol selected for quantification. A matrix-matching calibration using sunflower oil as analyte-free sample diluted with hexane was employed to compensate matrix effects. Samples were analyzed under optimized RP-DLLME conditions, i.e., extractant phase, 1 M HCl; extractant volume, 100 µL; extraction time, 2 min; centrifugation time, 10 min; centrifugation speed, 4000 rpm. The working range showed a good linearity between 0.075 and 2.5 mg L^{-1} (r=0.998, N=7) for caffeic acid, and between 0.075 and 3 mg L^{-1} (r=0.999, N=8) for tyrosol. The methodological limit of detection was empirically established at 0.022 mg L^{-1} for both analytes, which is significantly lower than average contents found in olive oil samples. The repeatability was evaluated at two different spiking levels (i.e., 0.5 mg L^{-1} and 2 mg L^{-1}) and coefficients of variation ranged from 8 to 11% (n=5). The applicability of the proposed method was

tested in olive oil samples of different quality (i.e., refined olive oil, virgin olive oil and extra virgin olive oil). Relative recoveries varied between 83 and 108% showing negligible matrix effects. Finally, fifteen samples were analyzed by the proposed method and a high correlation with the traditional Folin-Ciocalteu spectrophotometric method was obtained. Thereafter, the concentrations of the fifteen oil samples were employed as input variables in linear discriminant analysis in order to distinguish between olive oils of different quality.

Keywords: reversed-phase dispersive liquid-liquid microextraction, screen-printed electrodes, hydrophilic phenols, olive oil samples.

Introduction

Virgin olive oil (VOO) has become an essential component of the Mediterranean diet, having unique nutritional and organoleptic properties. Unlike other refined vegetable oils, VOO is produced exclusively by mechanical and physical means (e.g., cold-pressing, filtration, decantation, centrifugation) thus avoiding the oxidative degradation of bioactive compounds [1].

The chemical composition of VOO can be classified in majority and minority components. Majority components include monounsaturated and polyunsaturated fatty acids, mainly oleic and linoleic acids [2]. Minority components comprise a wide variety of chemical compounds such as carotenoids, phenols, aliphatic and triterpenic alcohols, sterols and hydrocarbons [2,3]. Carotenoids and phenols are the main components responsible for the antioxidant activity exerted by VOO, although carotenoids are present in significantly lower amounts. Lipophilic phenols (e.g., tocopherols)

can be found in other vegetables oils; however, hydrophilic phenols (also known as polyphenols) are typically found only in VOO [2,3]. Hydrophilic phenols play a key role in the oxidative stability and healthy properties of VOO (e.g., anti-inflammatory, chemopreventive, cardiovascular) [2,3]. In addition, these phenols contribute to sensory qualities, affecting the typically pungent and bitter tastes [2,3]. Many different compounds constitute the hydrophilic phenolic fraction, including phenolic acids, phenolic alcohols, hydroxyisochromans, flavonoids, lignans and secoiridoids. The qualitative and quantitative content of these compounds is strongly affected by different factors such as the olive cultivar, geographical origin, environmental conditions, olive ripening, harvesting, extraction methods and storage conditions [4,5].

Many efforts have focused on the characterization and quantification of the hydrophilic phenolic fraction in VOO samples. Powerful techniques for the separation, identification and quantification of individual compounds include liquid chromatography (LC) coupled to ultraviolet (UV), fluorescence (FL), mass spectrometry (MS) or nuclear magnetic resonance detector, with LC-MS being the most frequently employed combination [3]. However, the traditional Folin-Ciocalteu method, based on the colorimetric determination of total polyphenols, is still very useful to estimate the antioxidant capacity of VOO with a simple procedure and low cost [6,7]. Also, alternative electrochemical methods have been developed with the same purpose [8–17].

Inherent properties of olive oil samples (e.g., hydrophobicity, viscosity, complex chemical composition) make sample treatments necessary before instrumental analysis. Traditionally, solid-phase extraction (SPE) and liquid-liquid extraction (LLE) have been employed to isolate hydrophilic phenols prior

to LC-UV, LC-FL, LC-MS [3], spectrophotometry [3] or electrochemical analysis [8–11]. Nevertheless, recently reversed-phase dispersive liquid-liquid microextraction (RP-DLLME) has been introduced as valuable and green alternative to replace the aforementioned tedious and time-consuming techniques [18]. RP-DLLME is based on the dispersion in tiny droplets of a few µL of an aqueous solution in the hydrophobic sample. The cloudy solution presents a great contact surface area between the donor and acceptor phases, thus enhancing extraction efficiency [18]. After extraction (lasting a few seconds or minutes), phases are separated by centrifugation and the enriched aqueous phase is retrieved for subsequent analysis. RP-DLLME has been employed prior to LC analysis to determine hydrophilic phenols in VOO previously [18–22]; however, to the best of our knowledge, this miniaturized extraction technique in conjunction with electrochemical analysis has not been proposed to date.

Here we present for the first time an analytical method to assess the hydrophilic phenolic fraction in olive oils using RP-DLLME as sample preparation technique and screen-printed carbon electrodes (SPCEs) as electrochemical transducers. This association synergistically combines the advantages of RP-DLLME (i.e., speed and ease of use, low sample volume, reduced generation of wastes, ecological, high enrichment factors and affordability) with the rapid response, inexpensive instrumentation and portability of SPCEs. Electrochemical behavior of the main hydrophilic phenols (i.e., oleuropein, hydroxytyrosol, caffeic acid, ferulic acid and tyrosol) was evaluated with SPCEs, and subsequently caffeic acid and tyrosol were selected as model compounds. Parameters affecting RP-DLLME were studied using a

multivariate optimization strategy. The applicability of the proposed method was tested in olive oils of different quality. Finally, fifteen olive oil samples were analyzed using the proposed method and the results were compared with those obtained with the Folin-Ciocalteu spectrophotometric method. Thereafter, found concentrations by the proposed method were subjected to linear discriminant analysis (LDA) in order to distinguish between olive oils of different quality.

Experimental part

Reagents and oil samples

Oleuropein (≥ 98%), hydroxytyrosol (≥ 98%), caffeic acid (≥ 98%), ferulic acid (99%) and tyrosol (> 99.5%) standards were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions of individual compounds (1000 mg L^{-1}) were prepared in LC grade acetone from Sigma-Aldrich and stored in amber glass vials in the freezer (i.e., -18 ºC). Working solutions were prepared daily by proper dilution of stock solutions in LC grade hexane from Sigma-Aldrich. Fuming HCl (37%) from Merck (Darmstadt, Germany) was employed to prepare HCl aqueous solutions. The ultrapure water (resistivity of 18.2 MΩ cm at 25 ºC) employed to prepare aqueous solutions was obtained with a Millipore Direct System Q5™ purification system from Ibérica S.A. (Madrid, Spain). Analytes were dissolved in aqueous 0.1 M HCl solutions to study their electrochemical behavior with SPCEs.

Folin-Ciocalteu reagent from Fluka (Steinheim, Germany) and Na_2CO_3 (99%) from Prolabo (Paris, France) were employed in Folin-Ciocalteu assays.

Sunflower oil and fifteen olive oil samples of different trademark and quality, namely “olive oil”, VOO and extra virgin olive oil (EVOO), were purchased in

local supermarkets. It should be noticed that commercial oils labeled as “olive oil” consist of mixtures of refined olive oil (up to 90%) and VOO or EVOO. Hereafter, they will be named as refined olive oil (ROO) to avoid confusion. Samples were stored in the dark at room temperature and opened just before use to prevent the oxidative degradation of target analytes.

Instrumentation

A vortex mixer from Heidolph (Swabach, Germany) was used to assist RP-DLLME. A centrifuge from Selecta (Barcelona, Spain) was used for phase separation.

A Multi Autolab/M101 Potentiostat/Galvanostat from Metrohm Autolab B.V. (Utrecht, The Netherlands) controlled by NOVA software version 1.10 was used for electrochemical experiments. SPCEs (ref. DRP-110) with three-electrode configuration were purchased from DropSens (Oviedo, Spain). The working disk-shaped electrode, 4 mm in diameter, and the counter electrode were made of carbon ink whereas the pseudo-reference electrode was made of silver. Specific connectors obtained from DropSens (ref. DRP-DSC) were used to connect SPCEs to the potentiostat.

An ultraviolet-visible spectrophotometer from Thermo Scientific (Waltham, MA, USA) was employed in Folin-Ciocalteu assays.

RP-DLLME

Under optimized 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 aqueous 1 M HCl solution were added and the mixture was shaken for 2 min using vortex agitation. Next, phases were separated by

centrifugation for 10 min at 4000 rpm. The upper organic phase was carefully removed with a glass pipette and the remaining acidic aqueous phase (i.e., 40 µL) was retrieved with a syringe for final analysis by differential pulse voltammetry (DPV) using SPCEs. Fig. 1 shows a scheme of the overall procedure.

Electrochemical analysis

Cyclic voltammetry was employed to investigate the electrochemical behavior of hydrophilic phenols with SPCEs. Potential was recorded between 0.0 V and +1.2 V at 100 mV s^{-1} scan rate.

DPV was employed as electroanalytical technique after RP-DLLME. An aqueous 0.1 M HCl standard solution containing 10 mg L^{-1} of caffeic acid and tyrosol was employed to optimize DPV parameters. Potential was recorded between +0.2 V and +1.1 V. Optimum DPV parameters were: 100 mV modulation amplitude; 10 mV step potential; 0.05 s modulation time and 0.5 s interval time.

SPCEs were always discarded after a single use. All experiments were carried out in triplicate and at room temperature (i.e., 21 ºC).

Folin-Ciocalteu method

Hydrophilic phenols were also determined spectrophotometrically by the Folin-Ciocalteu method for comparative purposes. The calibration curve was constructed using caffeic acid aqueous standards from 0 to 300 mg L^{-1} (N=5) in 1 M HCl. 40 µL of each standard solution was mixed with 200 µL of Folin–

Ciocalteu reagent, 800 µL of 7.5% Na_2CO_3 aqueous solution and diluted up to 4 mL with deionized water. The mixture was manually shaken for a few seconds and, after a 2 h reaction in the dark at room temperature (i.e., 21 ºC), the absorbance was measured at 765 nm. Analytes were extracted from olive oil samples using RP-DLLME according to the procedure described in "RP-DLLME" section and 40 µL of final acidic aqueous extracts were subjected to the colorimetric assay (i.e., mixed with 200 µL of Folin–Ciocalteu reagent and 800 µL of 7.5% Na_2CO_3 solution, diluted up to 4 mL and incubated for 2 h in the dark before spectrophotometric determination). The concentration of total hydrophilic phenols was finally expressed as mg of caffeic acid equivalents per Kg of oil (i.e., mg_{CAE} Kg^{-1}) considering the preconcentration factor of RP-DLLME procedure and sample dilution.

Data processing

A multivariate optimization strategy was carried out to determine optimum conditions for RP-DLLME. The statistical software NEMRODW® ("New Efficient Methodology for Research using Optimal Design") from LPRAI (Marseille, France) was used to build the experimental design matrix and evaluate the results. The current peak of caffeic acid and tyrosol were individually used as response functions for optimization.

LDA was carried out using the Statgraphics statistical computer package "Statgraphics Plus 5.1." (Warrenton, VA, USA). The concentration of caffeic acid equivalents and tyrosol equivalents found during the analysis of ROO, VOO and EVOO samples (expressed in mg Kg^{-1} of oil) were used as input variables during LDA.

Results and discussion

Electroanalysis with SPCEs

Electrochemical behavior of hydrophilic phenols

Cyclic voltammograms of caffeic acid, hydroxytyrosol, oleuropein, ferulic acid and tyrosol are shown in Fig. 2a. Caffeic acid, hydroxytyrosol and oleuropein (i.e., ortho-phenols) showed one anodic peak and one cathodic peak after reversing the scan direction. The reversibility of the oxidation reaction can be explained considering their chemical structure. These compounds possess two hydroxyl groups attached to a benzene ring in ortho position, which can be reversible oxidized to ortho-quinones. Ferulic acid showed one oxidation peak and one smaller and broader reduction peak on the reverse scan. Although the mechanism underlying electrochemical oxidation of ferulic acid is still unclear, it is known to involve ortho-quinone moiety [23–25], whose reduction probably gave rise to the cathodic peak observed in the ferulic acid voltammogram. Finally, tyrosol showed a clearly irreversible process with one anodic peak, corresponding to the oxidation of the only hydroxyl group attached to the benzene ring, but no cathodic peak.

As also shown in Fig. 2a, the oxidation of ortho-phenols occurred at very near potentials whereas mono-phenols were oxidized at higher and separated potentials.

Selection of model compounds

A 10 mg L^{-1} mixed standard solution containing all phenols under study was prepared in aqueous 0.1 M HCl and analyzed by DPV. Then, RP-DLLME was applied to a VOO sample under the following conditions: 100 µL of aqueous 0.1

M HCl as extractant phase, 3 min of extraction time and centrifugation for 10 min at 4000 rpm. After RP-DLLME, the final acidic aqueous extract was also analyzed by DPV. Fig. 2b shows signals obtained with the mixed standard solution and the real sample after RP-DLLME for comparative purposes. As can be observed, ortho-phenols (i.e, oleuropein, hydroxytyrosol and caffeic acid) were simultaneously oxidized giving rise to an anodic peak at +0.5 V. At higher potential (i.e., +0.7 V), a peak was observed in the standard solution corresponding to ferulic acid oxidation, whereas this signal was almost negligible in the real sample. Finally, the oxidation peak of tyrosol was clearly distinguishable at +0.93 V in both voltammograms. It is important to point out that other minority mono-phenols (e.g., phenol, vanillic acid) could have a near oxidation potential to tyrosol, thus contributing to the total signal found at +0.93 V in the real sample [11,15]. Considering these results, caffeic acid was selected as reference compound to quantify total ortho-phenols as caffeic acid equivalents using the current peak at +0.5 V. Tyrosol was also included in subsequent experiments using the current peak at +0.93 V for quantification as tyrosol equivalents. On the contrary, ferulic acid was omitted in further investigations considering the low content of this compound in real samples.

Study of interferences

The effect of interferences on the simultaneous electrochemical determination of caffeic acid and tyrosol was evaluated. To this end, 10 mg L^{-1} caffeic acid solutions in 0.1 M HCl containing different amounts of tyrosol (i.e., 0, 10, 30, 50 and 90 mg L^{-1}) were analyzed by DPV. No effects were observed in the caffeic acid signal related to the presence of tyrosol (Fig. S1). A previous

publication reported an important effect of mono-phenols (i.e., phenol and tyrosol) on the electrochemical response of ortho-phenols (i.e., hydroxytyrosol) due to their adsorption on the electrode surface [11]. However, such an effect was not observed in our experiments with SPCEs.

The effect of caffeic acid upon tyrosol signal was also investigated analyzing 10 mg L^{-1} tyrosol solutions in 0.1 M HCl containing different amounts of caffeic acid (i.e., 0, 10, 30, 50 and 90 mg L^{-1}). Tyrosol current peak was maintained constant in all tested solutions (Fig. S2), revealing that neither caffeic acid nor its oxidation product (which is reversible reduced) blocked SPCEs surface.

Finally, we should mention that oxidation products of tyrosol were adsorbed onto SPCEs surface as a second use of the same electrode after tyrosol determination provided a significantly lower electrochemical response. Thus, SPCEs were always discarded after a single use.

RP-DLLME multivariate optimization

Fractional factorial designs are employed for screening purposes when a large number of factors can affect extraction yield. One particular strategy is the Plackett-Burman design, which studies up to k = N - 1 factors in N runs, where N is a multiple of 4 [26]. The Plackett-Burman design assumes that interaction between factors can be ignored so the main effects can be calculated with a reduced number of experiments, thereby saving time and resources. A Plackett-Burman design was used to construct the matrix of experiments, including five factors studied in eight runs. The five experimental factors selected at two levels were: HCl concentration, extractant volume, extraction time, centrifugation

speed and centrifugation time. Table S1 shows the experimental factors and levels considered in the Plackett-Burman design. The eight experiments were randomly performed using 5 mL of hexane standards with 1 mg L^{-1} of caffeic acid and tyrosol. DPV was selected as electroanalytical technique. The peak heights of caffeic acid and tyrosol were separately employed as response functions.

The data obtained were analyzed by ANOVA and the results were visualized with the Pareto charts shown in Fig. S3. The length of each bar was proportional to the influence of the corresponding factor, and the effects exceeding the reference vertical line can be considered significant with 95% of probability. In Fig. S3a, the reference vertical line does not appear meaning that factors are far from the significance level. In addition, negative and positive signals reveal whether the system responses decrease or increase, respectively, when passing from the lowest to the highest level of the corresponding factor.

As shown in Fig. S3, none of studied factors had a significant effect on the system responses. However, extractant phase HCl concentration and volume were the most important factors, having the same sign for both analytes studied here and, therefore, showing analogous behaviors during extraction. The positive effect of HCl concentration could be attributed to increased hydrogen-bonding interactions and thus, improved extraction performance. The negative effect of extractant volume can easily be explained considering that the smaller the volume of acceptor phase, the higher the concentration of the analyte in the extract. According to these results, HCl concentration was fixed at its highest level (i.e., 1 M) whereas extractant volume was fixed at the lowest level (i.e.,

100 µL). The other factors were fixed at the most convenient experimental level, namely: extraction time, 2 min; centrifugation speed, 4000 rpm; and centrifugation time, 10 min. Extraction time was fixed at its lowest level to reduce the length of the extraction procedure whereas centrifugation speed and time were fixed at the highest level to promote better phases separation during the analysis of olive oil samples. Further optimization was considered unnecessary since the limit of detection (LOD) of the proposed method was checked under the above mentioned conditions, being low enough to determine normal levels of hydrophilic phenols in olive oil.

Analytical figures of merit

Calibration curves were first constructed applying the proposed method (i.e., vortex-assisted RP-DLLME and electrochemical detection with SPCEs) to hexane standards of caffeic acid and tyrosol. However, important matrix effects were found when analyzing olive oil samples with relative recoveries ranging from 44 to 73%. The dispersion of the extractant phase in hexane was observed to be different from the dispersion of the extractant phase in olive oil samples diluted with hexane, affecting extraction procedure. Thus, matrix-matching calibration was proposed to correct matrix effects and evaluate quality analytical parameters. To this end, refined sunflower oil was employed as analyte-free sample matrix, where the dispersion of the extractant phase was very similar to the dispersion in olive oil samples. Standards of 1 g of sunflower oil diluted up to 5 mL with hexane were subjected to the proposed method under optimized conditions. The concentration range studied was from 0.075 to 3 mg L^{-1} of oil and the final working range is shown in Table 1. Other main analytical

parameters of the proposed method are also summarized in Table 1. The lowest concentration of working range was limited by the methodological limit of quantification (mLOQ), whereas the upper end for caffeic acid was established at 2.5 mg L^{-1} since the signals obtained with standards of 2.5 mg L^{-1} and 3 mg L^{-1} did not differ significantly. The resulting calibration curves possessed a high level of linearity (Table 1). The sensitivity was estimated by the slope of the calibration curves being (36.7±1.1) µA mg^{-1} L for caffeic acid and (26.4±0.8) µA mg^{-1} L for tyrosol. The repeatability of the proposed method, expressed as the coefficient of variation (CV), was evaluated by five consecutive extractions at concentrations of 0.5 and 2 mg L^{-1}, ranging between 8 and 11% (Table 1). The enrichment factor (EF) of RP-DLLME was evaluated through the slope ratio of calibration curves with and without preconcentration (Table 1). Calibration curves without RP-DLLME were performed using caffeic acid and tyrosol standards in 1 M HCl (aqueous acceptor phase solution), since the direct electrochemical determination of target analytes in sample solution was not feasible due to the complexity and low conductivity of the organic matrix. In addition, the organic drop spreads out of the electrode surface, also hindering the direct determination.

LOD and LOQ were determined for the proposed method including RP-DLLME and electrochemical detection, therefore, they are referred to as methodological LOD (mLOD) and mLOQ, respectively [27]. mLOD was empirically determined measuring progressively more diluted concentrations of caffeic acid and tyrosol. mLOD was the lowest concentration whose signal could be clearly distinguished from blank, namely 0.022 mg L^{-1} for the two analytes under study. Additionally, the mLOD was statistically evaluated using

three times the standard deviation of a sunflower oil standard solution containing very low concentrations of analytes. Obtained in this way, mLOD values were 0.006 mg L^{-1} and 0.003 mg L^{-1} for caffeic acid and tyrosol, respectively. The statistical estimation of the mLOD provided lower values than those obtained empirically. However, the empirical estimation is considered to provide much more realistic values and, therefore, the mLOD of the proposed method was established according to this approach. The mLOQ, defined as 3.3 times the mLOD [28], was 0.075 mg L^{-1}. It should be noted that both mLOD and mLOQ were lower than the average content of hydrophilic phenols commonly found in olive oil samples [2].

In order to assess the accuracy (i.e., trueness and precision) of the method, three oil samples were subjected to recovery studies. Samples of ROO, VOO and EVOO were diluted up to 5 mL with hexane, with a dilution factor depending on the phenolic content. Thus, 1 g of sample was employed when analyzing ROO whereas lower amounts of VOO and EVOO (i.e., 0.150 g) were necessary to fit the range of concentrations studied in calibration curves. Diluted olive oil samples were analyzed by the proposed method using matrix-matching calibration. Thereafter, the diluted olive oil samples were spiked with caffeic acid and tyrosol at three different concentration levels (i.e., 0.25, 0.5 and 1.5 mg L^{-1}) and also analyzed by the proposed method using matrix-matching calibration. Table 2 summarizes the results obtained. For EVOO, the highest spiking level (i.e., 1.5 mg L^{-1}) is omitted because, considering the original phenolic content of this sample, the addition of 1.5 mg L^{-1} resulted in a final concentration that exceeded the upper limit of the matrix-matching calibration curve (i.e., 2.5-3.0 mg L^{-1}). Relative recoveries (i.e., trueness) ranged between

83 and 108%, whereas the precision of the method expressed as CV ranged between 2 and 20%. According to these results, we can conclude that matrix effects were not significant in the three selected oil samples using the proposed matrix-matching calibration strategy.

Analysis of olive oil samples

Fifteen olive oil samples, including five ROOs, five VOOs and five EVOOs, were analyzed with the proposed method using matrix-matching calibration (see "Analytical figures of merit" section). As mentioned before, samples were diluted up to 5 mL with hexane, with a dilution factor depending on the phenolic content. Thus, 1 g of sample was employed when analyzing ROOs whereas lower amounts of VOOs and EVOOs (i.e., 0.150 g) were necessary to fit the range of concentrations studied in calibration curves. Found concentrations were expressed as mg Kg^{-1} of oil considering the dilution factors and results are shown in Table 3. As expected, the lowest content of hydrophilic phenols corresponded to ROO samples whereas the highest concentrations were found in EVOO samples.

Comparison with other electrochemical methods

For comparative purposes the characteristics of previously reported electrochemical methods for hydrophilic phenols determination in olive oil samples are summarized in Table 4. As can be seen, most of the reported methods involve slow and tedious sample preparation procedures, consuming large amounts of reagents and organic solvents. In addition, some methods use home-made electrochemical devices and complex modifications of electrode

surfaces, thus hindering their widespread laboratory use and reducing analysis throughput. By contrast, the proposed method combines a simple, fast and environmentally friendly sample preparation technique with electrochemical detection using unmodified, inexpensive and commercially available SPCEs, thus providing unique advantages. Finally, lower LOD values were obtained with the proposed method compared to those obtained in the previously reported works.

Comparison with the Folin-Ciocalteu method

The concentration of hydrophilic phenols found in the fifteen olive oil samples analyzed by the proposed method was expressed as the addition of mg of caffeic acid equivalents and mg of tyrosol equivalents per Kg of oil sample ($mg_{CAE+TYE}$ Kg^{-1}). Then, samples were analyzed by the Folin-Ciocalteu method according to the procedure described in "Folin-Ciocalteu method" section. A graphic comparison of the results of both procedures is shown in Fig. 3. As can be observed, lower concentrations were systematically found with the proposed method compared to those obtained with the reference method. This outcome could be explained considering the following: firstly, the Folin-Ciocalteu method estimates the total polyphenol content whereas the proposed electrochemical method only reflects the concentration of ortho-phenols and mono-phenols with oxidation potentials near to tyrosol; secondly, the Folin-Ciocalteu reagent is considered a non-specific reagent by many authors since it can be reduced by non-phenolic compounds [7]. Thus, the Folin-Ciocalteu method could also reflect the presence of other oxidizable species present in the sample extract. Despite these differences, Fig. 3 shows a high correlation

between the results obtained by the two methods. Accordingly, to estimate the antioxidant capacity of olive oil samples, we can conclude that RP-DLLME coupled to electrochemical detection with SCPEs is a valuable alternative to Folin-Ciocalteu. Finally, we should point out that electrochemical determination with SPCEs enables us to distinguish ortho-phenols from mono-phenols (mainly tyrosol), whereas a colorimetric method other than Folin-Ciocalteu is required to do so [3]. Therefore, the proposed method possesses unique advantages as it is simple, easy to handle and less-time consuming, given it does not require the incubation time (i.e., 2 h) inherent to colorimetric reactions.

Discriminant analysis

LDA was selected to assess the capability of the proposed method to distinguish olive oil samples of different quality. LDA is a supervised classification method whose main objective is to find a rule for allocating a new object of unknown group to the correct group, using a number of objects whose group membership is known [28]. With this aim, LDA maximizes the variation between pre-specified groups and minimizes the variation within a group, by the condensation of original variables into a set of orthogonal functions (i.e., linear discriminant functions, LDFs) with a minimum loss of information [28]. Thereby, the number of orthogonal LDFs is equal to the number of groups minus one.

LDA analysis was applied in order to find a predictive classification model able to separate olive oil samples according to their quality in three main groups, namely ROO, VOO and EVOO. Fig. 4 shows the graphical representation of the LDFs of the obtained classification model. As can be seen, LDF-1 possessed a higher discriminant capacity than LDF-2 since it completely

separated ROO from EVOO and VOO, whereas LDF-2 may help in separating the latter two types of olive oils. The higher discrimination capacity of LDF-1 was also revealed by the percentage of variance, being 97% for this function. Nevertheless, both LDFs possessed a *p*-value lower than 0.05 revealing their statistical significance with 95% probability.

The success of LDA at allocating oil samples correctly was tested using three samples of different quality. ROO and EVOO were correctly classified. However, VOO was classified as EVOO as a consequence of the overlap of these two groups observed in Fig. 4. According to these results, we can conclude that the proposed procedure is able to distinguish between ROO and olive oils of higher quality (i.e., VOO, EVOO) and could be used to detect adulterations.

Conclusions

For the first time, RP-DLLME has been successfully combined with SPCEs to determine hydrophilic phenols in olive oil samples. Thereby, the advantages of miniaturized systems, both in sample preparation and detection stage, have been synergistically exploited. On the one hand, RP-DLLME involves a fast and easy-to-handle procedure with a significantly low consumption of organic solvents compared to SPE or LLE techniques, thus making it environmentally friendly. On the other hand, unmodified and commercially available SPCEs provide a rapid and sensitive response with affordable and portable instrumentation.

The multivariate optimization strategy used here enabled us to rapidly and economically establish RP-DLLME operation conditions. The matrix-matching

calibration using refined sunflower oil as analyte-free sample resulted in a simple and suitable strategy to compensate matrix effects. The proposed method provided results that closely correlate with the well-established Folin-Ciocalteu method, which are useful to predict the results provided by time-consuming colorimetric assays. In addition, the proposed method is simpler, more time-efficient and enables us to distinguish ortho-phenols from mono-phenols. Finally, the proposed method in combination with LDA has resulted in a suitable strategy to discriminate between ROO and higher quality olive oils. Therefore, RP-DLLME coupled to SPCEs is a novel and promising alternative to determine hydrophilic phenols in olive oil samples, is affordable for any laboratory and has a potential application for the rapid assessment of olive oil quality and detect fraudulent practices (e.g., adulterations).

Acknowledgements

The authors would like to thank Spanish Ministry of Science and Innovation (project no. CTQ2011-23968), Spanish Ministry of Economy, Industry and Competitiveness (project no. CTQ2016-79991-R, AEI/FEDER, UE) and Generalitat Valenciana (Spain) (projects n. GVA/2014/096 and PROMETEO/2013/038) for the financial support. E. Fernández also thanks Spanish Ministry of Education for her FPU grant (FPU13/03125).

References

[1] M.Z. Tsimidou, D. Boskou, The health claim on “olive oil polyphenols” and the need for meaningful terminology and effective analytical protocols, Eur. J. Lipid Sci. Technol. 117 (2015) 1091–1094.

[2] M. Servili, B. Sordini, S. Esposto, S. Urbani, G. Veneziani, I. Di Maio, A. Taticchi, Biological activities of phenolic compounds of extra virgin olive oil, Antioxidants 3 (2014) 1–23.

[3] A. Segura-Carretero, A. Carrasco-Pancorbo, A. Bendini, L. Cerretani, A. Fernández-Gutiérrez, Analytical determination of polyphenols in olive oil, in: V.R. Preedy, R.R. Watson (Eds.), Olives and Olive Oil in Health and Dessease Prevention, 1st ed., Elsevier, London (UK), 2010: pp. 509–523.

[4] D. Krichene, M.D. Salvador, G. Fregapane, Stability of virgin olive oil phenolic compounds during long-term storage (18 months) at temperatures of 5-50°C, J. Agric. Food Chem. 63 (2015) 6779–6786.

[5] A.M. Gómez-Caravaca, R.M. Maggio, L. Cerretani, Chemometric applications to assess quality and critical parameters of virgin and extra-virgin olive oil. A review, Anal. Chim. Acta 913 (2016) 1–21.

[6] T. Gutfinger, Polyphenols in olive oils, J. Am. Oil Chem. Soc. 58 (1981) 966–968.

[7] L.M. Magalhães, M.A. Segundo, S. Reis, J.L.F.C. Lima, Methodological aspects about in vitro evaluation of antioxidant properties, Anal. Chim. Acta 613 (2008) 1–19.

[8] C. Capannesi, I. Palchetti, M. Mascini, A. Parenti, Electrochemical sensor and biosensor for polyphenols detection in olive oils, Food Chem. 71 (2000) 553–562.

[9] M.L. Rodríguez-Méndez, C. Apetrei, J.A. de Saja, Evaluation of the polyphenolic content of extra virgin olive oils using an array of voltammetric sensors, Electrochim. Acta 53 (2008) 5867–5872.

[10] M. Del Carlo, A. Amine, M. Haddam, F. della Pelle, G.C. Fusella, D.

Compagnone, Selective voltammetric analysis of o-diphenols from olive oil using Na_2MoO_4 as electrochemical mediator, Electroanalysis 24 (2012) 889–896.

[11] T.A. Enache, A. Amine, C.M.A. Brett, A.M. Oliveira-Brett, Virgin olive oil ortho-phenols electroanalytical quantification, Talanta 105 (2013) 179–186.

[12] C. Apetrei, M.L. Rodríguez-Méndez, J.A. de Saja, Modified carbon paste electrodes for discrimination of vegetable oils, Sens. Actuators B. 111–112 (2005) 403–409.

[13] M.S. Cosio, D. Ballabio, S. Benedetti, C. Gigliotti, Evaluation of different storage conditions of extra virgin olive oils with an innovative recognition tool built by means of electronic nose and electronic tongue, Food Chem. 101 (2007) 485–491.

[14] C. Apetrei, F. Gutierez, M.L. Rodríguez-Méndez, J.A. de Saja, Novel method based on carbon paste electrodes for the evaluation of bitterness in extra virgin olive oils, Sens. Actuators B 121 (2007) 567–575.

[15] S. Benedetti, M.S. Cosio, M. Scampicchio, S. Mannino, Nanoemulsions for the determination of the antioxidant capacity of oils by an electrochemical method, Electroanalysis 24 (2012) 1356–1361.

[16] I.M. Apetrei, C. Apetrei, Voltammetric e-tongue for the quantification of total polyphenol content in olive oils, Food Res. Int. 54 (2013) 2075–2082.

[17] N. Dossi, R. Toniolo, F. Terzi, E. Piccin, G. Bontempelli, Simple pencil-drawn paper-based devices for one-spot electrochemical detection of electroactive species in oil samples, Electrophoresis 36 (2015) 1830–1836.

[18] P. Hashemi, F. Raeisi, A.R. Ghiasvand, A. Rahimi, Reversed-phase dispersive liquid-liquid microextraction with central composite design optimization for preconcentration and HPLC determination of oleuropein, Talanta 80 (2010) 1926–1931.

[19] P. Hashemi, F. Nazari Serenjeh, A.R. Ghiasvand, Reversed-phase dispersive liquid-liquid microextraction with multivariate optimization for sensitive HPLC determination of tyrosol and hydroxytyrosol in olive oil, Anal. Sci. 27 (2011) 943–947.

[20] M.P. Godoy-Caballero, M.I. Acedo-Valenzuela, T. Galeano-Díaz, New reversed phase dispersive liquid-liquid microextraction method for the determination of phenolic compounds in virgin olive oil by rapid resolution liquid chromathography with ultraviolet-visible and mass spectrometry detection, J. Chromatogr. A 1313 (2013) 291–301.

[21] P. Reboredo-Rodríguez, L. Rey-Salgueiro, J. Regueiro, C. González-Barreiro, B. Cancho-Grande, J. Simal-Gándara, Ultrasound-assisted emulsification-microextraction for the determination of phenolic compounds in olive oils, Food Chem. 150 (2014) 128–136.

[22] S. Dairi, T. Galeano-Díaz, M.I. Acedo-Valenzuela, M.P. Godoy-Caballero, F. Dahmoune, H. Remini, K. Madani, Monitoring oxidative stability and phenolic compounds composition of myrtle-enriched extra virgin olive during heating treatment by flame, oven and microwave using reversed phase dispersive liquid-liquid microextraction (RP-DLLME)-HPLC-DAD-FLD method, Ind. Crops Prod. 65 (2015) 303–314.

[23] P. Hapiot, A. Neudeck, J. Pinson, H. Fulcrand, P. Neta, C. Rolando, Oxidation of caffeic acid and related hydroxycinnamic acids, J.

Electroanal. Chem. 405 (1996) 169–176.

[24] R. Petrucci, P. Astolfi, L. Greci, O. Firuzi, L. Saso, G. Marrosu, A spectroelectrochemical and chemical study on oxidation of hydroxycinnamic acids in aprotic medium, Electrochim. Acta 52 (2007) 2461–2470.

[25] M. Salas-Reyes, J. Hernández, Z. Domínguez, F.J. González, P.D. Astudillo, R.E. Navarro, E. Martínez-Benavidez, C. Velázquez-Contreras, S. Cruz-Sánchez, Electrochemical oxidation of caffeic and ferulic acid derivatives in aprotic medium, J. Braz. Chem. Soc. 22 (2011) 693–701.

[26] D.C. Montgomery, Design and Analysis of Experiments, 7th ed., Wiley, New Jersey (USA), 2009.

[27] M.S. Epstein, Comparison of Detection Limits in Atomic Spectroscopic Methods, in: L.A. Currie (Ed.), Detection in Analytical Chemistry, 1st ed., Washington DC, 1988: pp. 109–125.

[28] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 5th ed., Pearson Prentice Hall, London (UK), 2005.

Fig. 1 Vortex-assisted RP-DLLME coupled with SPCEs.

Fig. 2 (a) Cyclic voltammograms of 10 mg L^{-1} individual standards of hydrophilic phenols in aqueous 0.1 M HCl; and (b) DPV voltammograms of a 10 mg L^{-1} mixed standard solution in aqueous 0.1 M HCl (in red) and 0.1 M HCl aqueous extract after RP-DLLME of a VOO sample (in blue).

Fig. 3 Graphical comparison of the results obtained with the proposed method and the Folin-Ciocalteu method.

Fig. 4 Graphical representation of LDFs obtained during LDA. Groups are shown with different symbols: ROO, squares; VOO, triangles; EVOO, circles. Crosses mark the centroid of each group.

Table 1. Main analytical parameters of the proposed method obtained with the matrix-matching calibration using sunflower oil as analyte-free sample.

Analyte	Working range (mg L^{-1})	r[a]	CV[b] (%)		mLOD[c] (mg L^{-1})	mLOQ[d] (mg L^{-1})	EF[e]
			0.5 mg L^{-1}	2 mg L^{-1}			
Caffeic acid	0.075-2.5	0.998 (7)	11	10	0.022	0.075	38
Tyrosol	0.075-3.0	0.999 (8)	10	8	0.022	0.075	37

[a] Correlation coefficient: number of calibration points in parentheses.
[b] Coefficient of variation: mean value for 5 replicated analysis of 0.5 and 2 mg L^{-1} spiked oil solutions.
[c] Methodological limit of detection: experimentally obtained.
[d] Methodological limit of quantification: calculated as 3.3 times the methodological limit of detection.
[e] Enrichment factor: calculated as slope ratio between calibration curves with and without RP-DLLME.

Table 2. Concentrations added and found, relative recoveries and coefficients of variation (in parentheses) during recovery studies in different olive oil samples.

	Caffeic acid			Tyrosol		
	Added (mg L^{-1})	Found ± SD[a] (mg L^{-1})	Relative recovery[b] (%)	Added (mg L^{-1})	Found ± SD[a] (mg L^{-1})	Relative recovery[b] (%)
ROO	0	0.322 ± 0.015	-	0	0.343 ± 0.008	-
	0.25	0.59 ± 0.04	108 (17)	0.25	0.577 ± 0.015	94 (7)
	0.5	0.808 ± 0.018	97 (5)	0.5	0.84 ± 0.02	100 (4)
	1.5	1.87 ± 0.09	103 (6)	1.5	1.59 ± 0.03	83 (2)
VOO	0	0.907 ± 0.002	-	0	0.928 ± 0.002	-
	0.25	1.114 ± 0.013	83 (6)	0.25	1.178 ± 0.009	100 (4)
	0.5	1.37 ± 0.03	93 (6)	0.5	1.42 ± 0.03	98 (6)
	1.5	2.27 0.06	91 (4)	1.5	2.22 ± 0.11	86 (9)
EVOO	0	1.629 ± 0.007	-	0	2.316 ± 0.007	-
	0.25	1.85 0.04	87 (20)	0.25	2.57 ± 0.05	103 (19)
	0.5	2.12 ± 0.05	97 (10)	0.5	2.80 ± 0.05	97 (9)

[a] Standard deviation of three replicated analyses.
[b] Coefficient of variation in parentheses.

Table 3. Caffeic acid equivalents and tyrosol equivalents content found in fifteen olive oil samples of different quality analyzed by the proposed method.

Oil sample		Caffeic acid equivalents (mg Kg^{-1})	Tyrosol equivalents (mg Kg^{-1})
ROO	1	4.2 ± 0.7	5.2 ± 0.05
	2	4.32 ± 0.04	4.60 ± 0.10
	3	5.1 ± 0.5	4.58 ± 0.15
	4	10.5 ± 0.8	3.87 ± 0.05
	5	6.3 ± 0.5	5.38 ± 0.08
VOO	1	63 ± 6	44.8 ± 1.6
	2	64.4 ± 1.9	54.0 ± 1.8
	3	51 ± 8	55.1 ± 1.5
	4	60 ± 5	47 ± 4
	5	27.2 ± 0.8	41.2 ± 1.6
EVOO	1	77 ± 5	52.9 ± 1.9
	2	75 ± 3	51 ± 3
	3	72 ± 2	49.3 ± 1.2
	4	78 ± 3	51.7 ± 1.1
	5	69 ± 3	52 ± 2

Table 4. Electrochemical methods for hydrophilic phenols determination in olive oil samples.

Electrode	Sample preparation	Electrochemical technique	LOD	Ref.
SPCE	Extraction with glycine buffer 10 mM pH 2, NaCl 10 mM (oil:buffer, 1:10). Dilution of the final extract with glycine buffer (1:10)	DPV	0.25 mg Kg^{-1} (1)	[8]
Tyrosinase-based biosensor	-	Amperometric monitoring of O_2 consumption during phenols oxidation reaction catalyzed by tyrosinase. FIA system	4 mg Kg^{-1}(1)	[8]
Array of CPE (five modified with phthalocyanine derivatives, six modified with polypirrole and one unmodified)	7g of oil dissolved in hexane (10 mL) and extracted three times with 30 mL methanol:water (60:40, v:v). Evaporation of the extract until dryness and reconstitution in 25 mL of 0.1 M KCl aqueous solution	CV and SWV	-	[9]
SPGE	Solid-phase extraction with C18 cartridge	Amperometric detection in a FIA	-	[10]

		system		
SPCE	25 g of oil dissolved in hexane (25 mL) and extracted three times with 15 mL methanol:water (3:2, v:v). Dilution of the final extract with ultra-pure water up to 50 mL	SWV	1.25 mg $Kg^{-1(1)}$	[11]
CPE modified with oils as electroactive binder material	-	CV and SWV	-	[12,14]
GCE	Oil dilution with chloroform containing 2% acetic acid and 3.2% tetrabutylammonium bromide (oil: chloroform solution, 1:100)	Amperometric detection in a FIA system	-	[13]
GCE	Preparation of oil-in-water nanoemulsions using Tween 20 and SDS in 100 mM acetate buffer	Amperometric detection in a FIA system	0.5 mg $L^{-1(1)}$	[15]
SPCE modified with polypyrrole	Emulsions preparation by sonicating 25 mL of 0.2 M SDS aqueous solution with 5 mL of oil sample for 15 min	CV	-	[16]
Pencil-drawn paper-based carbon electrode	-	CV	-	[17]
SPCE	RP-DLLME	DPV	0.022 mg $L^{-1(2)}$	This work

SPCE, screen-printed carbon electrode; DPV, differential pulse voltammetry; FIA, flow injection analysis; CPE, carbon paste electrode; CV, cyclic voltammetry; SWV, square-wave voltammetry; GCE, glassy carbon electrode; SDS, sodium dodecyl sulfate; SPGE, screen-printed graphite electrode; RP-DLLME, reversed-phase dispersive liquid-liquid microextraction.

(1) Obtained as three times the standard deviation of the blank.

(2) Obtained empirically.

Highlights

Rapid, simple and sensitive determination of hydrophilic phenols in olive oil samples.

Reversed-phase dispersive liquid-liquid microextraction as ecological sample preparation.

Inexpensive and commercially available screen-printed electrodes for detection.

Matrix-matching calibration as suitable strategy to compensate matrix effects.

Results highly correlated with the well-established Folin-Ciocalteu method.

Fig. 1

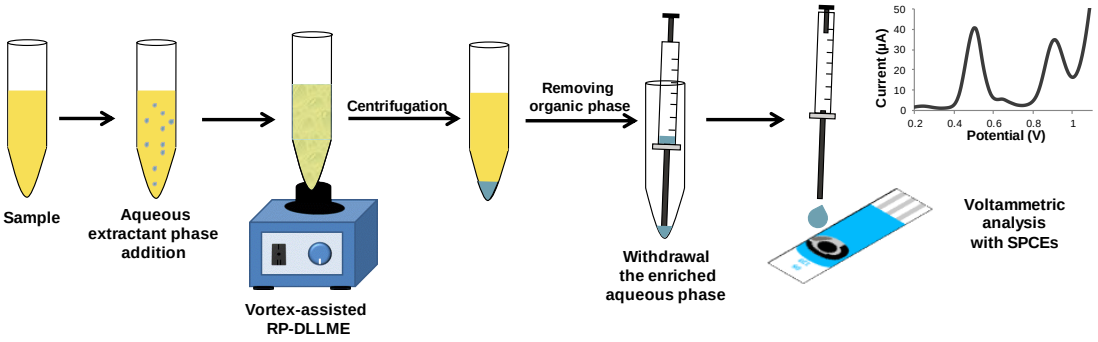


Fig. 2

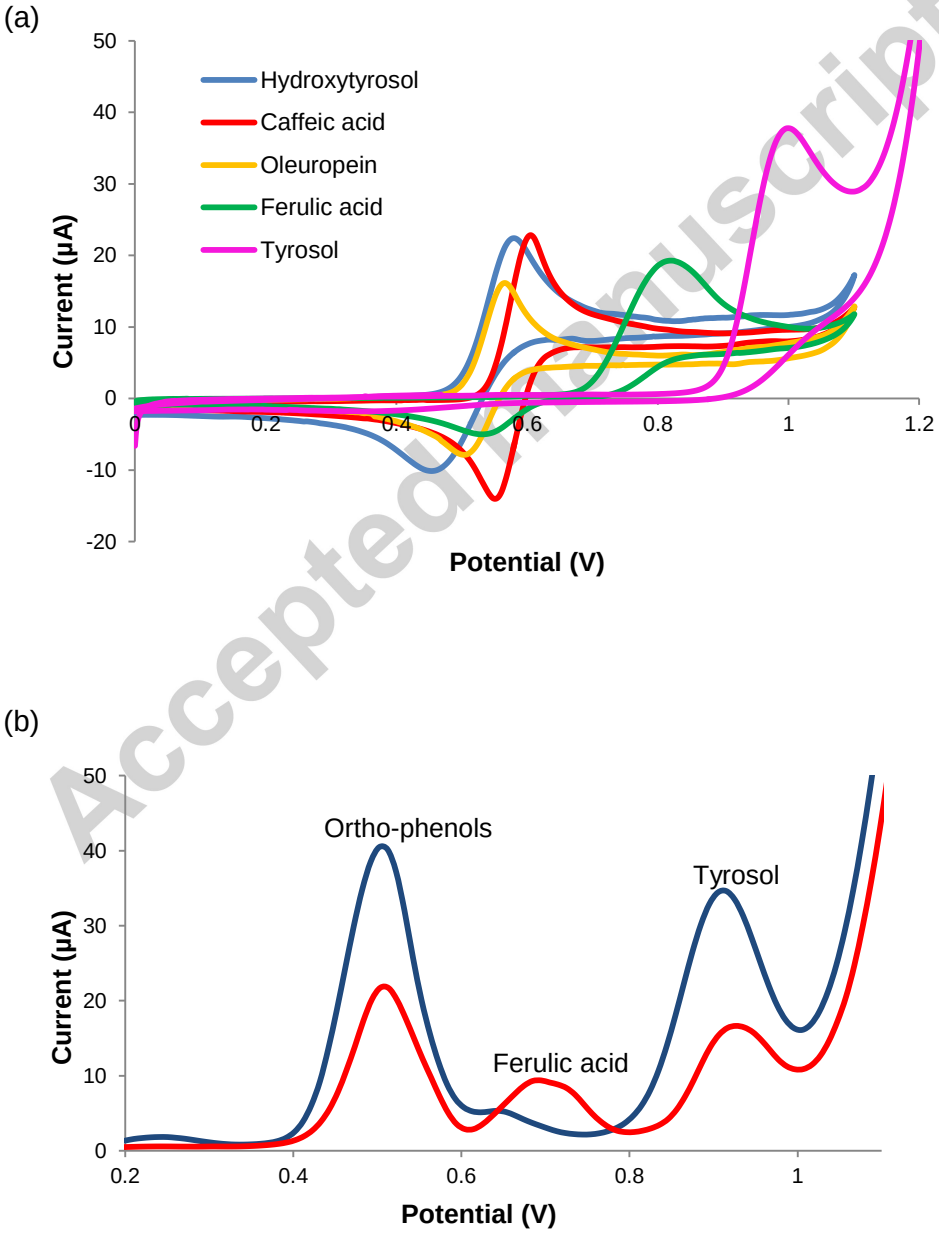


Fig. 3

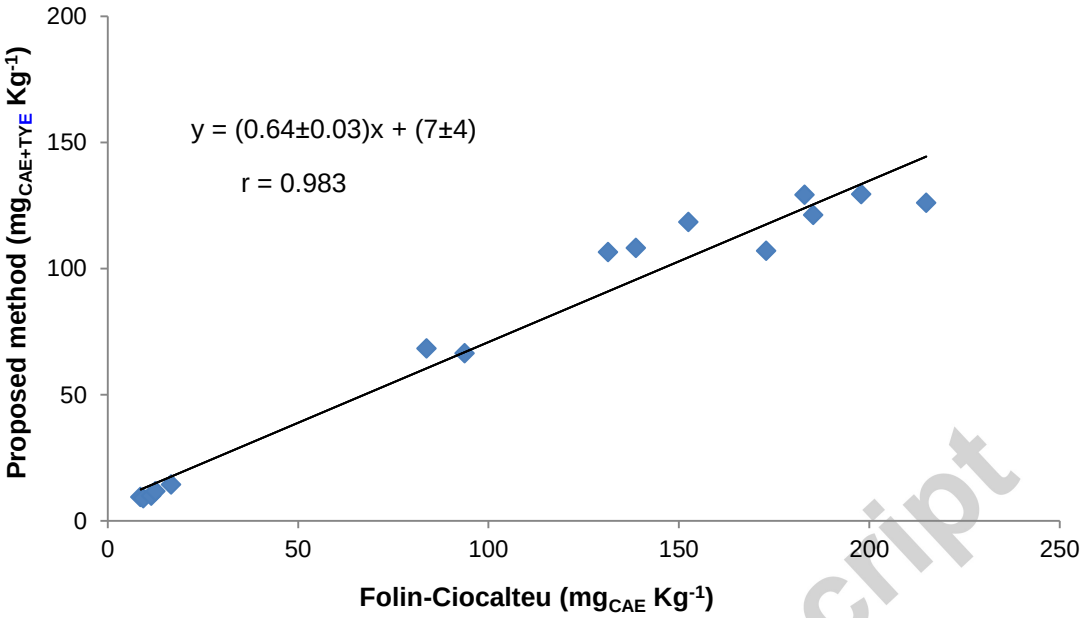


Fig. 4

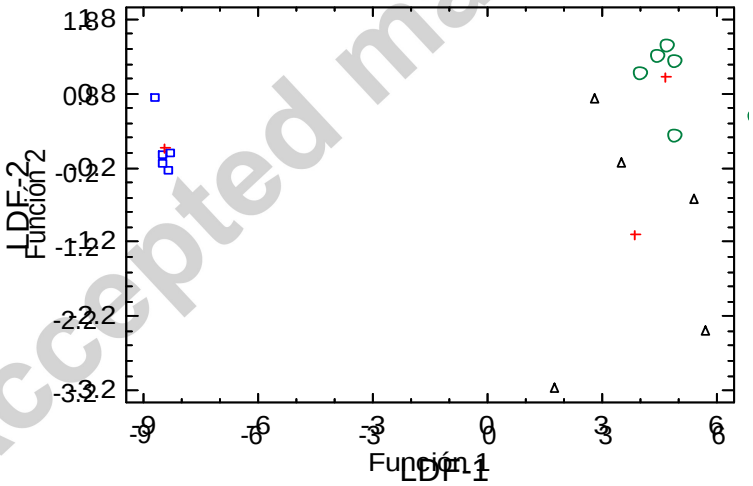


Fig. 1

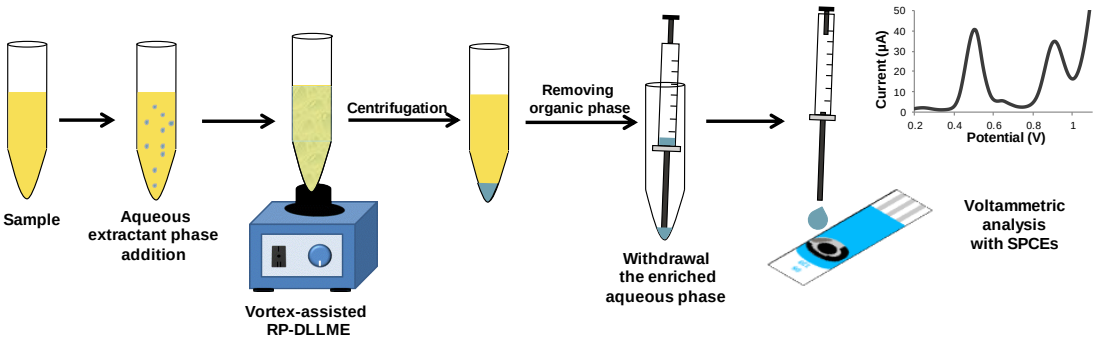
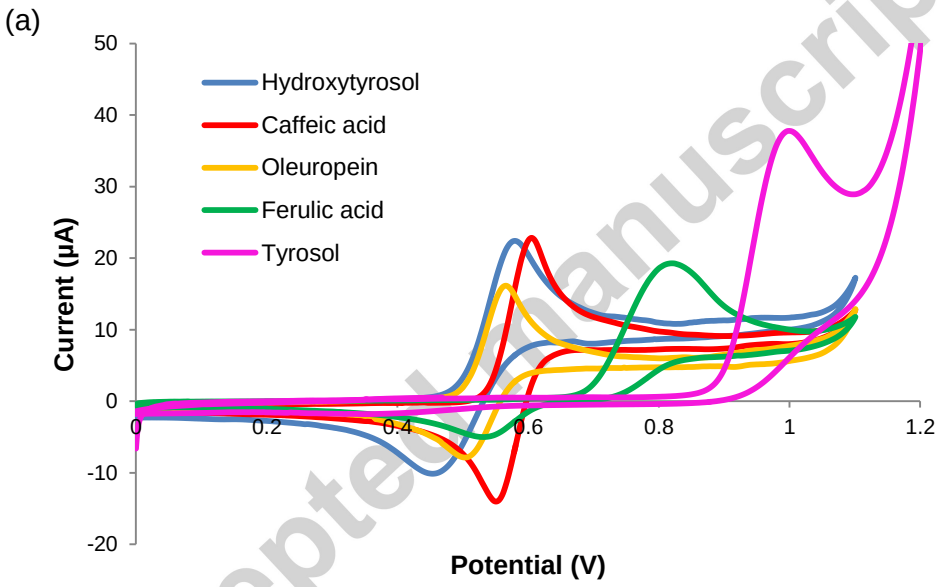


Fig. 2



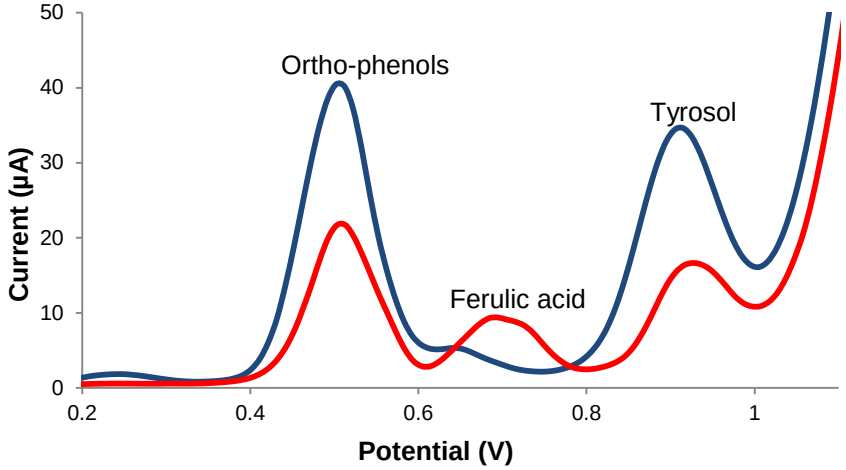


Fig. 3

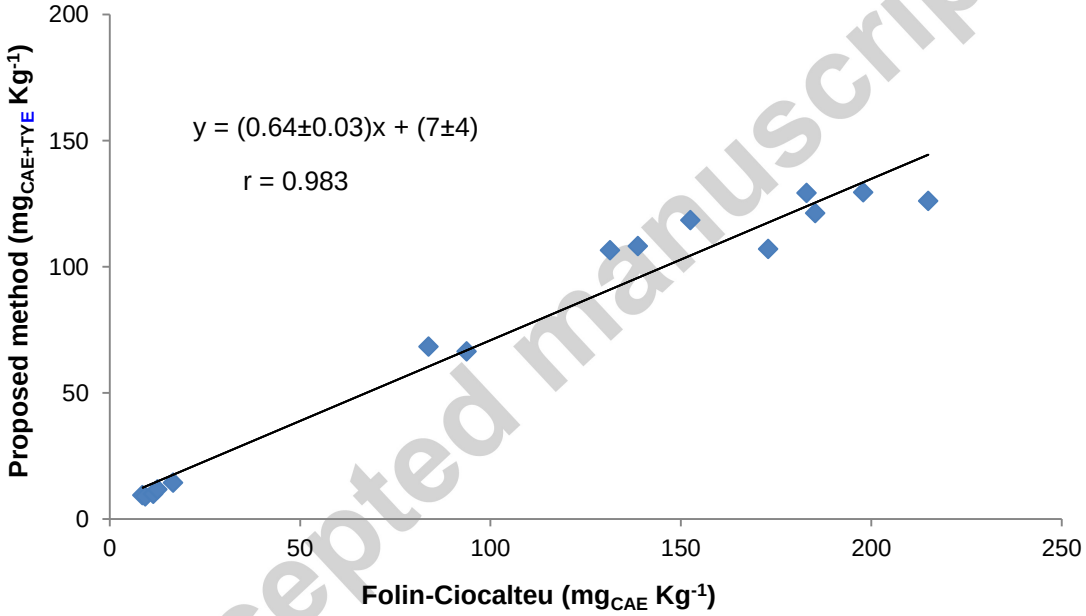
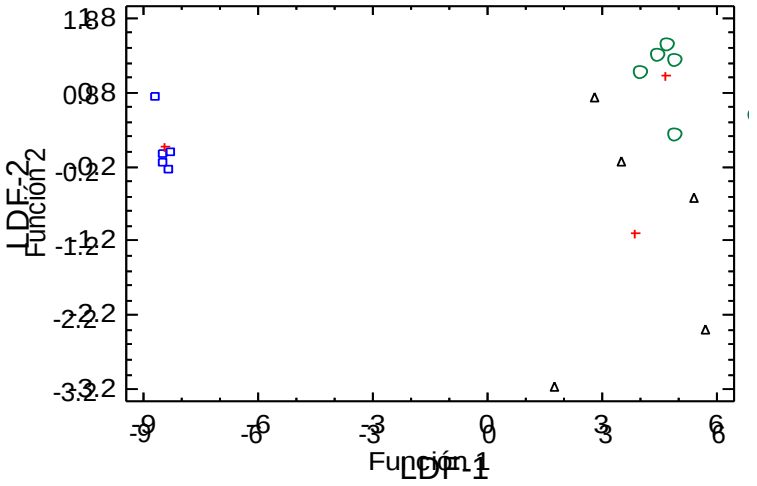


Fig. 4



Graphical abstract