(Bio)Sensor Approach in the Evaluation of Polyphenols in Vegetal Matrices

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Polyphenols are compounds widely distributed in the plant kingdom and have attracted much attention, because of their health benefits and important properties such as radical scavenging, metal chelating agents, inhibitors of lipoprotein oxidation, anti-inflammatory and anti-allergic activities. Due to their important role in the diet and in therapy, it is important to estimate their content in the different matrices of interest. Besides classical analytical methods, new emerging technologies have also appeared in the last decade aiming for simple and eventually cheap detection of polyphenols. This review focused on the recent applications of biosensing-based technologies for polyphenol estimation in vegetal matrices, using different transduction principles. These analytical tools are generally fast, giving responses in the order of a few seconds/minutes, and also very sensitive and generally selective (mainly depending on the enzyme used). Direct measurements in most of the investigated matrices were possible, both in aqueous and organic phases.

Keywords: Sensor, polyphenols, tyrosinase, laccase, peroxidase, plant tissue, vegetal matrices.

Polyphenols are widely distributed in the plant kingdom, including foods of vegetable origin, contributing to their taste and sensorial properties (such as olive oil and wine) and constituting an important role in human diet. These compounds are mainly represented by simple phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids and tannins (hydrolyzable tannins and condensed tannins) [1,2].

Recently, polyphenols have attracted much attention, because of their health benefits, being considered responsible for the majority of the antioxidant capacity in plant-derived products, with a few exceptions, such as carotenoids. It is reported that within the Mediterranean diet, the average daily intake of polyphenols is about 1 g, which is almost 10-fold the intake of vitamin C, 100-fold the intake of vitamin E, and 500-fold the intake of carotenoids [3]. Foods and beverages rich in polyphenols may have a large potential with respect to prevention of diseases, and fruits and vegetables are generally associated with the prevention of stroke [4] and cancers [5,6], including breast cancer [7,8]. Polyphenols act as free radical scavengers [9,10],

metal chelating agents [11], inhibitors of lipoprotein oxidation [12] anti-inflammatory agents [13] and have anti-allergic properties [14].

Due to their important role in food and in therapy, it is important that there is a simple and fast estimation of their content in the different matrices of interest. Several methods for polyphenols detection in plant sources are described in the literature, the most known and simplest approach being the Folin-Ciocalteu spectrophotometric method [15], but this presents limitations since it can estimate other different reducing non-phenolic compounds too. As an alternative to the Folin-Ciocalteu assay, a fluorimetric evaluation of the total phenol content in vegetal matrices and extracts was also proposed [16]. Other classic techniques used in the evaluation of polyphenols are UV-visible [17] and FT-NIR spectroscopies [18].

One of the most selective analytical methods is based on high-performance liquid chromatography (HPLC) combined with different detection methods: UV-Vis detection [19], chemiluminescence detection [20], fluorescence detection, DAD-ESI-MS detection, and

direct electrochemical detection [21]. However, these instrumental methods, although performing complete and sensitive analyses, require long sample processing (i.e. extraction, concentration, resuspension), massive use of organic solvents, costly instrumentation and skilled personnel.

In view of the need for simple and easy analytical methods for rapid estimation of polyphenolic content, one interesting approach is the electroanalytical techniques, due to the electrochemical behaviour shown by phenolic compounds. New emerging technologies have also appeared in the last decade aiming to provide simple, fast and eventually cheap detection of polyphenols. These are mainly based on electrochemical techniques, but some optical-based sensing has also been proposed.

In this review, some sensing approaches based on different transduction principles are reported, focusing on polyphenol characterization and quantification.

A chemical sensor is a device responding to a chemical stimulus, giving a recordable signal. When the sensor is coupled to a biological molecule, a biosensor is generated. Following the IUPAC definition, "A biosensor is a whole and integrated device providing analytical information (qualitative or semi/quantitative) by using a biomolecular recognition element (biochemical receptor) in close spatial contact with a transducer. The transducer converts the chemical event into a recordable signal."

Both sensors and biosensors can be considered innovative analytical devices able to detect different analytes in a qualitative and quantitative manner directly in complex matrices, without or with very little sample pre-treatment. They have been presented eventually as the "analyst dream" for the simplification they make in the analysis [22].

The paradigmatic example is represented by the glucose sensor, which represented a real revolution for diabetes patients; with this very small device (dimension of a pen, also called "glucose pen" in some versions) the glucose content in blood is estimated in a few seconds. The detection principle is amperometry, meaning that a current is the nature of the response, correlated to glucose concentration, and the selectivity is due to the enzyme glucose oxidase, which selectively uses glucose as substrate. The catalysis is at the base of the enzymatic event and for these reasons enzymatic based sensors are called also catalytic sensors. We can thus say that we could
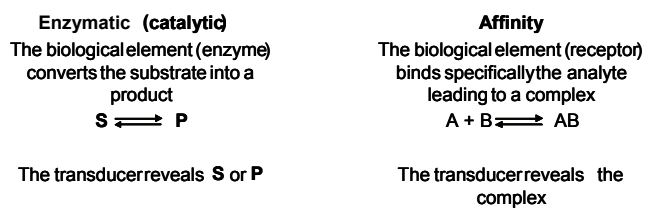

Enzymatic (catalytic)
The biological element (enzyme) converts the substrate into a product
$$S \rightleftharpoons P$$
The transducer reveals **S** or **P**

Affinity
The biological element (receptor) binds specifically the analyte leading to a complex
$$A + B \rightleftharpoons AB$$
The transducer reveals the complex

Figure1: Catalytic and affinity-based biosensors.

discriminate between mainly two different classes of biosensors: catalytic and affinity–based ones (Figure1).

The recognition element, immobilized on the sensing surface (i.e. an electrode, an optic fiber, a planar waveguide) is respectively an enzyme (or a system of enzymes) or a receptor able to form an affinity complex with the target analyte. To the affinity sensor category belongs: immunosensors, where the receptors are an antigen or an antibody, or a DNA sensor with suitable probes immobilized on the sensor surface. In Figure 2 are shown some examples of receptors employed both in catalytic and affinity sensors.

Relative to polyphenol analysis, catalytic biosensors are the most used. Different enzymes have been used over the years as a catalytic element coupled to electrochemical analysis, such as tyrosinase (also called polyphenol oxidase), laccase and peroxidase, using different electrode materials, flow systems and sample pre-treatment techniques since phenolic compounds can act as electron donors for these enzymes [23-26].

Phenol oxidases and peroxidases have different enzymatic mechanisms of action in the electrochemical biosensors. Enzyme molecules at the surface of the electrode are oxidized by oxygen (for phenol oxidases) or hydrogen peroxide (for peroxidase), followed by their re-reduction by phenolic compounds. The tyrosinase biosensors are restricted to the monitoring of phenolic compounds with at least one free *ortho*-position.

On the other hand, the laccase biosensor can detect free *para*- and *meta*-positions, but its catalytic cycle is complicated and in its major part is different from tyrosinase and still now not well understood. Peroxidases exhibit low specificity for electron donors as the phenolic compounds and can be used for phenol detection with certain selectivity and sensitivity.

Tyrosinase: The catalytic sensors reported in the literature use mostly the enzyme tyrosinase. The
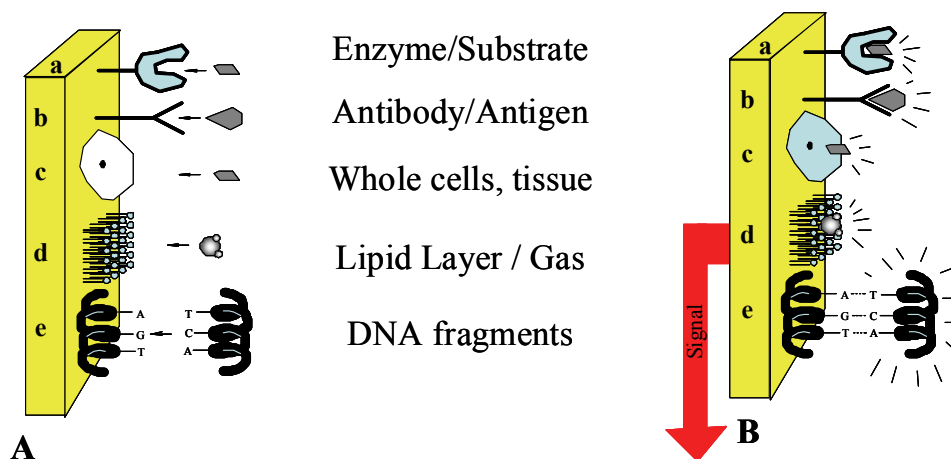
Figure 2: Examples of receptors.

enzyme catalyzes the oxidation of the phenolic substrate to a quinonic form that is reduced at the electrode polarized at a fixed potential. In the presence of oxygen, this enzyme is capable of catalyzing *ortho*-hydroxylations of monophenols and oxidation of the consequent *ortho*-dihydroxyphenols to *ortho*-quinones. Measurements can be carried out by recording the signal variation related to the dissolved oxygen consumption or to the formation of the relative quinone.

Among electroanalytical techniques, in particular, voltammetry has been successfully employed to detect phenols in water media [27-29]. Behaviour of the tyrosinase enzyme electrode has been investigated under different experimental conditions [30-33]. Several authors [34-39] have tested the performance of the tyrosinase electrode in different organic solvents and the effect of different additives has also been investigated [40,41]. Organic phase enzyme electrodes constitute a new class of biosensor applicable to the analysis of substrates or matrices insoluble or scarcely soluble in aqueous media.

Many biosensors have a limited lifetime due to enzyme inactivation by the bio-catalytically generated quinone products. For this reason, many studies were made concerning the development of good immobilization methods and materials to improve the biosensor stability. Recently, several amperometric biosensors based on the immobilization of tyrosinase on different electrode materials have been described in the literature. Glassy carbon electrodes modified with polymers [42], sol–gel materials [43], self-assembled monolayers (SAMs) on gold [44], Clark's electrodes [45,46], reticulated vitreous carbon [47] screen-printed [48] carbon paste [49,50], Nafion® membrane

[51], hydrogel [52], conducting polymers [53] and natural material such as chitosan [54], and other composite electrodes [55,56] have been used to prepare tyrosinase electrochemical biosensors.

Instead of conventional electrodes presenting the limitation of being poisoned after a certain use, recently screen-printed electrodes have been proposed to evaluate the polyphenol content [57]. Screen-printing technology is used for the production of disposable sensors which are very useful because during the oxidation process a polymeric film is formed on the electrode surface leading to electrode surface "inactivation" ("electrode fouling"), one of the main drawbacks of common graphite-based electrodes. Screen-printed electrodes have also been used for screening natural products using bare graphite [58]. The electrochemical device consists of three independent electrodes placed one next to another to form a rectangle 3 cm high and 1.5 cm wide comprising a screen-printed graphite working electrode, a silver reference, and a counter-electrode. Different compounds, including flavones, flavonols, catechins, tannins, and phenylpropanoids were tested with this system. Calibration was performed in a range between 20 and 80 μM of catechin. This method can be useful for a rapid and sensitive screening for polyphenols in plant matrices from grape, olive and green tea. Thus, many applications of biosensors, with different techniques of enzyme immobilization have been concerned with the determination of polyphenols in wine.

The use of gold nanoparticles is playing an increasingly important role for the preparation of biosensors [59] and recently, Sanz and coworkers reported the preparation of a tyrosinase biosensor based on the use of a glassy carbon electrode

modified with electrodeposited gold nanoparticles [57]. The enzyme, immobilized by cross-linking with glutaraldehyde, retains a high bioactivity on this electrode material, giving rise to fast, stable and sensitive responses to various phenolic compounds. The biosensor was applied to the amperometric estimation of the total content of phenolic compounds in red and white wines, which is of interest because of the correlation between wines' antioxidant capacity and their polyphenol content. The method used an extremely simple procedure involving the direct addition of a sample aliquot to the electrochemical cell. The response was given in a few seconds; the polyphenol concentration found in the wine samples ranged from 16 mg L^{-1} to 50 mg L^{-1}, expressed as caffeic acid. There was good correlation when the biosensor data were plotted versus the results achieved with the Folin–Ciocalteau method.

A different way to entrap the enzyme using electropolymerizing polymers was also reported [60-62]. In particular, Böyükbayram and coworkers [62] prepared graft copolymers by electropolymerization of pyrrole with thiophene capped polytetrahydrofuran and used these conducting copolymers to immobilize tyrosinase. The enzyme electrodes were used to determine the amount of phenolic compounds in two brands of Turkish red wine and found very useful owing to their high kinetic parameters and wide pH working range. Thiophene functionalized menthyl monomer with pyrrole (MM/ppy/Tyrosinase) represented another copolymer employed to immobilize tyrosinase [63]. Immobilization of enzyme was performed via entrapment in conducting copolymers during electrochemical polymerization of pyrrole. Maximum reaction rates, Michaelis–Menten constants and temperature, pH and operational stabilities of enzyme electrodes were investigated by the authors. The application of this sensor was again to evaluate the total amount of phenolic compounds in red wines. The immobilized enzyme was optimized at pH 9. MM/ppy/tyrosinase electrode showed stability up to 80°C, while free tyrosinase had an optimal temperature of 40°C and lost its activity completely at 50°C. The system was able to detect the total phenolic compounds present in two different red wines in a range of 3.3-6.0 g/L, expressed as gallic acid equivalents.

Different applications reported for tyrosinase deal with the development of artificial senses, such as electronic tongues, where the systems array of sensors with different selectivities are generally integrated to give a response "mimicking" the natural counterpart. A lot is known about artificial 'nose', but in the last years some work has appeared dealing with taste. In this regard, Gutés and coworkers [64] elaborated a simultaneous determination of different phenols (phenol, catechol, *m*-cresol), combining biosensor measurements with chemometric tools and artificial Neural Networks (ANN) analysis. Concentrations of the three phenols ranged from 0 to 130 µM for phenol, 0 to 100 µM for catechol and 0 to 200 µM for *m*-cresol. As the recognition-detection part, and working electrode, a tyrosinase-based biosensor was developed. The biosensor employs the concept of a graphite-epoxy biocomposite with bulk incorporation of enzyme.

Tyrosinase sensors are also employed for quantitative analyses of polyphenols in beer samples. Three amperometric biosensors are described based on immobilization of tyrosinase on a new Sonogel–Carbon electrode for detection of phenols and polyphenols [65]. The electrode was prepared using high energy ultrasound directly applied to the precursors. The first biosensor was obtained by simple adsorption of the enzyme on the Sonogel–Carbon electrode. The second and third ones, presenting sandwich configurations, were initially prepared by adsorption of the enzyme and then modified by mean of a polymeric membrane, such as polyethylene glycol for the second one, and the ion-exchanger Nafion in the case of the third biosensor. The optimal enzyme loading and polymer concentration, in the second layer, were found to be 285 U and 0.5%, respectively. All biosensors showed optimal activity under the following conditions: pH 7, −200 mV, and 0.02 mol L^{-1} phosphate buffer. Sensing performances and kinetic characterizations of the developed biosensors were investigated using some phenolic compounds (catechol, phenol, 4-chlorophenol, gallic acid, catechin). In the same paper, the tyrosinase-based Nafion modified Sonogel-Carbon electrode was used to quantify the polyphenol and phenol content of four beers (two lagers and two black) and four environmental water samples [65].

The same research group [66] produced a biosensor based on the bi-immobilization of laccase and tyrosinase. The biosensor employed as the electrochemical transducer the Sonogel-Carbon electrode. The immobilization step was accomplished by doping the electrode surface with a mixture of the enzymes, glutaricdialdehyde and Nafion-ion exchanger, as protective additive. The response of

this biosensor, carrying *Trametes versicolor* laccase (Lac) and Mushroom tyrosinase (Ty) based on Sonogel-Carbon detection, was optimized directly in beer samples and its analytical performance with respect to five individual polyphenols was evaluated. The electrode responds to nanomolar concentrations of flavan-3-ols, hydroxycinnamic acids and hydroxybenzoic acids. The limit of detection, sensitivity and linear range for caffeic acid, taken as an example, were 26 nM, 167.53 nA M^{-1}, and 0.01-2 µM, respectively. The Lac-Ty/sonogel-carbon electrode was stable in this matrix, maintaining 80% of its stable response for at least three weeks (RSD 3.6%). The biosensor was applied to estimate the total polyphenol index in ten beer samples and a correlation of 0.99 was obtained when the results were compared with those obtained using the Folin-Ciocalteu reagent.

Amphiphilic, tyrosinase-modified screen-printed carbon bioelectrodes were decrypted by Cummings [67] for the analysis of lager beers and were compared to the *p*-dimethylaminocinnamaldehyde (DAC) colorimetric method. Initially, the performances of the biosensors under flowing conditions were appraised using catechol as a model substrate. The electrodes displayed rapid response times and a high degree of sensitivity and reproducibility upon injection of catechol onto a single mainfold. In addition, simple flavanols, separated from barley, were utilized to assess the sensitivity of the biosensors afforded by the presence of the enzyme. The bioelectrode sensitivity decreased upon an increase in molecule size. Finally, using flow injection analysis, authentic beer samples were analyzed and compared to the DAC colorimetric method. A good correlation between the two methods of analysis was observed but, due to the lack of enzyme substrate specificity, the biosensor response did not decrease to the same extent as the colorimetric method; this can be attributed to the presence of interferents, for example, ferulic acid and p-coumaric acid.

An amperometric tyrosinase biosensor has also been used for detection of polyphenols in tea [68]. The system could detect tea polyphenols in the concentration range 10–80 mmol L^{-1}. Immobilization of the enzyme, by the crosslinking method, gave a good stable response to tea polyphenols. The biosensor response reached the steady state within 5 min. The voltage response was found to have a direct linear relationship with the concentration of polyphenols in black tea samples. Enzyme membrane fouling was observed with a number of analyses with a single immobilized enzyme membrane. The tyrosinase-based biosensor gave maximum response to tea polyphenols at 30°C. The optimum pH was 7.0. This biosensor system can be applied in evaluating tea polyphenols quality.

Campo Dall'Orto and coworkers have reported the polyphenol content, expressed as chlorogenic acid equivalents, in a variety of commercially available samples of yerba mate (*Ilex paraguaiensis*). The compounds were detected using a tyrosinase biosensor and comparing the results with a colorimetric method [69]. The 48% of analyzed samples presented a 92 ± 8 mg of extracted chlorogenic acid equivalents per gram of sample. The extracted chlorogenic acid, expressed as mg/g^{-1}, was evaluated by three methods in a unique yerba mate sample: biosensor (89.2 mg/g^{-1}), Folin (90.2 mg/g^{-1}), and HPLC analysis (21.0 mg/g^{-1}). Biosensing system validation was performed. Repetitiveness of genuine replicates was consistent with the nature of the samples. Discrimination between yerba mate and other plants can be made using principal component analysis (PCA) and the corresponding physical and chemical descriptors. Flavor and taste alterations can be studied by means of analytical methods that involve low-cost instrumentation.

Peroxidase: Horseradish peroxidase (HRP) has been eventually employed as another useful enzyme for polyphenol detection. In this approach, the reduction current of oxidized polyphenols, formed during the enzymatic oxidation of polyphenolic compounds in the presence of H_2O_2, is proportional to their concentration. In other words, the polyphenol content can be detected as the reduction current of the oxidized polyphenol generated by the enzyme reaction cycle of HRP with H_2O_2. The sensitivity of the detection of various polyphenols by the present method depends on both the electron-donating properties of polyphenols and the electron-accepting properties of oxidized polyphenols.

With this approach, Imabayashi [70] reports about the development of a sensor using horseradish peroxidise covalently immobilized on a self-assembled monolayer of mercaptopropionic acid on gold-electrode by the formation of the bond between amino groups on the HRP surface and carboxylic groups on the self-assembled monolayer. The electrode allows polyphenol detection down to 2 µM with a linear relationship up to 25 µM in standard

solutions. The reduction current of oxidized polyphenols, formed during the enzymatic oxidation of polyphenolic compounds in the presence of H_2O_2, is proportional to their concentration. The sensitivity of the detection of various polyphenols by the present method depends on both the electron-donating properties of polyphenols and the electron-accepting properties of oxidized polyphenols. When applied to real matrices, such as wine and tea, the total amounts of polyphenols, in the order of μM, were estimated, correlating well with the results determined by the Folin-Ciocalteu method.

In another paper [71], the decreased amount of H_2O_2 caused by the action of peroxidase was sensitively detected with a semipermeable-membrane-covered, HRP-entrapped, and ferrocene-embedded carbon paste electrode. This electrode allows the detection of (+)-catechin down to 0.3 μM and the response is linear up to 15 μM. The same linearity was obtained with other polyphenols found in wine and green tea, such as (-)-epicatechin, caffeic acid, tannic acid and gallic acid. The content of total phenolic compounds in wine [1-8 mM using (+)-catechin as the standard; 2-14 mM using gallic acid as the standard] and tea samples [1-3.5 mM using (+)-catechin as the standard; 0.6-6 mM using gallic acid as the standard] determined by the present method agrees well with results obtained by the Folin-Ciocalteu method.

The same enzyme has also been employed immobilized onto silica–titanium and it was applied to measure the polyphenol content of a plant extract without sample pretreatment, because no significant influence of the matrix was observed [72]. Silica-based materials have received greatest interest because they provide a suitable way for designing electrochemical biosensors. Among the silica-containing matrices, the silica gel modified with metal oxides has been used, not only to improve its amperometric detection, as increase in the internal electrical conductivity of the silica matrix, but also by providing a material with high chemical stability. A biosensor based on horseradish peroxidase and DNA immobilized onto silica–titanium is applied for measuring the polyphenol compounds in plant samples. In the study, various analytical parameters influencing the biosensor performance, such as working potential, type and concentration of the buffer, pH, response time and response in the presence of other compounds, have been investigated as a function of chlorogenic acid (CGA). In the optimized conditions, the biosensor presented a linear

response range for CGA from 1 to 50 μmol L^{-1}, applying a potential of −50 mV versus Ag/AgCl, with a sensitivity of 181 μmol^{-1} L nA cm^{-2} and detection limit of 0.7 μmol L^{-1}. The biosensor was used to determine the polyphenol content of extracts of coffee and mate. The experimental results showed good agreement with those from the Folin–Ciocalteau method. The polyphenol content in the aqueous extracts of coffee and mate tea ranged from 1.0 to 3.6 (mmol L^{-1})g^{-1} of sample.

Phenolic compounds are also important factors to be considered in order to evaluate the quality of an extra-virgin olive oil since they are partly responsible for its auto-oxidation stability and organoleptic characteristics. The phenolic content is correlated with many quality parameters, such as the oxidation level or free fatty acidity. Free fatty acids provide an index of the degree of lipase activity and can produce undesirable aromas in the oil; a high value for free fatty acid content indicates a high degree of lipase activity and hence a reduced antioxidant content. Moreover, the oxidation level is dependent upon the composition of the oil and, therefore, upon the degree of unsaturation and the presence of antioxidants, such as phenols. Free fatty acids are responsible for undesirable aromas in the oil. Thus, estimating polyphenol content could provide some indication of oil quality.

On the basis of this, some work dealing with olive oil as matrices for polyphenol content evaluation has been reported [73]. Monitoring the polyphenol content (oleuropein derivatives) in an extra-virgin olive oil with varying storage time and storage conditions was performed using two rapid procedures based on disposable screen-printed sensors (SPE) for differential pulse voltammetric analysis, and on an amperometric tyrosinase based biosensor operating in an organic solvent (*n*-hexane) and using an amperometric oxygen probe as transducer. Differential Pulse Voltammetry (DPV) parameters were chosen in order to study the oxidation of oleuropein, which was used as reference compound. A calibration curve of oleuropein was determined in glycine buffer [10 mM, pH = 2, NaCl 10 mM (D.L. = 0.25 ppm oleuropein, RSD= 7%]. In the case of the tyrosinase based biosensor, the calibration curves were realized using flow injection analysis with phenol as the substrate (detection limit = 4.0 ppm phenol, RSD = 2%). Both of these methods are easy to operate, require no extraction (biosensor) or rapid extraction procedure (Solid Phase Extraction, SPE) and the analysis time is short (min). The results were

comparable with those using Folin-Ciocalteau reagent and by HPLC analysis.

Campanella and coworkers [37] monitored the rancidification process of extra-virgin olive oil using a biosensor operating in organic solvent. The progressive rancidification of the oil was monitored by simultaneously using two different indicators: the peroxide number and an innovative one consisting of the progressive decrease in the content of polyphenols, the main natural antioxidants contained in the oil, as determined rapidly by means of a new organic phase enzyme electrode based on tyrosinase. The aim of the paper was to evaluate the 'genuineness' of the oil itself and then, above all, to check the correlation between the stability of an olive oil to an artificially induced process of rancidification. The main result of the research was to demonstrate the possibility of using the organic phase enzyme electrode based on tyrosinase to monitor the rancidification process occurring in any sample of olive oil. Indeed, a clear inverse correlation was found throughout the entire oxidation process between the classic indicator, namely the peroxide number, and the polyphenol content of the sample. The simplicity, together with the accuracy and precision of the polyphenol content measurements performed on the olive oil samples revealed the advantages offered by this biosensor. The total polyphenol content in various olive oils ranged from 15.3 to 114.2 mg kg^{-1} of oil, expressed as phenol.

Very recently, the thermal oxidative degradation process of polyphenols was studied, both in a synthetic mixture of five of the more readily available polyphenols contained in the extra-virgin olive oil (EVOO) (tyrosol, vanillin, caffeic acid, ferulic acid and oleuropein) dissolved in glyceryl trioleate, and commercial extra-virgin olive oil [74]. To this end, a series of oxidative degradation experiments was carried out on extra-virgin olive oil samples under isothermal conditions at 98, 120, 140, 160, and 180°C using a thermostatic silicon oil bath. The change in polyphenol concentration with time was monitored at selected temperatures using a tyrosinase biosensor operating in an organic phase (*n*-hexane). The EVOO rancidification process rate displayed good inverse correlation between the variation in the peroxide value, the more traditional index, and that of a more innovative index determined by the concentration of the "total polyphenols" (expressed in mol L^{-1} of phenol). In this paper, the authors analyzed the kinetic degradation process and the kinetic parameters of the process were determined through an isothermal study carried out at different temperatures (between 98 and 180°C).

Laccase: Some studies reported the use of laccase as a possible enzyme for development of biosensors for phenols and polyphenols. Laccase, a copper-containing oxidase, is widely distributed in fungi, higher plants and in some bacteria. The use of a laccase-modified electrode for detection of flavonoids was reported by Gorton's group [75]. In a recent study, the laccase from *Cerrena unicolor*, as a highly active enzyme, coupled to amperometric transduction was reported for the detection of flavonoids. The enzyme was adsorptively immobilized on the surface of a graphite electrode. In particular, catechin hydrate, epicatechin, epicatechin gallate, prodelphinidin, and caffeic acid were used as target compounds. Electrodes modified with laccase yield responses for both simple compounds and compounds with three or more phenolic and non-phenolic rings, but with different sensitivities.

Considering wine as a matrix of interest, another example of a laccase biosensor is given. A biosensor developed with Laccase *Coriolus versicolor* immobilized on derivatized polyethersulfone membranes and applied to a Pt–Ag, AgCl US electrode base was applied to evaluate several polyphenols usually found in red wine (caffeic acid, gallic acid, catechin, rutin, *trans*-resveratrol, quercetin and malvidin) [76,77]. It was observed that an amperometric response was obtained for catechin at +100 mV (versus Ag/AgCl) and caffeic acid at −50 mV in acetate buffer solutions (pH 4.5) having 12% ethanol. At pH 3.5 and +100 mV the biosensor was sensitive to both substrates and their response was additive. One limit of this biosensor is the necessity for a previous solid phase extraction of the matrix for polyphenol enrichment; large interferences can occur.

Amperometric determination using a biosensor based on immobilized laccase was applied for the analysis of tannins of tea at different stages of its production [78]. The enzymes were from *Coriolus versicolor*, *Coriolus hirsutus* and *Cerrena maxima*, and immobilized on threadlike DEAE-cellulose. The time needed for analysis in the flow injection mode was below 100 s. A column with immobilized enzyme could be used for up to 500 determinations of phenolic compounds (tannin content 100-199 mg/g of dry substance) without decrease of the enzyme activity.

The use of a laccase biosensor, both under batch and flow injection conditions, for a rapid and reliable amperometric estimation of the total content of polyphenolic compounds in wines is also reported [79]. The enzyme was immobilized by cross-linking with glutaraldehyde onto a glassy carbon electrode. Caffeic acid and gallic acid were selected as standard compounds to carry out such an estimation. Experimental variables, such as the enzyme loading, the applied potential, and the pH value, were optimized, and different aspects regarding the operational stability of the laccase biosensor were evaluated. Using batch amperometry at -200 mV, the detection limits obtained were 2.6×10^{-3} and 7.2×10^{-4} mg L^{-1} gallic acid and caffeic acid, respectively, which compares advantageously with previous biosensor designs. An extremely simple sample treatment consisting only of an appropriate dilution of the wine sample with the supporting electrolyte solution (0.1 mol L^{-1} citrate buffer of pH 5.0) was needed for the amperometric analysis of red, rosé, and white wines. Good correlations were found when the polyphenol indices obtained with the biosensor (in both the batch and FI modes) for different wine samples were plotted versus the results achieved with the classic Folin-Ciocalteu method. Application of the calibration transfer chemometric model (multiplicative fitting) allowed for the confidence intervals (for a significance level of 0.05) for the slope and intercept values of the amperometric index versus the Folin-Ciocalteu index plots ($r = 0.997$) including the unit and zero values, respectively. This indicates that the laccase biosensor can be successfully used for the estimation of the polyphenol index of wines and is comparable with the Folin-Ciocalteu reference method.

Plant tissue: An alterative approach to the use of purified enzymes is the employment of whole tissue which contains different enzymes sets. Whole tissue materials from plants or animals provide many advantages for the construction of biosensors.

In some cases, plant tissue containing polyphenol oxidase (e.g., banana, potato, apple, and burdock) has been used, coupled to electrodes, for the detection of catechol-related components, such as flavonols and catechins in beers and green tea [78,80-83]. The linear detection range of the plant-tissue electrodes, depending on the enzyme preparation used, was, on average, between 2 and 12 μM catechins.

Tissue sensors have been applied to the determination of flavonols in beers [81]. In particular, different plant tissues, banana, potato and apple, containing tyrosinase have been evaluated for their ability to detect catechol related components in beers. Calibration graphs were produced for each plant tissue with both catechol and (−)-epicatechin as standards. The response of the banana based sensor was rather erratic and showed a large zero error, probably due to flavanols in the banana. Potato, wet apple and dried apple were all satisfactory. The response with dried apple was 60% that of wet apple, but showed greater stability. The response of (−)-epicatechin was 1.4 to 1.5 times that of catechol with either wet or dried apple. Banana and potato based sensors were used for the determination of total flavanols in a range of commercial beers and lagers. Good analytical data were obtained with potato, comparable to those obtained using colorimetric or liquid chromatographic analyses. The best biosensors were from potato and apple. While there was slight loss of response due to the drying process, dried apple has greater longevity and excellent response characteristics. In preliminary experiments reported elsewhere, the same authors [82] have demonstrated that the flavanol components in beer can be determined with a 'bananatrode' biosensor based on the carbon paste electrode using catechol as the standard.

A burdock (*Arctium lappa* L., a biennial plant) tissue-based biosensor was applied for measuring total catechins in green tea infusions [83]. This catechin biosensor was found to respond to five catechins (catechin/epicatechin/epigallocatechin/epicatechin gallate/epigallocatechin gallate), gallic acid, catechol and ascorbic acid and to total catechins in green tea infusions. The precision of the measurements was good (< 3% RSD) and the biosensor showed no interference from major amino acids or carbohydrates in the infusions. One limitation of this approach, however, is that the biosensor is inadequate for accurate quantitation of total catechins because of the severe variability in the relative biosensor response to the different catechins.

Jewell and Ebeler developed a simple tyrosinase-based biosensor composed of 5% banana tissue, 10% mineral oil, and 85% carbon-containing ruthenium, mixed together in a small beaker to form a stiff paste, for the measurement in a winery or food setting for rapid and simple phenolics detection [84]. This was achieved by the design and construction of an operational amplifier-based tyrosinase biosensor. Excellent correlation was shown between the biosensor and the Folin-Ciocalteu assay for simple

phenolics (gallic acid, catechin, epicatechin, caffeic acid, quercetin, seed tannins) and for wines. The simple compounds and the seed tannins were chosen in order to examine their biosensor response, in correlation with different structure, OH groups and complexity of matrix. The varying signals observed were due to the chemical structure and variable number of OH groups associated with polyphenols, which caused different reaction with tyrosinase; this is also observed with all redox methods. No interferences were observed in a model wine solution made according to standard wine phenolic concentrations due to low pH, tartaric acid or ethanol present in the model wine. Finally, one white wine and three red wines, selected to examine the effects of aging and the different phenolic profiles, were analyzed with the biosensor. Also in this case, the response between Folin-Ciocalteau and biosensor methods was similar, and oak aging and varietal difference did not appreciably influence the biosensor response [84].

Behind electrochemical detection, optical sensing also represents an interesting feature for phenols detection. One particular application of this sensor was reported by Edelmann and Lendl, who developed an electronic tongue to evaluate the tannin content of red wine [85]. The interaction of tannins with proline-rich proteins (gelatin) was studied using an automated flow injection system with Fourier transform infrared spectroscopic detection to gain insight into chemical aspects related to astringency. The PRP gelatin was selected to mimic the parotid salivary proteins. In the perception of astringency, an interaction between proline-rich salivary proteins and tannins present in the sample takes place. To study this interaction, agarose beads carrying gelatin (a proline-rich protein) were placed in the IR flow cell in such a way that the beads were probed by the IR beam. Using an automated flow system, samples were injected in a carrier stream and flushed over the proteins in a highly reproducible manner. Simultaneously, any retardation due to tannin-protein interactions taking place inside the flow cell were monitored by infrared spectroscopy. Tannins of different sources (grapes, wooden barrels, formulations used in wine making) were investigated, and their flow-through behaviour was characterized. Significant differences in their affinity toward gelatin could be observed. Furthermore, because of small but characteristic differences in the IR spectrum, it is possible to distinguish condensed from hydrolysable tannins. The selectivity of the flow-through sensor

was also demonstrated on the examples of red and white wines [85].

Concluding remarks: A review of the literature concerning the application of biosensor technology to vegetal matrices has been reported and discussed. Special attention has been focused on the use of these devices for the identification and quantification of polyphenols and, in some cases, for the quality control of these matrices.

Usually the determination of total polyphenol content is performed by spectrophotometric or chromatographic methods, but in recent years, with the aim to develop methods capable of being employed also in situ, several biosensors have been developed to determine phenols in either aqueous or organic solvents. Organic phase enzyme electrodes constitute a new class of biosensor applicable to the analysis of substrates or matrices insoluble or scarcely soluble in aqueous media.

Many different sensors have been developed in the last 15 years for polyphenol detection and electrochemical transduction is the approach most applied. Different enzymes have been used over the years as the catalytic element coupled to electrochemical analysis, such as tyrosinase, laccase, and peroxidase, since phenolic compounds can act as electron donors for these enzymes. In some cases, plant tissue containing polyphenol oxidase (e.g., banana, potato, apple and burdock) has also been used for the detection of polyphenols in vegetal matrices.

The mostly investigated vegetal matrices are those with a great commercial interest such as olive oil, wine, beer and tea. By the use of screen-printed electrodes, different phenols, including flavones, flavonols, catechins, tannins, and phenylpropanoids were tested with these analytical tools. Some limitations occur with these devices in real matrices, such as the working conditions (pH, temperature) and the risk of enzyme inactivation. Many biosensors have a limited lifetime due to enzyme inactivation by the biocatalytically generated quinone products. For this reason, many studies were concerned with the development of good immobilization methods and materials to improve the biosensor stability. Recently, the immobilization of enzymes in electropolymerized conducting polymers, sol–gel materials, gold nanoparticles, Clark's electrodes, screen-printed, carbon paste, hydrogels, has received a great deal of interest.

Another limitation of this approach, however, is that sometime the biosensor is inadequate for accurate quantification of total polyphenols because of the severe variability in the relative biosensor response to the different phenol derivatives.

Nevertheless, biosensors may provide a promising competitive technology for a simple, fast and sensitive detection of polyphenolic compounds without any pre-treatment. In most of the cases successfully employed for the analysis of real samples, comparable results were obtained with conventional analysis by HPLC.

Thus, the impact of biosensors in food and plant matrices in the future will be enormous because they are a label-free screening system, which imparts flexibility to the process of assay design and facilitates successful integration with other technologies thanks to the increasing number of commercially available instruments having novel sensor surfaces, immobilization techniques and attachment chemistries.

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