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Chapter 3

DEVELOPMENT OF VOLTAMMETRIC TECHNIQUES AND SENSORS FOR THE DETERMINATION OF RESVERATROL

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ABSTRACT

Resveratrol is a polyphenolic compound of the viniferins family. Resveratrol exists in nature in both the *trans*- and *cis*-stereoisomeric forms. However, the *trans*-resveratrol or *trans*-3,4,5'-trihydroxystilbene (t-Res) is the most biologically active form. The t-Res was discovered by Michio Takaoka more than 70 years ago, in the resin of *Veratrum grandiflorum*. The t-Res occurs naturally in more than 80 plant species, such as berries, peanuts, groundnuts, tea, grapevines and red wines. However, the most abundant natural sources of t-Res are *Vitis vinifera*, *labrusca* and *muscadine* grapes, which are used to make wines. Besides its natural occurrence, t-Res is now available in tablets, being recommended as a dietary supplement.

Numerous previous studies have investigated many of its beneficial effects, such as an antioxidant, phytoestrogen, lifespan prolongation, anti-inflammatory, antiplatelet, antiatherosclerosis. In addition, t-Res is identified as a preventive agent against several pathologies: cancer, viral infection, neurodegenerative processes and cardiovascular diseases. Moreover, because of its high concentration in grape skin, red wines contain significant amount of t-Res, which it is thought to be responsible for the so called the "French paradox", i.e., low mortality due to coronary heart diseases as a result of a moderate consumption of red wines.

The growing interest in the determination of t-Res in food is directly related to the need of developing sensitive methods for its analysis. Thus, many methods have been

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developed for the determination of t-Res in food, mainly different chromatographic methods, particularly HPLC using different detectors (UV, fluorescence, capillary electrophoresis, electrochemical, mass spectroscopy and diode array detection).

Because of the t-Res electrochemical properties, it can also be quantified using different electroanalytical methods. The more attractive features of voltammetric methods are the high sensitivity, low cost, simplicity and relatively short measurement time. Voltammetric techniques in general and stripping techniques in particular, have been employed in food analysis, including wine among the considered matrices.

Another interesting proposal is the use of biosensors. According to the IUPAC definition, a biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals. The biosensors have been proposed as an effective analytical tool for the determination of polyphenolic compounds, exhibiting advantages such as the minimal preparation of the sample, selectivity, sensitivity, reproducibility, rapid time of response and simple use for continuous on-site analysis.

A. INTRODUCTION

1. Polyphenolic Compounds

Polyphenols are a wide range of biological molecules which play a protective role in plant-derived foods, particularly fruits, seeds and leaves. They are one of the main secondary metabolites synthesized by plants, both during normal development [1, 2] and in response to stress conditions such as infection, wounding and UV radiation, among others [3, 4]. Polyphenols are characterized by the presence of several phenol groups (i.e., aromatic rings with hydroxyls), which derive from L-phenylalanine [1, 5-10].

Plants may contain simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans and lignins. Phenolic acids are the most important polyphenol class, which include polymeric structures, such as hydrolyzable tannins, lignans, stilbenes, and flavonoids. Flavonoids include flavonols (i.e., quercetin and kaempferol, the most ubiquitous flavonoids in foods), flavones, isoflavones, flavanones, anthocyanidins).

In plants, phenolics may act as phytoalexins, antifeedants, and attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light, among others [10].

In food, phenolics may contribute to the bitterness, astringency, color, flavor, odor, and oxidative stability of food.

2. Resveratrol

2.1. Structure

Resveratrol exists in nature in both the *trans*- and *cis*-stereoisomeric forms (Figure 1). Resveratrol is a polyphenolic compound of the viniferins family. On the other hand, the *trans*-resveratrol or *trans*-3,4,5´-trihydroxystilbene (t-Res) is the most biologically active form.

The resveratrol has been classified as a phytoalexin for being synthesized in spermatophytes in response to injury, UV irradiation, fungal attack as well as to a variety of stress conditions, such as vicissitudes in climates, exposure to ozone, sunlight and heavy metal ions in soil [11-16].

The major part of the studies about resveratrol has been focused on the trans isomer, since the physiological activity of the cis isomer is not well elucidated. The trans isomer is thermodynamically more stable, due to the steric repulsion present in the cis molecule, and can be isomerized when exposed to intense UV irradiation, high temperature, or low pH [17, 18]. The cis-resveratrol has not been found in *Vitis vinifera* grapes, but it has been detected in wines produced with this grape [19], being formed during the fermentation step as a product of trans isomerization or decomposition of a resveratrol polymer [20].

2.2. History

The protective capacity of the health of some plants phenolic compounds and anti-nutritional properties of others are of great importance to both consumers and producers [10].

t-Res was discovered by Michio Takaoka more than 70 years ago, in the resin of *Veratrum grandiflorum* [21].

It was first isolated from the roots of white hellebore in 1940 [21], but the t-Res presence in *V. vinifera* grapes was discovered in 1976 [13]. t-Res attracted little interest until 1992, when it was postulated as a possible compound to explain some of the cardioprotective effects of red wines [22].

2.3. Occurrence

Both isomers can be present in variable amounts in plants, but amount of t-Res usually predominates. t-Res is a naturally occurring antioxidant found in grapes [23], grape products, including wine [22], peanuts [24], chocolate, and cocoa [25]. t-Res has often been reported in non-edible plants: vine, eucalyptus, spruce, and the tropical deciduous tree *Bauhinia racemosa*, and *Pterolobium* [26, 27].

On the other hand, t-Res, trans-piceid, and cis-piceid have been detected in hop, suggesting that stilbenes might also be found in beer [28]. High levels of t-Res have also been detected in leaves of *Veratrum grandiflorum* and in roots and rhizomes of *Veratrum formosanum*. These last plants have been extensively used in Japanese and Chinese folk medicine because their health properties [27].

2.4. Health Benefits and the "French Paradox"

In recent years, the research on t-Res has discovered several beneficial biological effects of this compound on human health. These include anticancer activity [29], cardioprotection [30], antioxidant activity [31, 32], inhibition of platelet aggregation [33], anti-viral [34], estrogenic [35], and, anti-inflamatory activities [36-38].

Moreover, the most recent data derived from animal studies open a new, promising perspective of the potential use of t-Res in preventing and/or treating serious metabolic disorders such as obesity and diabetes.

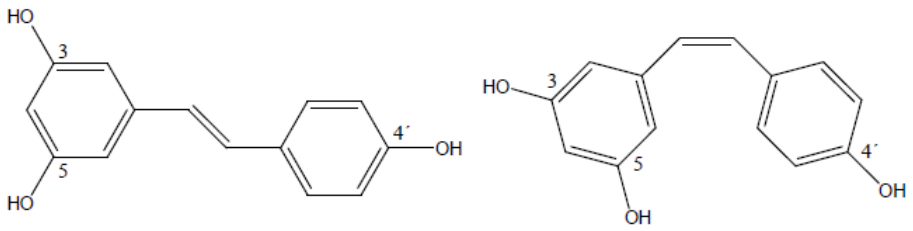


Figure 1. Stereoisomeric forms of resveratrol.

The prevalence of both these diseases is very high, especially in European Western countries, which tends to increase [39]. In addition, as it was recently reported, t-Res may also increase yeast lifespan and vertebrates [40]. For these reasons, the use of t-Res in the food additives industry is being studied and it is proposed as a fortifier and nutraceutical compound. Thus, a t-Res complex has recently been developed.

Moreover, due to the high concentration of t-Res in grape skin, red wines contain high amounts of t-Res, which it is thought to be responsible for the so called the "French paradox" i.e., the fact that the incidence of heart infarction in France is about 40% lower than in the rest of Europe, despite a diet being traditionally rich in saturated fat [41]. Wine with a higher content of t-Res might therefore be regarded as a "functional wine" according to the definition of Diplock at el. [42], due to the t-Res positive effect on health. Recently, a group of researchers from the Andalusian Agricultural Research Institute (IFAPA) has developed a methodology to produce wine with an enriched t-Res content. A significant increase in t-Res concentration (up to 3.2 times higher than in control) was achieved in bottled wine. Regarding the oenological parameters, wines obtained possessed good quality, apart from a herbaceous aroma [43].

B. Analytical Determinations of *T*-Res

1. Chromatographic Methods

During the last decade, it has been made a great effort related to the t-Res determination in different real samples, such as wines, peanuts, etc. At present, there are several analytical methods for the t-Res determination. The method most widely used for quantification of t-Res is High Pressure Liquid Chromatography (HPLC) with UV/VIS detection [44-50]. HPLC is regarded as a prime separation method and the most widely used even though it has some shortcomings, including a long analysis time, low resolution, and a short lifetime of columns [51]. The lowest detection limits are reached by employing chemiluminescent [52] or fluorimetric (FD) detections with on-line photochemical derivatization [53, 54]. The determination of t-Res has mainly been carried out by reversed phase HPLC using both UV [55-63] and mass spectrometry (MS) detection [64], and electrochemical detection (ED) [45]. Generally, HPLC methods use a C18 normal phase or a reverse phase column. These methods have also been used for a direct analysis of t-Res when the chromatographic separation was coupled with diode array detection (DAD) [65] either UV or ED [45, 66]. The high sensitivity FD [67] of stilbenes which is more specific than the UV detection was subsequently used to

determine the content of t-Res in grapes and wines. The chromatograms obtained using UV detection are very complex due to the enormous variety of compounds present in wines, making difficult the data treatment. Other detectors, more specific and sensitive are more used, such as MS, ED, and FD detection.

The quantification of t-Res in peanut kernels stressed by reverse phase HPLC was first performed by Cooksey et al. [68]. In wines, HPLC reverse-phase methods were typically more convenient and ‘‘robust’’ than other forms of liquid chromatography. Normal-phase methods have also been used to quantify t-Res in peanuts and wines. However, the methods of reverse phase are more commonly used. Vinas et al. [67] selected reverse-phase HPLC because polyphenols in wines are insoluble in water but soluble in alcohols and the stationary phase (Spherisorb ODS-2 column) permitted superior separation. The t-Res in peanuts by HPLC is also commonly analyzed using gradient elution rather than isocratic methods. The percent of mobile phase solvents and the gradient steepness (GS) varies greatly between the analysis methods. The percent of less polar solvents increases over time in reverse phase systems. Phenolic compounds, like t-Res, are highly soluble in reverse-phase HPLC common mobile phase solvents, such as methanol/water and acetonitrile/water solvent mixtures [69]. A dilute acid, such as acetic acid in water, is also commonly incorporated as one of the mobile phase solvents in reverse-phase systems. Therefore, the addition of an acid in the mobile phase improves the analysis by suppressing on-column ionic dissociation of the three acid phenolic hydroxyl groups of t-Res [70].

Gas chromatography (GC) is also widely used for the t-Res determination. A tandem MS is the detection method more used for GC [71-75]. Also, it is frequently used in liquid chromatography (LC) to determine t-Res. The LC with ED has proved to be selective and sensitive for the determination of t-Res and other phenolic compounds in natural sources [45, 76]. Reverse Phase Liquid Chromatography (RP-LC) by acid solvent gradient elution, either with photometric, FD, ED or MS detection has been the method most commonly used for the analysis of t-Res in wines.

On the other hand, capillary zone electrophoresis (CZE) with DAD [77] or ED [78] has been also employed for the analysis of t-Res with a sensitivity similar to the LC methods [79].

All the techniques previously mentioned need a clean-up step. Therefore, before performing the analysis the solution must be filtered [60] (separation column is generally preceded by a guard column). In addition, other clean-up steps include liquid– liquid extraction [56] (the extracts are generally dried and evaporated to dryness for concentration), solid extraction [59] or lixiviation followed by liquid–liquid extraction or solid extraction [57] in solid samples. These analysis techniques generally involve derivatization that requires extensive precautions such as creating exclusive nitrogen environment or ensuring protection from UV light.

2. Electroanalytical Methods

New techniques have been developed for the quantification of t-Res, such as electrochemical techniques. The excellent sensitivity and the wide linear range of electrochemical detection have attracted attention in recent years. The more attractive features of voltammetric methods are the high sensitivity, low cost, simplicity and relatively short measurement time. Voltammetric techniques in general and stripping techniques in particular

have been traditionally employed in food analysis [80]. Electrochemical methods, especially differential pulse voltammetry (DPV) and square wave voltammetry (SWV), make possible to decrease the analysis time as compared to the exhausted time of chromatographic methods [81, 82]. The advantages of SWV over other electroanalytical techniques are: a greater speed of analysis, lower consumption of electroactive species and less problems with blocking of the electrode surface. Antioxidants have traditionally been the most important target compounds apart of metals. Thus, Kilmartin et al. [83] ranked phenolic antioxidants by their reducing strength and characterized their electrochemical oxidation processes for reversibility, using cyclic voltammetry (CV) at a glassy carbon electrode(GCE). Also, the behavior of phenolic compounds with an ortho-diphenol group, gallic acid, myricetins and quercetin glycosides was reported in 2002 by the same authors [84] employing CV as a semi-quantitative technique to measure the level of galloyl and catechol groups present in wines, which correlated well with other total phenol measurements. Also, the appearance of individual phenolics, at electrode potentials expected for each one of them, was matched with peaks of cyclic voltammograms of wine samples.

Corduneanu et al. [85] determined t-Res in ethanol + pH 7.0 (0.2 M) phosphate buffer solution using DPV from to commercial reagent. The working electrode used was an unmodified GC electrode. These authors also studied the antioxidant properties of both t-Res and c-Res. The calibration curve was constructed in the concentration range from 0.3 to 41 µM. The detection and quantification limits were 0.178 µM and 5.86 µM, respectively.

Zhang et al. [86] developed a voltammetric method for the determination of t-Res on an unmodified carbon paste electrode (CPE). In this study, SWV was proposed as an alternative method to the HPLC techniques in therapeutic drug monitoring. The method was utilized for the determination of t-Res in Chinese patent medicine and diluted urine samples. The detection limit was 2×10^3 µM.

Recently, Airado-Rodríguez et al. [87] proposed a method for the determination of t-Res in red wine samples. Authors determine t-Res by adsorptive stripping square-wave voltammetry (Ad-SSWV) in an unmodified GCE. In this work, the extraction of t-Res from red wines was performed with diethyl ether and a posterior clean-up with C18 cartridges was carried out. The accumulation of the substrate onto the electrode was carried out in 0.10 M $HClO_4$ + 10% ethanol; while the measurement stage was performed after a medium exchange (0.10 M $HClO_4$ + 30% of ethanol). The calibration curve was constructed in the range from 0.022 to 0.15 µM, obtaining a detection limit of 0.018 µM.

3. Sensors

Wang and Zhang [88] developed a sensitive and inexpensively method based on molecular imprinted polymer (MIP). The t-Res bound to MIP was quantified by chemiluminescence, which allows the design of high throughput screening methods. The limit of detection was 0.1 µg mL^{-1}.

On the other hand, MIP were used to fabricate sensors for t-Res determination [89], since the MIP are an important class of synthetic materials mimicking the molecular recognition of low-weight molecules by natural receptors. The aim of this work was to develop the sensor for the sensitive and specific determination of t-Res based on fabricating t-Res-imprinted film on the indium tin oxide (ITO) electrodes via molecularly self-assembly process. The results

showed that the sensor exhibits good sensitivity and selectivity for t-Res. The molecularly imprinted self-assembled ITO electrode (MIP-Si-ITO) was prepared through in situ polymerization with t-Res as template, acryl amide (AA), ethylene glycol dimethacrylate (EDMA) and 2,2'-azobisisobutyronitrile (AIBN) as functional monomer, cross-linker and initiator, respectively. γ-methacyloxypropyl trimethoxysilane (γ -MPS) was chosen to form a self-assembly monolayer on ITO electrode through chemical interaction between the siloxane and the OH^- which resulted in an end alkenefunctionalized layer formed on ITO electrode surface. Then, t-Res-imprinted mixture containing AA, acetonitrile, EDMA and AIBN was coated on the modified surface and polymerized by thermal initiation. The sensor selectivity was confirmed using two similar molecules, polydatin (POL) and bisphenol A (BPA). These molecules were selected as the template analogues due to their electrochemical activity and structural similarity. The results showed a very good selectivity when t-res was determine in POL and BPA presence. The results showed that the rebound amounts of t-Res were increased with the increasing concentration of t-Res and the oxidation peak current was directly proportional to the concentration of t-Res in the range from 2.0 µM to 20µM. The prepared electrochemical sensor had a detection limit of 0.8 µM. The sensor can be easily cleaned by CV and then be used repeatedly, which offers a fast and easy method for the determination of t-Res in real samples like red wines with high sensitivity and selectivity.

Alternatively, Barroso et al. [90] proposed an electro-catalytic voltammetric method to assess the total antioxidant capacity (TAC) using DNA-modified CPE. The electrochemical oxidation of both adenine and guanine in neutral or alkaline conditions led to the formation of a common oxidized product that catalyzed the oxidation of NADH.

Therefore, the oxidative lesions generated after immersion of the DNA-CPE in the fenton mixture were indirectly quantified after the electrochemical oxidation of the adenines that remained un-oxidized on the electrode surface. The increase of this electrocatalytic current in the presence of several antioxidant species, including t-Res, was studied. The biosensor developed was used for the determination of TAC in several beverages and the results were compared with those attained using other methodologies to obtain an overall picture of the antioxidant profile.

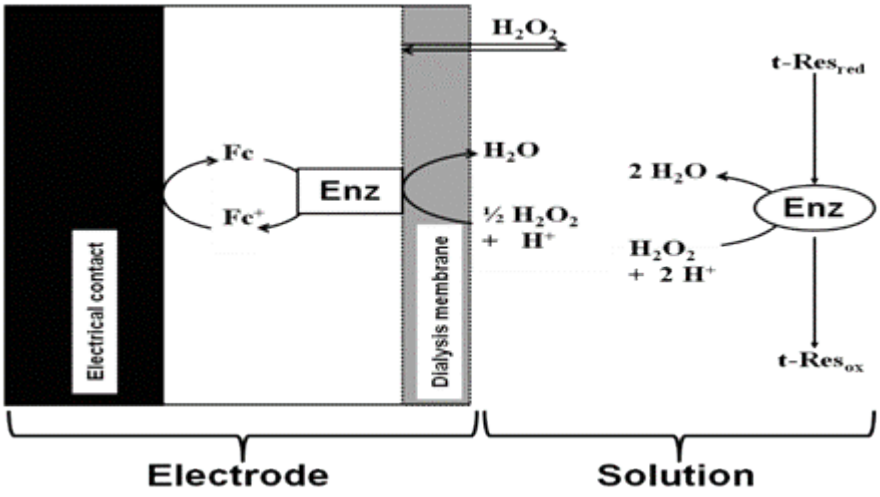
Molina-Garcia et al. [91] developed an optosensing device for the simultaneous determination of t-Res and piceid in red and white wines. The method makes use of a multi-commutated flow-through optosensor in which the resolution of t-Res and piceid is accomplished by means the sequential arrival of their photoproducts to the detection area, on-line generated by UV-irradiation. It has satisfactorily been applied to the simultaneous determination of piceid and t-Res in red and white wines. No matrix effect was observed in studied samples. The detection limits was 0.41 µM.

In our laboratory, we developed an amperometric biosensor employing the peroxidase basic isoenzymes (PBI´s) from *Brassica napus* as the biological component to determine t-Res in an aqueous medium [92]. The method employs CPE filled up with PBI´s and ferrocene (Fc) as a redox mediator. The biosensor was covered externally with a dialysis membrane (Scheme 1), which was fixed at the electrode body side part with a Teflon laboratory film and an O-ring.

The dialysis membrane function is to allow the passage of H_2O_2, but not any other substance with molecular weight higher than 100 g mol^{-1}. It is well known that phenolic and/or polyphenolic compounds can work as electron-donors for peroxidases in the catalytic reduction of H_2O_2. This approach allows detecting the decrease in H_2O_2 concentration in a

solution after the oxidation of t-Res produced by the PBI´s in the presence of H_2O_2, given that PBI´s acts in cascade in the solution and the electrode surface. The detection limit and the sensibility were 0.83 μM and (2.31 ± 0.05) x 10^6 nA M^{-1}, respectively.

Additionally, we have also developed an amperometric biosensor for determination of the total polyphenolic content (TPC) in wine and tea samples, following a procedure similar to that previously described (Scheme 1).



Scheme 1. Schematic representation of the operating principle of the biosensor, (Enz = PBI´S or PBHR).

This biosensor employs CPE filled up with peroxidases from *Brassica napus* hairy roots (PBHR), ferrocene (Fc), and multi-walled carbon nanotubes embedded in a mineral oil (MWCNT+MO). In this work, the TPC was estimated using the t-Res and caffeic acid (CA) as reference compounds to generate calibration curves. Therefore, the TPC in wine and tea samples was calculated from these curves. The lowest TPC concentration value measured experimentally for a signal to noise ratio of 3:1 was 0.1 μM for both t-Res and CA [93].

CONCLUSION

New techniques have been developed for the quantification of t-Res, such as electrochemical techniques. The excellent sensitivity and the wide linear range of electrochemical detection have attracted attention in recent years. The more attractive features of voltammetric methods are the high sensitivity, low cost, simplicity and relatively short measurement time. Voltammetric techniques in general and stripping techniques in particular have been traditionally employed in food analysis. Electrochemical methods, especially differential pulse voltammetry and square wave voltammetry, make possible to decrease the analysis time as compared to the exhausted time of chromatographic methods.

On other hand, in recent years there has been much progress in research and development of biosensors, supported by technological advances, developing new materials and computerization. Because of the multitude of fields that includes the development of

biosensors in the industry, success in research and development involve the participation of multidisciplinary teams formed among others by chemists, biochemists, food technologists, physicists, etc., who work together to solve technical problems involved in the development and the implementation of these devices. Finally, we can expect in the near future to develop biosensors that are suited to the needs of the food industry, which are economical and intelligent and allow detection of different substrates in real time.

Therefore, the electrochemical and biosensors are proposed as alternatives to chromatographic techniques in the short to medium time.

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