PROGRAMA DE DOCTORADO EN FÍSICA

TESIS DOCTORAL:

ELECTRONIC TONGUE TECHNOLOGY APPLIED TO THE ANALYSIS OF GRAPES AND WINES

Presentada por Celia García Hernández para
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A mis padres,

por su amor,

por dármelo todo.

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The Doctoral Thesis entitled *"Electronic tongue technology applied to the analysis of grapes and wines"* has been developed in the group of sensors UVaSens under the supervision of Prof. PhD. María Luz Rodríguez Méndez, from the Inorganic Chemistry and Physical Chemistry Department at the Engineers School, and Prof. PhD. Ana Cristina García Cabezón, from the Materials Science and Metallurgical Engineering Department at the Engineers School, both from the University of Valladolid (Spain). The author of this Thesis has carried out a research stay at the Missouri State University (Springfield, EE.UU.) under the supervision of Prof. PhD. Adam Wanekaya for 4 months to be able to obtain an international Ph.D. mention.

This dissertation contains research that studies the use of sensors and biosensors implemented as arrays of electrodes in electronic and bioelectronic tongues to analyze quality characteristics of wines and grapes of interest in the agrofood industry such as sugar and/or polyphenolic content. Different and novel electrocatalytic materials have been used and combined to construct the arrays of sensors and biosensors. The samples of wine and grape used in this study were provided by the wineries "Cooperativa de Cigales" and "Instituto Tecnológico Agrario de Castilla y León (ITACyL)", both located in the Castilla y León region (Valladolid, Spain). The chemical parameters of the samples were analyzed in the UVaSens laboratory, and also by ITACyL, following international regulations (OIV, The International Organisation of Vine and Wine).

The following provides an overview of the structure of this dissertation: Chapter 1 provides an introduction and background to this research. A literature review is presented in Chapter 2 including the state of the art, the applications of these devices in the food industry, the type of sensors used to develop electronic tongues, the electrochemical techniques applied, the electrocatalytic materials that help to improve the responses of the electronic tongues, the techniques used to fabricate nanostructured films to modify sensors, as well as the most common statistical tools implemented to analyze data obtained from electronic tongues. Chapter 3 looks at 9 research papers with the results obtained from the studies carried out and published in international journals with impact factor. An overview of the conclusions from the entire dissertation is presented in Chapter 4.

Finally, Annexes A, B, C and D contain a summary written in Spanish in accordance with the regulations of the University of Valladolid, the co-authored list of papers published during the Doctoral studies, the attestation letter of the international research stay carried out in the

Chemistry Department of the Missouri State University (Springfield, USA) signed by the supervisor Prof. Wanekaya and the assessment reports provided by the External Evaluators.

Tables

Figures

a*	Redness
AAS	Atomic absorption spectroscopy
AFM	Atomic force microscopy
ANNs	Artificial neural networks
ATR	Attenuated total reflection
AuNPs	Gold nanoparticles
b*	Yellowness
B%	Proportion of blue color
bioET	Bioelectronic tongue
BKG	Background
C*	Chroma or saturation
CA	Chronoamperometry
CD	Color density
CI	Color intensity
CNT	Carbon nanotube
CP	Chronopotentiometry
CPs	Conducting polymers
CPE	Carbon paste electrode
dA%	Proportion of red color produced by flavylium cations
DLS	Dynamic light scattering
D.O.	Denomination of Origin
DSA	1-decanesulfonic acid sodium salt
EDP	Electrodeposition
EIS	Electrochemical impedance spectroscopy
EM	Electrocatalytic material
EQCM	Electrochemical quartz crystal microbalance
ET, e-tongue	Electronic tongue
FIA	Flow injection analysis
FTIR, FT-IR	Fourier transform infrared spectroscopy
FT-NIR	Fourier transform near-infrared spectroscopy
h*	Hue angle
H	Hue
GC	Gas chromatography
GC-MS	Gas chromatography combined with mass spectrometry
GC-MS/MS	Gas chromatography combined with tandem mass spectrometry
GC-O	Gas chromatography combined with olfactometry

GOx	Glucose oxidase enzyme
HPLC	High-performance liquid comatography
HQ	Hydroquinone
IC	Ion chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
IRMS	Isotope ratio mass spectrometry
ISFETs	Ion-selective field effect transistors
ITO	Indium tin oxide
L*	Lightness
Lac	Laccase
LB	Langmuir-Blodgett
LbL	Layer by Layer
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography combined with tandem mass spectrometry
LDA	Linear discriminant analysis
$LnPc_2$	Lanthanides bis-phthalocyanine
LOD, LD	Limit of detection
LV	Latent variables
Lys	Lysozyme
MIP	Molecular imprinted polymer
MOFs	Metal–organic frameworks
MONPs	Metal oxide nanoparticle
MPcs	Metallophthalocyanines
MPs	Metalloporphyrins
MS	Mass spectrometry
MUA	11-mercapto-undecanoic acid
MWCNT	Multiwall carbon nanotube
NIR	Near-infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
N-PLS	N-way partial least squares
PA	Polyacetylene
PAH	Poly(allylamine hydrochloride)
PANI	Polyaniline
PARC	Pattern recognition
PARAFAC	Parallel factor analysis
Pc	Phthalocyanine
PC	Principal component

PCA	Principal component analysis
PCR	Principal component regression
PEDOT	Poly (3,4-ethylenedioxythiophene)
PEI	Poly(ethyleneimine)
pI	Isoelectric point
Ppy, PPy	Polypyrrole
PSS	Polystyrene sulfonate
PLS	Partial least squares
PLS-DA	Partial least squares discriminant analysis
PT	Polythiophene
PVC	Poly(vinyl chloride)
QCM	Quartz crystal microbalance
R%	Proportion of red color
RI	Refractive index
RMSE	Root mean square error
RSD	Relative standard deviation
SAM	Self-assembled monolayer
SEM	Scanning electron microscopy
S-CPE	Skin carbon paste electrode
SIMCA	Soft independent modeling of class analogy
SIRA	Stable isotope ratio analysis
SPE	Screen printed electrode
SVM	Support vector machines
SVMR	Support vector machines regression
SS	Stainless steel
TA	Total acidity
TEM	Transmission electron microscopy
TPI	Total polyphenol index, phenolic content
Tyr	Tyrosinase
UHPLC/Q-TOF-MS	Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry
UV-Vis	Ultraviolet and visible spectroscopy
VOCs	Volatile organic compounds
WE	Working electrode
Y%	Proportion of yellow color
ZP	Zeta potential

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1. Introduction

The concept of electronic tongues (ETs or e-tongues) or taste sensors has grown rapidly during recent years due to their large potential. They are based on electrochemical sensors combined with multivariate data analysis. The development of new methods of analysis to characterize food is of vital importance for improving the current quality control systems of food products. According to the IUPAC (International Union of Pure and Applied Chemistry), an electronic tongue is *a multisensor system, which consists of a number of low selective sensors and uses advanced mathematical procedures for signal processing based on the pattern recognition (PARC) and/or multivariate analysis [artificial neural networks (ANNs), principal component analysis (PCA), etc.]* [1]. Therefore, ETs are holistic systems that provide global and qualitative information about the sample instead of quantitative data about specific compounds. However, if the data matrix obtained by such multisensor systems is analyzed with adequate chemometric processing tools, descriptive or predictive information of particular parameters could be extracted [2,3]. There is a most recent term in the field of electronic tongues widely named *bioelectronics tongue* (bioET), which includes the use of one or several biosensors implemented in the ETs [4,5].

This research examines how ETs and bioETs can be applied to study red grapes and wines in order to better predict the harvesting time of grapes and quality parameters of interest to produce high quality wines. Routine analytical tests from the harvesting of grapes to the final bottled end-product include a number of varying chromatographic techniques, as well as more traditional ones (spectroscopy, photometry, enzymatic assays, etc.). These analytical techniques will be combined to provide an in-depth study of the composition of wines leading to analyses which are very expensive, time consuming and demand highly qualified personnel. It is therefore necessary to develop new technologies that allow us to obtain information concerning the spectrum of compounds, including global information about the sample instead of information about specific components [6]. ETs can become an alternative or complement to the traditional methods of wine and grape analysis that could be used, not only in the analytical laboratories, but also *in situ* as a quality control tool for the farmers and wine growers in the vineyards.

In addition, nowadays, we are immersed in the fourth revolution of the manufacturing industry, also called *Industry 4.0* (or smart factory, cloud-based manufacturing, factory of the future, smart manufacturing and digital manufacturing). This next big manufacturing revolution will demand benefits from joining multidisciplinary competences related to new sensing technologies,

big data, cloud computing, artificial intelligence, adaptive robots, smart valves and autonomous smart control applications to bring maximum added value in future smart processes. Existing technologies exploited by measurement, control and automation systems are mainly based on measuring physical parameters. The next big improvement will be to deploy real-time chemical information from the measurement objects to an automation database and use it in automated control decisions. Spectroscopic technologies are well-established in off-line analysis in chemical laboratories, but it is obvious that future smart factories will gain huge benefits when qualitative and quantitative material information can be generated with affordable and reliable sensors. Real-time chemical sensors will open up a new era of process control in several industries. Industrial processes of food and beverages will benefit more and more in the future from next generation smart sensor platforms [7,8].

On the other hand, there are not many places in the world as appropriate as Spain for studying high quality wines. Spain has a millenary tradition and history in the wine industry. Moreover, Spain has 69 wine Denominations of Origin, Rioja, Ribera del Duero and Rueda being some of the most relevant. Denominations of Origin are not solely an administrative status, but also the way used in Spain to recognize and protect a product with a high quality and unique characteristics, origin and tradition. According to the OIV Statistical Report 2018 [9], Spain was one of the largest producers of wine around the world, first in the ranking of planted surface area, third in production of wine excluding juice and musts (after Italy and France), fifth in grape production (after China, Italy, USA and France) and the largest exporter around the world in terms of volume (followed by Italy and France), third in terms of value. Additionally, Spain is not an importer of wine (neither in terms of volume nor value) as other important wine producer countries, are such as Italy or France.

However, besides being of great importance for the Spanish food industry, why has wine become so essential in the Mediterranean diet? This is due to the antioxidant properties of polyphenols which provide health benefits. It has been demonstrated that the consumption of antioxidants decreases the risk of cardiovascular diseases [10-12]. For this reason, international organisms recommend the daily ingestion of foods rich in antioxidants [13].

The primary goal of this research is to develop new electrochemical sensors to find appropriate electroactive and/or sensing materials able to interact with antioxidants and polyphenols contained in grape musts and wines to be implemented in electronic and bioelectronics tongues. Some candidates for this task will be conducting polymers, metal

nanoparticles, metal oxide nanoparticles, phthalocyanines and/or enzymes. In this aspect, the use of nanotechnology can play an important role in manufacturing nanostructured sensors by using different surface modification techniques that will provide chemically modified electrodes. Also, further improvements in sensors responses when combining some of the mentioned materials will be discussed. Then, the sensors will be combined to form an array of electrodes constituting an ET and/or bioET that will be used to analyze real samples of wines and grape musts. Finally, statistical analyses will be carried out to discriminate between samples, to establish correlations between the data obtained with the ET/bioET and the chemical parameters obtained by traditional analysis and, finally, to predict quality parameters of interest by using the data obtained with the ET/bioET.

1.2. Problem statement and justification

Wine, one of the oldest alimentary products known for more than 8000 years, is an alcoholic beverage obtained through the fermentation of grapes. The origin of viticulture dates back to 6000-5000 BC in Georgia and Iran. Winemaking culture still remains unchanged: modern wine is very similar to those our ancestors knew, while modern viticulture and oenology practices still refer to those of the ancient Greeks, preserved and later evolved by the Roman Empire [14].

The wine matrix has one of the most complex compositions, with more than 800-1000 volatile compounds ranging from alcohols, esters, acids, ketones, ethers, aldehydes, terpenes, lactones, phenolic compounds, etc., that arise from the grape and/or are formed during the fermentation process, with concentrations from a few parts per billion to a few percent in weight [14-17]. The abundance of these compounds depends on many factors: grape variety and origin, viticulture practices (blending, aging, yeast used during fermentation), production treatment, storage and oxygen exposure [17-19].

From the analytical point of view, wine analysis is a challenging task due to the complexity of the mixture and, moreover, minor differences in the concentration of certain compounds lead to wines with completely different organoleptic characteristics. Furthermore, the synergy between groups of compounds often has a stronger influence on the organoleptic characteristics than individual compounds [17].

Over the last few years, food control has become more appreciated by consumers. They have a right to expect that the foods they consume will be safe and of high quality. The term food

control includes a large number of factors such as: safety, setting standards for toxicological and microbiological hazards, implementing procedures and practices to ensure those standards; nutrition, ensuring the necessary nutrient levels and formulation in foods that contribute to a healthy diet; quality, which provides the expected sensory characteristics in foods, such as taste, aroma and/or appearance; and finally value, giving the consumers utility and economic advantages. Some of these factors, such as value, are exclusively in the domain of industry and consumers; whereas others, such as safety, are shared interests of government, industry and consumers [20].

Although there are several methods to determine quality parameters of interest in the wine industry, they are usually long, tedious and require very expensive laboratory equipment and qualified personnel. Moreover, the quality control that evaluates the organoleptic properties of wines, such as flavor, taste and color, is usually tested by a trained, expert human panel (wine-tasters) turning out a strong subjective element in the results that are influenced by their skills, mood and/or physical state [14]. Moreover, the number of tests that panelists can perform is limited to a few assessments per day, due to the saturation of tongue receptors. As time is a critical factor in the food industry, it is necessary to develop new, simple and fast analytic tools able to assess antioxidant activity and organoleptic properties, as well as the authentication of wines and grapes and geographic origin verification. These factors are becoming very important due to wine regulations, which include rules concerning the marketing, production, designations of origin and geographical indications, among others, becoming more restrictive than in the past with the final objective of protecting consumers in order to supply beverages and food that are safe, of good quality and exempt from fraud.

On the other hand, analytical tools for determining grape and wine characteristics are critical for oenologists. The standard analytical methods of alcoholic beverages, including wine, are based on traditional chemical analysis and on wet chemistry procedures or instrumental methods, involving such separation techniques as high-performance liquid chromatography (HPLC) or gas chromatography (GC) combined with mass spectrometry (MS), and such optical techniques as Fourier transform infrared spectroscopy (FTIR) [17,21-23].

A variety of new analytical tools are increasingly used to profile the volatile, non-volatile and elemental composition of grapes and wines in order to analyze aroma, taste, color, mouthfeel and even for authentication. Methods range from classical chemical analysis through to the use of modern analytical instrument techniques. For example, some improvements in standard

analytical tools that have been implemented are: gas chromatography combined with tandem mass spectrometry (GC-MS/MS), which offers significant advantages for trace quantification of aroma-active volatiles and contaminant compounds [24,25]; the ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) technique is a new approach in the chromatographic separations used for profiling non-volatiles and for varietal, geographic and vintage authentication purposes [26,27]; inductively coupled plasma mass spectrometry (ICP-MS) has been used to analyze metals that affect the chemical stability and oxidative reactions in wines [28]; nuclear magnetic resonance spectroscopy (NMR) has become popular for characterizing and profiling primary and secondary metabolites [29,30]; isotope ratio mass spectrometry (IRMS) is applied to detect the authentication of wines [31]; while, in connection with methods using IRMS, stable isotope ratio analysis (SIRA), based on the measurement of the ratios of the stable isotopes of carbon ($^{13}C/^{12}C$), hydrogen ($^{2}H/^{1}H$), and oxygen ($^{18}O/^{16}O$) within molecules and between different molecules, is one of the most powerful analytical tools for the authentication of wine [32,33]; and finally, gas chromatography combined with olfactometry (GC-O) for the characterization of aroma qualities of complex mixtures [34].

Table 1 collects the analytical methods, including botho classical and modern, traditionally used in the analytical chemistry of wine [24-37]. Although these techniques are sophisticated and provide accurate results, they only give information on individual wine components, but they cannot give a global assessment of the flavor or quality of wines that is produced by the synergistic interaction between several chemical compounds present in the complex chemical wine matrix [15,17]. Moreover, the equipment used is costly, large, requires calibration procedures, sample preparation, and the process is time-consuming and needs skilled and qualified personnel.

Table 1. Overview of classical chemical analysis and modern analytical instrument techniques usually applied to test wines.

Analysis	Compounds/feature	Analytical method
Characterization of wines	pH levels	pH meter
	Acid levels or tritatable acidity	- Classical titration (with an indicator or potentiometric titration) - Photometry
	Sugar levels	- Refractive index (RI) - Ion chromatography (IC) - FT-NIR, FT-IR
	Nitrogen	- Ammonia ion selective electrode - Enzymatic assay
	Ethanol	- Enzymatic assay - Gas chromatography (GC) - FT-NIR, FT-IR
	Glycerol	- Enzymatic assay - FT-NIR, FT-IR
	Acetaldehyde	- Enzymatic assay - Liquid chromatography (LC)
	Sulphite	- Distillation - Classical titration (iodometric or potentiometric) - Photometry - Flow Injection Analysis (FIA) - IC
	Organic acids	- Ion chromatography (IC)
	Metal contaminants (Cu, Zn, Fe)	- Atomic absorption spectroscopy (AAS) - ICP-MS
	Pesticides	- GC-MS - LC-MS/MS
Authentication of wine and fraud detection	Chaptalization (adding sugar to increase ethanol content)	SIRA (^{2}H/^{1}H-methyl and methylene in ethanol) (^{13}C in sugars/ethanol)
	Sugars (beet, cane and mixtures)	SIRA (^{2}H/^{1}H-methyl and methylene in ethanol (^{13}C in sugars/ethanol)
	Water (addition)	SIRA (^{18}O water ^{2}H/^{1}H-methylene in ethanol)
	Glycerol (synthetic or animal)	SIRA (^{13}C glycerol)
	Carbon dioxide (synthetic or fossil)	SIRA (^{13}C carbon dioxide)
	Tartaric acid (synthetic)	SIRA (^{13}C tartaric acid)
	Geographical origin mislabeling	- SIRA (^{2}H/^{1}H-methyl and methylene in ethanol (^{13}C ethanol ^{18}O water) - ^{1}H-NMR
	Vintage mislabeling	- SIRA (^{2}H/^{1}H-methyl and methylene in ethanol - SIRA (^{13}C ethanol ^{18}O water)

In order to reduce the cost and time-consumption of the previously described analytical procedures, electronic tongues (e-tongues) have emerged as a new analytical tool to assess a global, rapid and easy response with information concerning parameters related to the quality and organoleptic properties of grapes and wines, such as the phenolic content. E-tongues based on electrochemical sensors (potentiometric, amperometric, voltammetric or impedimetric) have been developed and used in food quality control [6,39-40]. In this sense, electrochemical techniques can lead to advantages in analytical chemistry due to their higher sensitivity and relatively low cost in comparison with the chromatography and spectroscopic methods, and they can be used as an alternative and/or complementary tool to the standard methods of analysis [41,42].

In addition, compared to conventional tasting panels, electronic tongues have the clear advantage that they can be applied in continuous-flow for quality control in industrial processes; whereas an expert taster cannot evaluate samples on a continuous basis. Table 2 shows the main advantages of electronic tongues over the use of tasting panels, traditional chemical analysis (gas or liquid chromatography, spectroscopy techniques, etc.) and modern analysis with high generation equipment (GC-MS/MS, UHPLC/Q-TOF-MS, ICP-MS, SIRA).

The development of new analytical tools includes the manufacture of portable devices conceived for use by wine farmers *in situ* in the vineyards. In this way, wine farmers could test their grapes with a simple, rapid experiment in the vineyard in order to decide the optimal point of maturity for harvesting grapes with no need to test samples in the oenological laboratory.

Finally, e-tongue technology has already been successfully applied to analyze such beverages as wines, beers and strong alcoholic beverages (spirits and liqueurs) as well as to evaluate the quality of non-alcoholic beverages [43-46].

Table 2. Advantages of electronic tongues over other methodologies.

	Electronic tongue	Classical chemical analysis	Modern analytical instrument techniques	Tasting panels
Simplicity	Yes	No	No	Yes
Design or acquisition costs	Low	Medium	High	High
Time consumption in measurements	Low	High	High	Medium
Need for highly qualified personnel	No	No	Yes	Yes
Contamination/ Destruction of the sample	No	Yes	Yes	Yes
Generation of waste	Low	High	Low	High
Possibility of on-site measurement	Yes	Yes	No	No
Possibility of incorporating the measuring system into production lines (e.g., food industry)	Yes	No	No	No

1.3. Overview of research

This research investigates the development of a quite new analytical tool, called electronic tongue, to analyze grapes and wines in terms of quality, vintage and/or geographical origin. The manufacture of new electrochemical sensors needs appropriate sensing materials able to interact with antioxidants, as well as making sensors with cross-selectivity and good stability in complex mixtures such as wines and grape musts. Therefore, two lines of research are adopted in this research. The first is basic research leading to the study of different sensing materials with interesting characteristics, such as electrocatalytic properties, to be used in the manufacture of new sensors, mainly based on thin films. The sensors were prepared by means of thin film manufacturing techniques such as *spin-coating*, electrodeposition and/or Layer by Layer. A range of sensing materials such as phthalocyanines, conducting polymers and nanoparticles are used in the construction of the electrochemical sensors. Enzymes have also been considered in this study to develop biosensors that will provide a certain specificity to the sensors.

In the second line of research, the as-manufactured sensors and biosensors were used to construct electronic and/or bioelectronic tongues to test standard solutions of interest in the food industry and to analyse real samples of wines and grapes. For this purpose, the study has counted on the support of important wineries that have provided wine and grape samples, as well as their chemical parameters analysed by means of standard methods. These collaborations have been of vital importance for this research, since it has been possible to establish correlations between the experimental data obtained with the electronic and/or bioelectronic

tongues and the chemical parameters provided by the wineries. For this purpose, a variety of multivariate data analyses have been applied. Princial component analysis (PCA) and parallel factor analysis (PARAFAC) have been used to discriminate between grape and wine samples, partial least squares (PLS-1) has been applied to construct linear regression models between the electrochemical data obtained with the electronic and bioelectronics tongues and the chemical parameters of interest of grapes and wines, such as the phenolic content, sugar content, alcohol degree, etc.; and finally, support vector machines (SVM) have been implemented to predict the quality parameters of wines from the data obtained in the grapes analyses.

In Chapter 3, an introduction to each paper is provided, discussing such topics as the advantages of the sensing materials used in this work, the techniques used to prepare the sensors, the sensitivity offered by the sensors and biosensors, the electrochemical technique used for the experimental acquisition of data, the expected results and the applied multivariate statistical analysis.

1.4. Research objectives

The research objectives proposed for the dissertation are:

1. To design and construct electronic and bioelectronic tongues specifically dedicated to quality analysis for wines and grapes using chemically modified electrochemical sensors.

2. To improve sensor performance by means of (a) new electrocatalytic materials, (b) sensors based on nanostructured films, (c) combining different electrocatalytic materials to increase the electrochemical capability of the sensors and (c) introducing enzymes to enhance sensor specificity to detect phenolic compounds and/or sugars.

3. To implement different surface modification techniques for sensing materials immobilization (e.g., electrodeposition, spin-coating and/or Layer by Layer) with the purpose of improving the sensitivity, reproducibility and/or life-time of sensors.

4. To apply different electrochemical techniques (e.g., voltammetric, impedimetric, electrochemical quartz crystal microbalance, etc.) to evaluate their versatility.

5. To discover ways to combine enzymes with nanomaterials, such as metal nanoparticles, using strong interactions.

6. To validate the electronic tongue as a tool for monitoring grape ripening from *véraison* to the optimal maturity date.

7. To implement the system for varietal authentication purposes in order to discriminate grape and red wine samples.

8. To establish correlations between the results obtained with the electronic and/or bioelectronic tongue in real samples of wines and grapes with the chemical parameters measured with traditional, standard analytical methods.

9. To evaluate the capability of the system to predict quality parameters of wines from the data obtained with the grapes used to elaborate wines.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction to electronic tongues

The main taste evaluation method in the food industry currently consists of a sensory analysis in which experienced evaluators, called sensory panelists, test the samples for evaluation [1-6]. However, in wine tastings, these analyses have some drawbacks, such as low objectivity and reproducibility, saturation of tongue receptors, as well as the high stress imposed on the panelists. Moreover, cross-mouth contamination between samples can compromise the validity of the sensory results [7-10].

A new sensory technology has been developed to evaluate the taste of food and beverages in an objective way. It is called the electronic tongue and it consists of a multisensor system of low selective sensors and uses advanced mathematical procedures for signal processing based on pattern recognition and/or multivariate analysis [11]. The concept of the electronic tongue was first developed by Toko, Vlasov and Winquist [12-14], although the first works concerning these systems were published mainly by Toko and Hayashi using the term *multichannel taste sensors* [15-17].

Electronic tongues are systems inspired to detect taste in similar manner to the human gustatory sensation [18]. The human tongue is equipped with different receptors (taste-buds) able to distinguish thousands of substances. The information on taste substances provided by the receptors is transduced into an electric signal and sensory nerves are responsible for transmitting it to the brain and then, our biological computer, trained with knowledge acquired earlier, is able to identify what we eat and also perceive information about the quantity of individual compounds (such as the amount of salt or sucrose). The performance of the taste system is attributed to the capability of taste-buds to respond to different stimuli, called cross-selectivity, and to the ability of analyzing the signal from all the tongue receptors at once. These two qualities (cross-selectivity and multivariate analysis) are the key concepts involving electronic tongue systems. In electronic tongues, sensors play the role of taste receptors and the brain is substituted by a computer equipped with appropriate data-processing techniques (Figure 1) [8].

As stated before, the IUPAC defines an electronic tongue as a *multisensor system, which consists of a number of low selective sensors and uses advanced mathematical procedures for signal processing based on the pattern recognition (PARC) and/or multivariate analysis [artificial neural networks (ANNs), principal component analysis (PCA), etc* [11]. In addition, many research groups working in this topic have summarized the definition of an electronic tongue as *an analytical instrument comprising an array of non-specific, poorly selective chemical sensors with*

partial specificity (cross sensitivity) to different compounds in a solution, coupled to an appropriate chemometric tool for data processing [19-21].
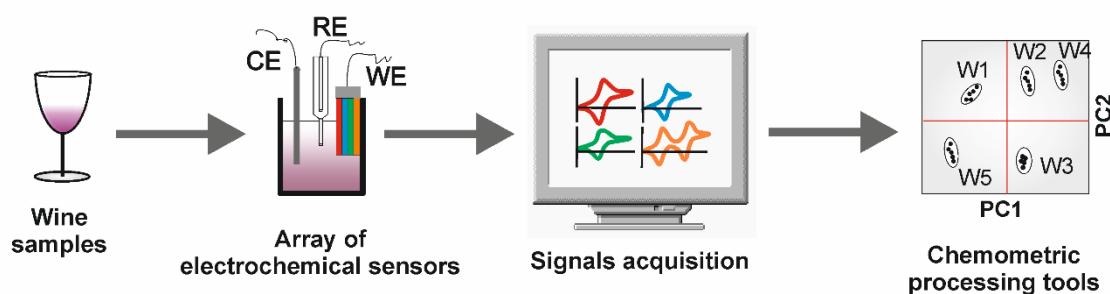
Figure 1. Schematic presentation of the electronic tongue system.

Although the concept of chemical sensor is generally used to detect a chemical substance at high sensitivity, human taste receptors do not necessarily recognize individual chemical substances. Each of the receptors for the five basic tastes simultaneously receives multiple chemical substances, showing a semi-selective response. It is practically impossible to measure the taste of foods containing hundreds of taste substances by chemical analysis methods, such as gas or liquid chromatography; although these techniques can measure the concentration of chemical substances. Therefore, in electronic tongues, sensors do not give the amounts of specific molecules that contribute to a certain taste, but rather the taste quality and intensity, in other words, a global evaluation of the analyzed matrix. Moreover, a taste sensor should be able to measure such interactions between taste substances as the synergistic effect and the suppression effect. The discrimination of each chemical substance is not an important task here, but recognition of the taste itself is, and its quantitative expression is necessary [8,11,13,18,19,22].

Arrays of sensors in electronic tongues combine non-specific chemical sensors with partial sensitivity that provides responses with cross-sensitivity. The difference in the electrical response of different sensors serves as a fingerprint for the analyzed sample. When combining these systems with pattern recognition software, we use the fingerprints of different samples to discriminate or classify them.

In the 1990s, while research was being carried out on the biological and molecular processes involved in taste receptors, sensitive technology was also being developed for objective evaluation in the discrimination and/or quantification of tastes, prior to the discoveries of taste receptors. Thus, in 1989, Toko and co-workers applied for a patent for their potentiometric taste sensor. They developed a taste sensor equipped in a multichannel electrode system using

an immobilized lipid membrane with a polymer as transducer [17]. This taste sensor is considered to be the first electronic tongue with global selectivity and has been applied to the analysis of numerous beverages such as beer, sake, coffee, tea or milk, among others [19].

After this first electronic tongue, and up to the present time, numerous papers have been published on this technology, mainly applied to the analysis of food and beverages, as it is considered a novel technology that can contribute greatly to quality purposes. Thus, in last few years, the design and application of these new systems has become very popular. One of the most important research groups in this field, which can also be considered as a pioneer in the design of electronic tongues, is Winquist and co-workers. In 1997, Winquist and Lundström developed an electronic tongue based on voltammetric techniques for the analysis of fruit juices, beverages and milk. They were able to detect the aging process of milk and orange juice with the developed system [14,23]. Following this work, in 2000, they combined potentiometry, voltammetry and conductivity measurements to produce a hybrid electronic tongue for the analysis of fermented milk [24]. Selective ion electrodes were used to measure pH, carbon dioxide and chlorine ion concentration. The electronic tongue consisted of six working electrodes of different metals. In 2002, Winquist and collaborators developed a measurement system based on the flow injection analysis technique applied to a voltammetric electronic tongue for the analysis of different standard solutions and for the classification of different apple juices [25].

Another research group dedicated to the design of electronic tongues since the 1990s is led by Prof. Vlasov, who together with the group of Prof. Di Natale and Paolesse, have made important advances in the development of potentiometric electronic tongues. In 1997, this group applied solid-state selective ion potentiometric sensors based on chalcogenide glass in an electronic tongue to detect heavy metal ions in solutions [26], in the analysis of beverages (tea, beer, coffee, juices, soft drinks, etc.) [27] and polluted waters [28]. Later, between 2003 and 2007, they published some works in which these electrodes were applied to the analysis of mineral waters, wines and alcoholic beverages [29,30].

One of the first works based on a voltammetric electronic tongue based on chemically modified electrodes was developed in 2003-2004 in the Laboratory of Inorganic Chemistry and Physical Chemistry of the University of Valladolid, directed by Prof. Rodríguez-Méndez. This electronic tongue was based on sensors prepared with graphite powder mixed with phthalocyanine and it was applied to the analysis of wine [31,32]. There are currently several international research groups of experts in electronic tongues that have made important

advances in the design and application of these systems in the field of food and beverages [33-50].

In the case of the wine industry, it is important to monitor changes occurring during grape ripening, wine fermentation and aging to ensure the quality of the wines, to assess the origin and authentication and to avoid falsifications or adulterations. Traditional techniques for analysing food and beverages, as well as human tasting panels, have some drawbacks and need new analytical tools. In this sense, electronic tongues are reliable and reproducible analytical tools, which have demonstrated their utility for the rapid and inexpensive assessment of different chemical matrices, including wines.

2.2. Principles of detection: Electrochemical methods

The heart of an electronic tongue is the array of sensors which captures physical or chemical signals from its surroundings and converts them into electrical signals. The sensors used in electronic tongues are mainly optical, electrochemical and gravimetric. In food analysis applications, the most commonly used are the electrochemical sensors (potentiometric, amperometric, voltammetric and impedimetric), due to their simple instrumentation, high sensitivity, fast response and ease of operation [51-53].

In *potentiometric sensors*, the voltage is measured at null current. A working electrode covered with a membrane is introduced into the sample to be analyzed. The potential is created at the membrane/solution interface, which is needed to retain the balance of the electrochemical process and depends on the nature of the electrode material and the composition of the solution. The advantages of potentiometric electronic tongues are the well-known principle of operation, low cost, ease of commercial production and possibility of obtaining selective sensors. Their disadvantages are the influence of the temperature on the measurements and the adsorption of solution components on the electrode surfaces that can modify the potentials. These sensors can be prepared from different materials, membranes and techniques [22,53,54].

In *voltammetric/amperometric sensors*, the measurements are based on the electric current established between the working electrode and the reference electrode in an electrochemical cell. In voltammetric sensors, a polarization voltage is applied while the current is measured. When a potential change occurs, the current flows through the working electrode, thus oxidizing or reducing the compound under analysis. The response current is the result of the

electrochemical reaction that occurs at the electrode/electrolyte interface layer. Voltamograms show peaks associated with the oxidation and reduction of electroactive species from the solution, and the intensity is a function of the analyte concentration. Moreover, voltammograms can show peaks associated to the electroactive material deposited on the electrode surface. In amperometric sensors, voltammetry is applied as a preliminary step to study the oxidation potentials of the analyte, and once established, amperometric measurements are carried out to measure the electrical current between the electrodes as a function of a constant potential applied to the working electrode. The current intensity signal from the oxidation or reduction of the substance analyzed is proportional to its concentration. These sensors are sensitive, very versatile and different materials can be used in the electrodes for sensing. Moreover, using voltammetric sensors, different techniques can be applied (cyclic voltammetry, linear sweep voltammetry, differential pulse voltammetry or square-wave voltammetry) [53,55-57]. The main disadvantage of such sensors is the lack of selectivity.

The ***impedimetric sensors***, which are considered part of the group of electrochemical methods, are based on impedance spectroscopy measurements. The main advantage over the previous sensors is that there is no need for a reference electrode. In this method, the impedance is measured at one constant frequency or for a frequency spectrum. This system can be described by an equivalent electrical circuit, in which the electrostatic double-layer formed at the electrode/electrolyte interface governs the response at low frequencies, the solution conductance and ultrathin films coating the electrodes rule the total impedance at intermediate frequencies and the geometric capacitance is most relevant at high frequencies. The advantage of these sensors is their high sensitivity [22,53]. They could be employed in an electronic tongue; however, in practice, the works reporting impedimetric electronic tongues are quite rare compared with potentiometric, amperometric or voltammeric systems.

Table 3 shows some examples of electrochemical electronic tongues based on potentiometric, amperometric, voltammetric and impedimetric sensors applied to food analysis. Hybrid systems have also been developed, mainly combining voltammetric and potentiometric sensors.

Table 3. Examples of electrochemical electronic tongues applied to food analysis.

Application	Sample	Type of e-tongue	Reference
Food process monitoring	Red wine	Potentiometric Voltammetric	58-60
	Beer	Potentiometric Voltammetric	46, 61
	Milk	Potentiometric Voltammetric	62,63
	Cheese	Potentiometric	64
Food quality and freshness	Meat	Potentiometric	37
	Fish	Potentiometric Voltammetric	65,66,67
	Non-alcoholic beverages	Potentiometric	48
	Milk	Voltammetric	68
	Rice	Potentiometric	69
	Olive oils	Voltammetric	33
	Tea	Voltammetric	34
	Apples	Hybrid	49
Authentication	Red wines	Voltammetric Potentiometric Impedimetric	70-72
	Beer	Potentiometric Voltammetric Hybrid	50,73-75
	Alcoholic beverages	Potentiometric Voltammetric	30,76,77
	Milk	Potentiometric Voltammetric	78
	Yogurt	Voltammetric	80
	Honey	Potentiometric	81,82
	Drinking water	Potentiometric	42
	Juices	Potentiometric Voltammetric Hybrid	14,38,83
	Tea	Potentiometric Voltammetric	84,85
	Olive oils	Voltammetric	86
	Apples	Potentiometric	87

2.3. Distinct materials for the sensing units

Sensing materials used for electrochemical sensors can be classified as: 1) materials for the electrode and supporting substrate, 2) materials for improving electroanalytical performances, 3) materials for the immobilization of biological recognition elements and 4) biological elements, the last two being applicable to electrochemical biosensors. Sensing units of traditional electronic tongues are mainly lipid membranes, ion-selective electrodes or noble metals functioning as working electrodes, with the voltage difference measured between the sensing unit and a reference electrode [22]. The choice of materials for the sensing units is crucial to obtain a high performance. Even though e-tongues do not require specific interactions with the analyte, the sensing units need to respond electrically to small changes in the liquid under analysis. Moreover, depending on the method of detection, materials with electrical conductivity and/or electroactivity might be required. Materials used for the electrode and supporting substrate are usually conductive materials exhibiting low currents in an electrolyte solution, free of any electroactive species, over a relatively wide potential window. Although these sensing materials play different roles in different electrochemical sensing systems, depending on their properties, their basic functions are mainly grouped as: 1) enhancement of electron transfer, 2) catalysis of electrochemical reactions, 3) immobilization of biomolecules, 4) labelling biomolecules and 5) acting as reactant.

Lipid membranes were one of the first class of materials used in potentiometric sensors for e-tongues to mimic the materials of the human tongue [13]. In addition, chalcogenide glasses were largely used in electrochemical measurements due to the ease of electrode preparation and cross-sensitivity [28]. However, due to the extensive use of electrochemical methods, research began to explore electroactive materials. Among the most frequently used materials for the electrode and supporting substrate, the following may be mentioned: phthalocyanines, porphyrins, metal nanoparticles, metal oxide nanoparticles, carbon-based materials and conducting polymers.

In recent years, **conducting polymers** (CPs), which consist of **organic π-conjugated** systems, have been employed in electrochemical sensors due to their light weight, workability, resistance to corrosion, low cost and excellent electrical, mechanical, optical and conducting properties [88,89]. CPs are generally semi-conductive in nature and have low conductivity, but can be made more conductive by doping. This means that the oxidation and reduction of a π-conjugated system generates p- and n-doped CPs. The doping process can be controlled by

chemical and electrochemical methods. Therefore, to overcome their low conductivity, composites of CPs have extensively been studied and applied in electrochemical applications [88]. Conducting polymers such as polypyrrole, polyaniline, PEDOT, or polythiophene have been used as sensing materials to construct electrochemical sensors [90-95].

Other sensing materials for electrochemical sensors are *metallophthalocyanines* (MPcs) and *metalloporphyrins* (MPs), which are organometallic compounds, also known as N4-macrocyclic metal complexes. They contain N4-ligands that co-ordinate to the metal ions. In their nature, the N4-ligands of phthalocyanine and porphyrin are 18-π conjugated ring systems with a highly conjugated structure. Metal ions are from the transition metals, group I-V metals, lanthanides and actinides. These MPc and MP complexes have a very rich electrochemistry owing to their ability to accept and donate electrons, either from central metal ions or 18-π-conjugated ring systems [96,97]. Phthalocyanines and porphyrins have been extensively used as sensing materials in electrochemical sensors due to their remarkable electrochemical and electrocatalytic properties [98-105].

Important advances have been made recently in the field of sensors thanks to the introduction of new platforms for sensors design, such as nanotechnological materials and nanostructured architectures (i.e., nanoparticles, carbon nanotubes and nanofibres, graphenes, nanostructured surfaces, etc.), which have improved their sensitivity. Many kinds of *nanoparticles*, including *metal nanoparticles*, *oxide nanoparticles*, *semiconductor nanoparticles* and even *composite nanoparticles* have been widely used in electrochemical sensors and biosensors. These nanoparticles exhibit unique optical, electrical, thermal and catalytic properties. Nanoparticles with different sizes and compositions are now available for electroanalysis applications. Metal nanoparticles have shown excellent conductivity and catalytic properties, which make them suitable for acting as "electronic wires" to enhance the electron transfer between redox centers in proteins and electrode surfaces and as catalysts to increase electrochemical reactions. Oxide nanoparticles are often used to immobilize biomolecules due to their biocompatibility while semiconductor nanoparticles are often used as labels or tracers for electrochemical analysis [106-110]. The use of nanoparticles applied to electrochemical sensors has been widely reported [106-114].

The combination of more than one sensing material to make *composite materials* can show excellent sensing and catalytic properties. The advantage of the synergistic interaction between different sensing materials produces a higher performance of electrochemical sensors.

This effect has been observed in the interaction between gold nanoparticles and nickel phthalocyanine [115]. A synergetic effect between silver-gold nanoparticles and zinc phthalocyanine has also been demonstrated in the electrocatalytic reduction of O_2 on vitreous carbon electrodes [116]. Gold nanoparticles-ZnO composite is one of the most widely discussed electrocatalytic materials with high catalytic activity, optical sensitivity, universal biocompatibility and high chemical stability [117]. Pt nanoparticles and PANI aero-gel have demonstrated a synergetic effect that results in the immobilization of a high density of GOx for an efficient oxidation of glucose [118].

Biochemical sensors are currently being successfully introduced into electronic tongue technology (bioelectronic tongues) by adding *enzymes* as a biorecognition element to the electrode surfaces. The biorecognition elements are classified into biological (enzymes, whole cells, cell organelles, tissue) and artificial (biomimetic) receptors (aptamers, ribosymes and moleculary imprinted polymers (MIPs)) [119]. Biosensors based on enzymes combine the advantages of classical electrochemical sensors, which provide high sensitivity and cross-selectivity, with the typical specificity of the biosensors [120,121]. In these biosensors, the selectivity for the target analyte is mainly determined by the biorecognition element, whereas the sensitivity of the biosensor is greatly influenced by the transducer [119]. Therefore, the electron transfer between the active sites of the enzyme and the electrode surface can be improved through an electronic mediator, such as the above mentioned sensing materials, acting as an electrical wire. Specific enzymes for the detection of phenols (e.g., tyrosinase, laccase or peroxidase) or sugars (e.g., glucose oxidase or fructose dehydrogenase) among others, have been incorporated into bioelectronic tongues [44,106,107,109,119-122].

2.4. Sensor and biosensor construction

Electrochemical sensors can be chemically modified with sensing materials by means of several techniques to improve their electroanalytical performance. E-tongue technology covers different types of sensors and biosensors, from classical sensors prepared by means of simple techniques (carbon paste electrodes, casting, electrodeposition, etc.) [31,32,123-127] to sophisticated and costly nanostructured sensors, including techniques of preparation such as self-assembled monolayer (SAM), Layer by Layer (LbL) or Langmuir-Blodgett (LB) [128-134].

Therefore, the major challenge in the field of electrochemical sensors, consisting in the improvement of their electroanalytical performance, as already mentioned, was addressed by using nanotechnology, which includes not only the use of sensing nanomaterials, such as nanoparticles, carbon nanotubes or graphene, providing higher electrocatalytic properties, but also the preparation of nanostructured architectures, where sensing materials can be deposited as nanostructured thin films. Sensors based on thin films at the nanoscale show high surface-to-volume ratio and small dimensions. The high surface ratio facilitates the interaction between the sensing material and the analyte, which leads to improvements in the sensitivity, and the small dimensions enable fast adsorption and desorption kinetics for the analyte in the sensing material and, therefore, providing a rapid response time [135].

Classical methods

Carbon paste-based methods: sensors are prepared by mixing a carbon-based material (graphite, carbon nanotubes, graphene, carbon nanoparticles) with the sensing material and a mineral oil. The carbon matrix acts as a support to prevent agglomeration and provides a large surface area, high mechanical strength, fast electron transfer rate, excellent thermal and electrical conductivities, which in turn enhance the electrocatalytic activity. These sensors are cheap, easy to prepare and have the great advantage that they can be easily recovered by cleaning the surface with a filter paper [31,32].

Coating-based methods: drop-casting, dip-coating or spin-coating are the most commonly used coating methods for modifying electrodes with nanomaterials. These methods are easy and low-cost in sensor manufacture and do not require complicated instruments. However, only soluble or solution-processible nanomaterials can be deposited onto an electrode surface [136].

Direct deposition-based methods: these methods are diverse, and include electrochemical, electrospinning, electrospray, sputtering and vapor deposition. Electrochemical deposition is one of the most used methods to deposit thin-films of polymers or polymer composites onto conducting electrode surfaces. This technique can be performed at room temperature and is carried out using three electrodes dipped into the solution at a potential (or current, depending on the method used) that is applied to polymerize the monomer and/or reduce metal ions in the simultaneous preparation of polymer composites with metal nanoparticles [136].

Printing-based methods: these methods include the well-known screen-printed method as well as newly developed methods, such as inkjet printing, nozzle-jet printing or the laser-scribing process. Printing-based methods have been used to deposit materials onto rigid and flexible substrates to make devices on a large-scale at low cost. One advantage of these methods is that they allow nanomaterials to be desposited directly onto predesigned patterns [136].

Methods based on nanostructured architectures

Self-assembled monolayer (SAM): this technique is used to form monomolecular films by the spontaneous adsorption of amphiphilic adsorbates in an orderly manner onto a solid support. It has been widely used to coat electrode surfaces. The initial driving force for the assembly is the chemical affinity between the adsorbates and the substrate. In the initial stage of SAMs, mainly alkyl compounds, such as alkylated thiols and silanols were used to construct well-organized SAM structures. However, there are various functional groups that can be used to generate SAMs [137].

Langmuir-Blodgett (LB): these films are formed by the deposition of a Langmuir film onto a solid substrate. Langmuir films are composed of an organized monolayer of amphiphilic molecules at a liquid/gas interface. These molecules with their hydrophobic tail and hydrophilic head are spontaneously oriented at a liquid/gas interface. Unlike the SAM technique, the LB method can provide multi-layered film structures with defined thickness and sequence, together with well-ordered molecular arrangement and orientation [137].

Layer by Layer (LbL): these films are formed by depositing onto a substrate alternating layers of oppositely charged materials. This technique has been given much attention as a versatile method for nanostructured films. Most of the LbL assemblies are based on electrostatic interactions between the layered materials. However, LbL assemblies can be driven by other interactions, such as hydrogen bonding, metal interactions, charge transfer complexation or bio-specific recognition, among others. A wide range of materials can be applied in the assembling process, such as polymers, biomaterials, supramolecular assemblies and inorganic substances. This method only requires the use of beakers and tweezers in the procedure [137].

Methods used for biosensor construction

The immobilization of enzymes to construct biosensors depends on the materials and procedure used and can include: 1) entrapment behind a permeable membrane as a thin film

covering the electrochemical detector; 2) entrapment within a conducting polymeric matrix or sol-gel; 3) entrapment within self-assembled monolayers or within LbL layers; 4) immobilization onto an LB structure; 5) covalent bonding on membranes or surfaces activated by means of bifunctional groups or spacers (glutaraldehyde, carbodiimide, silanization, etc.); 6) bulk modification of the electrode material (graphite epoxy resin or modified carbon paste, clays, zeolites, nanomaterials, organic polymers, complex compounds, etc.) [44, 101, 112, 122, 137-141].

2.5. Methods of data analysis

The correct utilization of e-tongues requires an appropriate data analysis. The main task of these systems is to classify the samples under study [8,22,53]. As the number of samples may be very large and many measurements are needed to distinguish between very similar samples – whose variability may also be considerable for complex liquids such as wines, juices, etc.– an enormous and complex amount of data is generated that must be processed using chemometric or pattern recognition methods. Four stages can be distinguished during data analysis: signal pre-processing, dimensionality reduction, prediction and validation [8]. Data pre-processing is carried out mainly to scale the data, discard outliers and extract representative parameters. Subsequently, a dimensionality reduction of variables is needed in many cases, for example when using cyclic voltammetry, since the information obtained is large and complex. Then, one solution is to simplify the high dimensionality, maintaining the meaningful information from the data and suppressing redundant and non-significant information without loss of information by using a feature extraction stage, such as Legendre polynomials, Fourier transform, kernel method, wavelet transformation or genetic algorithms. Depending on the type of application, different methods for dimensionality reduction can be applied. The most used process in the literature for e-tongues is principal component analysis (PCA), a non-supervised method used to discriminate between samples with different characteristics. PCA plots can be obtained in 2 or 3 dimensions (2D or 3D), depending on the need to distinguish the samples. This tool reduces the number of variables to new latent variables (principal components) in a reduced variable space to facilitate identification or classification. In addition, this new space simplifies the interpretation of the variability contained in the raw information. Therefore, PCA is a very powerful non-supervised linear pattern recognition method that reduces the dimensionality of multivariate data and helps to

visualize the different categories of multivariate profiles with high similarities and differences between sample clusters.

When the number of classes of samples is very large, for instance if one wishes to distinguish between a set of dozens of wines, the amount of points placed leads to overcrowding the 2D or 3D PCA plot. Hence, other methods to treat the data are required [22]. In this context, methods involving artificial neural networks (ANNs), parallel factor analysis (PARAFAC), soft independent modelling of class analogy (SIMCA), linear discriminant analysis (LDA) or support vector machines (SVM) are used.

The low dimensional feature may also be used for prediction and/or regression purposes, where the sensors respond as independent variables and a second set of variables (e.g., chemical parameters of the samples) as dependent variables are used to establish regression models that will be able to predict and validate some parameters of the analyzed samples. These models are usually established using partial least squares (PLS), principal component regression (PCR) or support vector machines regression (SVMR), among others.

2.6. Summary of the applications

Electronic tongues have several applications in various industrial areas, mainly in the pharmaceutical and food industry. In the pharmaceutical industry, it has been widely used to quantify the taste-making efficiency of medicines, as well as to analyze the stability of medicines regarding taste [142,143]. In addition, e-tongues have also been applied to the field of medicine for non-invasive diagnostics (urine, sweat, saliva, etc.) or clinical analysis [144,145], and in the field of the environment to analyze water contamination [146,147].

In the food industry, this new technology has been used in quality control during production, in food storage stages, in the control of aging processes, in the analysis of tastes, to evaluate authentication, to identify brand and/or geographical origin, or to detect adulterations (see Table 3).

Given the advantages and numerous applications of electronic tongue technology, many efforts have been made to make miniaturized and commercial prototypes of electronic tongue devices at a low cost. In this field, the main issues to overcome are miniaturization and the integration with existing silicon technology for microelectronics [22]. The first commercial

electronic tongues, SA402B and TS-5000Z Taste Sensing Systems, based on 7 potentiometric electrodes with lipid-polymeric membranes, were developed by Toko and co-workers (Intelligent Sensor Technology Inc., Japan) [13,19]. These devices have been used to determine and quantify the intensity of taste in food products and pharmaceuticals [148-152].

Another commercial e-tongue is Astree II (Alpha MOS, France), which is based on 7 ion-selective field effect transistors (ISFETs) and has been applied to quality control, food recognition, taste assessment, process monitoring and the pharmaceutical industry [152-156]. Other existing commercial systems, although their applications are rather scarce compared with those already mentioned, are: the Multiarray Chemical Sensor (McScience Inc., Korea) built of PVC and polyurethane membranes selective to ions (H^+, Na^+, Ca^{2+}, NH_4^+, NO_3^-, Cl^-) [157] and Sensor System (St. Petersburg, Russia) comprising 7 potentiometric ion-selective sensors [158].

2.6.1 Electronic tongues in the wine industry

The wine production process, as shown, comprises a series of steps that need to be carefully managed and for which continuous checking and measurements are required. Electronic tongues have been applied to the study of grapes, musts and wines in different stages of the winemaking process.

The chemical composition of wines has a direct influence on their organoleptic properties. Each wine has a different chemical composition that depends on the variety and state of ripeness of the grapes, the extraction of different components in the must and the reactions that occur during the winemaking process, the post-fermentation treatment and the aging of wine. The ripeness and quality of the grapes used to elaborate wines is usually established on their external appearance or the taste of the grape juices. The analysis of sugar and phenolic content is also a common practice to establish the quality [20,159].

Electronic tongues have been applied to discriminate between grapes according to their variety and vintage [44]. After crushing, during maceration, the contact between the must and
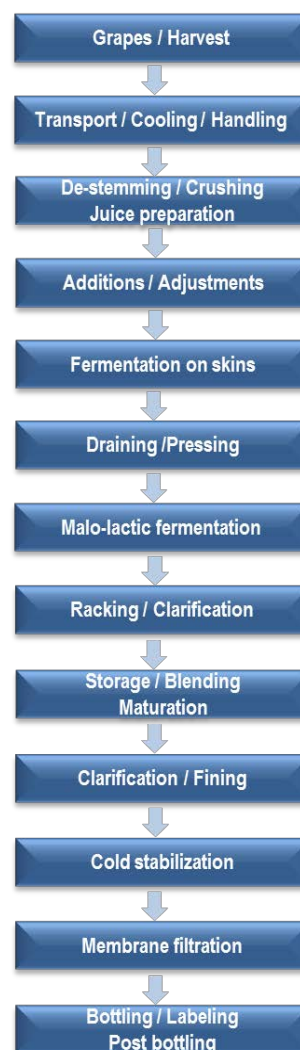
the skins increases the concentration of phenols. Moreover, by applying different pressing techniques, the extraction of phenols can be increased. E-tongues have been used to study the increase in phenol concentration after using Flash Release and micro-oxygenation pressing techniques [160].

The fermentation of fresh grape juice or must transforms sugars into alcohols, producing wine. In white wines, only one fermentation is needed, which is the alcoholic fermentation producing ethanol, whilst red wines need two fermentation processes, the alcoholic fermentation followed by the malolactic fermentation, where malic acid is transformed into lactic acid. Here, the control by means of chemical sensors is quite difficult, because fermentation is a turbulent process that produces changes in temperature that affect the performance of the sensors. For this reason, only a few papers have described the use of e-tongues to monitor wine fermentation. Using infrared spectroscopy, combined with an e-nose and e-tongue, the kinetics of the fermentation process of musts has been analyzed and acceptable correlations with sugar consumption and alcohol production have been found [35].

After the fermentation of the grape must, the final product, the wine, is obtained. It is then aged in oak barrels for 1-2 years before bottling. This stage has a great influence on the organoleptic characteristics that wine acquires, influenced by the retention and release of volatile compounds from the lees during ageing. The oxygen that diffuses through the small pores of the barrels, the origin of the oak wood and its degree of toasting also influence the final taste of the wine. Subsequently, the wine continues to be aged in bottles in a reducing environment that further improves its organoleptic properties. Electronic tongues have been applied to discriminate red wines according to their ageing [161] and the origin of oak barrels and toasting level [58,162].

In addition, as the ageing stage in barrels requires a lot of time and high costs, innovative techniques are applied to accelerate the ageing. One of these techniques consists of adding pieces of wood of different sizes in stainless steel tanks with built-in micro-oxygenation. An electronic tongue has been applied to discriminate between wines aged by traditional techniques and wines aged with alternative practices [60].

After ageing, the wines are bottled. Traditionally, the bottles are sealed with stoppers made of natural cork, ideal for sealing the liquid while allowing oxygenation from the outside through small pores. Traditional natural cork stoppers are associated with high quality wines. Nowadays, the wine industry uses synthetic stoppers, made of polymers, with a degree of

porosity. The effect of oxygen transfer in synthetic closures has been studied using an electronic tongue [59, 160].

The organoleptic properties of final wines produced with different varieties of grape, vintage, appellation, etc., have been evaluated with e-tongues. The grapes used to make wine will determine both the taste and the final organoleptic characteristics of the product. There are many grape varieties and each winery uses different oenological techniques to produce different styles of wine with different flavours and characteristics. An electronic tongue has been used to discriminate wines with different Spanish appellations (Rioja, Rueda, Ribera de Duero) [31] and, in addition, this e-tongue was also used to determine differences in these wines from vintage to vintage [57]. Bioelectronic tongues have also been used to analyze cava wines [163] or to discriminate grape juices prepared with different varieties of grape [44].

Weather conditions or improper storage, among other variables, can spoil the final wine. E-tongues have been used to monitor the spoilage of wines by monitoring their acidity levels [164].

Finally, e-tongues have been used to evaluate possible adulterations or frauds in wines [70,165]. Some adulteration practices consist in dilution with water, blending with wine of lower quality or made with grapes from a different region, the use of prohibited ageing techniques and the addition of ethanol or other substances (tartaric acid, tannic acid, SO_2, acetic acid or sucrose).

2.7. References

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CHAPTER 3

RESULTS AND DISCUSSION

3.1. Introduction

In this chapter, a selected number of papers published in international journals as a result of the research work carried out during the doctoral studies in the group UVaSens from the University of Valladolid are presented. The papers appear as follows:

Paper I: *Multisensor systems based on phthalocyanines for monitoring the quality of grapes.*

Paper II: *Monitoring the phenolic ripening of red grapes using a multisensor system based on metal-oxide nanoparticles.*

Paper III: *A different approach for the analysis of grapes: Using the skin as sensing element.*

Paper IV: *Electrochemical behavior of polypyrrol/AuNP composites deposited by different electrochemical methods: sensing properties towards catechol.*

Paper V: *Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator.*

Paper VI: *Layered composites of PEDOT/PSS/nanoparticles and PEDOT/PSS/phthalocyanines as electron mediators for sensors and biosensors.*

Paper VII: *Impedimetric electronic tongue based on nanocomposites for the analysis of red wines. Improving the variable selection method.*

Paper VIII: *An electrochemical quartz crystal microbalance multisensor system based on phthalocyanine nanostructured films: Discrimination of musts.*

Paper IX: *In situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological applications.*

UVaSens is an expert multidisciplinary research group in the development of electrochemical sensors, mainly voltammetric, for their application in electronic tongues. Its research work includes the manufacture and characterization of classical sensors based on CPE, spin-coating or casting, but also the preparation of nanostructured thin films using Layer by Layer or Langmuir-Blodgett techniques. In addition, for the construction of the sensors, the group has widely used sensing electrocatalytic materials, such as phthalocyanines and bisphthalocyanines, in both nanostructured and non-nanostructured sensors. The group has also worked with other

interesting materials in the manufacture of sensors, such as conducting polymers, when they began to be applied in electrochemical sensors. The UVaSens group was one of the first to develop modified voltammetric electronic tongues based on CPE and phthalocyanines to improve the electrochemical responses. Thus, paper I, *"Multisensor systems based on phthalocyanines for monitoring the quality of grapes"*, is a review article that gives a general overview of electronic tongues based on phthalocyanines for the analysis of grapes in which the use of non-nanostructured and nanostructured sensors and biosensors is discussed.

Research papers II to VII are based on electrochemical sensors prepared by classical techniques, such as CPE, electrodeposition and spin-coating; whereas, in papers VIII and IX, we have applied nanoscience and nanotechnology, concretely, the LbL technology.

In papers II and III, we have used sensors based on CPE which are easy to prepare and are low cost. In paper II, *"Monitoring the phenolic ripening of red grapes using a multisensor system based on metal-oxide nanoparticles"*, graphite was mixed with different metal oxide nanoparticles to construct an array of sensors. This array, containing metal oxide nanoparticles, has been used as a voltammetric electronic tongue for the first time and was applied to monitoring grape ripeness according to their phenolic maturity. In addition, the voltammetric responses obtained with this electronic tongue have shown good correlations with the phenolic content parameter of the grapes. Moreover, these sensors have the advantage that they can easily be recovered by peeling the surface with a filter paper, which makes them highly appropriate for industrial applications.

In paper III, *"A different approach for the analysis of grapes: Using the skin as sensing element"*, we have developed an original work never proposed before, in which the sensing material is the grape skin itself. Thus, the skin was deposited onto a CPE electrode with an intimate contact between the graphite and the inner side of the skin. Considering that grape skin contains phenols that are released to the pulp during the ripening process and that these influence the color, sensory sensations and quality of both the grapes and future wines, by analysing the skin, we can study grape ripeness directly. However, some issues must be improved and taken into account in order to get better correlations between the sensor responses and the chemical parameters obtained by traditional methods.

Papers IV to VII are based on sensors modified with well-known conducting polymers. In paper IV, *"Electrochemical behavior of polypyrrol/AuNP composites deposited by different electrochemical methods: sensing properties towards catechol"*, we have electrodeposited

a film of polypyrrole onto platinum and stainless steel substrates and also incorporated AuNPs in the polymeric films to enhance the electrocatalytic performance of the electrochemical sensors. Sensors as-prepared were characterized by using SEM, EIS and cyclic voltammetry. Then, they were applied to the analysis of the polyphenol catechol, which is a phenolic group of interest in the oxidation process in wine. These sensors were used later in paper V, "*Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator*", to develop a voltammetric electronic tongue for the first time to analyse must and the subsequent wines. Furthermore, in this work, we used two enzymes, tyrosinase and glucose oxidase, to improve the specificity of the sensors to phenols and sugars. The enzymes were deposited onto the electrode surface by means of the drop-casting technique. The novelty of this work lies in the fact that, never before, have the quality parameters of interest in wines been predicted from the previously obtained voltammetric data in musts. Therefore, oenologists could know the quality of their wines from the beginning of the vinification process.

Papers VI and VII are based on the highly electrical conducting polymer PEDOT:PSS. Thus, in paper VI, "*Layered composites of PEDOT/PSS/nanoparticles and PEDOT/PSS/phthalocyanines as electron mediators for sensors and biosensors*", we have used the spin-coating technique to prepare ITO substrates modified with a polymer layer of PEDOT:PSS and we have also incorporated a secondary layer of electrocatalytic material, such as AuNP, copper phthalocyanine and lutetium bisphthalocyanine to improve the electrochemical performance of the sensors. In addition, the enzymes tyrosinase and laccase were deposited onto the electrode surface by drop-casting to improve the specificity. These sensors and biosensors have shown excellent limits of detection and reproducibility for catechol and hydroquinone. After these excellent results, in paper VII, "*Impedimetric electronic tongue based on nanocomposites for the analysis of red wines. Improving the variable selection method*", we decided to apply electrochemical sensors based on PEDOT:PSS in an impedimetric electronic tongue for the authentication of wines elaborated with different varieties of grape and from different Denominations of Origin. This application is quite novel, since only one work has reported the use of an impedimetric tongue to analyze red wines and, in general, the use of impedimetric electronic tongues for food authenticity is rather scarce. Moreover, in this work, we used different strategies to find the best variables for further chemometric analysis, including the classical method of measuring resistance and capacitance data at a fixed

frequency, selecting parameters from the electrochemical impedance spectroscope analysis, and using the parameters from an equivalent electrical circuit model.

Finally, papers VIII and IX are based on the LbL technology. In paper VIII, "*An electrochemical quartz crystal microbalance multisensor system based on phthalocyanine nanostructured films: Discrimination of musts*", nanostructured LbL films based on phtahlocyanines were deposited onto QCM substrates to be used as voltammetric EQCM sensors. This electronic tongue was used for the first time in the food industry recording voltammetric data and mass changes simultaneously. Musts of grapes of different varieties were analyzed and discriminated according to their sugar and phenolic content. In the field of food, a piezoelectric immunosensor based on QCM has been developed to determine pesticide residues and metabolites in fruit juices; however, most applications for QCM sensors are in electronic noses for the analysis of volatile organic compounds (VOCs), as piezoelectric immunosensors for the recognition of biomolecular interactions and as gravimetric sensors in several electrochemical studies (EQCM). The main advantage of EQCM sensors over traditional electrochemical sensors is that they provide more information (mass and electrochemical) in a single experiment. Moreover, in comparison with the works mentioned above, sensors based on LbL nanostructures are more sophisticated and provide a higher surface area to volume ratio, but are still easy to prepare and inexpensive.

In the last paper, "*In situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological applications*", the LbL technique has also been used although with a dissimilar purpose. In this case, LbL was applied to prepare a nanostructured carrier system based on AuNPs, where the enzyme lysozyme has been immobilized using stronger interactions. This work was developed at the Missouri State University during a short research stay of 4 months. Although this work is not based on the development of sensors to be applied in electronic tongues or even in the food industry, it can be extended to that field, since the reported nanostructured assembly of the enzyme can be performed for others of interest in the food industry, such as tyrosinase, laccase or GOx, which have already been used in the works mentioned above and with the possibility of creating many designs thanks to the versatility of the LbL technique.

3.2. Paper I

Multisensor systems based on phthalocyanines for monitoring the quality of grapes.

By María Luz Rodríguez-Méndez, Celia García-Hernández, Cristina Medina-Plaza, Cristina García-Cabezón and José Antonio de Saja.

3.2.1. Motivation and objective

In last few years, the control of phenolic maturity in grapes has become more essential for quality control purposes. Traditionally, wine growers have estimated the optimal harvesting date by evaluating the sugar content and acidity of grapes, but other compounds like phenols are also as important as sugars and acids present in grapes, due to the fact that phenolic composition will affect the final organoleptic characteristics of the wines.

The use of quick, reliable and cheap sensors for monitoring the quality of grapes represents an important need in the wine sector. Electronic tongues are good candidates due to their advantages of higher sensitivity and relatively low cost in comparison with other methods like spectroscopy (FTIR, FT-NIR) or gas and liquid chromatography (GC, HPLC). Electronic tongues have been widely used in food quality control but, in the case of grapes and wines, there are many more research works dedicated to the analysis of wine than the number of works dedicated to the analysis of grapes or musts.

In addition, electrochemical sensors can be chemically modified with sensing materials by means of several techniques to improve their sensitivity and specificity. Among the variety of sensing materials that have been used in the modification of sensors, metallophthalocyanines (MPc) have been demonstrated to be a good choice, due to their excellent electrocatalytic properties. The catalytic activity of MPc for electrochemical reactions is due to their macrocyclic nature with extended π-systems (or conjugated systems) where electrons are delocalized about the system. In this way, MPc are capable of undergoing fast redox processes with minimal reorganizational energies and can act as electron mediators in transfer processes involving a great variety of molecules. There are many methods to incorporate MPcs to an electrode surface: the adsorption of the MPcs onto graphite or carbon materials, adsorption of monolayers of MPc by dipping the electrode into a solution containing the complexes, dropping the solution of the complexes onto the electrode surface, by self-assembled monolayers (SAMs) of thiol-derivatized phthalocyanines on gold substrates, by means of the electropolymerization of a functionalized monomeric phthalocyanine, by coating the electrode with a preformed polymer containing MPcs, or by using thin films techniques like Langmuir-Blodgett (LB) or Layer by Layer (LbL).

The modified electrode can be characterized by simple electrochemical techniques, such as cyclic voltammetry, which allows the estimation of the formal potentials of redox processes

involving the immobilized MPc and also the amount of active species present on the electrode surface.

In this review, the state of the art of electronic tongues based on phthalocyanines applied in the analysis of the quality of grapes is described. Particular attention is paid to voltammetric sensors.

3.2.2. Summary of the research work

Carbon paste electrodes were used to prepare the simplest sensor array based on MPc to analyze grapes of different variety. Cobalt phthalocyanine, copper phthalocyanine, zinc phtahlocyanine and lutetium bis-phthalocyanine were used to modify the graphite electrodes. The sensors were prepared by mixing graphite, mineral oil (nujol) and MPc forming a homogeneous paste that was later placed in a plastic syringe. A copper wire was used as electrical contact. The voltammetric responses of the sensors showed anodic peaks at 0.4-0.8 V related to phenols present in musts and a cathodic peak at -0.5 V that could be associated to glucose or protons. Lutetium bis-phthalocyanine and cobalt phthalocyanine CPEs evidenced a higher electrocatalytic effect, with intensities for catechol and glucose much higher than copper phthalocyanine and zinc phathalocyanine CPEs. In addition, the variety in the electrochemical responses of the different mettallophthalocyanines used provided a degree of cross-selectivity that allowed us to discriminate between grape varieties, by means of PCA, according to their sugar and polyphenolic content.

On the other hand, other alternatives to traditional electrodes are nanostructured thin films based sensors consisting in preparing homogeneous surfaces with an increased number of active sites, thus improving in the sensitivity of the electrochemical sensors. The analysis of different grape varieties was carried out using a multisensor system based on LbL films of iron, nickel and copper tetrasulfonic phthalocyanins. The LbL films were deposited onto quartz crystal microbalance sensors (QCM) in order to simultaneously register the electrochemical response and mass changes occurring during redox processes. This work allowed us to use these sensors as an electronic tongue based on EQCM sensors to discriminate between grapes with different sugar and phenolic contents.

Finally, going one step further, the specificity of electrochemical sensors can be improved by incorporating enzymes to develop biosensors. The immobilization of enzymes onto electrode surfaces is not easy to achieve. It is important to deposit the enzyme in a way that will still maintain the enzymatic activity. LbL or Langmuir-Blodgett (LB) techniques can be used to adsorb enzymes in lipidic membranes, creating a kind of biomimetic environment. Moreover, the electron transfer between the enzymes and the electrode surface can be improved through a mediator such as phthalocyanines. Grapes with different phenolic content were analyzed by means of a voltammetric electronic tongue based on nanostructured sensors, prepared by the LB technique and made up of one non-specific sensor based on a phthalocyanine and two biosensors based on arachidic acid, a phthalocyanine and the enzymes laccase and tyrosinase. These two enzymes are phenoloxidases that catalyze the oxidation-reduction of phenols and improve the specificity of the sensors for phenols present in the grapes. The voltammetric responses of the sensors for grapes of different varieties allow us to discriminate between the samples according to their phenolic content by using PCA and, moreover, using PLS-1, good correlations with the chemical parameters of total polyphenol Index, acidity and pH (obtained by traditional methods) were found, showing high correlation coefficients and low errors for a small number of latent variables.
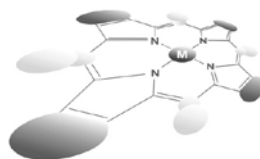
Multisensor systems based on phthalocyanines for monitoring the quality of grapes

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Dedicated to Professor Tomás Torres on the occasion of his 65th birthday

ABSTRACT: Arrays of phthalocyanine-based sensors with complementary activity have been used to develop voltammetric electronic tongues. Such systems have demonstrated to be useful in enology for the evaluation of quality of wines in different production stages, from grapes to bottles. In this paper, the state of the art of multisensor systems based on phthalocyanines dedicated to the analysis of musts (juices obtained from crushed grapes) is described. Such multisensor systems cover different types of sensors from simple Carbon Paste Electrodes, to sophisticated nanostructured sensors, including Langmuir–Blodgett or Layer by Layer thin films and biomimetic biosensors where phthalocyanines play a crucial role as electron mediator between enzymes and electrodes. In all cases, multisensor systems based on phthalocyanines have been able to discriminate musts prepared from different varieties of grapes. The performance of these systems can be improved by combining non-specific sensors with biosensors containing enzymes selective to phenols. In this case, excellent relationships have been found between the responses provided by the array and the content in phenols and acids provided by traditional chemical analysis.

KEYWORDS: electronic tongue, phthalocyanines, voltammetric sensors, wines, grapes.

INTRODUCTION

Phthalocyanines have been extensively used as sensing materials due to their physicochemical properties [1–8]. A variety of sensors based on phthalocyanines has been developed using different strategies that include resistive, chemical, optical or mass transduction [9–11]. Phthalocyanines are particularly useful in the field of electrochemical sensors (potentiometric, amperometric, voltamperometric or electrochemical impedance) [9–13]. From the large family of phthalocyanine molecules, metalophthalocyanines (MPc) have been the most widely

studied derivatives. Double decker derivatives (LnPc₂) have also attracted considerable interest as voltammetric sensors [9, 12–14].

Voltammetric sensors modified with phthalocyanines are commonly used to analyze compounds with redox activity. The number of papers published in this field is quite large and include a variety of phthalocyanine compounds, electrode designs, and target molecules [9–14]. Many papers have been dedicated to the analysis of compounds of interest in the food industry such as antioxidants, sugars and organic acids among many others, because the electrocatalytic properties of electrodes chemically modified with phthalocyanines decrease in a large extent the oxidation potential of these natural reducing agents [15–19]. Phthalocyanines have

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also obtained great success in the field of multisensor systems, the so-called electronic tongues (ET). According to the IUPAC, an ET is a multisensor system formed by a number of non-selective sensors combined with multivariate chemometric tools [20]. ETs try to mimic the biological taste systems which contain sensors that non-selectively interact with flavor molecules to produce electronic signals. Such signals are processed using pattern recognition software. Using non-supervised methods such as Principal Component Analysis (PCA) samples with different characteristics can be discriminated. Using supervised techniques such as Artificial Neural Networks (ANN) it is possible to classify samples belonging to different classes. The produced signals can also be correlated to the composition of the studied solution. ETs are used to analyze complex mixtures and provide global information about the sample instead of giving information of particular constituents. Due to this holistic approach, ET are particularly interesting for the analysis of complex liquids such as drinks and beverages [18–24].

Arrays of phthalocyanine-based sensors with complementary activity have been used in voltammetric electronic tongues dedicated to the analysis of saffron [25], oils [26, 27], beers [28], milk [29] and wines [24, 30–32]. The complexity of these liquids makes them ideal samples to be analyzed with multisensor systems that will give global information of the sample as the human senses do.

The analysis of grapes is becoming more and more important because the final quality of wine is directly related to the quality of grapes. In the vineyard, the quality of grapes is usually established by measuring their sugar content and acidity. Nowadays, it is becoming evident that the quality of grapes is also closely related to many other parameters such as their phenolic composition [33, 34]. This makes necessary to develop new methods to assess the quality or the ripeness of grapes. ETs could be able to provide the necessary complementary information needed to decide important issues such as the retail price (marked by the quality) or the appropriate date for harvesting.

During the last few years our group has been involved in the design of ETs. In this paper, the state of the art of ETs based on phthalocyanines devoted to the analysis of grapes will be presented. Examples will be given to illustrate how multisensor systems based on phthalocyanines can be used to analyze the quality of grapes efficiently. The examples of ETs will include from arrays formed by simple and cheap electrodes to sophisticated nanostructured sensors and biosensors.

ELECTRONIC TONGUES BASED ON PHTHALOCYANINES FOR THE ANALYSIS OF GRAPES

Electronic tongues have been widely used to analyze wines, however, the number of works dedicated to the analysis of grapes or musts (the juice obtained after pressing grapes) is quite small. ETs can use conventional ISFETS [35], but most work use voltammetric sensors. Voltammetric electrodes chemically modified with phthalocyanines are particularly suitable to discriminate grapes [36]. When phthalocyanine based electrodes are immersed in a must which is a complex solution containing ions (Na$^+$, K$^+$, H$^+$, *etc.*) and redox active species (antioxidants, sugars, *etc.*), voltammograms show peaks of two different origins: peaks associated with the oxidation-reduction of the redox species present in the solution and the transient responses associated with the electrode material. In addition, the interactions between the electrode and the solution can improve the selectivity of the electrodes. These interactions include: (i) antioxidants present in musts can modify the oxidation potential of the phthalocyanine deposited at the electrode surface; (ii) the electrocatalytic activity of the phthalocyanine can facilitate the oxidation of the redox species present in must; (iii) the response of the electrode material is related to the ability of ions to diffuse inside the phthalocyanine layer, an influx necessary to preserve the macroscopic electroneutrality of the electrode; and (iv) changes in pH can produce modifications in the redox potentials of both the phthalocyanine (due to protonation) or the solution (changes in the redox potential of phenols). All these redox processes and
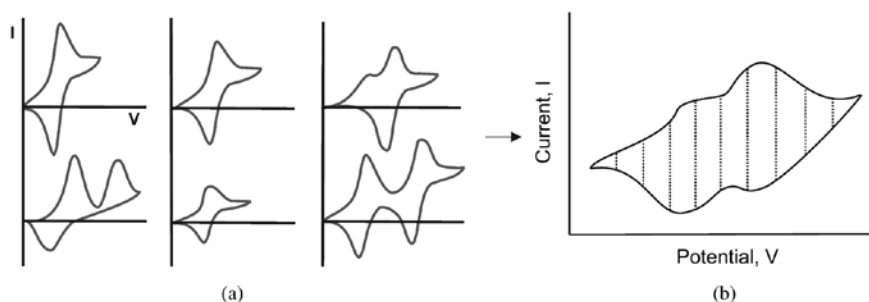

Fig. 1. Scheme of the principles of a voltammetric electronic tongue. (a) Variety of voltammetric signals from an array of sensors and (b) Pre-treatment of a voltammetric signal which is divided into 10 parts to obtain 10 variables

interactions give rise to rich voltammograms that differ from one phthalocyanine to another and from one must to another, increasing the degree of selectivity (Fig. 1a) [24]. The whole voltammogram can be used as the output of each sensor device (instead a single peak as other electrochemicals sensors do). In consequence, a pre-treatment step to reduce the number of variables (without loss of information) is required. Using the Kernel method, voltammograms are multiplied by a 10 smooth, bell-shaped windowing functions, and integrated with respect to potential. The idea behind this pre-processing technique is to capture the information throughout the global response to obtain 10 parameters per curve (Fig. 1b) [30, 37]. Once the voltammograms have been pre-processed and the number of variables reduced, such variables are used as the input for statistical analysis using for instance Principal Component Analysis, Parallel Factor Analysis (PARAFAC) or Partial Least Squares Discriminant Analysis (PLS-DA) as discrimination and classification tools.

Besides their electrocatalytic properties, another important advantage of phthalocyanines is that they can be deposited onto electrode surfaces using a variety of techniques from simple and cheap procedures to advanced methods that produce nanostructured electrodes with enhanced performances [16, 17].

The next sections will describe ETs based on different types of voltammetric sensors based on phthalocyanines dedicated to the analysis of grapes.

Electronic tongue based on carbon paste electrodes (CPE)

Sensors based on phthalocyanines can be prepared using the carbon paste technique by mixing graphite powder and the corresponding phthalocyanine (15%, w/w). The paste is formed using a binder such as Nujol. Pastes are then packed into the body of a plastic tube and compressed. A metallic copper wire is used as a contact. Carbon Paste Electrodes (CPE) thus prepared are ideal for industrial applications because they are cheap and can be easily recovered by cleaning the surface with a filter paper.
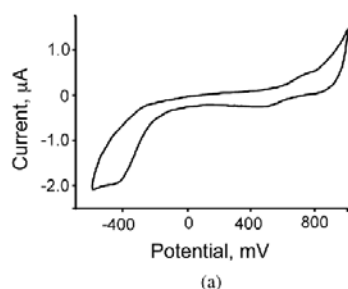
An array was formed by combining five CPE electrodes including CoPc (CoPc–CPE), CuPc (CuPc–CPE), ZnPc (ZnPc–CPE), one electrode modified with LuPc$_2$ (LuPc$_2$–CPE) and one unmodified carbon paste electrode (C–CPE) [38, 39]. The system was used to analyze grapes of five different varieties (Tempranillo, Garnacha, Cabernet, Prieto Picudo and Mencía) harvested in 2012 in the D.O. Ribera de Duero region (Spain).

The electrochemical responses towards musts were dominated by the redox response of the phenolic compounds present in musts that appear as anodic peaks in the 0.4–0.8 V region. A cathodic peak in the -0.5 V region was also observed that could be associated with the glucose but also with the presence of protons. In Fig. 2, the voltammetric response of the CPE electrodes is illustrated for a CuPc electrode immersed in a must obtained from Mencía variety. The important degree of cross-selectivity of the MPc–CPE array was related to the different electrocatalytic properties of the phthalocyanines used. For instance, the voltammetric responses obtained with CoPc and LuPc$_2$ sensors towards catechol and glucose showed higher intensities than the obtained with ZnPc and CuPc sensors, confirming their superior electrocatalytic behavior.

Since voltammetric responses were related to the content of polyphenols, voltammograms could be used to discriminate must samples according to their composition. Using Principal Component Analysis (PCA) it was demonstrated that the array could be used to discriminate grapes. Clusters were located according to the composition of the musts in terms of polyphenolic content and sugar concentration, two of the main components responsible for their quality (Fig. 2b).

Arrays of sensors based on Layer by Layer (LbL) nanostructured films

The roughness of the sensors surface and the number of active sites depend on the technique used to prepare the electrodes, and these characteristics have a direct effect on the intensity of the responses. Nanostructured thin films show highly homogeneous surfaces with an increased number of active sites, improving the sensitivity

Fig. 2. (a) CuPc –CPE immersed in must prepared from grapes of the variety Mencia (diluted in water 1:2). (b) PCA obtained from the five musts analyzed

Chapter 3: Results and discussion

Paper I: Multisensor systems based on phthalocyanines
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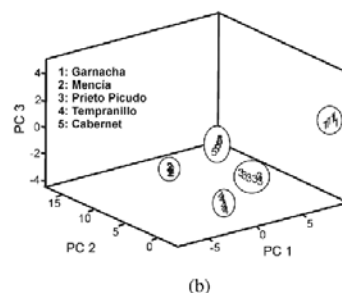of chemical sensors and facilitating the adsorption-desorption processes of the analyzed molecules [40].

Typical methods used to fabricate nanostructured phthalocyanine films include electropolymerization [18], Layer by Layer (LbL) [41] and Langmuir–Blodgett technique (LB) [42].

The analysis of grapes was attempted with an array of voltammetric sensors formed by nanostructured LbL films of iron, nickel and copper tetrasulfonic phthalocyanines by alternating layers of the corresponding phthalocyanine with layers of poly-allylamin hydrochloride [36].

The secondary objective of this work was to combine electrochemical and mass measurements to study the mass changes accompanying the oxidation of electroactive species present in the sample. In fact, in previous works it was established that Quartz Crystal Microbalances (QCM) sensors covered with phthalocyanines could detect the presence of certain gases due to the mass changes that occur when gases are adsorbed on the phthalocyanine coated QCM sensors [43].

In our case, LbL films were deposited on Pt/QCM substrates through the Layer by Layer technique. The response of the array of sensors towards the must samples was studied by recording cyclic voltammetry and mass changes in synchronicity (Fig. 3). The combination of electrochemical and mass measurements (the so-called Electrochemical Quartz Crystal Microbalance, EQCM) was a new class of array of sensors that provided further information about the must samples.

Voltammograms registered by immersing the array in grape juices of six Spanish varieties of grapes (Juan García, Prieto Picudo, Mencía Regadío, Cabernet Sauvignon, Garnacha and Tempranillo) showed the characteristic features already described using CPE sensors. The peak positions associated with the phenolic content shifted to higher values when advancing in the transition metal series. In addition, because the polyphenolic content varied from one must to another, the positions, broadness and intensities of the peaks were different. The massograms registered simultaneously to the cyclic voltammetry are illustrated in Fig. 3. All massograms showed a progressive increase in mass, but also in this case, each must produced a different signature. The change in mass could be attributed to the adhesion of sugars and/or to other complex processes and polymerizations.

The mass and voltammetric outputs processed using PARAFAC demonstrated the capability of both types of signals to discriminate grapes according to their chemical composition: voltammograms discriminated according to the phenolic content in musts and massograms according to their sugar content. In fact, N-PLS (multi-way partial least squares) evidenced the good correlations existing between voltammetric data and Total Polyphenol Index measured by chemical methods. Similarly N-PLS showed a good relationship between mass outputs and sugar content. These results evidenced that multisensory systems
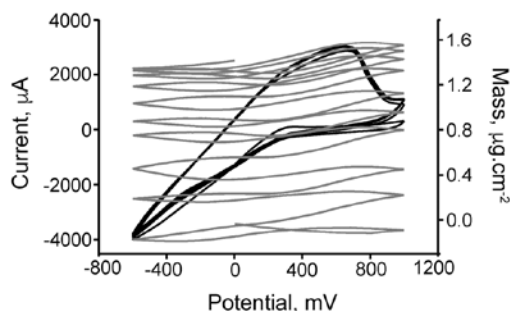
Fig. 3. Voltammetric (black line) and mass response (grey line) of the CuPc^{SO3}/PAH sensor towards a must obtained from Cabernet grapes

formed by EQCM sensors are superior over arrays of mass or voltammetric sensors used separately [36].

Arrays of biomimetic nanostructured biosensors using phthalocyanines as electron mediators

As already stated in precedent paragraphs, the quality of the grapes is typically established on the basis of their phenolic and sugar content. The performance of a multisensor system dedicated to the analysis of grapes can be improved by combining non-specific sensors with biosensors containing enzymes selective to phenols or sugars. It has been claimed that arrays formed by biosensors can be advantageous because they combine the advantages of classical arrays of electrochemical sensors (that provide global information about the sample), with the specificity of the enzyme-substrate reaction typical of biosensors.

The immobilization of the enzyme onto the substrate is a key step in the development of efficient biosensors with high enzymatic activity. Layer by Layer (LbL) [41, 44] or Langmuir–Blodgett (LB) [45–51] techniques can be used to adsorb enzymes in lipidic membranes formed by fatty acids or phospholipids. This biomimetic environment contributes to the preservation of the enzymatic activity facilitating the electron transfer process. Furthermore, the biosensor sensitivity can be enhanced by the introduction in the LB or LbL film an appropriate electron mediator, which can facilitate the electron transfer between the redox enzyme and the electrode [52]. Phthalocyanines have demonstrated to be efficient electron mediators [48, 53].

A bioelectronic tongue based on nanostructured biosensors was developed and used to discriminate grapes according to their phenolic content. The multisensor system of three voltammetric sensing units was prepared using LB technique: one non-specific sensor based on a phthalocyanine and two biosensors formed by films of arachidic acid, a phthalocyanine (as the electron mediator) and the corresponding enzyme [54].

MULTISENSOR SYSTEMS BASED ON PHTHALOCYANINES FOR MONITORING THE QUALITY OF GRAPES   893

Table 1. Results of the PLS-1 analysis

Parameter	RMSEC[a]	RMESP[b]	R^2_C[c]	R^2_P[d]	LV[e]
pH	0.05800	0.06997	0.9822	0.9740	3
Total acidity	0.04724	0.05948	0.9948	0.9918	3
TPI	0.59340	0.67736	0.9731	0.9649	3

[a] Root mean square error of calibration; [b] Root mean square error of prediction; [c] Squared correlation coefficient in calibration; [d] Squared correlation coefficient in prediction; [e] Number of latent variables.

Tyrosinase and laccase were selected because they are phenoloxidases involved in the oxidation of phenols to the corresponding quinoid form [55].

When the array of sensors was immersed in grape juices, each sensor showed a characteristic electrochemical response towards musts obtained from five different varieties of grapes (Tempranillo, Garnacha, Cabernet-Sauvignon, Prieto Picudo and Mencía). The voltammetric signals provided global information about the sample (as in classical multisensor systems) combined with specific information due to the specificity of the enzyme-substrate interaction. PCA of the obtained signals allowed a clear discrimination of the musts according to the variety of grape. Moreover, using PLS-1, excellent relationships were found between the responses provided by the biosensors and phenols and acids content provided by traditional chemical analysis carried out following international regulations [56]. As shown in Table 1, correlation coefficents (R^2) of *ca.* 0.99 were found in both calibration and validation, with residual errors close to zero and with an small number of latent variables.

CONCLUSION

Multisensor systems (Electronic Tongues) based on phthalocyanines are a good alternative to analyze grapes. Important differences between ETs are attributed to the type of sensor. CPE electrodes are simple to prepare and cheap, but their repeatability and sensitivity are low. In contrast, nanostructured LB or LbL sensors are highly sensitive but at a high cost.

The cross-selectivity of a multisensor system can be improved by constructing hybrid arrays combining non-specific sensors with biosensors containing enzymes selective to specific compounds of interest, for instance phenoloxidases able to detect phenols. This can be of particular importance in the field of viticulture and enology where the content of certain compounds is directly related to the quality of wines and grapes.

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3.3. Paper II

Monitoring the phenolic ripening of red grapes using a multisensor system based on metal-oxide nanoparticles.

By Celia García-Hernández, Cristina Medina-Plaza, Cristina García-Cabezón, Yolanda Blanco, José A. Fernández-Escudero, Enrique Barajas-Tola, Miguel A. Rodríguez-Pérez, Fernando Martín-Pedrosa and Maria Luz Rodríguez-Méndez.

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3.3.1. Motivation and objective

The performance of electrochemical sensors and biosensors has been greatly improved by using nanotechnology. This field has received increasing attention from researchers in analytical chemistry applications. There are many nanomaterials which have been implemented in many applications, such as sensing or drug delivery, among others. On this matter, nanoparticles are excellent candidates, due to their small size, as they provide unique physical, chemical and electronic properties not found in bulk materials. For instance, metal nanoparticles exhibit excellent conductivity and electrocatalytic properties related to the formation of mixed valence states on their surface that help to enhance the surface area, facilitate the electron transfer between redox species and electrode surface, improve the sensitivity and selectivity of sensors, as well as the signal-to-noise ratio; metal oxide nanoparticles can be used to immobilize biomolecules due to their biocompatibility; while semiconductor nanoparticles have been applied as sensor labels for electrochemical detection.

Therefore, when using nanoparticles in electrochemical sensors, it is understandable to expect a decrease in the redox potentials of electrochemical reactions, improved reversibility or increased responses. Moreover, for biosensing applications with enzymes, nanoparticles can ameliorate the lack of electrical contact between the enzyme and the electrode, which is practically non-existent as enzyme active sites are impeded by insulating protein shells. The conductivity of metal, metal oxide and semiconductor nanoparticles enhances the electron transfer between the active sites and the surface electrodes acting as mediators or electrical wires.

In short, metal nanoparticles, metal oxide nanoparticles and semiconductor nanoparticles have mainly been used in electrochemical sensor systems for the following purposes: catalysis of electrochemical reactions, immobilization of biomolecules, improvement of electron transfer, labelling biomolecules and acting as reactant.

They also have the advantage that they can be deposited onto electrode surfaces by many methods, including covalent bond, electrodeposition or physical adsorption, among others.

As previously stated, the optimal date of harvesting is a point that concerns wine growers. The main parameter to monitor the maturity of grapes is the sugar content (°Brix), although other parameters, such as the phenolic content (TPI) and total acidity (TA), are also

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relevant as they can strongly influence the final organoleptic characteristics of wines. For example, many oenologists use the relation ºBrix/TA to decide the optimal maturity day, although there is no biochemical relationship between the acidity loss and the sugar increase.

Metal oxide nanoparticles have been widely used in the detection of many analytes, such as H_2O_2, glucose, dopamine, bis-phenol A, catechol, ascorbic acid, fructose, cysteine, etc., but they have never been applied to electronic tongues, which is the novelty of this work, together with the purpose of implementing a multisensor system to study grape ripeness.

Therefore, in this work, we have developed an electronic tongue based on metal oxide nanoparticles (CeO_2, NiO and TiO_2) to monitor grape ripeness from *véraison* to over-ripening in terms of phenolic maturity. Statistical methods were used to discriminate between grape samples by means of PCA and, finally, correlations were established between the chemical parameters of the analysed grapes and the data obtained with the electronic tongue using PLS-1.

3.3.2. Summary of the research work

Sensors were prepared by using a simple, previously reported method based on carbon paste electrodes (CPE), where phthalocyanines were mixed with graphite powder and a mineral oil (nujol). In this case, phthalocyanines were replaced by metal oxide nanoparticles. Therefore, the array of sensors that formed the electronic tongue was composed of nickel (II) (NiONP-CPE), titanium (IV) (TiO_2NP-CPE) and cerium (IV) (CeO_2NP-CPE) oxide nanoparticles and one non-modified carbon paste electrode (C-CPE). Plastic syringes were used as support for the carbon paste. TEM images showed that MONPs were spherical, with a homogeneus distribution and an average size ranging from 20 to 50 nm.

Grape samples were provided by the vineyards Bodega Cooperativa de Cigales and the Instituto Tecnológico Agrario de Castilla y León (ITACyL). Eight different varieties were analyzed: Tempranillo, Garnacha, Cabernet, Prieto Picudo, Juan García, Mencía Secano, Mencía Regadío and Rufete. All samples were collected weekly from *véraison* to maturity, and several grapes bunches were also left in the vineyards and were collected two extra weeks after maturity (over-ripeness). The varieties Tempranillo, Garnacha and Cabernet were considered mature 6 weeks after *véraison* and the rest of the varieties after 5 weeks. The Oenological Centre of Castilla y

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León analyzed the chemical parameters of the grapes and the Brix degree, TPI and total acidity were provided. These parameters were analyzed weekly as was the ºBrix/TA, since this relationship is used by several wine growers to establish the optimal harvesting date. The results showed that TA decreased during the ripening process, sugar content increased and TPI varied irregularly, but increased drastically just after *véraison.* For Garnacha, Cabernet and Tempranillo grapes, the ºBrix/TA ratio increased weekly and reached stabilization after week 5, indicating the proximity of grape maturity and grapes were then harvested in week 6. Mencía Secano, Mencía Regadío, Prieto Picudo, Juan García and Rufete ripened faster and were harvested in week 5.

The changes due to over-ripening demonstrated that TA values were almost constant, but the ºBrix continued to increase, so the optimal ripening date was not reached until week 7 or 8 (depending on the variety). As the TPI trend was so irregular, this result showed that, using traditional methods, we are not able to assess phenolic maturity.

In order to find out if the developed electronic tongue based on metal oxide nanoparticles was able to monitor the phenolic ripening, the e-tongue was tested in catechol, vanillic acid, caffeic acid and pyrogallol, which are all phenols present in grapes. The voltammetric responses showed that metal oxide nanoparticles induced an increase in the intensities and/or a decrease in redox potentials, making the process more easily reversible. Each sensor showed a characteristic response that provided a degree of cross-selectivity in the responses, which is advantageous for its application as a multisensor system. The electrocatalytic effect was more strongly pronounced in the mono-phenol (vanillic acid) and di-phenols (catechol and caffeic acid) than in the tri-phenol (pyrogallol). The detection limits were found to be 10^{-8} M, which is much lower than those reported for traditional carbon paste electrodes, but are similar or comparable to detection limits obtained with more sophisticated sensors constructed with nanostructured Langmuir-Blodgett or LbL films. Additionally, PCA analysis evidenced that the electronic tongue was able to discriminate phenols according to the number of hydroxyl groups present in the molecule (mono, di or tri).

The electronic tongue was then immersed in grapes collected weekly from *véraison* during 7 or 8 weeks from *véraison* (8 weeks for Cabernet, Garnacha and Tempranillo and 7 weeks for the rest). Voltammograms showed oxidation peaks related to redox species present in the musts (mainly phenols). The peaks observed at negative potentials were due to the acidity of the samples. The different electrocatalytic properties of the metal oxide nanoparticles used was

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reflected in the responses. For example, NiONP-CPEs were more sensitive to phenol redox processes, whereas TiO_2NP-CPEs were sensitive to the acidity.

PCA analysis was carried out for each variety of grape and the results were very interesting. For the varieties Mencía Secano, Mencía Regadío, Prieto Picudo, Juan Garcia and Rufete, the clusters corresponding to the first 4 weeks were located drawing a kind of circle that was "closed" passing through the sample of week 5 (ripening week), located relatively near the week 1 sample, and then the samples of weeks 6 and 7 were located near weeks 2 and 3, respectively, following a kind of spiral tendency. This result was also observed for the Cabernet, Garnacha and Tempranillo varieties, where the week 6 sample (ripening week) was repeating the same behavior as the week 5 sample described above, with the over-ripening samples also following a kind of spiral tendency. These results indicated that the optimal phenolic maturity point is connected to when the circle is closed and before samples start to follow a spiral tendency.

Finally, regression models were performed by PLS-1 to find linear correlations between the chemical parameters (ºBrix, TA and TPI) and the data obtained with the electronic tongue. For each grape variety, good correlations were found between the voltammetric signals and the phenolic content (TPI), with a low number of latent variables (2-4), correlation coefficients of about 0.8 and low errors. ºBrix and TA, which are the parameters usually used to monitor the maturity of grapes, gave regression models with lower correlations, showing a higher number of latent variables, but with low errors and similar coefficient of correlations.

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Monitoring the Phenolic Ripening of Red Grapes Using a Multisensor System Based on Metal-Oxide Nanoparticles

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The maturity of grapes is usually monitored by means of the sugar concentration. However, the assessment of other parameters such as the phenolic content is also important because the phenolic maturity has an important impact on the organoleptic characteristics of wines. In this work, voltammetric sensors able to detect phenols in red grapes have been developed. They are based on metal oxide nanoparticles (CeO_2, NiO, and TiO_2,) whose excellent electrocatalytic properties toward phenols allows obtaining sensors with detection limits in the range of 10^{-8} M and coefficients of variation lower than 7%. An electronic tongue constructed using a combination of the nanoparticle-based sensors is capable to monitor the phenolic maturity of red grapes from véraison to maturity. Principal Component Analysis (PCA) can be successfully used to discriminate samples according to the ripeness. Regression models performed using Partial Least Squares (PLS-1) have established good correlations between voltammetric data obtained with the electrochemical sensors and the Total Polyphenolic Index, the Brix degree and the Total Acidity, with correlation coefficients close to 1 and low number of latent variables. An advantage of this system is that the electronic tongue can be used for the simultaneous assessment of these three parameters which are the main factors used to monitor the maturity of grapes. Thus the electronic tongue based on metal oxide nanoparticles can be a valuable tool to monitor ripeness. These results demonstrate the exciting possible applications of metal oxide nanoparticles in the field of electronic tongues.

Keywords: electronic tongue, grape, ripening, phenolic maturity, metal oxide nanoparticles

INTRODUCTION

Grapes must be harvested at the optimal maturity point. The sugar content has a direct influence on the alcoholic degree of wines and it is the analytical indicator commonly used to decide the harvest date (OIV, 2013). It is periodically assessed using fast and simple density measurements and it is expressed as degree Brix (°Brix). The variation of the acidity along ripening is also an important parameter which is expressed as Total Acidity (TA) (Boss et al., 2014).

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The phenolic content of grapes also changes during ripening and the phenolic maturity has a direct impact on the organoleptic characteristics of wines, and it would be desirable to assess it routinely. However, the main changes in the phenolic content take place in skins and seeds, and the assessment of phenolic maturity requires the previous separation of seeds and skins using long and complex methods (Xu et al., 2011; Meléndez et al., 2013; Nogales-Bueno et al., 2014; Sharma et al., 2015). Oenologists require new methods capable to measure the phenolic maturity of grapes with simple and direct methods.

Besides, it would be of great interest to develop new technologies able to detect simultaneously the sugar content, acidity and phenolic composition. Such a method would help to take faster decisions about the optimal harvest date. One possible approach can be the use of holistic methods, where complex signals obtained from an instrument such as GC-MS, FTIR or NMR, are processed using multivariate methods. These holistic methods have been successfully used to classify wines according to quality, to monitor fermentation, to detect wine spoilage, etc. (Buratti et al., 2011; Cozzolino et al., 2012; Godelmann et al., 2014).

Electronic tongues (ET) are a new class of instruments which are gaining interest in the food industry (Cosio et al., 2012; Kirsanov et al., 2012; Lvova et al., 2014; Rodríguez-Méndez, 2016). They are multisensor systems where an array of sensors is combined with a multivariate data software. The most common sensors used in ETs dedicated to the analysis of wines are electrochemical (amperometric or voltammetric). They have been widely used to discriminate wines according to the variety of grape, the vintage, the type of barrel used for aging etc. (Gutiérrez et al., 2010; Prieto et al., 2011; Apetrei et al., 2012; Cetó et al., 2017; Rudnitskaya et al., 2017). In spite of the interest of winemakers to have available objective methods to determine the harvesting date and the quality of grapes, ETs have been rarely applied to the quality control of grapes or their musts (Codinachs et al., 2008; Gutiérrez et al., 2011; Campos et al., 2013; Medina-Plaza et al., 2014a,b).

Nowadays, it is clear that the performance of ETs can be improved by introducing in the array sensing units specifically dedicated to a particular application. Electrodes with enhanced performances can be obtained using new sensing materials linked to nanotechnology. Metal nanoparticles have been used in the fabrication of electrochemical sensors due to their electrocatalytic properties which are related to the formation of mixed valence states on their surface (Campbell and Compton, 2010; Saha et al., 2012; Lin et al., 2013). Metal nanoparticles have demonstrated their capability to catalyze the oxidation/reduction of organic acids and phenols commonly present in wines. The oxidation of phenols occurs at lower potentials, whereas the presence of metallic nanoparticles increases the reactivity of acids and enhances the intensity of the anodic wave at negative potentials (Medina-Plaza et al., 2015a,b). In spite of this interest, metal nanoparticles have rarely been included as sensing materials in electronic tongues (Gutiérrez et al., 2013; Sharpe et al., 2014; Mercante et al., 2015). Up to now, the sensing capabilities of metal oxide nanoparticles (MONPs) have never been tested in electronic tongues.

The aim of this work was to develop an electronic tongue (ET) formed by sensors based on metal oxide nanoparticles (CeO_2, NiO, and TiO_2,) and to evaluate their capabilities to detect phenols. Using chemometric techniques such as Principal Component Analysis (PCA), the ET was used to monitor the ripeness of 8 different varieties of grapes, from *véraison* (the onset of ripening) to post-maturity. Mathematical models were built to establish correlations between the phenolic index measured by traditional chemical techniques and the results of the ET. PLS-1 was used to predict the polyphenol index of the grapes along ripening. The possibility to obtain information about other classical indicators of maturity such as the °Brix and Total Acidity was also analyzed.

MATERIALS AND METHODS

Metal oxide nanoparticles of Titanium (IV), Cerium (IV), and Nickel (II) (TiO$_2$NP, CeO$_2$NP, NiONP) were purchased from Sigma-Aldrich. All reagents and solvents were of high purity (Sigma–Aldrich). Deionized water (18.2 MΩ·cm^{-1}) was obtained from a Millipore MilliQ purifier.

Electron Microscope TEM images were recorded using a high-resolution electron microscope (HRTEM: JEOL JEM 2200) (Tokyo, Japan) operating at an accelerating voltage of 200 kV. Sample images were processed using Image J image processing software (public software).

Eight varieties of red grapes typical from the Castilla-Leon region (Spain) were included in the study: *Tempranillo, Garnacha, Cabernet-Sauvignon, Prieto Picudo, Mencia Regadio, Mencia Secano, Rufete,* and *Juan Garcia.* Grapes were grown and harvested by the Agrotechnological Institute of Agriculture of the Regional Government (ITACyL) and the cellar Bodega Cooperativa de Cigales. Berries were collected in a weekly basis from *véraison* until grapes were completely mature. Ripeness rate was different from one variety to another. For this reason, the official harvest date indicated by traditional chemical parameters, varied from one variety of grape to another. Grapes from Tempranillo, Garnacha, and Cabernet Sauvignon were considered matured 6 weeks after *véraison.* The rest of varieties attained the optimal ripeness 5 weeks after *véraison.* In all cases several grape bunches were left in the vine and two extra samplings were collected in successive weeks in order to analyze over-ripened grapes.

Musts were prepared from 100 berries representative of the ripening state of the vineyard. Once collected, grapes were crushed, and peels and seeds were separated by decantation. 50 mL aliquots of musts thus obtained, were frozen at −20°C until used. Total Polyphenol Index (TPI), Brix degree (°Brix) and Total Acidity (TA) were analyzed following international standard methods (OIV, 2013; Boss et al., 2014). TPI was determined by diluting the samples with distilled water in a 1:100 ratio and measuring the absorbance directly at 280 nm (using a quartz cuvette of 10 mm optical path). The value of the I280 nm (TPI) was calculated by multiplying the absorbance × 100. The total acidity is the sum of its titratable acidities when it is titrated to pH 7 against a standard alkaline solution. Carbon dioxide is not included in the total acidity. The total acidity is usually expressed in grams of tartaric acid per liter.

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Brix measurements are taken with an Atago WM-7 digital refractometer.

A multisensor system consisting of 4 Carbon Paste Electrodes (CPE) was formed. Three electrodes were modified with metal oxide nanoparticles including NiO (NiONP-CPE), TiO$_2$ (TiO$_2$NP-CPE), and CeO$_2$ (CeO$_2$NP-CPE). One unmodified carbon paste electrode (C-CPE) was also included in the array. Electrodes were prepared following the classical method by mixing graphite with the corresponding metal oxide nanoparticle (15% w/w of the MONP) Nujol was used as the binder until a paste was obtained. The paste was packed in a 1 mL plastic syringe and compressed. A metallic copper wire was used as a contact (Apetrei et al., 2011). The size of MNOPS ranged from 20 to 50 nM. **Figure 1** illustrates the shape and size of TiO$_2$MNPs. As observed in the TEM image, MONPs were spheric with an homogeneous distribution of sizes that coincided with the size labeled by the supplier.

Electrochemical experiments were carried out using a potentiostat (Autolab. Metrohm, USA) combined with a multiplexor system. CPEs (surface of 2 mm in diameter) served as working electrodes, with an Ag|AgCl/KCl 3 mol·L^{-1} electrode acting as a reference electrode and a platinum wire as the counter

electrode. The MONPs sensors were able to provide responses toward aqueous solutions of phenolic acids (vanillic and caffeic acids) and polyphenols (catechol and pyrogallol) present in wines. The electrocatalytic properties and the detection limits (LD) were evaluated. The calibration curves were constructed measuring standard solutions with concentrations ranging from 10^{-4} to 10^{-6} mol·L^{-1}. LDs were calculated following the 3 SD/m criterion. The reproducibility of data provided by the MONPs sensors was evaluated by comparing data provided by three sensors measuring standard solutions in different days. Cyclic voltammograms were registered at a sweep rate of 0.1 V·s^{-1} from -0.8 to $+1.0$ V. Four replicas of each sample were measured. Principal Component Analysis (PCA) and Partial Least Squares (PLS) models were built using the software Matlab v5.3. (The Mathworks Inc., Natick, MA, USA).

Prior to perform the statistical analysis, the number of variables was reduced using a data reduction technique based on predefined response "bell-shaped-windowing" curves called "kernels" (Medina-Plaza et al., 2015b). In this method, each voltammogrammetric curve is multiplied by 10 smooth, bell-shaped windowing functions defined as:

$$K_i(V_j) = \frac{1}{1 + (\frac{V_j - c_i}{a_i})^{2b_i}}$$

where a_i, b_i, and c_i define the width, shape, and center of the different windowing functions K_i. Subsequently, data were integrated with respect to voltage. As a result, each voltammogram was reduced to a vector of 10 variables that were used as the input data source for statistical analysis.

RESULTS AND DISCUSSION
Monitoring Ripeness Using Chemical Parameters

Ripening was monitored by measuring °Brix, TA, and TPI in a weekly basis (from *véraison*). These parameters were used by oenologists to establish the optimal date of harvest. The °Brix/TA ratio was also calculated. This parameter is also commonly used to decide the optimal maturity date even if there is no biochemical relationship between the acidity loss and the sugar increase. The complete list of parameters is collected in Supplementary Table. Details can be observed in **Figure 2** for variety Tempranillo.

As observed in the **Figure 2**, TA and °Brix followed the expected trend: TA decreased along the ripening process, while sugar content increased. °Brix/TA ratio increased accordingly. These values were stabilized after week 5, indicating the proximity of the end of ripeness, and grapes were considered mature and harvested in week 6. Similar trends were observed in Garnacha and Cabernet-Sauvignon that also reached the maturity in week 6. Mencia Secano, Mencia Regadio, Rufete, and Juan Garcia gapes, matured at a faster rate, and were considered ripened and harvested in week 5.

In order to study the changes in over-ripened grapes, some bunches were kept in the plant during 2 more weeks. During

FIGURE 1 | TEM image of the TiO$_2$NPs.

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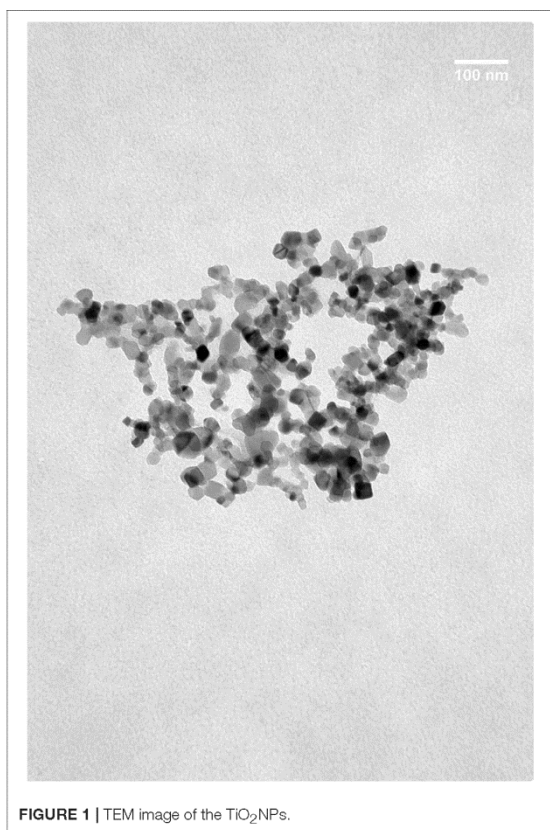
these 2 extra weeks, TA values were almost constant, whereas
°Brix raised drastically in Cabernet and Garnacha grapes,
indicating that optimal ripening was not attained until weeks 7
or 8.

TPI values increased sharply just after the *véraison*. The
following weeks, TPI varied irregularly. These results evidenced
the difficulty to assess the phenolic maturity using traditional

FIGURE 2 | Variation of the °Brix (g sugar/ 100 g solution), TA (g/L), Brix/TA
and TPI for juices obtained from the variety Tempranillo harvested weekly form
véraison to over-ripening.

TPI measurements in pulps. It is therefore important for wine
producers to develop new methods to define the peak of ripeness
and in particular of the phenolic ripeness (which nowadays is
difficult to assess).

Electrochemical Characterization of the NP-Based Electrode. Electrocatalytic Activity Toward Phenols

The objective of this work was to develop a multisensory system
based on metal oxide nanoparticles to monitor the phenolic
ripening. As a first task, the response of the MONP-CPE
electrodes toward four phenols present in musts (vanillic acid,
catechol, caffeic acid, and pyrogallol 10^{-4} M), was evaluated
(**Figure 3**). The response of the C-CPE electrode is also shown
for comparison purposes.

The responses on MONP-CPEs were consistent with those
already reported in bare CPE electrodes (Kilmartin et al., 2001),
but the presence of MONPs caused shifts in the position of
the peaks to lower potentials and/or increases in the intensity
of the peaks. This effect could be attributed to the excellent
electrocatalytic activity of MONPs due to the mixed valence
state found at the nanoparticle surface (Medina-Plaza et al.,
2015b). Depending on the nature of the MONPs used as
modifiers, the responses toward a certain compound was more
or less enhanced. For instance, the intensity of the redox
peaks produced by the oxidation/reduction observed in vanillic

FIGURE 3 | CV of **(A)** vanillic acid, **(B)** catechol, **(C)** caffeic acid, and **(D)** pyrogaloll at C-CPE, CeO₂NP-CPE, NiONP-CPE, and TiO₂NP-CPE electrodes.
Concentration of 10^{-4}M. Scan rate 100 mV·s^{-1}.

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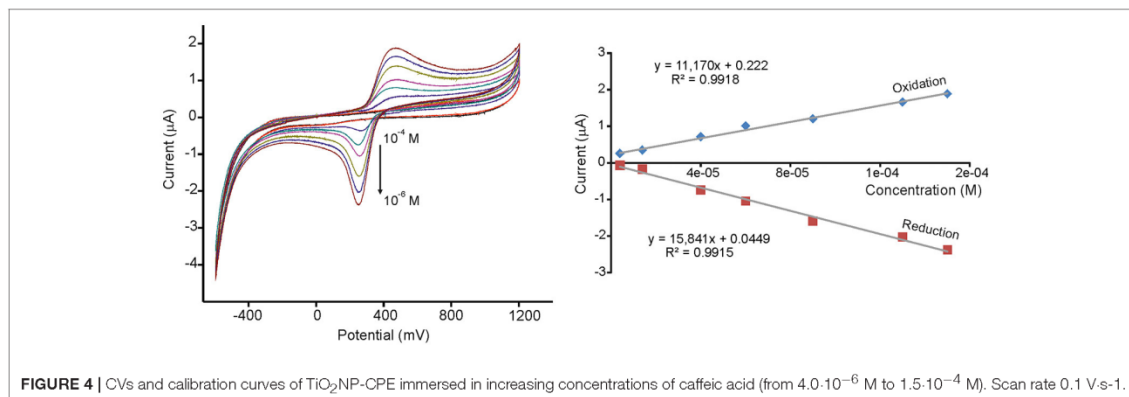
FIGURE 4 | CVs and calibration curves of TiO_2NP-CPE immersed in increasing concentrations of caffeic acid (from $4.0 \cdot 10^{-6}$ M to $1.5 \cdot 10^{-4}$ M). Scan rate 0.1 V·s-1.
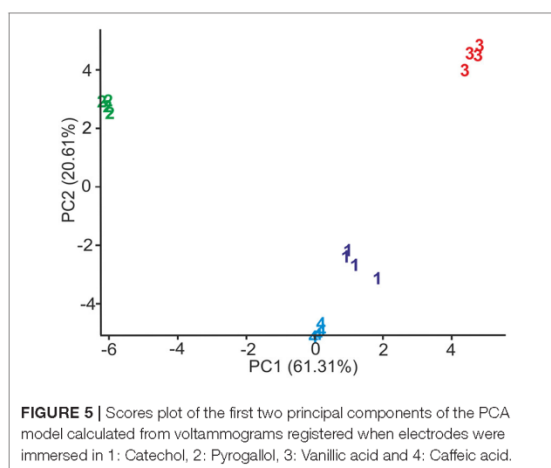
FIGURE 5 | Scores plot of the first two principal components of the PCA model calculated from voltammograms registered when electrodes were immersed in 1: Catechol, 2: Pyrogallol, 3: Vanillic acid and 4: Caffeic acid.

acid and catechol at the graphite electrode surface increased drastically in the presence of NiONPs. The electrocatalytic effect was not so remarkable at TiO_2NP-CPE or CeO_2NP-CPEs electrodes. It is also worth noting that the electrocatalytic effect was more intense in the mono-phenol (vanillic acid) or di-phenols (catechol and caffeic acid) than in the tri-phenol (pyrogallol).

Limits of detection (LD) were calculated from the slope of the curves of the redox peaks registered at concentrations ranging from 10^{-4} to 10^{-6} M, using 3 *SD*/m method, where *SD* is the standard deviation of the current density. LDs as low as 10^{-8} M could be attained, which are much lower than those typically found in carbon electrodes (Kilmartin et al., 2001). The results are illustrated in **Figure 4** for TiO_2NP-CPE immersed in caffeic acid. Reproducibility was also evaluated by measuring repeatedly the standard solutions and Variation coefficients (CV) lower than 7% were found in all cases. From the above results it can be concluded that modification with metal oxide nanoparticles drastically increases the

sensibility of voltammetric electrodes toward phenols present in musts.

As stated before, each sensor showed a characteristic response which is linked to the electrocatalytic properties of the type of nanoparticle introduced in the carbon paste. This cross-selectivity can be used to construct a multisensor system coupled to a pattern recognition software able to provide a pattern or "digital fingerprint" for each sample studied.

Figure 5 shows the Principal Component Analysis (PCA) scores plot obtained from the responses of the array to 10^{-4} M solutions of phenols. The PCA showed four distinct clusters in the plot of the two first principal components (PC1 61.31% and PC2 20.61%). The position of the clusters was related to the number of hydroxyl groups in the molecule. The monophenol (vanillic acid) was located at positive PC1 values, diphenols (catechol and caffeic acid) in the central part of the diagram and the triphenol (pyrogallol) on the left side corresponding to positive PC2 values.

Monitoring the Ripening of Grapes With an Array of MONP-Based Electrodes

The capacity of the electronic tongue to monitor the phenolic maturity of grapes was evaluated by analyzing juices obtained from grapes collected from *véraison*, in a weekly basis, during 7–8 weeks. Due to the complexity of the samples and in order to improve the repeatability, musts were diluted 1:2 with deionized water. Coefficients of variation lower than 7% were obtained in all cases.

Figure 6 illustrates the responses obtained using the NiONP-CPE and TiO_2NP-CPE sensors immersed in musts obtained from grapes of the variety Garnacha collected during 7 weeks after *véraison*.

As illustrated in the figure, voltammograms registered in must showed peaks in the 0.6–0.8 V region, produced by components with redox activity (mainly phenols). Other components present in musts also influenced the overall response. For instance, the peaks observed at negative potentials were affected by the acidity of the samples. The array of sensors showed an interesting cross-selectivity that was due to the different catalytic properties of

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FIGURE 6 | Voltammetric responses of **(A)** TiO$_2$NP-CPE and **(B)** NiONP-CPE electrodes toward Garnacha grapes collected weekly after *véraison*.
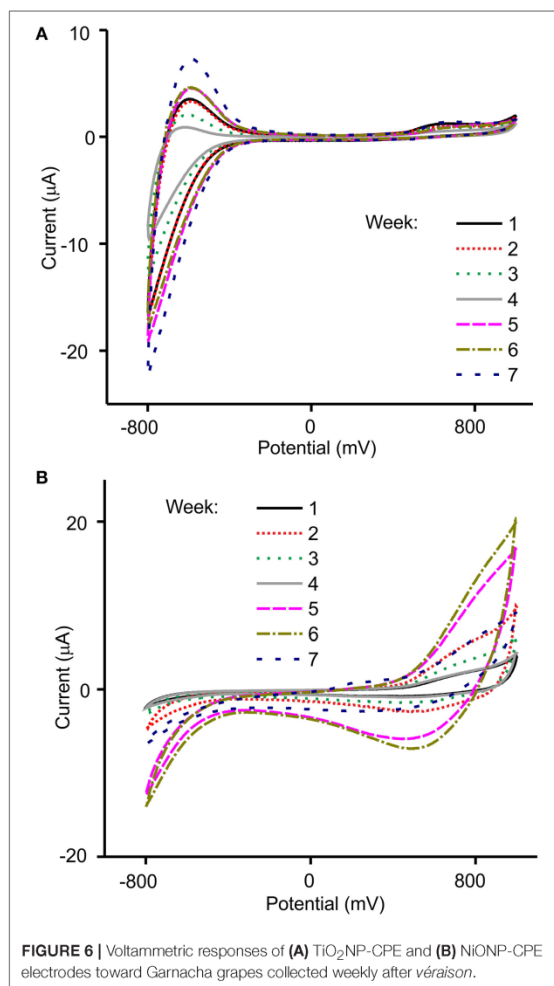
the MONP selected. For instance, NiONP-CPEs were highly sensitive to phenols, whereas TiO$_2$NP-CPEs were sensitive to the acidity.

The chemical changes occurring during the ripening process could be followed using the MONP-based sensors. First, the intensity of the peaks increased, then intensity decreased and finally increased according with the tendency shown in **Figure 2** where TPI values varied similarly. The irregular increase stopped when optimal ripeness was attained. Simultaneously, a decrease in the total acidity corresponded with a progressive decrease in the intensity of the peaks at negative potentials. As already stated in the introduction, multivariate methods have demonstrated to be a good strategy for the analysis of wines. It can be expected that they can also be useful to monitor the ripeness of grapes.

When PCA was carried out using the data obtained from the electronic tongue, it was observed that clusters corresponding to grapes collected in the first 4 weeks after *véraison*, were positioned in a counterclockwise organization "drawing" a circle (**Figure 7A**). In sampling number 5, the circle was closed. Surprisingly, in next samplings the positions were repeated and clusters corresponding to next specimens were situated continuing the counterclockwise trend following a "screw" structure. According to PCA, grapes could be considered matured when the clusters reached the original positions. Similar results have been obtained for all the grape varieties analyzed in this work. Similar results have also been observed in previous works where ETs have been used to monitor maturity of grapes (Medina-Plaza et al., 2016)

Figure 7B show the contribution of the variables in a loading plot. The loadings of each sensor (ten variables per sensor) spread along the four quadrants. This graph shows the high cross-selectivity achieved by the array of sensors.

Table 1 shows the statistical parameters obtained from the PLS-1 regression models (leave one out validation) correlating the output of the array of sensors with the chemical parameters (TPI, °Brix, TA). PLS-1 builds regression models to establish a linear relationship between the set of predictors (X-variable: Voltammetric signals obtained from the array of sensors) and the

FIGURE 7 | **(A)** 3D Score plot, **(B)** Loading plot of PCA performed from grape juices from the variety Juan Garcia harvested weekly from *véraison* to over-ripening (1: First week, 2: Second week, etc. 7: Over-ripening week).

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TABLE 1 | Results of the PLS-1 regression models correlating the chemical parameters and the signals of the sensors.

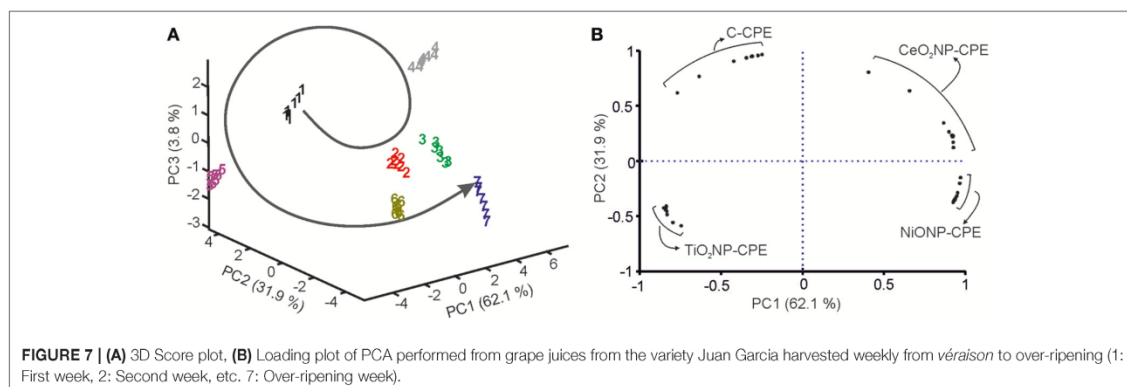| Grape	Parameter	R_C^2 (a)	RMSE$_C$ (b)	R_P^2 (c)	RMSE$_P$ (d)	Number of components
Cabernet	°Brix	0.87	0.86	0.85	0.95	**3**
	TA (g/L)	0.87	0.81	0.81	1.02	7
	TPI	0.86	2.13	0.83	2.42	**2**
Garnacha	°Brix	0.92	0.91	0.82	1.40	6
	TA (g/L)	0.85	1.24	0.73	1.72	6
	TPI	0.86	2.25	0.77	3.01	**3**
Juan Garcia	°Brix	0.86	0.79	0.79	1.00	5
	TA (g/L)	0.80	1.08	0.70	1.39	6
	TPI	0.84	1.54	0.79	1.84	**4**
Mencia Regadio	°Brix	0.97	0.22	0.96	0.26	5
	TA (g/L)	0.89	0.69	0.86	0.77	5
	TPI	0.88	1.08	0.83	1.33	5
Mencia Secano	°Brix	0.86	0.58	0.79	0.74	6
	TA (g/L)	0.81	0.84	0.71	1.06	6
	TPI	0.86	1.57	0.82	1.79	**3**
Prieto Picudo	°Brix	0.87	0.73	0.63	1.25	7
	TA (g/L)	0.88	0.93	0.70	1.52	7
	TPI	0.83	1.33	0.81	1.43	**3**
Rufete	°Brix	0.85	0.49	0.83	0.53	**3**
	TA (g/L)	0.82	0.91	0.76	1.09	5
	TPI	0.91	1.51	0.90	1.64	**3**
Tempranillo	°Brix	0.81	0.89	0.76	1.01	6
	TA (g/L)	0.91	0.56	0.76	0.95	6
	TPI	0.87	2.05	0.75	2.94	**4**

Models were established for each variety of grape considered separately.
(a) Squared correlation coefficient in calibration; (b) Root mean square error of calibration; (c) Squared correlation coefficient in prediction; (d) Root mean square error of prediction.

set of responses (Y-variable: Chemical parameters obtained with traditional methods). PLS-1 finds the latent variables (indicated as number of components) in X that will best predict the latent variables in Y.

In all varieties of grapes, good correlations were found with the Total Polyphenol Index. Using a low number of components, both the calibration and the prediction showed Squared Correlation Coefficients in calibration (R^2C) and in prediction (R^2V) of ca. 0.8. Additionally, low Root Mean Square Error of calibration (RMSEC) and of prediction (RMSEp) were accomplished. These results confirm that the ET can be used to monitor the phenolic maturity of grapes. The °Brix and Total Acidity which are the parameters commonly used to evaluate the maturity of grapes, could also be predicted using the PLS-1 models but with lower correlation coefficients in prediction

($\sim$0.7) and the models need a higher number of components. In spite of the lower correlation, the models are good enough to confirm that the electrochemical responses also reflected the acidity and the sugar concentration. This indicates that it is possible to obtain simultaneous information of the three main parameters used to monitor the maturity of grapes.

CONCLUSIONS

The advantages of metal oxide nanoparticles as sensing materials for the detection of phenols has been evidenced. They rely on the excellent electrocatalytic properties of the metal oxide nanoparticles, that improved the sensitivity and limits of detection toward phenols with respect to unmodified sensors. Using these exceptional sensing properties, and the cross-selectivity shown by different MONPs, an electronic tongue based on metal oxide nanoparticles has been developed. The chemical changes occurring during ripening could be followed using the MONP-based multisensor system. The sensors based on different MONP show an excellent degree of cross-selectivity. Using PLS-1, correlations have been established between the signal of the multisensor system and the Total Polyphenol Index TPI, the °Brix, and the Total Acidity.

According to this, simultaneous analysis of three important markers of maturity can be achieved using a multisensory system based on MONPs. This means that the ET can be a useful tool to establish with a better accuracy the optimal harvesting date in a single experiment. This method can be used as a supplementary tool to classical analytical techniques.

The results shown here demonstrate the excellent opportunities offered by metal oxide nanoparticles as sensing materials in electronic tongues.

AUTHOR CONTRIBUTIONS

MR-M: conceived the experiments an wrote the paper; CG-H, CM-P, CG-C, and YB: carried out the experiments; JF-E and EB-T: provided de grape samples and analyzed the grapes by chemical methods; MR-P and FM-P: carried out the chemometric analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2018.00131/full#supplementary-material

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3.4. Paper III

A different approach for the analysis of grapes: Using the skin as sensing element.

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3.4.1. Motivation and objective

Phenolic compounds (phenolic acids, flavonoids and tannins) are important components of wines that can strongly influence their final organoleptic properties. In fact, the characteristic color in red wines is, to a large extent, ascribable to the phenolic substances present in the grape skin cells, which are transferred to the must during the maceration step. The optimal point of grape harvesting is traditionally indicated by measuring the sugar content and acidity of the grapes. However, the phenolic content is a factor that must be considered to obtain wines with a high quality.

Grape skins contain phenols bounded to polysaccharides by means of hydrophobic interactions and hydrogen bonds (called cell-wall phenols), and phenols inside the vacuoles of the plant cells or associated with the cell nucleus (called non-cell-wall phenols). The phenolic composition of wines will determine such factors as color, sensory characteristics and the healthy quality of wines. The phenols are transferred from the grapes into the must, so the wine depends on how those phenols are bound in the grape skins. During the maceration step in the winemaking process, the phenolic compounds are transferred from the grape skins to the must.

The polyphenolic and antioxidant content in food and beverages can be assessed by recognized traditional spectrophotometric methods, such as Total Polyphenol index (TPI), FRAP, DPPH, ORAC and/or Folin-Ciocalteu Indexes, which are methods based on the evaluation of the capabilities of an oxidizing agent to induce oxidative damage to a substrate, and also by using multi-parametric methods such as infrared spectroscopy (FTIR, NIR) or gas and liquid chromatography (HPLC) combined with chemometric tools. However, electronic tongue technology is emerging as a supplementary or alternative method to assess food quality. In the case of grapes and wines, as phenolic compounds are redox species, they can be analyzed using electrochemical methods by means of electronic tongues. Unlike the spectroscopy methods, ETs can help to improve the sensitivity at a relatively low cost.

The aim of this work is to develop a new and alternative voltammetric sensor based on a carbon electrode covered with the skin of grapes to monitor their phenolic maturity by improving the intimate contact between the analyte and the electrode surface so that there is no need for sensing material to enhance the electron transfer. By using PLS-1, correlation models were

performed between the voltammetric responses obtained with the skin-modified sensors and the phenolic (Glories or TPI) and sugar (ºBrix, density, sugar content) parameters.

3.4.2. Summary of the research work

Carbon paste electrodes were prepared by mixing graphite powder with a mineral oil (nujol) as already mentioned. The carbon paste was put inside a plastic syringe and a copper wire was used as electrical contact. Then, a piece of grape skin was carefully placed on the surface of the CPE and fixed with a rubber O-ring, maintaining the inner side of the skin in contact with the carbon paste. The sensors were prepared using 3 grape varieties: Prieto Picudo, Mencía (Regadío) and Juan García, collected weekly from *véraison* to harvesting date. In order to evaluate the repeatability and reproducibility of the skin-carbon paste-based sensors (S-CPE), 3 sensors were prepared for each sample, using 3 different grapes from each bunch, making a total of 9 electrodes for each week and 45 electrodes for the whole experiment (9 S-CPE sensors x 5 weeks).

Grapes samples were provided by the vineyard of the Instituto Tecnológico Agrario de Castilla y León (ITACyL). All samples were collected weekly from *véraison* to harvesting date. The Oenological Centre of Castilla y León provided the chemical parameters of TPI, Brix degree, density, sugar content, degree 16.8 and degree 17.5. In addition, the Glories method was carried out to evaluate the phenolic maturity of the grapes at pH 1, providing information of total potential in anthocyanins, and at pH 3, giving information of the total potential in extractible anthocyanins at the pH of the wine.

Once the S-CPE sensors had been prepared, they were tested in phosphate buffer to evaluate their electrochemical responses. Voltammetric responses showed anodic peaks in the range from 0.5 to 0.7 V and cathodic peaks ranging from -0.5 to -0.8 V. These peaks were related to the phenols present in the skin, mainly anthocyanins and proanthocyanins. The electrochemical response for each grape showed that each grape skin has a specific phenolic composition. It is important to notice that the structure and mechanical properties of each type of skin, such as porosity, thickness or hardness, might also influence the electrochemical responses, giving a distinctive response for each grape. Thus, grape skins were monitored in phosphate buffer from week 1 (just one week after *véraison*) to week 5 (harvesting date). The variations in skin composition during ripening were observed. The main characteristic was

observed in the intensity and position of the peaks related to phenols in the collected voltammograms. Peaks increased in intensity from week 1 until a maximum was attained just before harvesting time, after which the intensities decreased. This result may be associated with the fact that the level of anthocyanins increases during ripening and decreases slightly in ripened grapes. Shifts in the position of the cathodic and anodic waves are usually related to polymerization processes. The Mencía sample was the slowest to reach phenolic skin maturation, showing the highest intensity in the week 4, whereas the Prieto Picudo and Juan García ripened faster, with a maximum intensity in week 3. Comparing these results with such traditional methods as the Glories method and TPI, in which the evaluation of phenolic content includes polyphenols extracted from the whole grape (skin, seeds and flesh), the results showed a similar trend to that observed in the Glories results at pH 3.0 and at pH 1.0. However, the differences were more easily observable using S-CPE sensors than the Glories method, while the decrease observed prior to maturation can be detected more easily. TPI values did not show any inflexion and were mainly stable from week 1 to week 4, suddenly increasing in the harvesting week. Therefore, using S-CPE, we can predict the harvesting date one or two weeks before the date by observing the inflexion point in the electrochemical responses.

Using PCA analysis, we obtained information about the ripening over weeks. The PCA loading plots from weeks 1, 3 and 5 showed a progressive change in the places where the samples were located. In week 1 samples, Prieto Picudo and Mencía appeared to overlap and were separate from Juan García, whereas in week 2, the Mencía and Juan García samples appeared to overlap and were separate from the Prieto Picudo. However, in week 5, each sample appeared to be located in a different region in the diagram, demonstrating that the phenolic maturity had been reached and that each grape shows a specific, characteristic phenolic composition.

The progressive maturing of the grapes was analyzed by means of partial least squares-discrimination analysis (PLS-DA). It was observed that the first Principal Component shifted to negative values as the ripening advanced. Then, in week 5, the PC1 returned to positive values, illustrating a circular behavior. This trend has already been described using multisensor systems based on metal oxide nanoparticle CPEs.

Finally, using PLS-1 regression models, we established linear correlations between the data obtained weekly using S-CPE and the chemical parameters obtained by classical methods.

Since voltammograms contain information not only about polyphenols, but also about other components present in the skin, such as sugars, acids or ions, the correlations were performed for each chemical parameter. The Juan García and Prieto Picudo skin sensors showed a good correlation with Glories at pH 1, considering the total extractable polyphenols present in the skin, whereas at pH 3, only the easily extractable polyphenols were considered. The Juan García sensors also had a good correlation with the TPI; however, this result was isolated and the correlations with the rest of the parameters could be established, but with low correlation coefficients (lower than 0.70) and high latent variables. In the case of the Mencía skin sensors, no correlations could be established. These results are, to a large extent, determined by the different properties of the skins, such as porosity or hardness, which can affect the extractability of phenol compounds and may be due to environmental factors. For instance, in this study, we found it difficult to separate the skin from the flesh in the Mencía grape variety. In this sense, the degree of adhesion between skin and flesh during ripening was more variable than for the Juan García and Prieto Picudo varieties, where the skin and flesh were properly separated in every sampling. Moreover, in week 3, the Mencía grapes were collected after heavy rain, and skin and flesh could hardly be separated. These facts might have interfered with the permeability of the skin and therefore with the diffusion of the ions and electron transfer from the skin to the electrode surface.

A different approach for the analysis of grapes: Using the skin as sensing element

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ABSTRACT

In this work, an alternative method to monitor the phenolic maturity of grapes was developed. In this approach, the skins of grapes were used to cover the surface of carbon paste electrodes and the voltammetric signals obtained with the skin-modified sensors were used to obtain information about the phenolic content of the skins. These sensors could easily detect differences in the phenolic composition of different Spanish varieties of grapes (*Mencía*, *Prieto Picudo* and *Juan García*). Moreover, sensors were able to monitor changes in the phenolic content throughout the ripening process from *véraison* until harvest.

Using PLS-1 (Partial Least Squares), correlations were established between the voltammetric signals registered with the skin-modified sensors and the phenolic content measured by classical methods (Glories or Total Polyphenol Index). PLS-1 models provided additional information about Brix degree, density or sugar content, which usually used to establish the harvesting date. The quality of the correlations was influenced by the maturation process and the structural and mechanical skin properties. Thus the skin sensors fabricated with *Juan García* and *Prieto Picudo* grapes (that showed faster polyphenolic maturation and a higher amount of extractable polyphenols than *Mencía*), showed good correlations and therefore could be used to monitor the ripening.

1. Introduction

Harvesting grapes in the optimal point of maturity is a main concern for winemakers (Jackson, 2014). It is therefore important to develop chemical markers to define the optimal ripeness. The physicochemical and biochemical processes occurring during ripening process, produce a continuous rising of the sugars concentration and a decrease in the acid levels. For this reason, the classical parameters measured to monitor grape ripeness and to determine the harvesting date are the berry weight, the must density and the relation between sugars content and total acidity (Robredo, Junquera, González San José, & Barren, 1991) in musts.

To elaborate a high quality red wine, grapes need to reach an appropriate phenolic maturation. To elaborate a high quality red wine, grapes must reach an appropriate phenolic maturation. According to the literature, phenols accumulate in the berries during ripening.

Therefore, an increase in the total phenolic content is a good indicator of the grape maturity (González-San José, Barren, & Díez, 1990; González-San José, Barren, Junquera, & Robredo, 1991; Mahmood, Anwar, Abbas, & Saari, 2012).

The phenolic maturity has been measured using different methods and TPI is one possible approach (Adams, 2006; Garrido & Borges, 2013; Kennedy, 2008; Nogales-Bueno et al., 2017).

In spite of the variety of methods mentioned, the assessment of the grape maturity is difficult task because each parameter changes with ripeness in a different manner (since they depend on different biochemical pathways). Moreover, the optimal maturity values at harvest depend on the variety of grape, the type of wine being made, weather or seasonal factors, or viticultural practices among many other factors (Lohitnavy, Bastian, & Collins, 2010). Another difficulty is that the active metabolism of the skin during grape development has an essential effect on the final quality of the grape berries and wine (Negri

Table 1
Results of the chemical analysis carried out by traditional chemical methods.

Sample	Week	Glories pH 3	Glories pH 1	TPI	°Brix	Density (g/mL)	Sugars (g/L)	Degree 16.8	Degree 17.5
Mencía	S1	0.255	0.396	11	18.71	1.0760	177.2	10.53	10.15
	S2	0.340	0.573	11	20.16	1.0822	193.1	11.47	11.05
	S3	0.454	0.579	11	21.65	1.0889	210.3	12.49	12.00
	S4	0.394	0.508	9	20.58	1.0840	197.7	11.75	11.30
	S5	0.244	0.452	17	20.99	1.0857	202.2	12.01	11.55
Prieto Picudo	S1	0.322	0.376	9	19.56	1.0795	186.3	11.07	10.65
	S2	0.474	0.586	9	20.93	1.0857	202.2	12.01	11.55
	S3	0.443	0.824	11	22.65	1.0933	221.7	13.17	12.70
	S4	0.357	0.662	9	21.05	1.0862	203.3	12.08	11.60
	S5	0.341	0.523	19	22.89	1.0943	224.1	13.31	12.80
Juan García	S1	0.361	0.396	14	17.30	1.0699	161.5	9.59	9.25
	S2	0.376	0.480	14	19.80	1.0809	189.7	11.27	10.80
	S3	0.593	0.609	18	21.59	1.0884	209.1	12.42	11.95
	S4	0.458	0.583	17	20.70	1.0849	200.0	11.88	11.45
	S5	0.316	0.418	29	22.18	1.0911	216.0	12.83	12.35

et al., 2008; Rockenbach et al., 2011; Sokolowsky, Rosenberger, & Fischer, 2015). In fact, the majority of the phenolic compounds present in wine are derived from skin which contains anthocyanins, flavonols and proanthocyanidins (tannins) (Jara-Palacios, Hernanz, Escudero-Gilete, & Heredia, 2014; Nogales-Bueno et al., 2017). So, methods are needed that analyze the phenolic composition of skins. Phenolic compounds are redox active compounds that can be analyzed by means of electrochemical methods (Blasco, González-Crevillén, González, & Escarpa, 2007; Hoyos-Arbelaez, Vazquez, & Contreras-Calderon, 2017; Kilmartin, 2013; Kirsanov, Mednova, Vietoris, Kilmartin, & Legin, 2012; Rudnitskaya et al., 2017). For instance, using simple electrodes (graphite, platinum or ITO glass) the antioxidants present in teas (Buratti, Scampicchio, Giovanelli, & Mannino, 2008; Piljac-Žegarac, Valek, Stipčević, & Martínez, 2010), natural juices (Bordonaba & Terry, 2012; Pisoschi, Cheregi, & Danet, 2009), wines (Makhotkina & Kilmartin, 2010), or onions (Zielinska, Wiczkowski, & Piskula, 2008) can be analyzed.

Chemically modified electrodes can also be used for the detection of antioxidants. In such sensors, the electrode surface is covered with a sensing material that facilitates the charge-transfer reactions between the electrode and the solution (Barroso, Santos-Alvarez, Delerue-Matos, & Oliveira, 2011; Durst, Baumner, Murray, Buck, & Andrieux, 1997; Ziyatdinova, Kozlova, & Budnikov, 2016). Many sensing materials have been used as chemical modifiers, for instance, carbon nanotubes, graphene, nanoparticles, porphyrins, phthalocyanines or conducting polymers among others (García-Hernández, García-Cabezón, Martín-Pedrosa, de Saja, & Rodríguez-Méndez, 2016).

Electrochemical sensors can be combined to form Electronic tongues (ET) According to the IUPAC, an electronic tongue is a multisensor system, which consists of a number of low-selective sensors and cross-sensitivity to different species in solution, and an appropriate method of pattern recognition and/or multivariate calibration for data processing (Rodríguez-Méndez, 2016). Such systems provide global information about complex samples. For instance, the electrochemical responses of the multisensor system, contain information not only of the electro-active components of the sample (i.e. phenols) but also, about other components such as protons, ions, etc. The responses of the sensors are analyzed using a pattern recognition software that allows discriminating samples with different characteristics. In addition, after an appropriate training, mathematical models can be built to correlate the measures carried out using the multisensor system and measures carried out by traditional chemical analysis.

Inspired by the concept of ETs, he aim of this work is to develop a new method to monitor the phenolic maturity of grapes based on Carbon Paste Electrodes (CPEs) that were modified with a piece of the skin of the corresponding grape. Three different varieties of *Vitis vinifera*

grapes were included in this study (*Mencía*, *Prieto Picudo* and *Juan García*). Grape samples were collected weekly along their ripeness process, starting one week after *véraison* and until complete maturity. The electrochemical response of the skin-sensor (*S*-CPE) was registered in phosphate buffer (pH 7.0) using cyclic voltammetry. The discrimination capability of the electrodes and the ability to monitor the phenolic ripening, were analyzed by means of Principal Component Analysis (PCA) and Partial Least Squares (PLS-1). In addition, the electrochemical response of the electrodes was analyzed and compared with chemical data measured by classical chemical methods.

2. Materials and methods

2.1. Reagents and solutions

All reagents were of high purity and purchased from Sigma–Aldrich. Solutions were prepared using deionized water (Milli-Q, Millipore). Phosphate buffer solution 0.01 M (pH 7.0) was prepared from potassium monobasic and dibasic phosphate salts.

2.2. Grape and must samples: selection and chemical analysis

Samples of three different varieties of red grapes: *Mencía*, *Prieto Picudo* and *Juan García* were harvested in September–October 2013 in the vineyards of the Bodega Cooperativa de Cigales and of the Instituto Tecnológico Agrario de Castilla y León, both located in the Castilla y León region (Spain). 200 berries of each variety were collected on a weekly basis, starting one week after *véraison* until harvest (12, 20 and 26 of September, and 4, 8 of October 2013, samples numbered S1 to S5 respectively).

The polyphenolic content of the grapes was estimated using Total Polyphenol Index (TPI) measured as the Absorbance at 280 nm and the Glories method (Glories & Augustin, 1993). Brix, Density, sugar content, Degree (16.8) and Degree (17.5) were analyzed following the international regulations (OIV, 2013). All chemical parameters are shown in Table 1.

2.3. Skin-carbon paste electrodes (S-CPE)

Carbon paste electrodes were prepared by mixing graphite powder (High purity Ultracarbon®, Ultra F purity. Bay City, MI, USA) with the mineral oil Nujol (Fluka), in 1.5:1 (w/w) ratio. The obtained paste was packed in a 1 mL PVC (polyvinyl chloride) syringe (Yang, Denno, Pyakurel, & Venton, 2015). A copper wire, inserted into the syringe, was used as the electrical contact. The voltammetric behavior of the carbon paste electrodes was analyzed in 0.01 M phosphate buffer

Table 2
Skin-CPE sensors prepared in this work.

Sampling date	*Mencía*	*Prieto Picudo*	*Juan García*
September 12, 2013	S1-*M*-CPE	S1-*PP*-CPE	S1-*JG*-CPE
September 20, 2013	S2-*M*-CPE	S2-*PP*-CPE	S2-*JG*-CPE
September 26, 2013	S3-*M*-CPE	S3-*PP*-CPE	S3-*JG*-CPE
October 4, 2013	S4-*M*-CPE	S4-*PP*-CPE	S4-*JG*-CPE
October 8, 2013	S5-*M*-CPE	S5-*PP*-CPE	S5-*JG*-CPE

adjusted to pH 7.0.

A piece from the skin of a fresh grape was carefully peeled using a cutter, placed on the surface of the CPE and fixed with a rubber O-ring. It is important to mention that the inner side of the skin was placed in contact with the carbon paste. Sensors were prepared using grapes from three different varieties that were collected periodically (S1 to S5). Sensors prepared are collected in Table 2.

In order to evaluate the repeatability and reproducibility of the method, devices were prepared using three different grapes from each bunch, choosing those grapes representative of the general ripening state of the vineyard. Moreover, three pieces of skin were cut from each grape and used to prepare electrodes. Using this method, nine electrodes (3 grapes × 3 pieces of skin) were prepared from each type of grape.

2.4. Electrochemical measurements

Cyclic voltammetry was performed using a three electrode configuration, in an EG&G PARC Model 263 potentiostat/galvanostat (Princeton Applied Research Corp., NJ, USA) controlled by the Echem software. *S*-CPEs were used as the working electrode and the counter electrode was a platinum wire. Potential values were referred to an Ag/AgCl electrode. All measurements were carried out in 20 ml of 0.01 M phosphate buffer adjusted to pH 7.0. Cyclic voltammograms were registered from −1.1 to +1.1 V (the scan started at 0 V) at a sweep rate of 0.1 V/s. Voltammograms shown in this work correspond to the average of nine replicas of each grape sample.

2.5. Statistical analysis

Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA), a non-supervised and supervised multivariate method respectively, were used to analyze and compare the voltammetric results of the different samples along maturation. Mathematical correlations between the signals obtained using the sensors and data obtained by classical chemical analysis were established using PLS-1 models.

In order to reduce the number of variables used in multivariate analysis, feature extraction was carried out using the kernel method to compress the information from the original signals and to extract meaningful data from the readings (Medina-Plaza et al., 2015). Using this method, voltammograms were multiplied by 10 smooth, bell-shaped windowing functions defined as:

$$K_i(V_j) = \frac{1}{1 + \left(\frac{V_j - c_i}{a_i}\right)^{2b_i}}$$

where a_i, b_i and c_i define the width, shape and center of the different windowing functions K_i. Subsequently, data were integrated with respect to voltage. Thus, each curve provided ten variables which were used as the input data source for statistical analysis. The analysis was carried out using the software Matlab v5.3 (The Mathworks Inc., Natick, MA, USA).
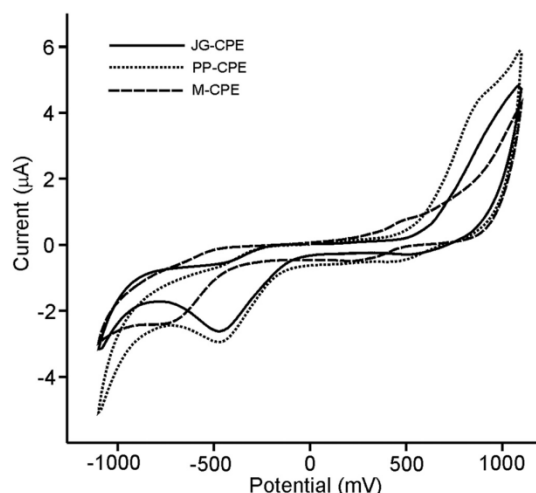
Fig. 1. Cyclic voltammetry of electrodes immersed in 0.01 M phosphate buffer pH 7.0. Scan rate 0.1 V/s. The grapes correspond to the second sampling on 20th September.

3. Results and discussion

As described in the Experimental section, a new class of sensors dedicated to the analysis of grapes was developed. Pieces of the skin grape were cut and placed on the top of the carbon surface of a CPE electrode to obtain Skin-CPE (*S*-CPE) sensors.

The voltammetric behavior of *S*-CPE sensors modified with the skin of grapes obtained from three varieties *Mencía*, *Prieto Picudo* and *Juan García* (denoted as *M*-CPE, *PP*-CPE and *JG*-CPE) was tested in 0.01 M phosphate buffer pH 7.0 in the potential range −1.1 to +1.1 V. Nine replicas of each sensor were measured to study the repeatability of the voltammograms registered. Fig. 1 illustrates the electrochemical responses of *S*-CPEs modified with skins of the three varieties of grapes. In this example, grapes were collected the same day (corresponding to the second sampling collected fifteen days after *véraison*). Voltammograms showed an anodic wave at ca. 0.5 to 0.7 V and a cathodic wave in the region −0.5 to −0.8 V. According to the literature, these peaks can be associated to phenols present in grapes which are mainly anthocyanins and proanthocyanidins (Apetrei, Apetrei, de Saja, & Rodriguez-Méndez, 2011; Blasco et al., 2007; Kilmartin, 2013; Makhotkina & Kilmartin, 2013; Rodriguez-Mendez et al., 2014). Moreover, the position of redox peaks observed in diluted wines carried out using carbon electrodes, depends on the nature of the phenols present in the solution. In our case, due to the complexity of the electrode surface and the variety of phenols present on the skin, redox peaks were wider than those observed in standard aqueous solutions or in diluted wines.

S-CPEs prepared from different varieties of grape showed characteristic electrochemical responses, indicating that each skin grape has a specific phenolic composition which involves anthocyanin content and extractability (Rio Segade, Rolle, Gerbi, & Orriols, 2008). Moreover, because the skin is a part of the sensitive layer, the electrochemical responses could also be influenced by structural and mechanical (porosity, hardness, thickness, etc.) properties of the skin. All these factors produced distinctive responses and justify why these *S*-CPE sensors can be used to discriminate grapes of different varieties or grapes with distinct phenolic content in its skin.

Next objective was to evaluate the capability of the *S*-CPE sensors to monitor the changes taking place during ripening. For this purpose, sensors were prepared using the skin of grapes collected during five consecutive weeks starting from *véraison*.

Fluctuations in the berry compositions occurring during ripening
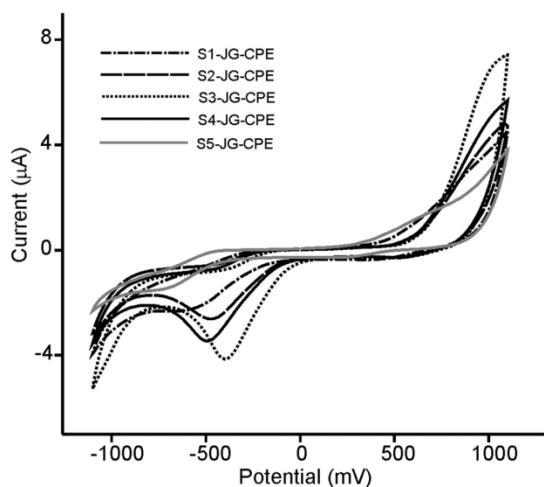
Fig. 2. Electrochemical response of *JG*-CPE electrodes along maturation. Cyclic voltammograms registered in 0.01 M phosphate buffer pH 7.0, Scan rate 0.1 V/s.

Table 3
Voltammetric parameters for the three skin grape-CPEs sensors: *Mencía*, *Prieto Picudo* and *Juan García* obtained in each sampling time.

	Mencía-CPE		*Prieto Picudo*-CPE		*Juan García*-CPE	
Anodic peak	E (mV)	Imax (μA)	E (mV)	Imax (μA)	E (mV)	Imax (μA)
S1-*S*-CPE	455	0.73	475	1.12	851	2.7
S2-*S*-CPE	467	0.72	885	4.43	938	3.66
S3-*S*-CPE	462	0.88	926	5.59	1030	7.09
S4-*S*-CPE	744	1.89	916	5.50	1008	5.05
S5-*S*-CPE	698	1.47	685	2.19	590	1.1
Cathodic peak	E (mV)	Imax (μA)	E (mV)	Imax (μA)	E (mV)	Imax (μA)
S1-*S*-CPE	−706	−1.73	−648	−1.87	−581	−2.24
S2-*S*-CPE	−731	−2.36	−468	−2.94	−468	−2.61
S3-*S*-CPE	−693	−1.24	−453	−3.15	−395	−4.14
S4-*S*-CPE	−726	−2.13	−465	−3.14	−493	−3.45
S5-*S*-CPE	−699	−1.27	−685	−2.19	−694	1.43

caused important changes in the position and intensities of the peaks (Fig. 2). The main changes corresponded to the intensity of the peaks associated to phenols that increased in intensity from *véraison* until a maximum was attained. Then values decreased slightly near harvesting time. This result is in good agreement with variations measured using classical chemical techniques that establish that the level of anthocyanes increases during grape ripening and decreases slightly in ripened grapes (Cadot, Chevalier, & Barbeau, 2011; Conde et al., 2007; Liang et al., 2011; Smith, 2011). The shift of the anodic wave towards higher potentials might be a demonstration of a higher polymerization level of the skin polyphenols. This may be corroborated by the shift of the cathodic wave to less negative potentials.

The extension of the changes detected depended on the variety of grape. The voltammetric parameters obtained from the three varieties of grapes, along maturation, are summarized in Table 3. The maximum
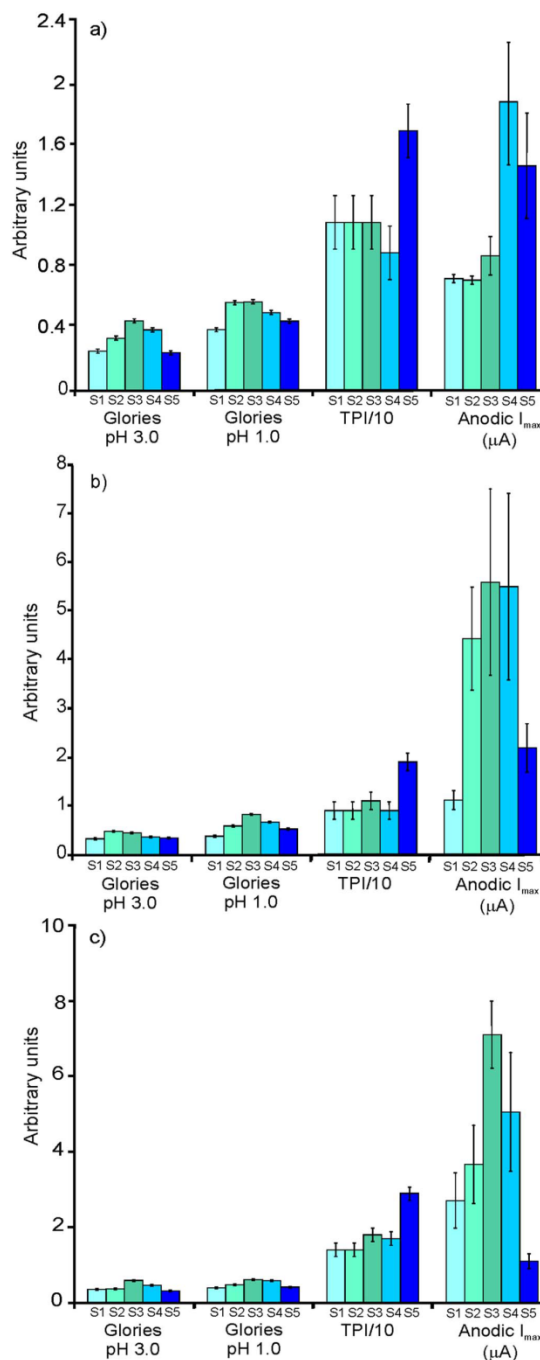
Fig. 3. Representation of the results obtained using Glories at extracts pH 3.0 and pH 1.0, IPT and intensity of the anodic peak for (a) *M*-CPE; (b) *PP*-CPE and (c) *JG*-CPE.
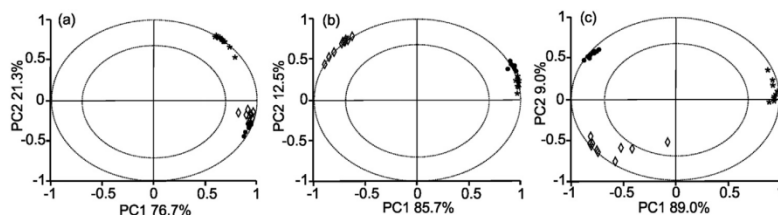
Fig. 4. Loading plot of the PCA. CPE-*Mencía* (●), CPE-*Prieto Picudo* (◊) and CPE-*Juan García* (*) (ten kernels per sensor were extracted) represented at different maturation states (a) S1; (b) S3 and (c) S5.
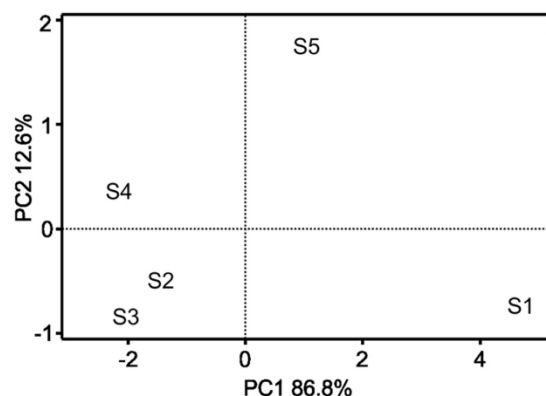
Fig. 5. Partial Least Squares Discriminant Analysis (PLS-DA) of *JG*-CPEs analyzed along maturation.

Table 4
Results of the PLS-1 analysis for *JG*-CPE.

Parameter	Rc[a]	RMSEC[b]	Rp[c]	RMESP[d]	LV[e]
Glories pH 3	0.7204	0.0516	0.6718	0.0572	3
Glories pH 1	0.7858	0.0397	0.7511	0.0437	3
TPI	0.8376	2.2304	0.8017	2.5207	3
°Brix	0.6967	0.9414	0.6262	1.0687	3
Density (g/mL)	0.6913	0.0041	0.6200	0.0047	3
Sugars (g/L)	0.6920	10.5732	0.6209	11.9976	3
Degree (16.8)	0.6918	0.6287	0.6205	0.7135	3
Degree (17.5)	0.6927	0.6023	0.6224	0.6828	3

[a] Squared correlation coefficient in calibration.
[b] Root Mean Square Error of Calibration.
[c] Squared correlation coefficient in prediction.
[d] Root Mean Square Error of Prediction.
[e] Latent variables.

Table 5
Results of the PLS-1 analysis for *PP*-CPE.

Parameter	Rc[a]	RMSEC[b]	Rp[c]	RMESP[d]	LV[e]
Glories pH 3	0.6177	0.0371	0.5616	0.0406	3
Glories pH 1	0.8464	0.0581	0.8175	0.0648	3
TPI	0.6740	2.2142	0.5606	2.6292	4
°Brix	0.6598	0.7149	0.5632	0.8284	5
Density (g/mL)	0.6410	0.0033	0.4875	0.0040	4
Sugars (g/L)	0.6419	8.3456	0.4888	10.1972	4
Degree (16.8)	0.6416	0.4950	0.4884	0.6049	4
Degree (17.5)	0.6431	0.4792	0.4903	0.5857	4

[a] Squared correlation coefficient in calibration.
[b] Root Mean Square Error of Calibration.
[c] Squared correlation coefficient in prediction.
[d] Root Mean Square Error of Prediction.
[e] Latent variables.

intensity is indicated in the table using a grey background.

The peak potentials and intensities of the anodic and cathodic waves of the three varieties of *S*-CPE sensors increased with time and decreased in the last sampling corresponding to the official date of harvest, decided by oenologists, using the Brix degree and the sugar content.

Comparing the three varieties of grapes, *Mencía* showed the slower polyphenolic skin maturation and the maximum redox intensity was observed in the S4 sampling. The skins of *Prieto Picudo* and *Juan García* grapes reached the maximum redox intensity in the S3 sampling, indicating a faster ripening. As shown in Fig. 3, the intensity of the redox peaks obtained from the three types of skin-based sensors along maturation, followed a similar trend than the absorbance value of grape extracts at 280 nm at pH 3.0 and pH 1.0 obtained with the Glories method, in which polyphenols are extracted from the whole grape. The advantage of the *S*-CPE sensors is that variations are more marked than in Glories and the decrease previous to maturation is easier to detect. This fact was specially observed in *JG*-CPE, corresponding to *Juan García* skin grapes, that seems to show faster polyphenolic skin maturation and a higher amount of extractable polyphenols. The variations of the Total Polyphenol Index (TPI) follow a different trend and tend to increase from *véraison* to complete maturity. So, TPI does not show an inflexion point that could be used to establish the optimal harvesting date. According to these results, the use of the skin of grapes as the sensitive layer provides an easy and direct method to assess the phenolic maturity of grapes.

Inspired in holistic analytical methods where arrays of sensors are combined with a pattern recognition software (Merkyte, Morozova, Boselli, & Scampicchio, 2017; Pigani et al., 2018; Rodriguez-Méndez, de Saja, Medina-Plaza, & Garcia-Hernandez, 2016; Sliwinska, Wisniewska, Dymerski, Namiesnik, & Wardenci, 2014), the electroanalytical capacity of the *S*-CPE electrodes to monitor grape maturation was evaluated using Partial Least Squares-Discriminant Analysis (PLS-DA) and Principal Component Analysis (PCA).

PCA loading plot showed that *M*-CPE, *PP*-CPE and *JG*-CPE prepared in S1 (just one week after *véraison*), appeared in similar areas of the diagram (Fig. 4). Ripening caused progressive changes in the molecular and mechanical properties of skins and in their porosity. In the last sampling corresponding to mature grapes, the signals of *M*-CPE, *PP*-CPE and *JG*-CPE skin-based sensors appeared in different regions of the diagram, confirming that each grape possess a characteristic phenolic composition and the three varieties of grapes could be clearly discriminated.

Fig. 5 shows the Partial Least Squares-Discriminant Analysis (PLS-DA) for *PP*-CPE samples collected along the maturation (five samplings). It was observed that the first Principal Component shifted to negative values as the ripening advanced. Then, five weeks after *véraison*, the PC1 returned to positive values. This circular behavior has already been observed using multisensors systems based on electrochemical electrodes (Medina-Plaza et al., 2016). PCA results found in *Mencía* and *Prieto Picudo* were similar and the clusters corresponding to the progressive maturation of grapes turned clockwise and tended to return to the initial position (data not shown).

A PLS-1 multiparametric model was established to find correlations between the results obtained with our skin sensors and the chemical

Table 6
Results of the PLS-1 analysis for *M*-CPE.

Parameter	Rc[a]	RMSEC[b]	Rp[c]	RMESP[d]	LV[e]
Glories pH 3	0.5129	0.0561	0.2143	0.0728	10
Glories pH 1	0.3603	0.0563	0.0336	0.0708	10
TPI	0.6916	1.5065	0.5394	1.8829	5
°Brix	0.3472	0.7962	−0.1142	1.0638	10
Density (g/mL)	0.3379	0.0035	−0.1231	0.0047	10
Sugars (g/L)	0.3386	8.9652	−0.1239	11.9524	10
Degree (16.8)	0.3401	0.5305	−0.1198	0.7067	10
Degree (17.5)	0.3379	0.5012	−0.1257	0.6684	10

[a] Squared correlation coefficient in calibration.
[b] Root Mean Square Error of Calibration.
[c] Squared correlation coefficient in prediction.
[d] Root Mean Square Error of Prediction.
[e] Latent variables.

parameters obtained by classical chemical techniques. Voltammograms can bring information not only about the polyphenolic content, but also about other components present in the skin (sugars, acids or ions) that can affect the electrochemical response. For this reason, attempts were made to use the *S*-CPEs to assess not only the phenolic content, but also to determine simultaneously other indicators of maturity.

Tables 4, 5 and 6 show the statistical parameters for the PLS-1 regression for calibration and validation (cross-validation). The root mean square errors and the correlation coefficients at calibration (RMSEC) and prediction (RMSEP) are shown in the tables. Rc (and Rp) are correlation coefficients of calibration (and prediction). LV indicates the number of latent variables used in the calculations.

Good relationships were found between the responses provided by the *JG*-CPE (Table 4) and *PP*-CPE sensors (Table 5), and the phenolic content measured by Glories and TPI. Higher correlation coefficients were found with Glories at pH 1.0 than at pH 3.0. The extracts at pH 1.0 take account of the total extractable polyphenols, whereas at pH 3.0 only easily extractable polyphenols are measured. Phenolic extractability depends of their cellular localization and degree of polymerization (Gagné, Saucier, & Gény, 2006). These data indicate that *JG*-CPE and *PP*-CPE sensors can detect most of the polyphenolic content of the skin. In the case of TPI, correlations coefficients were not so high because this method has interferences from non-phenolic compounds that contain aromatic rings (nucleotides, aromatic amino acids, peptides or proteins). Moreover, because the whole voltammograms was used to build the mathematical model, electrochemical data bring information not only about phenols but also about other components of the skin. For this reason, good correlations were also found with other parameters of interest in oenology including sugar, degree brix, grade and density.

However, low correlations were found using the *M*-CPE (Table 6). This lack of correlation might be attributed to the delay in the phenolic maturation process of *Mencía* grapes and to the different properties of their skins. In addition, it is known that the porosity and the structural properties, such as number of cell layers from cuticle to flesh, and mechanical properties of the skins, such as hardness, vary from one variety of grape to another and are also influenced by environmental factors (Rolle, Gerbi, Schneider, Spanna, & Río Segade, 2011). The evolution of skin hardness during the ripening period is not completely known, although it considered an adequate parameter for the estimation of the extractability of phenol compounds (Rolle et al., 2011). An important observation in our study is the higher difficulty to separate skin and flesh in *Mencía* grapes. In this sense, the degree of adhesion between skin and flesh, along ripening, was more variable in this variety that for *Juan García* and *Prieto Picudo*, varieties where the skin and flesh were properly separated in every sampling. For instance in sampling S3, *Mencía* grapes were collected after a heavy rain, and skin and flesh could hardly be separated. These facts might have interfered in the permeability of the skin and in consequence with the diffusion of

ions and electron transfer from the skin to the electrode.

4. Conclusions

Skin-CPE electrodes fabricated by covering a carbon electrode with the skin of grapes can be used to evaluate the phenolic content of grapes. The redox processes at electrode surface, provided information about phenolic compounds present in the skin. The electrochemical response of the three different skin-sensors electrodes along the maturation, could be used to monitor the ripening process. Good correlations were found with other parameters suh as sugar content or °Brix usually used to establish the optimal maturity. The phenolic maturation process of the vintage and the structural and mechanical skin grape properties, are essential factors, to found good correlations between the responses provided by *S*-CPE sensors and chemical data measured by classical chemical methods. Thus the skin sensors fabricated with *Juan García* and *Prieto Picudo* grapes, that showed a faster polyphenolic skin maturation and a higher amount of extractable polyphenols than *Mencía* variety, showed a very good correlations and therefore could be used to monitor the ripening of *Juan García* and *Prieto Picudo* grapes. Althought the presented work brings a new and original approach to monitor the phenolic maturity, it might be noticed that the elaboration of the sensors must be carried out very carefully when peeling off the grape skin.

Conflict of interest

The authors report no conflict of interest.

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3.5. Paper IV

Electrochemical behavior of polypyrrol/AuNP composites deposited by different electrochemical methods: sensing properties towards catechol.

By Celia García-Hernández, Cristina García-Cabezón, Cristina Medina-Plaza, Fernando Martín-Pedrosa, Yolanda Blanco, José Antonio de Saja and María Luz Rodríguez-Méndez.

Chapter 3: Results and discussion

*Paper IV: Electrochemical behavior of polypyrrol/AuNP composites deposited
 by different electrochemical methods: sensing properties towards catechol.*

3.5.1. Motivation and objective

Organic π-conjugated polymers have received much attention for sensing applications. Some of the most used CPs for industrial applications or fundamental research have been polyacetylene (PA), polyaniline (PANI), polypyrrole (Ppy) and polythiophene (PT). In general, CPs have attractive properties, such as electrical conductivity, workability, low cost, light weight, resistance to corrosion, high electron affinity, redox activity and excellent mechanical and optical properties.

In a neutral state, conjugated polymers behave as insulators; however, their conductivity can be increased by doping through the oxidation (p-doping) and reduction (n-doping) using chemical or electrochemical methods. For example, the backbone of Ppy is neutral in the reduced state and positive in the oxidized state, needing a counterion that diffuses into the polymer during charging and out during neutralization, maintaining electroneutrality. When the conducting polymers are exposed to higher oxidative potentials than their standard, an overoxidation is produced, causing a de-doping and therefore loss of electrical conductivity.

Ppy films have been prepared by means of electrochemical reactions more than by chemical synthesis. However, other polymerization methods that have been reported for Ppy preparation include photochemistry, solid-state, plasma, pyrolysis, metathesis and/or concentrated emulsion. However, the electrochemical methods are the most suitable to prepare thin solid films due to various factors: the simplicity of the technique, easy control of film thickness, facility of doping during the synthesis, the availability of several dopant ions and the formation of films with a good quality. In this way, polypyrrole is electrodeposited onto a positively polarized working electrode by a condensation reaction from the bulk solution phase of monomer units, which also contains a negatively charged counterion that maintains the electroneutrality along the Ppy backbone that will have positive charges.

Conducting polymers have been widely used to prepare composites materials, along with other sensitive materials with electrochemical properties, such as metal nanoparticles, carbon materials, metal oxide nanoparticles, MOFs, dyes and/or enzymes to be implemented in several applications, including electrochemical sensors. For example, composites of CPs and carbon-based materials can improve selectivity for target molecules in sensor devices. Moreover, the combination of metal and metal nanoparticles with conducting polymers for electrochemical

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device purposes can increase the electron transfer between the electrolyte and the electrode. Metal and metal oxide nanoparticles exhibit a high performance with respect to their bulk state as electrode materials. For instance, AuNPs have unique electrochemical properties for sensing applications and can be synthesized by means of several methods, including wet chemical synthesis, electrochemical deposition or monolayer formation. Therefore, conducting polymer/AuNPs composites are good candidates to modify electrode surfaces and improve the electrochemical performance of sensors.

The objective of this work is to develop new Ppy/AuNPs composites and study the factors that can influence their conductivity and electrochemical behavior, such as the electrodeposition technique used to prepare Ppy films (chronoamperometry or chronopotentiometry), the way of incorporating AuNPs into the polymeric matrix, the importance of the selected substrate (platinum or stainless steel), as well as the influence of the electrolyte solution in sensor performance. The electrochemical responses of the as-prepared sensors were studied for catechol and the selected sensors with the best performances will be further implemented in another experimental work to analyze grapes and wines.

3.5.2. Summary of the research work

The electrochemical sensors were prepared by electrodepositing Ppy films from an aqueous solution containing pyrrole and sodium 1-decanesulfonate (DSA), which was used as the counterion to maintain the electroneutrality of the Ppy films. Two electrochemical techniques were tested: chronopotentiometry (CP) at a constant potential of 0.8V for 300 s, and chronoamperometry (CA) at a constant current of 0.02 mA for 300 s. Ppy/AuNPs were prepared following two strategies: in the first, referred to as the trapping method, AuNPs were previously synthesized using the well-known procedure proposed by Slot and Geuze and then mixed with the solution of pyrrole and DSA before the electrodeposition. In the second, referred to as the cogeneration method, a solution of tetrachloroauric acid ($HAuCl_4$) was mixed with the solution containing pyrrole and DSA and further electropolymerization was carried out to form *in situ* AuNPs simultaneously with the deposition of the film. Moreover, Ppy and Ppy/AuNPs films were deposited onto platinum and stainless steel substrates.

Electropolymerization experiments, both chronopotentiomtry and chronoamperometry, evidenced that the presence of AuNPs or tetracloroauric acid in the solution of pyrrole and DSA impeded the oxidation of the monomers, making the final oxidation potential stay over the potential at which the monomer is oxidized. AuNPs previously synthesized impeded the oxidation of the monomer even more than the AuNPs generated *in situ*. The structural characterization by SEM confirmed the incorporation of AuNPs with an average size of 30-40 nm, uniformly dispersed in the Ppy matrix. The structures of films deposited onto platinum (Pt) and stainless steel (SS) were almost identical. However, the number of AuNPs incorporated into the Ppy matrix was higher when using CP and the cogeneration method.

Electrochemical impedance spectroscopy was carried out in order to study the conductivity of the films. In platinum substrate, AuNPs inserted into a Ppy matrix evidenced a change in the electrical behavior of the sensors, reducing the resistance by facilitating the electron transfer. Moreover, the conductivity of films improved in those prepared by chronopotentiometry and inserting AuNPs by the cogeneration method, which turned out to be the most suitable for inserting a higher amount of AuNPs. In stainless steel substrate, EIS experiments of films deposited by CA were irreproducible, indicating that films were unstable. Results obtained by CP produced reproducible responses, but the electron-transfer resistance and impedance values were higher for SS than for those obtained in platinum substrate, corroborating the fact the conductivity of films on Pt substrate was higher.

The electrochemical behavior of Ppy and Ppy/AuNP sensors was studied by cyclic voltammetry in a KCl electrolyte solution. The first scan was always different from the subsequent cycles. Ppy sensors showed two redox processes in the first scan, corresponding to the polaron and bipolaron of Ppy. In the next cycles, a broad oxidation peak and a cathodic peak were observed. Results were similar for both CA or CP; however, the substrate played an important role, the intensities obtained when using SS substrate were lower than in the case of using Pt.

The electrocatalytic performance of AuNPs was also confirmed by cyclic voltammtrey. In platinum substrate, Ppy/AuNP films generated by CP and CA showed an important increase in the intensities compared to Ppy films. Moreover, the separation between the anodic and cathodic peaks was slightly reduced. In stainless steel substrates, a decrease in the intensities for Ppy/AuNP films compared to Ppy/AuNP films on Pt was observed. This result points to an interference factor between AuNPs and SS. Moreover, Ppy/AuNP films deposited on Pt substrate

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were more stable and reproducible over time than films obtained onto SS substrates. However, that irreproducibility was assigned to the electrolyte solution, specifically to a pitting process produced by chloride ions. Changing the electrolyte solution to phosphate buffer did improve the reproducibility of films deposited onto SS substrates.

The electrochemical sensing behavior of the Ppy and Ppy/AuNP films was also investigated for catechol. The electrochemical response was characterized by the two-electron redox process, due to the oxidation of catechol to benzoquinone. AuNPs increase the intensities of the peaks and also their reversibility. This effect was stronger when using CP instead of CA as the amount of AuNPs was higher using the chronopotentiometry method. The method of inserting AuNPs (trapping or cogeneration) did not strongly influence the results, probably due to the minimal differences in the concentration of the AuNPs. Limits of detection in catechol were in the range from 10^{-5} to 10^{-6} mol/L, lower than concentration values found in foods and beverages. AuNPs in Ppy films improved the limits of detection by almost one order of magnitude and, in general, the limits were lower when using Pt substrate, showing higher sensitivities and regression coefficients.

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Electrochemical behavior of polypyrrol/AuNP composites deposited by different electrochemical methods: sensing properties towards catechol

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catechol; conducting polymers; electropolymerization; gold nanoparticles (AuNPs); polypyrrole

Abstract

Two different methods were used to obtain polypyrrole/AuNP (Ppy/AuNP) composites. One through the electrooxidation of the pyrrole monomer in the presence of colloidal gold nanoparticles, referred to as trapping method (T), and the second one by electrodeposition of both components from one solution containing the monomer and a gold salt, referred to as cogeneration method (C). In both cases, electrodeposition was carried out through galvanostatic and potentiostatic methods and using platinum (Pt) or stainless steel (SS) as substrates. Scanning electron microscopy (SEM) demonstrated that in all cases gold nanoparticles of similar size were uniformly dispersed in the Ppy matrix. The amount of AuNPs incorporated in the Ppy films was higher when electropolymerization was carried out by chronopotentiometry (CP). Besides, cogeneration method allowed for the incorporation of a higher number of AuNPs than trapping. Impedance experiments demonstrated that the insertion of AuNPs increased the conductivity. As an electrochemical sensor, the Ppy/AuNp deposited on platinum exhibited a strong electrocatalytic activity towards the oxidation of catechol. The effect was higher in films obtained by CP than in films obtained by chronoamperometry (CA). The influence of the method used to introduce the AuNPs (trapping or cogeneration) was not so important. The limits of detection (LOD) were in the range from 10^{-5} to 10^{-6} mol/L. LODs attained using films deposited on platinum were lower due to a synergy between AuNPs and platinum that facilitates the electron transfer, improving the electrocatalytic properties. Such synergistic effects are not so pronounced on stainless steel, but acceptable LOD are attained with lower price sensors.

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Introduction

Polypyrrole (Ppy) is one of the most extensively studied, conducting polymers due to its good electrical conductivity and redox properties [1,2]. Ppy films can be easily generated by electropolymerization and used as a strong adherent layer using different electrochemical techniques [3]. Electrodes that are chemically modified with Ppy have good electrocatalytic activity. For this reason, they have been widely used as chemical sensors for the detection of a variety of substances. The structure and sensing properties of the Ppy films are considerably influenced by the electrochemical method used for the polymerization (potentiostatic, galvanostatic or potentiodynamic), by the electrochemical conditions (such as voltage, intensity, or scan rate), and by other experimental conditions such as the nature and concentration of the doping agent or the nature of the substrate [4]. This versatility can be used to better control the development of electrochemical sensors with the appropriate selectivity, reproducibility and sensibility towards a particular application.

Recently, composite nanomaterials based on conducting polymers and metal nanoparticles (NPs) of different metals have been developed. Gold nanoparticles (AuNPs) have attracted considerable interest because of their unique optical, electronic and catalytic properties [5-8]. Conducting polymer–gold nanoparticle composites exhibit improved physical and chemical properties over their single-component counterparts and are the focus of intensive research [9-12]. In the case of sensors, it has been reported that the insertion of NPs into the sensing layer provides remarkable properties compared to conventional polymeric matrices. Several examples have been reported in the literature. For instance, electrochemically deposited Ppy/AuNP films have demonstrated a great potential to detect DNA [13], ammonia gas at room temperature [14], caffeine [15] or hydroxylamine [16] among others.

Ppy/AuNP composites can be prepared by chemical and electrochemical polymerization. Electrochemical methods provide a better control of the structure and properties of the composite by controlling the electrochemical conditions during film generation [17]. The electrodeposition of the composite can be achieved using different strategies [18], mainly through the electrooxidation of the monomer in the presence of colloidal gold nanoparticles and the corresponding doping agent [19] but also by electrodeposition of polymer and metal from two separate solutions [20,21] or by electrodeposition of both components from one solution containing a monomer and a metal salt [17]. Finally, layers of electrodeposited polypyrrole and gold nanoparticle films can also been obtained from a single solution where PPy chains served as the reductant of tetrachloroauric acid [22].

Most of the works devoted to the electrosynthesis of Ppy/AuNPs films, are often limited to establish recipes to prepare the films and to tests their electrocatalytic or sensing properties. It could be expected that the electrocatalytic and the sensing properties of the Ppy/AuNPs films directly depend on the polymerization conditions. However, the influence of the polymerization conditions in the properties of Ppy/AuNPs electrodes has not been yet studied.

One of the fields where electrochemical sensors are having an important success is in the detection of phenolic compounds, which are strong antioxidant reagents present in foods, with beneficial effects on human health [23]. As phenols are electroactive compounds, they can be detected by amperometric or voltammetric techniques using graphite or platinum electrodes [24-26]. In addition, electrodes chemically modified with a variety of sensing materials (e.g., phthalocyanines or conducting polymers) have been successfully used as voltammetric sensors for the detection of antioxidants [27]. It has also been demonstrated that the combined use of electrocatalytic materials such as phthalocyanines and nanoparticles, can induce synergistic effects that increase the sensitivity of the sensors [28]. Following this idea, Ppy/AuNPs composites could be good candidates as electrocatalytic materials for the detection of phenols.

The objective of this work was to develop new voltammetric sensors based on electrodeposited Ppy/AuNps for the detection of catechol (an antioxidant of interest in the food industry) and to evaluate the influence of the electrodeposition method in their performance. For this purpose Ppy/AuNp films doped with 1-decanesulfonic acid (DSA) were deposited using different methods. The first approach consisted on the electrodeposition of the Ppy/AuNPs films from a solution containing the monomer and tetrachloroauric acid (denoted as "cogeneration", C). The second approach consisted of the electrodeposition of the Ppy/AuNPs composited from a solution containing the monomer and gold nanoparticles previously formed (denoted as "trapping" method, T). In both methods, electrodeposition was carried out by chronoamperometry (CA) and by chronopotentiometry (CP). Particular attention was paid to the study of the influence of the substrate used for the electrodeposition that was carried out onto classical platinum electrodes and on stainless steel substrates. This aspect could play a crucial role not only in the structure, properties and performance of the sensor but also in the final price.

The structure and sensing properties of voltammetric sensors modified with Ppy/AuNPs films prepared under different conditions were evaluated and compared.

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Results and Discussion

PPy/AuNPs films were prepared using two different approaches referred as "trapping method" and "cogeneration method", which are described in the Experimental section. The electropolymerization of pyrrole was generated under potentiostatic and galvanostatic conditions on both platinum and stainless steel substrates, resulting in the formation of nanocomposites based on gold nanoparticles within the polypyrrole layer.

Electropolymerization of Ppy/AuNPs

Figure 1 shows the potential (E) vs time (t) curves registered during the electrodeposition PPy/AuNPs films using a galvanostatic process. The figure compares the results obtained by the trapping and the cogeneration methods. The CP registered for Ppy (in the absence of AuNPs) is also shown for comparison. As expected, as the current pulse was applied, a sharp decrease in the potential was observed. This was due to the charge of the double layer capacitance that produces a nucleation process at the electrode surface. Then, at the potential at which the monomer is oxidized, a stabilization and growth step was attained, which was characterized by a "plateau", where the potential varied only slightly.

Figure 1: Chronopotentiometric curves obtained during the polymerization of (a) Ppy-CP$_{Pt}$, (b) Ppy-CP$_{SS}$ (c) Ppy/AuNPs-T-CP$_{SS}$ (d) Ppy/AuNPs-T-CP$_{Pt}$, (e) Ppy/AuNPs-C-CP$_{Pt}$ and (f) Ppy/AuNPs-C-CP$_{SS}$.
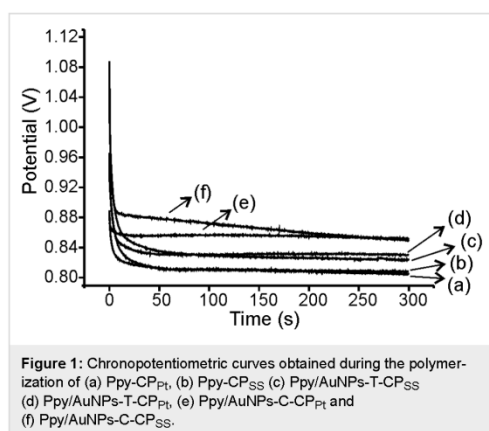
The highest nucleation rate (faster electrode potential variation) was observed when Ppy was polymerized in the absence of gold nanoparticles or tetrachloroauric acid. At the same time, the final potential (at which the monomer is oxidized) was clearly lower for Ppy films. According to these results, it can be assumed that, the the presence of AuNPs affects the nucleation of Ppy, making impeding the oxidation of the monomers.

The final potential attained when polymerization was carried out in the presence of previously formed AuNPs (trapping), was lower than the potential obtained when AuNPs were generated

in situ (cogeneration). This result seems to confirm that AuNPs affect the nucleation process. Only a small difference was found in the final potential attained by Ppy/AuNPs deposited on Pt or on SS.

Nanocomposites Ppy/AuNP were also prepared by trapping and cogeneration using CA. Curves show the characteristic stepped shape of the potentiostatic polymerization: After a short induction period where diffusion controls the monomer oxidation, the current increased rapidly with time, where polymer started nucleating and growing on the electrode surface. Finally, the current reached a plateau coinciding with a continuous and gradual polymer growth [29,30]. The calculated charges are shown in Table 1.

Table 1: Polymerization charges calculated for Ppy and Ppy/AuNPs composites prepared by chronoamperometry.

Sample	Q (C/cm^2)	
	SS	Pt
Ppy-CA	0.62	0.62
Ppy/AuNPs-T-CA	0.07	0.08
Ppy/AuNPs-C-CA	0.12	0.22

In good accordance with results shown in previous paragraphs, also when using CA, the polymerization charge was strongly dependent on the presence of AuNPs and the mass deposited in the absence of AuNPs was higher than the mass deposited in the presence of gold. The charge calculated for films obtained by cogeneration was higher than that of the films obtained by trapping. That is, the amount of polymer deposited followed the same trend regardless whether CP or CA was used (Ppy > Ppy/AuNP-C > Ppy/AuNP-T). This result also points to the role of AuNPs in the nucleation of Ppy, which impede the the oxidation of the monomers. The coefficients of variation (% CV) were always lower than 2% regardless of the electropolymerization method or the susbstrate used.

Structural characterization: SEM studies

The microscopic structure of the Ppy/AuNP films analyzed by scanning electron microscopy confirmed the incorporation of the AuNPs into the Ppy films (Figure 2). They were uniformly dispersed in the typical granular raspberry PPy matrix. The structures of films deposited onto SS or Pt were almost identical.

The average size of the AuNPs was between 30 and 40 nm (regardless of the method used), which is consistent with the absorbance at 540 nm observed by colloid that was used to obtain the nanocomposites by trapping. The number of AuNPs

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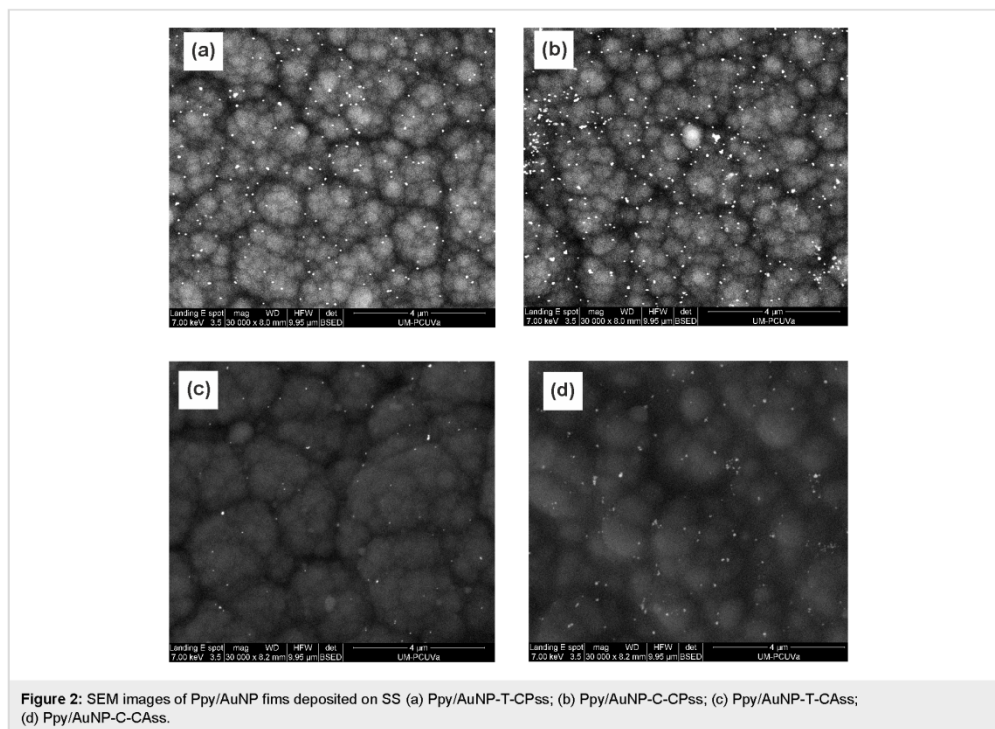
Figure 2: SEM images of Ppy/AuNP fims deposited on SS (a) Ppy/AuNP-T-CPss; (b) Ppy/AuNP-C-CPss; (c) Ppy/AuNP-T-CAss; (d) Ppy/AuNP-C-CAss.

incorporated in the Ppy films was higher when using CP than that when using CA. In turn, using cogeneration, the amount of nanoparticles incorporated was higher than using trapping.

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) can provide information about the conductivity changes resulting from the insertion of AuNPs in the Ppy films. The complex impedance can be plotted as the real (Z_{real}) vs imaginary ($Z_{imaginary}$) components (Nyquist plot), which are related to the resistance and capacitance of the cell, respectively. At high frequencies (left part of the diagram) the semicircular part is associated to electron-transfer limited processes. The diameter of the semicircle is equivalent to the electron-transfer resistance (R_{ct}). The linear part that appears at lower frequencies is related to diffusion limited processes. In the case of Ppy deposited by CA, the Nyquist plot (Figure 3a) was a semicircle (R_{ct}, 45.54 kΩ). The electrochemical process was thus, dominated by electron transfer.

The insertion of AuNPs in the Ppy films clearly modified the electrical behavior. In effect, the Nyquist plot of Ppy/AuNP-T-CA$_{Pt}$ films obtained by trapping (Figure 3b)

showed a semicircle with a smaller R_{ct} (13.52 kΩ) in the high frequencies region. At low frequencies a straight line with a slope of 45° was observed indicating a contribution of both electron transfer and diffusion processes. In Ppy/AuNPs-C-CA$_{Pt}$ films obtained by cogeneration R_{ct} was practically zero and only the linear part corresponding to diffusion control was observed (Figure 3c).

These results confirm the ability of AuNPs to reduce the resistance by facilitating the electron transfer. In fact, as observed in SEM images the number of AuNPs inserted in the films was higher using cogeneration, explaining the drastic decrease in the resistance. This is in good agreement with previous published results that indicated that the presence of AuNPs in the polymer matrix resulted in an increase in conductivity [31].

EIS results of Ppy/AuNPs films deposited by CP showed similar trends, but resistance and impedance values were clearly smaller than those observed in films deposited by CA. For instance, the impedance values of Ppy/AuNP-C-CP$_{Pt}$ were one third smaller than those obtained by CA (Figure 3d). Again, the high number of AuNPs inserted in the nanocomposite by CP, explains the improvement in the conductivity.

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Figure 3: Nyquist plot of films deposited onto Pt registered in 0.1 mol/L KCl. Frequency swept from 105 to 0.1 Hz (a) Ppy-CA$_{Pt}$; (b) Ppy/AuNP-T-CA$_{Pt}$; (c) Ppy/AuNP-C-CA$_{Pt}$; (d) Ppy/AuNP-C-CP$_{Pt}$.
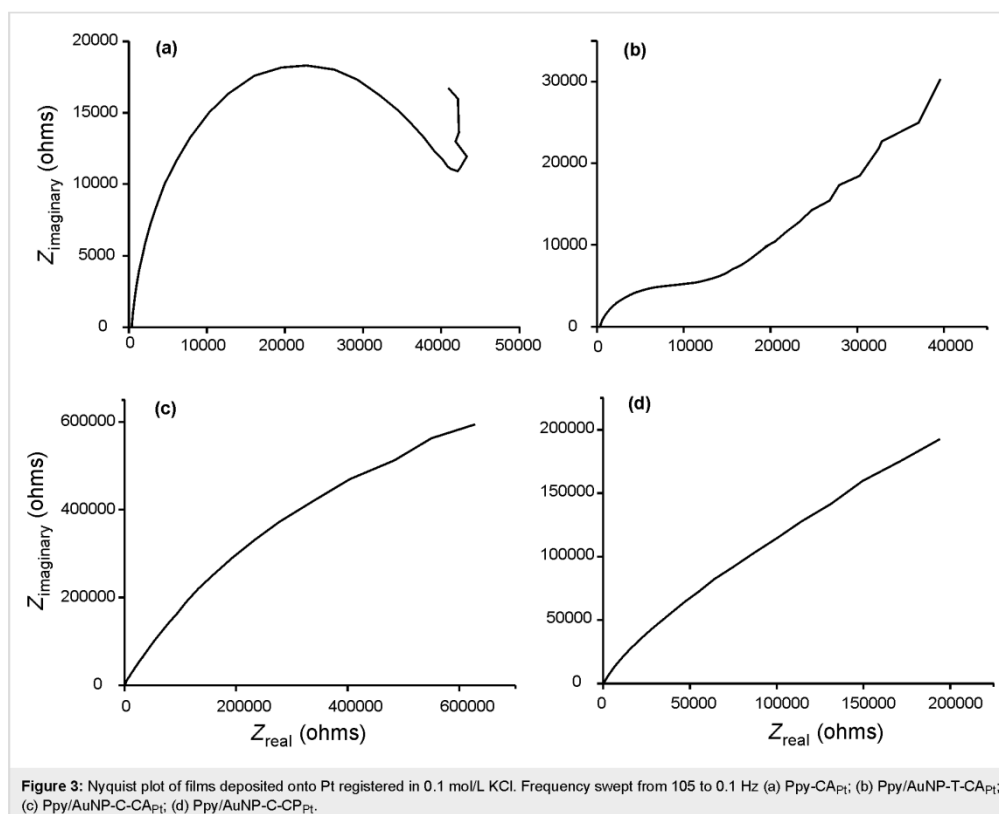
It is important to point out, that EIS measurements carried out in films deposited on SS by CA where irreproducible, indicating that the films obtained were unstable. Films deposited on SS by CP produced reproducible results but with higher R_{ct} and impedance values than those found on the platinum substrate. In fact, in the Nyquist plot for bare Ppy-CP$_{SS}$ the R_{ct} was so high that the semicircle was not completed.

According to these results, and taking into account that lower R_{ct} values correspond to an increase of the voltammetric signal [32] the cogeneration combined with chronopotentiometry seems to be the most suitable electrodeposition technique to prepare voltammetric sensors.

Electrochemical behavior of Ppy/AuNPs prepared using different techniques

The electrochemical behavior of Ppy and Ppy/AuNP films was analyzed using cyclic voltammetry in 0.1 mol/L KCl solution. The responses are influenced by the polymerization method, the deposition technique and the type of substrate. Before going

into the details, it is important to notice that, in good accordance with previously published results, the first scan was always different from the subsequent cycles. Subsequent cycles were highly reproducible [22]. For this reason, in the next figures, the fifth scan will be displayed.

For Ppy films deposited on platinum using CA or CP, the first cycle showed two redox processes corresponding to the polaron and bipolaron. In successive cycles one single process (anodic wave at −0.35 V and the corresponding cathodic peak at around −0.5 V) was found. When deposition was carried out on SS, voltammograms showed lower intensities and in the case of Ppy-CA$_{SS}$, a certain irreproducibility.

When AuNPs were introduced in the films (Ppy/AuNPs), the preparation method induced important differences. In films deposited on platinum, the insertion of AuNPs caused an increase in the intensity of the peaks. Simultaneously the separation between the anodic and the cathodic waves was reduced. This is illustrated in Figure 4 for films deposited on Pt by CP.

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According to this, it can be concluded that the reversibility of the redox processes is improved in Ppy/AuNP composites. The increase was more pronounced in films deposited by CP than in films deposited by CA. As the number of AuNPs inserted in the films was higher in films deposited by CP (Figure 2), the electrocatalytic effect of the AuNPs is confirmed. This is also in agreement with EIS results that demonstrated that the insertion of AuNPs increased the conductivity.
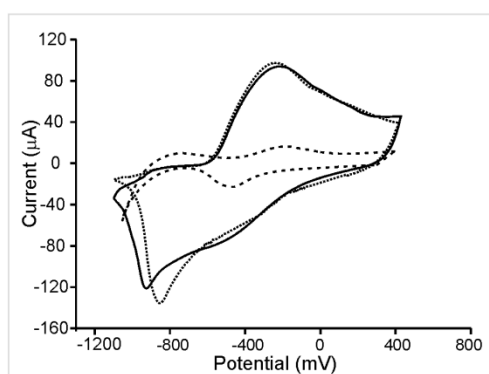
Figure 4: Cyclic voltammograms of Ppy-CP$_{Pt}$ (dashed line), Ppy/AuNPs-T-CP$_{Pt}$ (dotted line) and Ppy/AuNPs-C-CP$_{Pt}$ (solid line) immersed in 0.1 mol/L KCl. Scan rate 0.1 V/s.

It is important to point out that, when the deposition was carried out on SS, a decrease in the intensity of the peaks accompanied by a separation between the anodic and cathodic waves was observed. This behavior pointed to the interference between SS and AuNPs. In addition, some irreproducibility was observed (as it also happened in EIS experiments).

In fact, a part from the differences already commented in the electrochemical behavior of Ppy/AuNps films deposited onto Pt and SS, the most remarkable difference was related to their stability and lifetime. We already mentioned that the first cycle was different from the subsequent ones, but the changes occurring in successive cycles were more pronounced in films deposited on stainless steel substrates. The variation coefficients calculated in films deposited on Pt by CP or CA were less than 2% and 5%, respectively. The %CV calculated from films deposited on SS were 8% for CP and 15–20% for CA. Moreover, when electrodes were withdrawn from the solution and reintroduced in the tested solution, electrodes deposited onto SS, changed completely their electrochemical response and could not be further used.

The above results established the important influence of the electropolymerization method (CA or CP) and of the nature of

the substrate in the electrochemical properties of the films. The influence of the method used to introduce the AuNPs (trapping or cogeneration) was not so important. In fact, when films were deposited onto Pt, the differences in the voltammograms prepared by trapping or by cogeneration were minimal. In contrast, when SS was used as the substrate, the differences observed between trapping and cogeneration could be ascribed to the irreproducibility and therefore conclusions could not be deduced.

The irreproducibility observed in stainless steel can be clearly attributed to pitting processes produced by chloride ions. In consequence, reproducibility could be improved by changing the supporting electrolyte.

According to this idea, the influence of the supporting electrolyte was further investigated using phosphate buffer. As expected, the large size and high charge of the phosphate anions, made difficult the diffusion of anions inside the polymeric film producing a broadening of the peaks and the increase in the separation between the anodic and the cathodic waves that appeared at −0.15 and −0.8 V, respectively [33] . A part from the broadening of the peaks, the effects caused by AuNPs were similar to those observed in KCl (e.g., increase in the intensity of the peaks accompanied by a decrease in the separation between anodic and cathodic waves.

Using phosphate buffer, the pitting processes were avoided and the reproducibility of films deposited on SS was clearly improved and was similar to that calculated in films deposited on platinum (CV less than 5%).

Electrocatalytic and sensing behavior towards catechol

Once stable Ppy/AuNP electrodes were obtained, their electrocatalytic and sensing properties towards catechol (a phenolic compound of interest in the food industry), were analyzed in terms of signal amplification and peak shifts. Experiments were carried out in the range between −0.1 and 0.8 V at a scan rate of 0.1 V/s in phosphate buffer. Under these conditions, SS could be used as a substrate due to the absence of pitting processes. Notice also that the polaron–bipolaron response of pyrrole occurs out of this range at negative potentials.

Catechol produced the expected well-shaped redox pair generated by the two-electron oxidation/reduction of the *ortho*-dihydroquinone to benzoquinone [26]. The reversibility of the peaks was improved with the incorporation of the AuNPs. Simultaneously, the intensity of the peaks increased with the concentration of AuNPs. This is illustrated in Figure 5 for electrodes deposited on SS by CP. As observed in the Figure, the

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separation between the anodic and cathodic waves was 300 mV in Ppy-CPss films and only 100 mV in Ppy/AuNP-T-CP$_{SS}$.

These effects were stronger in films deposited by CP than in films deposited by CA, due to the higher concentration of nanoparticles. In contrast, the method to insert the nanoparticles (trapping or cogeneration) only produced small changes in the intensities and positions of the peaks, probably due to the minimal differences in the AuNPs concentration.

The electrocatalytic effect was stronger in films deposited on platinum than in SS. This is in good accordance to previously published reports that have established that AuNPs exhibit a catalytic behavior when deposited onto platinum due to the synergy between both metals [34].

The effect of the concentration of catechol was studied by immersing the electrodes prepared by CP in $1 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ mol/L catechol solutions. A linear increase in the intensity of the peaks with the concentration was observed in the studied range. The limit of detection (LOD) was calculated from the calibration curves following the "3sd/m" criterion. As observed in Table 2, the LODs were in the range from 10^{-5} to 10^{-6} mol/L. The LOD obtained using Ppy/AuNP composite films was almost one order of magnitude lower than the one observed in Ppy films. The synergy between platinum and AuNPs increased the sensitivity, allowing a further decrease in the LODs. This synergy is not so important when using SS. Therefore, the use of SS as a substrate, provides stable sensors with good LODs while decreasing the price of the devices considerably.

Conclusion

Ppy/AuNP nanocomposites have been successfully prepared employing in situ polymerization of pyrrole using tetrachloroauric acid as an oxidant in the presence of gold ions and by trapping AuNPs in a Ppy matrix during the electropolymerization. SEM images confirmed the formation of uniform nanocomposites on smooth platinum and stainless steel substrates.
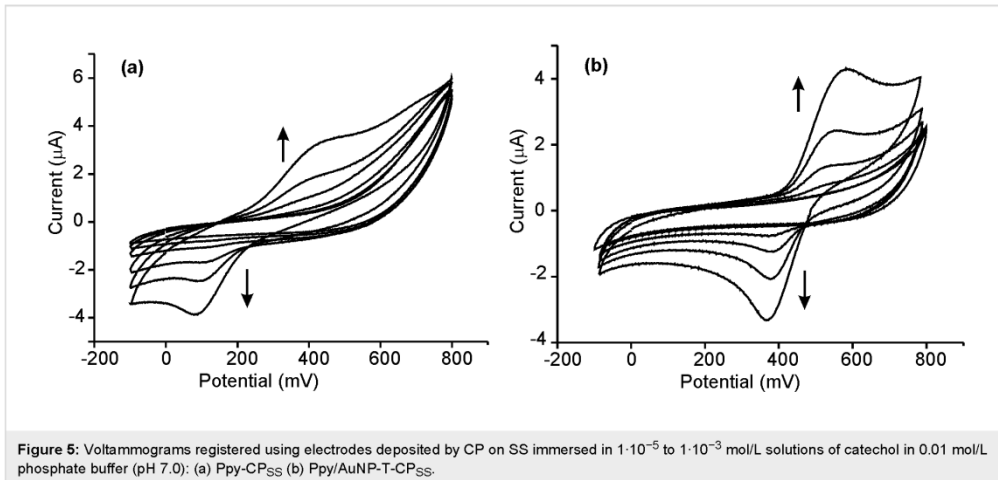
Figure 5: Voltammograms registered using electrodes deposited by CP on SS immersed in $1 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ mol/L solutions of catechol in 0.01 mol/L phosphate buffer (pH 7.0): (a) Ppy-CP$_{SS}$ (b) Ppy/AuNP-T-CP$_{SS}$.

Table 2: LOD, sensitivity and regression coefficients calculated from the anodic and cathodic peaks of catechol.

Sensor	LOD (mol/L) (cathodic peak)	R^2	LOD (mol/L) (anodic peak)	R^2
Ppy-CP$_{Pt}$	$9.1 \cdot 10^{-5}$	0.977	$5.3 \cdot 10^{-5}$	0.989
Ppy/AuNP-C-CP$_{Pt}$	$2.4 \cdot 10^{-5}$	0.976	$8.8 \cdot 10^{-5}$	0.996
Ppy/AuNP-T-CP$_{Pt}$	$0.9 \cdot 10^{-5}$	0.984	$0.3 \cdot 10^{-5}$	0.981
Ppy-CP$_{SS}$	$8.9 \cdot 10^{-5}$	0.956	$7.2 \cdot 10^{-5}$	0.975
Ppy/AuNP-C-CP$_{SS}$	$4.3 \cdot 10^{-5}$	0.977	$3.1 \cdot 10^{-5}$	0.971
Ppy/AuNP-T-CP$_{SS}$	$3.2 \cdot 10^{-5}$	0.968	$1.1 \cdot 10^{-5}$	0.975

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The presence of AuNPs in the polymer matrix resulted in an increase in the conductivity and in the intensity of the voltammetric signals. These variations in conductivity and intensity of voltammograms are directly related to the number of AuNPs inserted in the Ppy films.

Irreproducibility observed in the EIS and voltammetric measurements carried out in KCl using films deposited on stainless steel, caused by pitting process can be avoided by using phosphate buffer as supporting electrolyte.

As an electrochemical sensor, the Ppy/AuNP deposited on platinum exhibited important electrocatalytic activity towards the oxidation of catechol. The effect was higher in films obtained by CP than in films obtained by CA. The influence of the method used to introduce the AuNPs (trapping or cogeneration) was not so important. The detection limits were in the range of 10^{-5} to 10^{-6} mol/L, which is lower than the concentration usually found in foods and beverages such as wines and musts. The synergy between Pt and Au nanoparticles gave rise to lower LODs. In turn, stainless steel can be used as the substrate in the absence of KCl, with a LOD only slightly higher than those obtained in sensors deposited on Pt, but at a lower cost.

Experimental
Reagents and solutions
All experiments were carried out in deionized Milli-Q water (Millipore, Bedford, MA). Pyrrole, tetrachloroauric acid, 1-decanesulfonic acid (DSA), potassium chloride, sodium phosphate, potassium phosphate and catechol were purchased from Sigma-Aldrich. Commercially available reagents and solvents were used without further purification. 10^{-3} mol/L stock solutions of catechol were prepared by solving the corresponding compound in KCl solution (0.1 mol/L) or phosphate buffer solution (pH 7.0; 0.1 mol/L). Solutions with lower concentration were prepared from the stock solutions by dilution.

Preparation of the Au colloidal suspension
The synthesis of AuNPs colloids was carried out according to the procedure proposed by Slot and Geuze [35]. Two solutions were prepared: (1) $HAuCl_4$ ($0.25 \cdot 10^{-3}$ mol/L) in deionized water and (2) sodium citrate dehydrate ($17 \cdot 10^{-3}$ mol/L) in deionized water. 20 mL of solution (1) was heated until boiling on a hot plate, then 1 mL of solution (2) was quickly added to the $HAuCl_4$ solution while stirring. The mixture was then boiled for 20 min. Using this procedure, a red colloid with a UV absorbance maximum at $\lambda = 540$ nm was obtained.

Instruments
Electropolymerizations and electrochemical studies were carried out at room temperature in an EG&G Parstat 2273

potentiostat/galvanostat using a three-electrode configuration. The same instrument was used for the EIS experiments. UV–vis spectra were recorded on a Shimadzu UV-2600 model spectrometer. A SEM-FEI (QUANTA 200F) was used to record the images of the electrode surfaces.

Electropolymerization methods
The auxiliary electrode was a conventional Pt electrode. The reference electrode was an Ag/AgCl electrode in a 3 mol/L KCl solution. Pt and stainless steel 316L (SS) disks (1 mm diameter) were used as working electrodes. The disks were polished with 0.3 μm alumina suspension using a microcloth polishing pad and rinsed with deionized water in an ultrasonic bath.

Electropolymerization of Ppy films
The Ppy films were obtained by electropolymerization from a solution containing 0.1 mol/L pyrrole and 0.05 mol/L 1-decanesulfonic acid (DSA) using two electrochemical techniques: chronopotentiometry (CP) using a constant potential at 0.8 V over a period of 300 s, and chronoamperometry (CA) using 0.02 mA over a period of 300 s (except otherwise indicated). Films were deposited onto Pt and SS.

Electropolymerization of Ppy/AuNPs films
Ppy/AuNPs films were obtained using two different approaches. On one hand, Ppy/AuNPs films were synthesized by the "trapping method" from a solution containing 0.2 mol/L pyrrole, 0.1 mol/L DSA. This solution was mixed (1:1) with a solution containing AuNPs previously formed (Au colloidal suspension). Films were polymerized by chronoamperometry using a constant potential at 0.8 V over a period of 300 s, and by chronopotentiometry using 0.02 mA over a period of 300 s. Sensors obtained by trapping were termed as Ppy/AuNP-T-CA (obtained by chronoamperometry) and Ppy/AuNP-T-CP (obtained by chronopotentiometry).

Ppy/AuNPs films were also synthesized using the "cogeneration method" by mixing a solution containing tetrachloroauric acid 10^{-3} mol/L and a solution containing pyrrole and DSA. In this method, and according to the oxidation potentials of pyrrole (0.7 V vs SCE) and the reduction potential of $AuCl_4^-$ (1 V), the AuNPs where generated in situ and inserted in the polymeric film during the electrochemical growth. Also in this case, electropolymerization was carried out by CA and CP under the same conditions used for trapping. Sensors obtained by cogeneration were termed as Ppy/AuNP-C-CA (obtained by chronoamperometry) and Ppy/AuNP-C-CP (obtained by chronopotentiometry).

In all cases, films were deposited onto Pt and SS disks. The type of substrate will be denoted using a subscript (i.e., Ppy/AuNPs-

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C-CP$_{Pt}$ or Ppy/AuNPs-T-CP$_{SS}$). Once prepared, the polymeric films were extracted from the generation solution and washed thoroughly with water.

Electrochemical impedance spectroscopy (EIS) characterization

EIS was performed in a 0.1 mol/L KCl solution with a frequency range from 105 to 0.1 Hz and a signal amplitude of 10 mV, at a working potential of 0.0 V.

Tests of the voltammetric sensors

The Ppy and Ppy/AuNPs films were used as working electrodes in electrochemical experiments. The reference electrode was Ag/AgCl/KCl 3 mol/L and the counter electrode was a platinum wire.

Cyclic voltammetry was carried out at room temperature with a scan rate of 0.1 V/s in the potential range between −1.0 V and 0.8 V (vs Ag/AgCl) except otherwise indicated.

Calibration curves were constructed from catechol solutions with concentrations ranging from $1 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ mol/L. The limits of detection (LODs) were calculated following the "3sd/m" criterion, where "m" is the slope of the calibration graph, and "sd" was estimated as the standard deviation ($n = 5$) of the voltammetric signals at the concentration level corresponding to the lowest concentration of the calibration plot [36,37].

Acknowledgements

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3.6. Paper V

Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator.

By Celia García-Hernández, Cristina García-Cabezón, Fernando Martín-Pedrosa and María Luz Rodríguez-Méndez.

Chapter 3: Results and discussion

Paper V: Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator.

3.6.1. Motivation and objective

With the aim of elaborating a wine of good quality, it is very important for vineyard growers to harvest grapes at the optimal point of ripeness, the point at which the grapes have reached an appropriate phenolic maturation. As the grapes ripen, many chemical, physical and microbiological processes may occur that can strongly influence the quality of the future elaborated wines. Moreover, wine quality will also depend on many other factors, such as grape variety, wine characteristics, weather factors or viticulture practices.

In recent years, multi-parametric methods have been applied in the food industry for quality control purposes. Infrared spectroscopy (FTIR, NIR) combined with chemometric methods is emerging as a useful technique to analyze grapes and wines. It is rapid, versatile and requires minimal sample preparation. For instance, sugar content or acidity has been analyzed by means of these methods. In addition, the polyphenolic and antioxidant content in wine samples can be assessed by other recognized traditional spectrophotometric methods, such as Total Polypneol index (TPI), FRAP, DPPH, ORAC, and Folin-Ciocalteu Indexes, among others. However, up to the present time, a single method has not been recognized as the most adequate, and the results obtained depend on the method used. Therefore, the recommendation when approaching the study of the antioxidant activity of wines is to use more than one method.

On this matter, electronic and bioelectronic tongues based on electrochemical sensors have been developed and used in food quality control. In this sense, electrochemical techniques can represent an advantage thanks to their higher sensitivity and relatively low cost in comparison with the spectroscopic methods. However, experiments with ETs and bioETs provide a vast amount of data that must be analyzed with the appropriate multiparametric statistical tools. So it is very important to select statistical studies that allow us to extract the maximum information from each electrochemical analysis carried out with ETs/bioETs.

In the case of wines and grapes, there are several research studies that have applied the technology of electronic tongues to analyze parameters of interest in terms of quality control. The UVaSens group is one of the most active research groups that have implemented ETs and bioETs in this field. However, up to the present time, no research work in the field of grapes and wines has implemented an electronic tongue able to assess the quality of wines from the data

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obtained when analyzing the grapes that will be used to elaborate those wines and this is the proposed objective for this work.

In this work, we have developed an electrochemical bioelectronic tongue based on Ppy and Ppy/AuNP films with the enzymes tyrosinase and glucose oxidase to analyze red grapes and their corresponding elaborated wines. The Ppy based-sensors were reported in a previous work, selecting the chronopotentiometry as the electrodeposition method, incorporating the AuNPs *in situ* during the electrodeposition using tetreacloroauric acid as precursor, and the use of two substrates, platinum and stain steel, to increase the cross-selectivity of the responses. In this case the counterion used was dodecylbenzenesulphonic acid (DBSA) instead of DSA, which acted in a similar way, but at a lower price. The results obtained from the electrochemical responses evidenced that the conducting polymer and AuNPs are effective electron mediators to be used with enzymes. Principal component analysis (PCA) and parallel factor analysis (PARAFAC) were used to discriminate samples of grapes with different sugar and polyphenolic contents and wines with different alcoholic degrees and polyphenolic content. Moreover, partial least squares (PLS-1) was capable of constructing regression models with good correlations between the data obtained with the bioET and the chemical parameters obtained by traditional methods. Further, using support vector machines (SVM), we were able to predict the alcoholic degree and polyphenolic content of the wines from the data obtained from red grapes with the bioET.

This result is of great interest for the food industry as it opens up the possibility of predicting some of the characteristics of the final wine from the beginning of the vinification process, allowing us to take actions to obtain high quality wines.

3.6.2. Summary of the research work

Biosensors inspired by sensors of Ppy and Ppy/AuNPs, reported before, were prepared. For this purpose, we selected the experimental conditions that improved the electrochemical responses of Ppy and Ppy/AuNP sensors. Therefore, the bioET consisted of eight biosensors of Ppy and Ppy/AuNP films prepared onto substrates of Pt and SS by chronopotentiometry. AuNPs were formed and introduced in the polymeric matrix during the electrodeposition experiments (previously mentioned as the cogeneration method). The average size of the as-prepared AuNPs

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has already been studied by SEM, resulting in about 30-40 nm. The enzymes, tyrosinase (Tyr) and glucose oxidase (GOx), were immobilized onto the sensor surfaces by casting the enzyme solutions and then cross-linking with glutaraldehyde. Tyrosinase is a phenoloxidase specific for the detection of phenols, whereas glucose oxidase is used for the detection of sugars.

Red grapes and their corresponding elaborated red wines were provided by the vineyards Bodega Cooperativa de Cigales and the Instituto Tecnológico Agrario de Castilla y León (ITACyL), both located in the Castilla y León region. The varieties of grapes analyzed were: Cabernet, Garnacha, Tempranillo, Juan García, Mencía Secano, Mencía Regadío, Prieto Picudo y Rufete. The Oenological Centre of Castilla y León analyzed the chemical parameters of the grapes and wines. The Brix degree and TPI of grapes and the alcoholic degree and TPI of wines were provided.

Grapes and wines were analyzed by cyclic voltammetry. The electrochemical responses of musts showed anodic peaks at positive potentials whereas, in the case of wines, the responses showed a redox process. However, voltammograms responses depended on the type of substrate (Pt or SS), the modifier (Ppy or Ppy/AuNP) and enzyme (Tyr or GOx). For instance, in the case of using platinum substrate, the voltammograms in wines showed a redox pair at negative potentials whereas, by using SS, the redox peak appeared with an anodic peak at the positive potential and the cathodic peak at the negative potentials, so the responses were more irreversible than for Pt substrates. Moreover, the responses in wines and musts were completely dissimilar, reflecting the fact that the composition of samples is different due to the concentration of ionic and redox species (e.g., polyphenols), which affects the interchange of ions in and out of the polymeric matrix during the redox process. The enzymatic activity of enzymes allowed us to obtain an important degree of selectivity in the responses, especially in the intensities and positions of the cathodic waves. Ppy/AuNPs and platinum substrate evidenced an efficient electron tranfer performance, increasing the intensities of the voltammetric responses. In conclusion, the responses showed a high degree of cross-selectivity.

Further, the voltammetric responses were used as input data for several statistical treatments. On the one hand, we used two different discrimination methods on the results, PCA and PARAFAC. The PCA score plots for wines showed samples located in space depending on their alcoholic degree and TPI values. In the case of musts, we were not able to discriminate the grapes according to their Brix degree or TPI value; however, samples were located in space in a

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similar way to that in which wines were located in the PCA score plot. This result indicates that the bioET might be able to establish interesting correlations between the grapes and wines.

With PARAFAC analysis, we gave a three-way nature to the date. The PARAFAC results for wines corroborate those obtained by the two-way PCA method; however, in the case of musts, the samples appeared to be discriminated according to their Brix degree. The PARAFAC analysis showed an important difference with respect to PCA, since we can obtain further information by analyzing the Mode plots obtained in PARAFAC. Therefore, Mode 1 gave information about the contribution of the samples, Mode 2 about the contribution of kernels (a reduction of variables method used to extract the information from voltammograms) and Mode 3 about the contribution of the sensors and their cross-selectivity.

On the other hand, we established correlations between the voltammograms obtained from the samples and the chemical composition. Using PLS-1, we obtained regressions models that correlated the data obtained in must and wines with their chemical parameters measured by traditional methods. The data obtained from musts were well correlated with the TPI and Brix degree, with only 3 components with low errors; however, the correlation coefficient obtained for the TPI results were lower than 0.90. In the case of the wines, the voltammetric data also correlated with the TPI and alcoholic degree, also with 3 components, low errors, while the correlation coefficients were higher than in the case of must.

Finally, in order to predict values of TPI and alcoholic degree in wines, we carried out a supervised statistical method called support vector machine regression (SVMR). In this analysis, we introduced the voltammetric data obtained from musts with the bioET as the X-matrix and the chemical parameters of the wines elaborated from those musts as the Y-matrix. We then created correlation models to obtain a predictor model, after which, by applying the corresponding predictor model, we were able to predict values of the TPI and alcoholic degree in wines with low relative errors from the data obtained in the must with the bioET. This means that, from the data obtained in must, we are able to predict two chemical parameters that point to the quality of wines before having elaborated them.

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Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator

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ABSTRACT

A bioelectronic tongue (bioET) based on combinations of enzymes (tyrosinase and glucose oxidase) and polypyrrole (Ppy) or polypyrrole/AuNP (Ppy/AuNP) composites was build up and applied to the analysis and discrimination of musts and wines. Voltammetric responses of the array of sensors demonstrated the effectiveness of polymers as electron mediators and the existence of favorable synergistic effects between Ppy and the AuNPs. Using Principal Component Analysis and Parallel Factor Analysis it was possible to discriminate musts according to the °Brix and TPI (Total Polyphenol Index), and wines according to the alcoholic degree and TPI. Partial Least Squares provided good correlations between the bioET output and traditional chemical parameters. Moreover, Support Vector Machines permitted to predict the TPI and the alcoholic degree of wines, from data provided by the bioET in the corresponding grapes. This result opens the possibility to predict wine characteristics from the beginning of the vinification process.

1. Introduction

During the last years, multi-parametric techniques are gaining interest in oenological applications because they make possible to assess many parameters in a single experiment. The most commonly used techniques are Near Infrared, FTIR or NMR which have been used to determine sugar content, acidity or sulfurose content, among other parameters (Buratti et al., 2011; Friedel, Patz, & Dietrich, 2013; Hayasaka, Black, Hack, & Smith, 2017; Musingarabwi, Nieuwoudt, Young, Eyéghè-Bickong, & Vivier, 2015). Electronic tongues (ETs) are also multi-parametric systems that combine a multisensor system formed by a number of non-selective sensors with chemometric tools (Baldeón et al., 2015; Lvova et al., 2016; Rodríguez-Méndez, 2016; Peris & Escuder-Gilabert, 2016; Smyth & Cozzolino, 2013). ETs based on voltammetric sensors have been successfully used to analyze wines at different levels (fermentation, aging, variety of grape or fraudulences among many others) (Apetrei et al., 2012; Cetó et al., 2017; Gil-Sánchez et al., 2011; Giménez-Gómez et al., 2016; Pigani et al., 2011; Prieto et al., 2011; Rudnitskaya, Rocha, Legin, Pereira, & Marques, 2010; Rodríguez-Méndez et al., 2014). Some works have also been dedicated to the analysis of grapes and their phenolic maturity (Medina-Plaza et al., 2016). However, there are no reports on the capability of an ET to

establish correlations or predictions between the characteristics of grapes and that of wines elaborated from them. Such a difficult task requires the development of an improved electronic tongue with enhanced capabilities. In order to improve the sensitivity and cross-selectivity of the sensors, a possible strategy is to introduce bioelectrochemical sensors, to form the so-called bioelectronic tongues (bioET). BioETs combine the advantages of classical ETs, which provide overall information about the sample, with the specificity induced by biosensors (Ghasemi-Varnamkhasti et al., 2012; Medina-Plaza et al., 2016; Toko, 2013; Zeravik, Hlavacek, Lacina, & Skládal, 2009). BioETs dedicated to wines include phenoloxidases specific for the detection of phenols (e.g. tyrosinase, laccase or peroxidase) and enzymes specific for the detection of sugars (glucose oxidase or fructose dehydrogenase) (Cetó, Capdevila, Mínguez, & del Valle, 2014; Gutiérrez-Capitán et al., 2014; Medina-Plaza et al., 2015). However, in these works, the enzymatic activity is not very high and the peaks corresponding to the enzymatic process are weak.

In order to enhance the enzymatic signals, enzymes must be combined with appropriate electron mediators. Conducting polymers or gold nanoparticles have demonstrated to be excellent electron mediators in biosensors (Apetrei & Apetrei, 2013; Mavrikou, Flampouri, Iconomou, & Kintzios, 2017). In previous works, we have demonstrated

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that phthalocyanines and AuNPs combined with conducting polymers improved electrochemical responses in sensors and biosensors for phenols analysis (García-Hernández et al., 2015; García-Hernández, García-Cabezón, Martin-Pedrosa, De Saja, & Rodríguez-Méndez, 2016). Moreover, electropolymerization of conducting polymers onto electrode surfaces accompanied by in situ synthesis of AuNPs offers an easy way to fabricate composites combining both electron mediators that further will facilitate transfer kinetics process.

In this work, a bioET dedicated to the analysis of grapes and wines has been build up. For this purpose, an array of biosensors containing enzymes (glucose oxidase and tyrosinase) specialized in the detection of two of the main components usually analyzed to establish the quality of grapes and wines (glucose and phenols) was developed. The multi-sensor system was formed by tyrosinase or glucose oxidase biosensors containing polypyrrole (Ppy) and polypyrrole/AuNP composites (Ppy/AuNP) as electron mediators. In order to further increase the cross-selectivity, sensors were prepared in two different substrates, platinum (Pt) and Stainless Steel (SS).

The bioET was used to establish correlations between the quality of grapes and the characteristics of the wines obtained from those grapes. Different chemometrics tools –Principal Component Analysis (PCA), Parallel Factor Analysis (PARAFAC), Partial Least Squares regression (PLS-1) and Support Vector Machine (SVM) were used to discriminate between grape juices (musts) and wines elaborated from different varieties of grapes, to establish correlations between the results obtained from the electronic tongue and the chemical parameters obtained using classical chemical methods and to predict the quality of the wines from the data obtained in musts.

2. Materials and methods

2.1. Reagents and solutions

Pyrrole, dodecylbenzenesulfonic acid sodium salt (DBSA), tetrachloroauric acid and potassium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents were of reagent grade and used as supplied. Solutions were prepared by dissolving substances in deionized water (resistivity of $18.2\,M\Omega\cdot cm^{-1}$) obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Glucose oxidase (GOx) (from *Aspergillus niger*, type VII, activity $\geq 100\,U\cdot mg^{-1}$) and tyrosinase (Tyr) (from mushroom, activity $\geq 1000\,U\cdot mg^{-1}$) were purchased from Sigma-Aldrich. $5\,mg\cdot mL^{-1}$ solutions of enzymes were prepared in buffer phosphate $0.01\,mol\cdot L^{-1}$ (pH 7.0).

2.2. Grapes, musts and wines samples

Red grapes were harvested in September 2014 from the vineyards "Bodega Cooperativa de Cigales" and "Instituto Tecnológico Agrario de Castilla y León (ITACyL)", both located in Castilla y León area (Valladolid, Spain). Varieties under study were Cabernet, Garnacha, Tempranillo, Juan García, Mencía Secano, Mencía Regadío, Prieto Picudo and Rufete.

Musts were prepared by crushing 200 berries during one minute. Then, musts were fermented and bottled. Musts and the corresponding wines elaborated from these musts were analyzed in The Oenological Centre of Castilla y León (Valladolid, Spain). Brix degree, alcoholic degree and the Total Polyphenol Index (TPI) were analyzed following international regulations (International Organisation of Vine and Wine, 2011). Results are collected in Table 1.

2.3. Preparation of the electrochemical sensors and biosensors

The bioET consisted of an array of eight electrodes based on Polypyrrole (Ppy) films doped with dodecylbenzenesulphonic acid (DBSA) obtained by electropolymerization on both platinum (Pt) and

Table 1
Average chemical parameters measured in musts and wines following international procedures.

Sample #	Variety	Musts		Wines	
		°Brix	TPI	°Alcoholic	TPI
1	Cabernet	22.4 ± 2	24 ± 3	13.0 ± 0.8	57 ± 3
2	Garnacha	20.2 ± 2	17 ± 2	11.4 ± 0.8	41 ± 3
3	Juan García	19.8 ± 2	24 ± 2	11.4 ± 0.8	41 ± 3
4	Mencía Regadío	21.9 ± 2	19 ± 2	12.0 ± 0.8	44 ± 3
5	Mencía Secano	22.7 ± 2	19 ± 2	12.8 ± 0.8	54 ± 3
6	Prieto Picudo	22.0 ± 2	26 ± 2	12.5 ± 0.8	46 ± 3
7	Tempranillo	22.8 ± 2	24 ± 2	13.3 ± 0.8	53 ± 3
8	Rufete	20.2 ± 2	27 ± 2	11.1 ± 0.8	39 ± 3

stainless steel 316L (SS) substrates (García-Hernández et al., 2015). Electropolymerizations and voltammetric measurements were carried out using a Parstat 2273 potentiostat/galvanostat (EG&G, Oak Ridge, TN, USA).

Pt and SS disks (1 mm diameter) were used as working electrodes, the reference electrode was $Ag|AgCl/KCl\,3\,mol\cdot L^{-1}$ and the counter electrode was a platinum sheet with a surface of $1\,cm^2$. The disks were polished with $0.3\,\mu m$ alumina suspension using a microcloth polishing pad and rinsed with deionized water in an ultrasonic bath.

The Ppy films were deposited onto the electrode surface from a solution containing $0.2\,mol\cdot L^{-1}$ pyrrole and $0.1\,mol\cdot L^{-1}$ DBSA using chronopotentiometry (CP) with a constant current of $0.02\,mA\cdot cm^{-2}$ during 300 s. In the case of Ppy/AuNP composites, AuNPs were generated in situ and inserted in the polymeric film during polymerization and growth, using the pyrrole-DBSA solution mixed 1:1 with a solution containing $10^{-3}\,mol\cdot L^{-1}$ tetrachloroauric acid and applying the same experimental conditions than for Ppy films ($0.02\,mA\cdot cm^{-2}$ during 300 s) Once prepared, films were washed thoroughly with deionized water. Morphology and size of the AuNPs in the as-preapared Ppy/AuNP films have been studied by scanning electron microscopy (SEM). As expected, the average size of the AuNPs was about 30–40 nm and the nanoparticles were uniformly dispersed in the polymeric matrix showing a spherical shape. (García-Hernández et al., 2015).

Biosensors were prepared by depositing the enzymes GOx or Tyr onto the Ppy and Ppy/AuNP films by casting $5\,\mu L$ of $0.01\,mol\cdot L^{-1}$ phosphate buffer (pH 7.0) containing $5\,mg\cdot mL^{-1}$ of the corresponding enzyme. After drying at room temperature, the process was repeated. Then, biosensors were immersed in glutaraldehyde (2.5% v/v, buffer solution) for 5 min and dried in air at room temperature. The biosensors were then rinsed with phosphate buffer to remove any unbound enzyme and stored at 4 °C.

Using these procedures Ppy-GOx, Ppy/AuNP-GOx, Ppy-Tyr and Ppy/AuNP-Tyr biosensors were prepared onto two types of substrates, platinum (Pt) and stainless steel (SS), obtaining thus, an array formed by eight sensors.

2.4. Voltammetric measurements

Voltammetric measurements were carried out in a three conventional cell using the sensors and biosensors as working electrodes, the reference electrode and the counter electrode were the same as in the case of the polymerization procedure. Cyclic voltammetry was carried out from $-1.0\,V$ to $+0.8\,V$ (vs Ag|AgCl) at a scan rate of $0.1\,V\cdot s^{-1}$. Musts and wines were diluted 1:1 in potassium chloride solution ($0.3\,mol\cdot L^{-1}$). Four replicas per sample were carried out (16 samples × 4 repetitions = 64 samples).

2.5. Chemometrics

Voltammograms were normalized and pre-processed with an adaptation of a data reduction technique based on 'kernels' (Gutiérrez-

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Osuna & Nagle, 1999; Prieto et al., 2011). Using this method, voltammograms were "sliced" in 10 variables that were used as the input for statistical analysis. Thus, the array provided 80 data from each sample (8 sensors × 10 kernels).

The statistical analysis was performed by using Matlab v2014b (The Mathworks Inc., Natick, MA, USA) and The Unscrambler v9.7. (CAMO Software AS, Oslo, Norway). Two non-supervised multivariate methods, Parallel Factor Analysis (PARAFAC) and Principal Component Analysis (PCA) were used to evaluate the discrimination capability of the electronic tongue and to compare the results obtained in grapes and wines. Partial Least Square Regression-1 (PLS-1) was used to establish correlations between the results obtained from the bioET and the chemical parameters given by the Oenological Centre of Castilla y León (Valladolid, Spain). Finally, Support Vector Machine (SVM) was used to predict the quality of the wines from the measurements made in musts.

3. Results and discussion

With the aim to obtain a bioET dedicated to the analysis of musts and wines, biosensors specialized in the detection of phenols and sugars (two of the main indicators of the quality of grapes and wines) were developed. The array of sensors was used to analyze grape juices (musts) prepared from different varieties of grapes and the corresponding wines elaborated from those musts.

Voltammograms were characterized by broad peaks whose intensities and positions were determined by the nature of the sensor (enzyme, modifier and substrate) and of the type of sample. The cross-selectivity of the sensors included in the array is illustrated in Fig. 1. It is important to remark that the first cycle was always different from the rest, but after 5 cycles, the signals were stabilized and a decrease lower than a 5% in 15 consecutive cycles was observed. However, once the electrodes were withdrawn from the must/wine solution, proteins, sugars and other wine components were adhered to the sensor surface and sensors could not be reused.

As shown in Fig. 1a, the enzymatic activity of tyrosinase and glucose oxidase induces an important degree of selectivity in the sensor responses. In particular, in the intensity and position of cathodic wave that reflects the consumption of O_2 to produce H_2O_2 at $-0.6\,V$ (in the case of GOx) (Jugović et al., 2016) or the reduction of the oxidized quinoid species to the phenolic form at $-0.8\,V$ in the case of Tyr

(Kirsanov, Mednova, Vietoris, Kilmartin, & Legin, 2012). Fig. 1b illustrates the remarkable increase in intensity observed when the composite Ppy/AuNPs was used as electron mediator demonstrating the existence of synergistic effects when two electrocatalytic materials are mixed. Finally, in Fig. 1c, the influence of nature of the substrate is illustrated. As observed in the figure, platinum facilitates the electron transfer causing an increase in the intensity of the electrochemical response.

Fig. 1d and e illustrate the responses obtained from wines and musts. The electrochemical responses of musts were characterized by a broad anodic peak at ca. 0.2–0.5 V. The response of wines showed a redox pair at negative potentials with a large separation between the anodic and cathodic waves. The dissimilar responses in wines and musts are due not only to their different chemical nature but also to their different viscosity and conductivity.

The positions of the peaks changed from one must to another (or one wine to another), depending on the variety of grape. Voltammograms thus reflect the different composition of the samples, mainly in concentration of redox components such as polyphenols and also differences in ionic composition (including protons) which affect the interchange of ions across the polymer/solution interface during oxidation/reduction process. The variety of responses obtained, allowed us to obtain an array of biosensors with a high degree of cross-selectivity.

3.1. Chemometric analysis

The outputs of the array of sensors were preprocessed to obtain 10 variables per sensor (Gutiérrez-Osuna & Nagle, 1999). These variables were used as the input for statistical analysis.

3.1.1. Discrimination capability of the array: PCA and PARAFAC

Principal Component Analysis (PCA) was used to evaluate the capability of the bioET to discriminate wines and musts according to the variety of grape. PCA scores plots for wines (Fig. 2a) showed well-defined and separated clusters for each variety of grape. Clusters were distributed in the plot according to the alcoholic degree and TPI (Table 1). Thus, Rufete (8) wine (with the lowest values of alcoholic degree and TPI) was clearly separated from the rest of wines. Garnacha (2) and Juan García (3) varieties with the same values of alcoholic
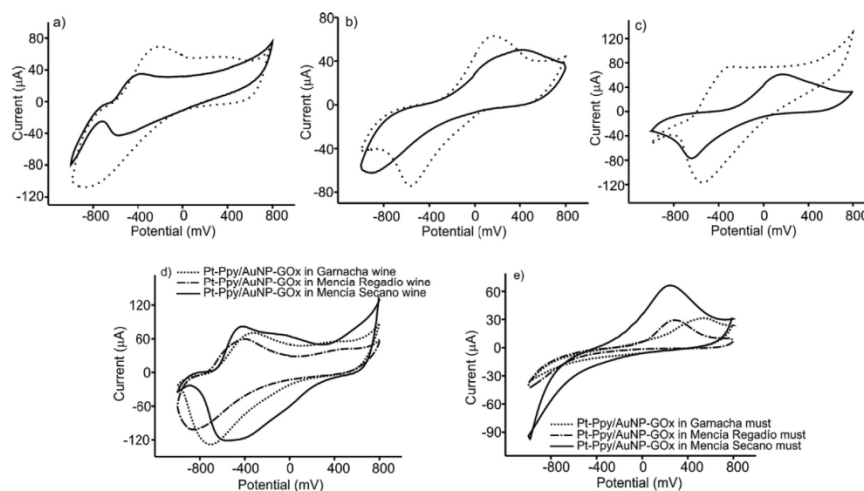
Fig. 1. (a) Pt-Ppy/AuNP-Tyr (dotted line) and Pt-Ppy/AuNP-GOx (solid line) immersed in Rufete wine; (b) SS-Ppy/AuNP-GOx (dotted line) and SS-Ppy-GOx (solid line) immersed in Rufete wine and (c) Pt-Ppy/AuNP-GOx (dotted line) and SS-Ppy/AuNP-GOx (solid line) immersed in Cabernet wine. (d) Voltammetric responses obtained in wines of different varieties of grapes and (e) Voltammetric responses obtained in musts of different varieties of grapes.

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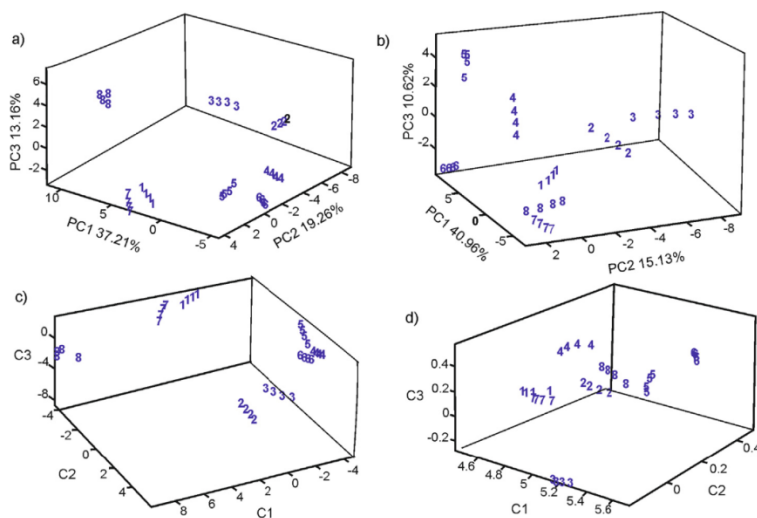

Fig. 2. (a) 3D PCA score plots of the bioET obtained from voltammetric responses in wines; (b) 3D PCA score plots of the bioET obtained from voltammetric responses in musts; (c) 3D PARAFAC score plots of the bioelectronic tongue obtained from voltammetric responses in wines and (d) 3D PARAFAC score plots of the bioelectronic tongue obtained from voltammetric responses in musts. 1: Cabernet, 2: Garnacha, 3: Juan García, 4: Mencía Regadío, 5: Mencía Secano, 6: Prieto Picudo, 7: Tempranillo and 8: Rufete.

degree and TPI were in the same region. Cabernet (1) and Tempranillo (7) varieties with the highest values in TPI and alcoholic degree also appear closer and separated from the rest. The captured information was 69.64% (PC1 = 37.21%; PC2 = 19.26%; PC3 = 13.16%).

In the case of musts (Fig. 2b), first three principal components explained the 67.2% of the information (PC1 = 40.96%; PC2 = 15.13%; PC3 = 10.62%). The position of the clusters could not be unequivocally related to the °Brix and/or TPI. However, interesting similarities between the relative positions observed in musts with the positions of the clusters obtained in wines were observed. For instance, also in musts, Cabernet (1) and Tempranillo (7) appear close together. Mencía Regadío (4), Mencía Secano (5) and Prieto Picudo (6) were also located nearby. Garnacha (2) and Juan Garcia (3) also appeared together.

This similarity in the results obtained in wines and in musts used to prepare the wines could indicate that the bioET could be used to establish correlations between the quality of grapes and the wines produced form them.

Given the three-way nature of the data (samples × kernels × sensors), the information contained in the voltammetric responses was extracted by multi-way modelling using PARAFAC (Kirsanov et al., 2012). Using the core consistency diagnostic tool and the corresponding residual sum of squares (Bro & Kiers, 2003) three components were able to describe our PARAFAC model. Fig. 2c and 2d show the PARAFAC score scatter plot on three factors, obtained for wines and musts respectively. In the case of PARAFAC the error, in terms of root-mean-square error (RMSE), is the parameter used to confirm the model. In our case low RMSEs were found when performing PARAFAC: 0.687 in the case of wines and 0.089 in the case of musts.

PARAFAC results corroborate the outputs obtained with the two-way PCA. For instance, scatter plot for wines (Fig. 2c) showed that samples were grouped according to their TPI and alcoholic degree. In the case of PARAFAC (Fig. 2d) the PARAFAC model also corroborated the results obtained with PCA, but some explicit differences could be observed because in this case, discrimination was clearly linked to the °Brix. For example, Cabernet (1) and Tempranillo (7) musts with the same TPI and °Brix values appear so close that seem to be overlapped, whereas the must Juan Garcia (3) that shows the same TPI but different °Brix appeared separated. Similarly, Garnacha (2) and Rufete (8) samples with the same °Brix but a very different TPI, appeared close one from each other. Finally, Juan Garcia with the lowest °Brix was located quite separated from the rest.

PARAFAC analysis was also used to obtain information about the data structure by means of Mode plots. Mode plots display in a simple and interpretable manner the relevant information and the sources of data variability. Given the three-way nature of the bioET data (samples × kernels × sensors) three Mode plots are analyzed: Mode 1 gives information about the contribution of the samples to the three components, Mode 2 about the contribution of kernels and Mode 3 about the contribution of the sensors and the cross-selectivity. Fig. 3 corresponds to Mode plots obtained for wines (upper figures) and musts (lower figures) samples and lines black, grey and dotted refer to first, second and third component, respectively.

Mode 1 plots (Fig. 3a and d) indicate that the first Principal Component (black line) is bringing the most relevant information. The information contained in the second (grey line) and third (dotted line) components is relatively small. Mode 2 shows the contribution of the 10 kernels (the 10 sections in which voltammograms are divided after applying Kernel functions). A difference between wines and musts can be observed (Fig. 3b and e). In the case of wines (Fig. 3b) kernels 3–10, are the most important contributors to the first principal Component. This means that almost the whole voltammogram (except kernels 1 and 2, corresponding to the left part of the voltammograms) contribute to the capability of discrimination of the bioET. This is in good agreement with the shapes of voltammograms shown in Fig. 1 for wines, were it can be observed that positions and shapes of the peaks depend on the type of wine and the variety of grape. In the case of musts, Mode 2 (Fig. 3e), kernels 5 to 10 bring more information than kernels 1–4. Again, this can be easily explained taking into account that at positive potentials, the oxidation of many components of musts such as phenols takes place. Finally, Mode 3 (Fig. 3c and f) demonstrates that all sensors contribute equally to the first principal component, and confirms the important degree of cross-selectivity of the sensors forming the array.

3.1.2. Correlations of the chemical composition: Partial Least Squares and Support Vector Machines regression

As it has been stated before, ETs give an overall view of the samples, however, the signals provided by ETs, can be correlated with the chemical composition of the samples using regression techniques. Table 2 collects the statistical parameters obtained from the Partial Least Squares PLS-1 regression models correlating the output of the bioET with chemical analytical parameters measured in musts and wines. Models were validated by means of the "leave-one-out" method. Good

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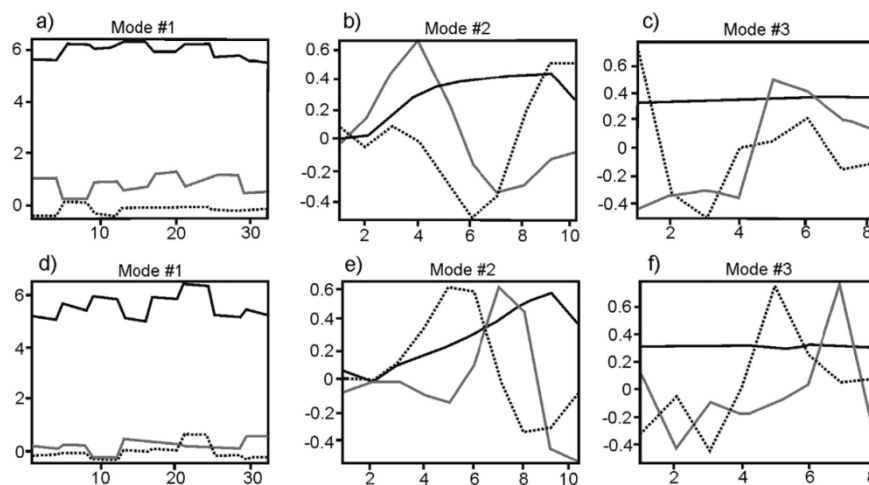
Fig. 3. Mode plots obtained from PARAFAC analysis in wines (a-c) and musts (d-f). Mode #1: Contribution of the samples, Mode #2: Contribution of the 10 kernels, Mode #3: Contribution of the sensors. Red: Component 1, Blue: Component 2 and Yellow: Component 3.

Table 2

Statistical parameters obtained for the PLS-1 regression models established between the chemical parameters and the bioET.

Must					
Parameters	R_C^{2a}	$RMSE_C^b$	R_P^{2c}	$RMSE_P^d$	Number of components
TPI	0.8751	1.5379	0.7687	2.1602	3
Brix	0.9390	0.2845	0.8455	0.4675	3
Wines					
Parameters	R_C^{2a}	$RMSE_C^b$	R_P^{2c}	$RMSE_P^d$	Number of components
TPI	0.9442	1.5192	0.8874	2.2273	3
Alcoholic degree	0.9268	0.2103	0.8615	0.2985	3

[a] Squared correlation coefficient in calibration.
[b] Root mean square error of calibration.
[c] Squared correlation coefficient in prediction.
[d] Root mean square error of prediction.

correlations (in both calibration and prediction) were found for the TPI, the Brix degree, which are chemical parameters usually used to evaluate the quality of grapes. Similarly, correlations were found with the alcoholic degree and the TPI which are usual markers of the quality of wines. The good correlations can be attributed to the working principle of our bioET that shows specificity towards phenols (improved by the presence of tyrosinase) and sugars (improved by the presence of GOx). The good correlation with the alcoholic degree of a wine can be due to the fact that the alcoholic degree is directly related with the sugar content of the grapes used to elaborate them.

3.1.3. Support Vector Machine (SVM)

It is well known that the characteristics and quality of a wine depend in a large extent on the characteristics and quality of the grapes used in the elaboration. In this work, the bioET was used to predict chemical characteristics of wines from voltammograms collected in grapes. For this purpose, Support Vector Machine Regression (SVMR) was used to predict the total polyphenol index (TPI) and the alcoholic degree of wines from data measured using the bioET in grapes. Two data matrices were developed: the "X" matrix (predictors) was constructed using the data from musts registered using the bioET and a "Y" matrix (responses) contained data of chemical parameters (TPI or alcoholic degree) of wines. The SVMR models were created using SVM type: Regression (epsilon SVR), kernel type: Linear, C value: 1, weights: All 1.0 and cross validation segments size: 10, in all cases. Prediction values of TPI and alcoholic degree obtained after creating the regression models were close to those obtained by means of traditional methods. SVMR models showed coefficients of correlation of 0.9899 in the calibration and 0.9469 in the prediction in the case of TPI, with errors of 0.7095 in the calibration and 1.6353 in the prediction. In the case of the alcoholic degree, the coefficients of correlation were 0.9910 in the calibration and 0.9536 in the prediction with errors of 0.0836 in the calibration and 0.1833 in the prediction.

After the SVMR model was constructed, the regression models were used to predict the TPI and the alcoholic degree of wines samples that were not included in the creation of the model. Results are shown in Table 3 versus the experimental results obtained by traditional

Table 3

TPI and alcoholic degree predicted in wine samples after applying the regression models previously performed by SVM.

Sample	TPI predicted in wine by means of SVMR	TPI obtained by traditional methods	|Relative error| %	Alcoholic degree predicted in wine by means of SVMR	Alcoholic degree obtained by traditional methods	|Relative error| %
Cabernet	56.9	57	0.2	13.00	13.03	0.2
Garnacha	43.7	41	6.6	11.69	11.36	2.9
Juan García	40.3	41	1.7	11.39	11.40	0.1
Mencía Regadío	44.1	44	0.2	12.04	12.01	0.2
Mencía Secano	53.1	54	1.7	12.66	12.81	1.2
Prieto Picudo	46.2	46	0.4	12.52	12.48	0.3
Rufete	41.5	39	6.4	11.42	11.13	2.6
Tempranillo	52.8	53	0.4	13.24	13.28	0.3

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tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator.*

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chemical methods.

4. Conclusions

A bioET combining biosensors sensitive to phenols and sugars has been developed. The use of Ppy/AuNPs as the electron mediator increases the sensitivity and cross-selectivity of the sensors, improving in this way the performance of the array of biosensors. The results revealed that using PCA and PARAFAC it was possible to discriminate samples according to the variety of grape. PLS-1 regressions showed good correlations with the chemical parameters. Using SVMR it has been possible to predict the TPI and the alcoholic degree of wines, from data provided by the bioET in the corresponding grapes. This result is of great interest for the food industry and has advantages with respect to already existing methods because it opens the possibility to predict some of the characteristics of the final wine, from the beginning of the process and in a single measurement.

Acknowledgements

Conflict of interest

The authors declare that they have no conflicts of interest in the research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.107.

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3.7. Paper VI

Layered composites of PEDOT/PSS/nanoparticles and PEDOT/PSS/phthalocyanines as electron mediators for sensors and biosensors.

By Celia García-Hernández, Cristina García-Cabezón, Fernando Martín-Pedrosa, José Antonio de Saja and María Luz Rodríguez-Méndez.

Chapter 3: Results and discussion

Paper VI: Layered composites of PEDOT:PSS/nanoparticles and
PEDOT:PSS/phthalocyanines as electron mediators for sensors and biosensors.

3.7.1. Motivation and objective

The food industry requires the development of new sensors with improved performance in terms of sensitivity, detection limit and selectivity. In the last few years, the conducting polymer PEDOT:PSS (poly(3,4-ethylenedioxythiophene:poly(styrenesulfonate)) has received much attention for manufacturing flexible or rigid structures of nanocomposites with improved characteristics for sensing applications. This polymer consists of a mixture of two ionomers, poly(poly(3,4-ethylenedioxythiophene), which is a conjugated polymer carrying a positive charge, and sodium polystyrene sulfonate, where some sulfonyl groups are deprotonated and carry a negative charge. Both together, they form a macromolecular salt, PEDOT:PSS, with such properties as high electrical conductivity, biocompatibility, mechanical elasticity and high transparency, along with the reversible charge/discharge ability from the doping/de-doping of the polymer chain. These characteristics make this polymer very attractive for developing new electrochemical sensors over other organic conducting polymers.

However, the applications of organic semiconductor PEDOT:PSS film-based sensors might be limited in aqueous solutions due to the highly hydrophilic property caused by the presence of PSS, which acts as a dopant to stabilize the PEDOT:PSS to avoid the poor solubility and processability of PEDOT. This fact gives the conducting polymer a degree of water solubility which leads to the film breaking on the electrode surface and, therefore, reducing the electrical conductivity and electrochemical performance. To solve this limitation and make the polymer less hydrophilic, researchers have combined PEDOT:PSS with other sensing materials, such as graphene or carbon nanotubes, and they have added a binder to strengthen the adhesion of the parts. Gold nanoparticles and metalphthalocyanines are classical electrocatalytic materials that might be good candidates to form composites with PEDOT:PSS in electrochemical sensors for applications in the food industry. In addition, the strong electrochemical properties of the as-fabricated sensors can be used to make biosensors in which the PEDOT:PSS composites will act as mediators to shuttle the electrons between the enzyme and the electrode. Thanks to its high conductivity, stability, organic nature and good compatibility with such biomolecules as enzymes, PEDOT is one of the most suitable materials for bioelectronics among the conducting polymers.

The objective of this work is to design new electrodes based on composites of PEDOT:PSS with a secondary electrocatalytic material, such as gold nanoparticles (AuNPs), lutetium bisphthalocyanine (LuPc$_2$) and copper phthalocynine (CuPc), to enhance the

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electrocatalytic activity for two diphenols, such as catechol and its isomeric form hydroquinone. Polyphenol compounds with the catechol group are expected to represent the main polyphenol type in red wines. Furthermore, the enzymes tyrosinase and laccase have been implemented in the mentioned composite-based sensors to improve their electrochemical performance. Tyrosinase is selective to the oxidation of orto-diphenols, whereas laccase catalyzes the oxidation of a larger variety of polyphenols.

3.7.2. Summary of the research work

PEDOT:PSS-based sensors were made using the spin-coating technique. The conducting polymer was first deposited onto an ITO surface and then spin-coated. After the deposition, the electrodes were annealed to improve adhesion onto the ITO surface. Then, the secondary electrocatalytic material (EM) (e.g., copper phthalocyanince, lutetium phthalocyanine or gold nanoparticles) was deposited onto the previously modified ITO surface and spin-coated with the same experimental conditions as before to obtain PEDOT:PSS/CuPc, PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNP sensors. AuNPs were previously synthesized following the Turkevich method.

Enzymatic biosensors were prepared using tyrosinase (Tyr) for catechol detection and laccase (Lac) for hydroquinone detection. For this purpose, the enzyme solution was drop-casted onto the modified ITO surface and dried at room temperature to obtain PEDOT:PSS/EM-Tyr and PEDOT:PSS/EM-Lac biosensors. Glutaraldehyde was used as the cross-linker during enzymes deposition.

The surface morphology of the PEDOT:PSS sensors was studied by SEM, evidencing a regular, smooth and homogeneous structure for PEDOT:PSS, PEDOT:PSS/CuPc and PEDOT:PSS/ LuPc$_2$ films. In the PEDOT:PSS/AuNP films, SEM images showed AuNPs located in clusters with an average size of 40-60 nm.

Conductivity measurements (resistance and resistivity) demonstrated that PEDOT:PSS/EM presented a higher conductivity than the non-modified PEDOT:PSS sensor following the sequence: PEDOT:PSS/LuPc$_2$ > PEDOT:PSS/AuNPs > PEDOT:PSS/CuPc. This result implies that the electron transfer will be improved with the layered composites. This fact

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was later corroborated by the electrochemical impedance spectroscopy (EIS), where it was observed that the value of the electron-transfer resistance in PEDOT:PSS/EM sensors was about half that observed in the PEDOT:PSS sensor.

The electrochemical behavior of PEDOT:PSS-based sensors for di-phenols was analyzed by means of cyclic voltammetry. Voltammograms registered in catechol showed a shift of the cathodic peak to lower potentials when using PEDOT:PSS/EM. In the case of PEDOT:PSS/LuPc$_2$, the electrocatalytic effect was stronger than for the rest, showing more reversible peaks and higher intensities. Although the response of the sensors for hydroquinone did not show a decrease in the cathodic peak to lower potentials, the intensities did increase on both redox peaks.

Biosensors PEDOT:PSS/EM-Tyr and PEDOT:PSS/EM-Lac showed higher intensities in the cathodic wave than non-biosensors when analyzing catechol and hydroquinone. However, the oxidation peaks were not observed. This result evidences the electron mediator performance of the PEDOT:PSS/EM composites.

The limits of detection obtained with the biosensors were in the range of 10^{-6}-10^{-7} mol·L^{-1}, one order of magnitude lower than those obtained with non-biosensors 10^{-5}-10^{-6} mol·L^{-1}.

The dynamic behavior of sensors for catechol and hydroquinone was studied by varying the scan rate and registering the corresponding voltammograms. Results showed that the cathodic peak potentials shifted to more negative values when the scan rate was increased, evidencing the irreversibility of the redox process. Moreover, the cathodic peak currents varied linearly with the square root of the scan rate, corroborating the diffusion-controlled nature of the electrode reaction. The slope values calculated in the PEDOT:PSS biosensors were higher than those observed in non-biosensors, so this result confirmed the improvement in the charge transfer due to the enzymatic activity.

Finally, the repeatability and reproducibility of the as-prepared sensors and biosensors was studied, resulting in coefficients of variation lower than 5% in both tests.

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Layered composites of PEDOT/PSS/nanoparticles and PEDOT/PSS/phthalocyanines as electron mediators for sensors and biosensors

Celia García-Hernández, Cristina García-Cabezón, Fernando Martín-Pedrosa, José Antonio De Saja and María Luz Rodríguez-Méndez[*]

Full Research Paper

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Abstract

The sensing properties of electrodes chemically modified with PEDOT/PSS towards catechol and hydroquinone sensing have been successfully improved by combining layers of PEDOT/PSS with layers of a secondary electrocatalytic material such as gold nanoparticles (PEDOT/PSS/AuNPs), copper phthalocyanine (PEDOT/PSS/CuPc) or lutetium bisphthalocyanine (PEDOT/PSS/LuPc$_2$). Layered composites exhibit synergistic effects that strongly enhance the electrocatalytic activity as indicated by the increase in intensity and the shift of the redox peaks to lower potentials. A remarkable improvement has been achieved using PEDOT/PSS/LuPc$_2$, which exhibits excellent electrocatalytic activity towards the oxidation of catechol. The kinetic studies demonstrated diffusion-controlled processes at the electrode surfaces. The kinetic parameters such as Tafel slopes and charge transfer coefficient (α) confirm the improved electrocatalytic activity of the layered electron mediators. The peak currents increased linearly with concentration of catechol and hydroquinone over the range of 1.5×10^{-4} to 4.0×10^{-6} mol·L^{-1} with a limit of detection on the scale of µmol·L^{-1}. The layered composite hybrid systems were also found to be excellent electron mediators in biosensors containing tyrosinase and laccase, and they combine the recognition and biocatalytic properties of biomolecules with the unique catalytic features of composite materials. The observed increase in the intensity of the responses allowed detection limits of 1×10^{-7} mol·L^{-1} to be attained.

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Introduction

The assessment of phenols has been successfully achieved using electrodes chemically modified with a variety of materials [1-10]. In addition, some mixtures of electrocatalytic materials such as polyaniline or polypyrrole with graphene can enhance the sensitivity of the sensors while lowering the detection limits [11-13].

Poly(3,4-ethylene dioxythiophene)/poly(styrene sulfonic acid) (PEDOT/PSS) is an appealing electrocatalytic material due to its high conductivity and low redox potential. PEDOT/PSS is soluble in water and the polymer can be mixed with water-soluble electrocatalytic materials or colloidal metal nanoparticles providing an excellent method to modulate the sensing properties by means of a synergistic effect [14,15]. However, a large number of electrocatalytic materials are soluble in organic solvents and this fact limits the number of electrocatalytic materials that can be used to modulate the PEDOT/PSS properties. One possible strategy to avoid this problem is to develop layered composites where a film of a secondary electrocatalytic material is deposited on top of a PEDOT/PSS layer.

The first objective of this work was to develop novel electrochemical sensors based on layered composites formed by alternating layers of PEDOT/PSS and a secondary electrocatalytic material (EM) (PEDOT/PSS/EM) and to evaluate the existence of synergistic effects. As EMs, three different materials with different characteristics and electrocatalytic activity towards phenols were tested, including gold nanoparticles (AuNPs), a copper phthalocyanine (CuPc) (a p-type semiconductor) and a lutetium bisphthalocyanine (LuPc$_2$) (a sandwich-type derivative with free radical character which is an intrinsic semiconductor) [9,16-18].

On the other hand, enzymatic electrochemical biosensors based on phenol oxidases are a good alternative to analyze phenols due to their high sensitivity and selectivity. Tyrosinase oxidizes monophenols and o-diphenols to the corresponding quinone, whereas laccase catalyzes the oxidation of a larger variety of aromatic compounds such as substituted mono- and poly-phenols, with subsequent formation of radicals, which are converted to quinones in the second stage of the oxidation [19]. Tyrosinase (Tyr) and laccase (Lac) must be combined with electron mediators to facilitate the transfer of electrons from the enzyme to the electrode [20]. PEDOT/PSS is becoming popular as an electron mediator in biosensing [21,22]. Gold nanoparticles and phthalocyanines have also been positively demonstrated as electron mediators in tyrosinase biosensors [16,23-26]. Also, in the case of biosensors, an improvement of the electron mediator activity could be expected by using layered composites formed by two electron mediators. For this reason, the second objective of this

work was to develop phenol biosensors containing Tyr or Lac using layered composites of PEDOT/PSS and AuNPs, CuPc or LuPc$_2$ as electron mediators and to investigate the existence of synergistic effects in the electron transfer occurring during biosensing.

In order to pursue the two objectives proposed, PEDOT/PSS/EM electrodes were prepared by depositing a layer of AuNPs, CoPc or LuPc$_2$ on the top of the PEDOT/PSS film by means of spin coating to obtain PEDOT/PSS/AuNP, PEDOT/PSS/CuPc and PEDOT/PSS/LuPc$_2$ sensors, respectively. The electrodes were used as the working electrodes in cyclic voltammetry. The sensing properties and any synergistic effects in the layered composite electrodes were evaluated towards catechol (1,2-dihydroxybenzene) and hydroquinone (1,4-dihydroxybenzene) which are two dihydroxybenzene isomers with many ubiquitous industrial applications [27,28]. Additionally, the kinetic parameters and limit of detection were calculated.

Further studies were carried out using the modified electrodes combined with tyrosinase (PEDOT/PSS/EM-Tyr) and laccase (PEDOT/PSS/EM-Lac) enzymes. The electron mediator properties of the layered composites with respect to the performance of biosensors containing tyrosinase or laccase were evaluated.

Results and Discussion
Structure and conductivity of the PEDOT/PSS/EM sensors

ITO electrodes were modified by depositing a PEDOT/PSS layer by means of spin coating. Layered composite electrodes were prepared by depositing layers of a secondary electrocatalytic material (AuNPs, CoPc or LuPc$_2$) on top of the PEDOT/PSS film to obtain PEDOT/PSS/AuNP, PEDOT/PSS/CuPc and PEDOT/PSS/LuPc$_2$, respectively. The surface morphologies of the modified electrodes were analyzed by SEM. As observed in Figure 1, the PEDOT/PSS film showed a regular, smooth and homogeneous structure. Further modification with CuPc and LuPc$_2$ did not affect the morphology. SEM images of the PEDOT/PSS/AuNP electrode confirmed that small clusters of 40–60 nm diameter AuNPs were homogeneously distributed on the surface of the film. The increase in the effective surface area due to the AuNPs could be highly beneficial for electrochemical applications in sensors.

The square resistance and resistivity were measured with the four-point probe test (Table 1). The current–voltage curves obtained from PEDOT/PSS and PEDOT/PSS/EM electrodes exhibited a good linear fit with correlation coefficients higher than 0.999.

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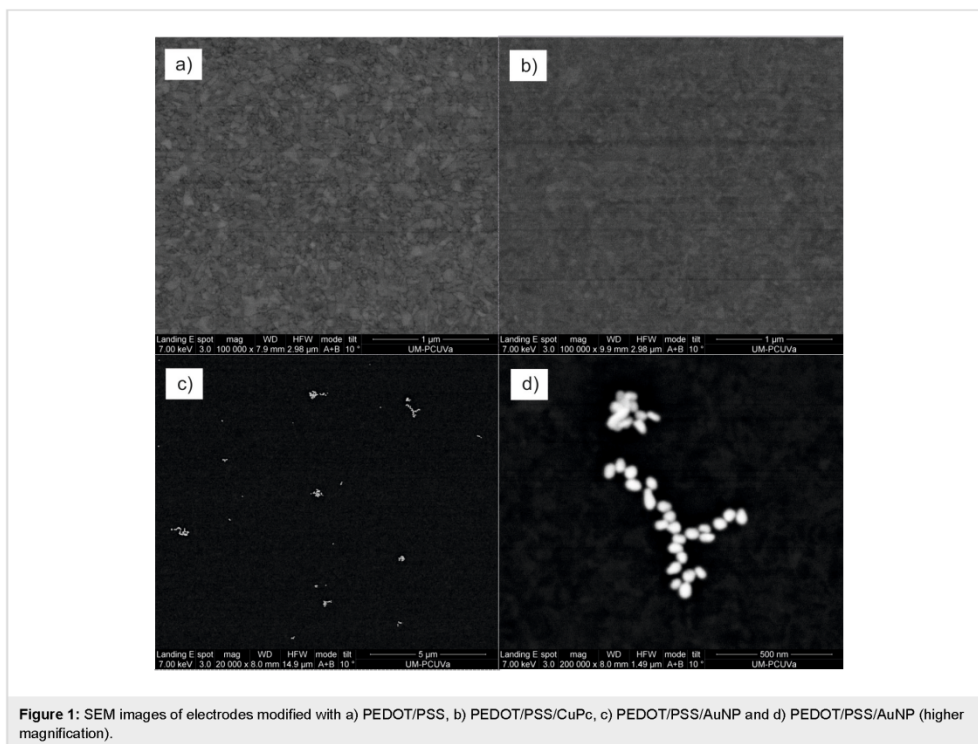
Figure 1: SEM images of electrodes modified with a) PEDOT/PSS, b) PEDOT/PSS/CuPc, c) PEDOT/PSS/AuNP and d) PEDOT/PSS/AuNP (higher magnification).

Table 1: Square resistance (R_{sq}) and conductivity (ρ) of the PEDOT/PSS and PEDOT/PSS/EM electrodes.

	R_{sq} ($\Omega \cdot sq^{-1}$)	ρ ($\times 10^{-6}$ $\Omega \cdot m$)
PEDOT/PSS	13.9	27.9
PEDOT/PSS/LuPc$_2$	6.4	15.7
PEDOT/PSS/CuPc	9.6	22.4
PEDOT/PSS/AuNP	7.9	19.7

It has been reported that the addition of different dopants to an aqueous solution of PEDOT/PSS produces an enhancement in the conductivity of PEDOT/PSS films obtained from the solution [29,30]. In our case, the additives were deposited as a thin film on the top of the PEDOT/PSS layer and the same effect was observed. Layered composites exhibited better conductivity than PEDOT/PSS following the order PEDOT/PSS/LuPc$_2$ > PEDOT/PSS/AuNPs > PEDOT/PSS/CuPc. This enhanced conductivity can contribute to the observed improvement of the electron transfer rate of the sensors that will be shown in the following.

The mechanism of the conductivity enhancement can be different depending on the material deposited on the PEDOT/PSS layer [29-31]. In the case of CuPc or AuNPs, the improvement in the conductance can be due to the increase in the charge carrier mobility and/or in the large effective surface provided by the metallic AuNPs. In the case of LuPc$_2$, which is an intrinsic semiconductor and has a free radical character [32], the enhancement can be due to the increase in charge carriers produced by the interaction between the PEDOT/PSS chains and the free carriers in LuPc$_2$.

Electrochemical characterization of the PEDOT/PSS/EM sensors

The electrochemical characteristics of PEDOT/PSS/EM electrodes were analyzed using cyclic voltammetry. Voltammograms of PEDOT/PSS, PEDOT/PSS/AuNPs, PEDOT/PSS/CuPc and PEDOT/PSS/LuPc$_2$ electrodes immersed in catechol and hydroquinone 1.5×10^{-4} mol·L^{-1} with 0.01 mol·L^{-1} phosphate buffer as the supporting electrolyte are shown in Figure 2. The response of ITO glass is not shown in the figure due to the low intensity, characterized by one cathodic peak at -0.4 V. At positive potentials, the oxidation of phenols could not be ob-

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Figure 2: Cyclic voltammograms of PEDOT/PSS, PEDOT/PSS/LuPc$_2$, PEDOT/PSS/CoPc and PEDOT/PSS/AuNP sensors in (a) catechol and (b) hydroquinone 1.5 × 10^{-4} mol·L^{-1} with 0.01 mol·L^{-1} phosphate buffer as the supporting electrolyte. Scan rate 0.1 V·s^{-1}.

served because it occurs at potentials higher than the working range.

In the case of PEDOT/PSS modified electrodes, catechol and hydroquinone were oxidized at the working electrode to form 1,2-benzoquinone and 1,4-benzoquinone, respectively. During the reaction, oxygen and hydrogen also form H_2O_2. During the reverse scan, quinones were reduced to the phenolic compound.

PEDOT/PSS and PEDOT/PSS/EM electrodes showed much larger current response (one order of magnitude) towards catechol than bare ITO glass. In addition, the presence of nanoparticles or phthalocyanines produced a shift of the cathodic wave to lower potentials (−0.4 V in unmodified ITO, −0.2 V in PEDOT/PSS and −0.01 V in PEDOT/PSS/EM). The electrocatalytic effect was stronger on the PEDOT/PSS/LuPc$_2$ electrode were a drastic decrease in the oxidation potential (from 1 V in PEDOT/PSS to 0.65 V in PEDOT/PSS/LuPc$_2$) was observed. The improvement of the performance observed in PEDOT/PSS/EM, where two electrocatalytic materials are combined, can be attributed to the enhanced electron transfer rate provided by the composites due to the interactions between two electrocatalytic components. In particular, the excellent response of the PEDOT/PSS/LuPc$_2$ electrode can be due to the interaction between the free radical LuPc$_2$ and the radical intermediate produced during the oxidation of catechol. In addition, the aromatic structure of catechol can also establish strong π–π interactions with phthalocyanines, thus facilitating the electron transfer.

The interaction of catechol and hydroquinone with electrodes was similar. However, a clear difference was observed with PEDOT/PSS/LuPc$_2$ electrodes where a strong decrease in the oxidation peak voltage was not observed [33].

The electrocatalytic properties of the layered composite PEDOT/PSS/EM electrodes were further investigated using electrochemical impedance spectroscopy (EIS). At −0.5 V, a RMS sine wave was applied with frequencies varying logarithmically from 10^{-2} to 10^5 Hz. Typical Nyquist plots obtained from a 10^{-3} mol·L^{-1} catechol solution are displayed in Figure 3. R_s represents the solution resistance. The semicircular part of the diagram at high frequencies corresponds to electron-transfer-limited processes, and the diameter is equivalent to the electron transfer resistance (R_{ct}).

Figure 3: Nyquist plots collected at −0.5 V using (a) PEDOT/PSS; (b) PEDOT/PSS/CuPc; (c) PEDOT/PSS/LuPc$_2$; and (d) PEDOT/PSS/AuNP. Electrodes were immersed in catechol 10^{-3} mol·L^{-1} with 0.01 mol·L^{-1} phosphate buffer (pH 7.0) as the supporting electrolyte. The frequency was swept logarithmically from 10^{-2} to 10^5 Hz.

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The impedance parameters were derived using a Randels equivalent circuit. The whole interface impedance was modelled by a constant phase element (CPE) (Equation 1 and Table 2):

$$Z = \frac{1}{C_{\text{CPE}}(jw)\alpha_{\text{CPE}}} \qquad (1)$$

where C_{CPE} and α_{CPE} are the capacitance and coefficient of the constant phase element, respectively.

Layered composites showed low R_{ct} values ($\approx$4300–5300 Ω) which is about half of the R_{ct} observed in PEDOT/PSS (11500 Ω), indicating that charge transfer rates are higher in PEDOT/PSS/EM sensors. This agrees well with the aforementioned resistivity measurements. The smaller R_{ct} values revealed a low electron-transfer resistance which might be caused by the synergetic enhanced effect between PEDOT/PSS and the secondary EM.

Electrochemical characterization of PEDOT/PSS/EM enzymatic biosensors

PEDOT/PSS/EM composites were also used as electron mediators in tyrosinase (PEDOT/PSS/EM-Tyr) and laccase-based (PEDOT/PSS/EM-Lac) biosensors. As tyrosinase is selective to

the oxidation of o-diphenols and laccase catalyzes the oxidation of a larger variety of polyphenols, PEDOT/PSS/EM-Tyr sensors were used to detect catechol and PEDOT/PSS/EM-Lac sensors were used to detect hydroquinone.

In PEDOT/PSS/EM-Tyr biosensors immersed in catechol, a drastic increase in intensity of the reduction peak at −0.05 V was observed (Figure 4). This increase is due to the simultaneous reduction of o-quinone formed by the electrochemical oxidation at positive potentials plus the reduction of the o-quinone formed by enzymatic oxidation. The electron transfer was promoted in the presence of PEDOT/PSS and further improved in the presence of a secondary electron mediator at PEDOT/PSS/EM electrodes. The presence of LuPc$_2$ produced the largest increase in the peak intensity (−30 µA in PEDOT/PSS, −80 µA in PEDOT/PSS-Tyr and −132 µA in PEDOT/PSS/LuPc$_2$-Tyr). No amplification was observed in the oxidation peak and the current showed the same values as those observed in nonenzymatic sensors (due to the increase in the scale, oxidation peaks cannot be clearly seen in the Figure). When biosensors containing laccase were immersed in the hydroquinone solution, a drastic increase in intensity of the reduction peak at −0.2 V was also observed (−20 µA in PEDOT/PSS, −85 µA in PEDOT/PSS-Lac and −130 µA in PEDOT/PSS/LuPc$_2$-Lac). In this case, the intensity of the signal at positive potentials was also ampli-

Table 2: Results obtained from the impedance spectroscopy measurements.

	R_s (Ω)	C_{CPE} (F)	α_{CPE}	R_{ct} (Ω)
PEDOT/PSS	170.8	1.70×10^{-5}	0.91	11430
PEDOT/PSS/LuPc$_2$	127.4	2.23×10^{-5}	0.92	4734
PEDOT/PSS/CuPc	120.3	2.62×10^{-5}	0.92	5375
PEDOT/PSS/AuNPs	148.4	3.2×10^{-5}	0.93	4346

Figure 4: Cyclic voltammograms of (a) PEDOT/PSS/EM-Tyr immersed in catechol 1.5×10^{-4} mol·L^{-1} and (b) PEDOT/PSS/EM-Lac immersed in hydroquinone 1.5×10^{-4} mol·L^{-1}. Scan rate 0.1 V·s^{-1}.

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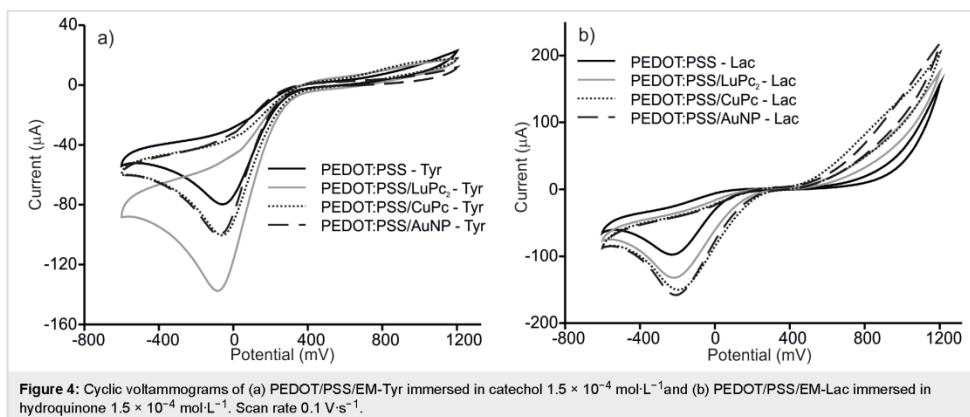
fied. The results demonstrate that the layered composites developed in this work facilitate the electron transfer and the synergistic effect between the components was evidenced.

Repeatability and reproducibility

Repetitive measurements were carried out in 10^{-3} mol·L^{-1} solutions to study the repeatability of the voltammograms. The results of five consecutive measurements showed a coefficient of variation lower than 2% in all cases. Additionally, the reproducibility of the electrodes was examined by cycling the electrodes in 10^{-3} mol·L^{-1} solutions using three electrodes prepared using the same method. The coefficient of variation in both cathodic and anodic peaks were found to be less than 4%, confirming the reproducibility of the method.

It is worth mentioning that the PEDOT/PSS/EM electrodes could be used up to 50 times with a decrease in the intensity of less than 5%. The PEDOT/PSS/EM enzymatic electrodes were found to be highly stable for ten scans. After that their functionality progressively decreased and could no longer be used.

Scan rate dependence study

In order to further analyze the electron transfer and to evaluate the dynamic behavior of the electrodes, voltammograms were collected at different scan rates. The responses are illustrated in Figure 5 for the PEDOT/PSS/AuNP electrode immersed in catechol 10^{-3} mol·L^{-1}. The cathodic peak potentials shifted to more negative potentials with increasing scan rate (Figure 5a). This suggests the involvement of a kinetic limitation between the electrode and the phenol. The cathodic peak currents (I_c) varied linearly with the square root of the scan rate ($v^{1/2}$), demonstrating the diffusion-controlled nature of the electrode reaction (Figure 5b). The slopes and correlation coefficients for the measurement of all the sensors and biosensors immersed in catechol are collected in Table 3. The results obtained in hydroquinone are presented in Table 4. According to the slope values, the reduction of hydroquinone proceeds more rapidly than that of catechol. The slope values calculated in layered PEDOT/PSS/EM composites were higher than those observed in PEDOT/PSS, confirming the improvement of the charge transfer rates. It is also worth noting that the slopes found in

Figure 5: Effect of the scan rate in PEDOT/PSS/AuNP immersed in catechol 10^{-3} mol·L^{-1}. (a) CVs collected at 0.025, 0.05, 0.1, 0.2, 0.5 and 1.0 V·s^{-1}; (b) linear relationship between I_c and the square root of the scan rate; (c) linear relationship between E_c and the log of the scan rate and (d) Tafel plot representing the logarithm of the intensity vs the overpotential.

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Table 3: Relationship between scan rate in sensors immersed in catechol 10^{-3} mol·L^{-1} calculated in the cathodic peak.

Sensor	I_c (µA) vs $v^{1/2}$ (V/s)$^{1/2}$		E_c (V) vs log v (V/s)			Log I (µA) vs η (V)		
	Slope	R^2	Slope	R^2	$α_n$	Slope	R^2	n
PEDOT/PSS	−115.71	0.946	−0.117	0.993	0.50	−4.536	0.998	1.87
PEDOT/PSS/LuPc$_2$	−169.33	0.947	−0.144	0.994	0.41	−4.256	0.991	1.95
PEDOT/PSS/CuPc	−185.97	0.953	−0.135	0.997	0.44	−3.781	0.988	1.97
PEDOT/PSS/AuNP	−257.53	0.960	−0.173	0.999	0.34	−3.324	0.989	1.73
PEDOT/PSS-Tyr	−268.38	0.980	−0.169	0.985	0.35	−2.823	0.975	2.10
PEDOT/PSS/LuPc$_2$-Tyr	−309.65	0.977	−0.184	0.992	0.32	−2.361	0.977	2.30
PEDOT/PSS/CuPc-Tyr	−230.25	0.981	−0.174	0.984	0.34	−2.632	0.968	2.20
PEDOT/PSS/AuNP-Tyr	−231.99	0.984	−0.181	0.984	0.33	−2.402	0.963	2.30

Table 4: Relationship between scan rate in sensors immersed in hydroquinone 10^{-3} mol·L^{-1} calculated in the cathodic peak.

Sensor	I_c (µA) vs $v^{1/2}$ (V/s)$^{1/2}$		E_c (V) vs log v (V/s)			Log I (µA) vs η (V)		
	Slope	R^2	Slope	R^2	$α_n$	Slope	R^2	n
PEDOT/PSS	−247.88	0.947	−0.168	0.992	0.50	−4.607	0.997	1.84
PEDOT/PSS/LuPc$_2$	−299.33	0.954	−0.199	0.995	0.41	−3.247	0.999	2.10
PEDOT/PSS/CuPc	−270.75	0.967	−0.170	0.994	0.44	−3.680	0.996	2.02
PEDOT/PSS/AuNP	−300.56	0.966	−0.210	0.993	0.34	−3.391	0.997	1.70
PEDOT/PSS-Lac	−411.98	0.975	−0.143	0.988	0.41	−3.050	0.998	2.3
PEDOT/PSS/LuPc$_2$-Lac	−511.99	0.978	−0.206	0.984	0.29	−2.004	0.999	2.4
PEDOT/PSS/CuPc-Lac	−532.85	0.968	−0.214	0.982	0.29	−2.003	0.999	2.4
PEDOT/PSS/AuNP-Lac	−463.63	0.969	−0.189	0.990	0.31	−2.254	0.999	2.3

enzymatic PEDOT/PSS/EM biosensors were higher than the values found in nonenzymatic PEDOT/PSS/EM electrodes. This result indicates that the charge transfer within the composite film and/or through the electrode interface is facilitated in biosensors and this improvement should be related with the enzymatic activity.

In the scan rates ranging from 0.025 to 1 V·s^{-1}, the cathodic peak potential (E_c) showed a linear relationship with the logarithm of scan rate (log v) (Figure 5c) according to Laviron's equation (Equation 2) for a totally irreversible diffusion-controlled process [34,35].

$$E_c = E^0 - \frac{2.3RT}{(α_c)nF} \log υ, \qquad (2)$$

where α is the transfer coefficient, v is the scan rate (expressed in V·s^{-1}), n is the number of electrons involved in the rate-determining step, R is the ideal gas constant (8314 J·mol^{-1}·K^{-1}),

T is the temperature (298 K) and F is Faraday's constant (95,484.56 C·mol^{-1}).

The regression coefficients calculated for the analysis of all the sensors and biosensors immersed in catechol and hydroquinone are presented in Table 3 and Table 4, respectively.

The linear variation of the peak potential, E_c, as a function of log v suggests that the electrode process can be regarded as a totally irreversible reaction. From the slope of the graph, $α_n$ can be determined, where α is the transfer coefficient and n is the number of electrons transferred in the rate-determining step.

The slope indicated that the values $α_n$ have values between 0.3–0.5 for the cathodic peak. These values indicate the total irreversibility of the electron transfer process and also confirm the ideal diffusion-controlled mechanism [36].

The simplified Butler–Volmer equation can be applied to calculate the α values (Equation 3) [35]. The Tafel slope was calculated from the representation of log I vs the overpotential (η)

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obtained from a voltammogram collected in catechol or hydro-quinone 10^{-3} mol·L^{-1} at a scan rate of 0.1 V·s^{-1} (Figure 5d). Then, using the results obtained from the Laviron equation for α_n, the number of electrons transferred can be calculated.

$$\log I = \log I_0 - \frac{\alpha F}{2.3RT}\eta \qquad (3)$$

The calculated Tafel slopes, the α values and the number of electrons are listed in Table 3 and Table 4. As can be seen from these results, the Tafel slopes showed a less negative value in the electrodes modified with the composite PEDOT/PSS/EM. The values were even lower in the presence of an enzyme, confirming the improved electrocatalytic activity. This result further confirms that the combination of PEDOT/PSS with AuNPs and phthalocyanines can promote electron transfer in the active centers of biological molecules, increasing the relative activity of the enzymes.

The number of electrons involved in the reaction calculated from the Tafel slope and Equation 2 confirms a two electron process in all electrodes, confirming the proposed mechanism.

Limit of detection

The limit of detection (LD) was evaluated by analyzing the response of the sensors towards phenol solutions with concentrations ranging from 4.0×10^{-6} mol·L^{-1} to 1.5×10^{-4} mol·L^{-1}. The results are illustrated in Figure 6 for PEDOT/PSS/LuPc$_2$ and PEDOT/PSS/LuPc$_2$-Tyr immersed in catechol.

In PEDOT/PSS/EM electrodes, a linear relationship was observed between the current response signals and the concentration of catechol and hydroquinone, confirming that they may reliably be used for the determination of both compounds in this concentration range. The limit of detection calculated from the anodic peak (LD$_A$) and the cathodic peak (LD$_C$) (using the 3σ criteria) and correlation coefficients are collected in Table 5.

The results confirmed that the LD towards catechol was improved almost one order of magnitude when using layered composite electrodes and attained the µmol·L^{-1} range. The improvement was not so marked in hydroquinone, but also in this case, the LD calculated from the cathodic peak was in the µmol·L^{-1} range.

The improved performance can be attributed to synergistic interactions between PEDOT/PSS and the additives similar to the interactions already described between PEDOT/PSS and graphene [37]. In our composites, PEDOT/PSS acts as the electron mediator and the additive further enhances the electron transfer due to the high electrical conductivity demonstrated in the impedance experiments.

In the case of PEDOT/PSS/EM enzymatic biosensors, the main amplification occurred in the cathodic wave. For this reason, the study of the LD was carried out only in the cathodic reduction peak.

When representing the peak current vs catechol concentration obtained from PEDOT/PSS/EM-Tyr, a linear relationship was obtained in the concentration range from 4.0×10^{-6} to
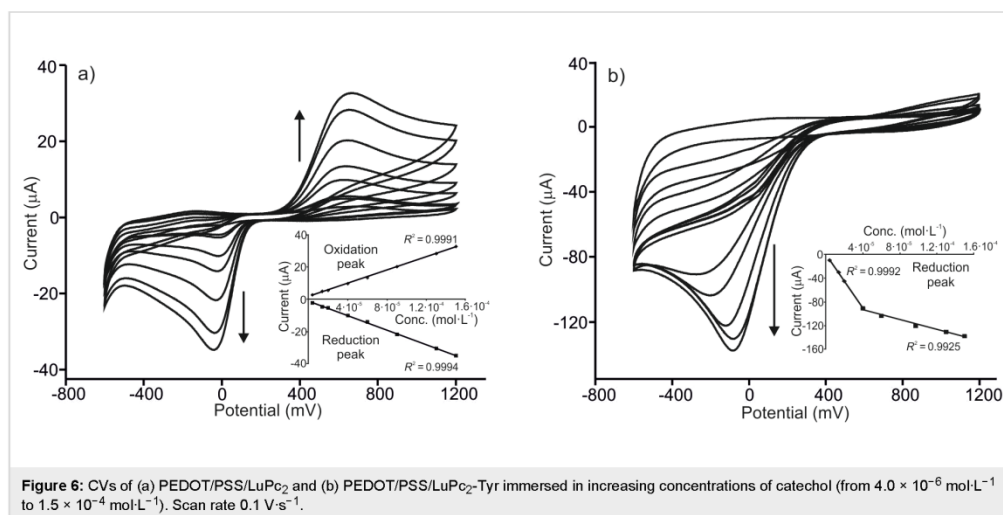

Figure 6: CVs of (a) PEDOT/PSS/LuPc$_2$ and (b) PEDOT/PSS/LuPc$_2$-Tyr immersed in increasing concentrations of catechol (from 4.0×10^{-6} mol·L^{-1} to 1.5×10^{-4} mol·L^{-1}). Scan rate 0.1 V·s^{-1}.

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Table 5: Limit of detection and regression coefficients obtained for catechol and hydroquinone using the anodic (LD$_A$) and the cathodic (LD$_C$) peaks.

Sensor	LD$_A$ (mol·L^{-1})	R^2 (anode)	LD$_C$ (mol·L^{-1})	R^2 (cathode)
Catechol				
PEDOT/PSS	4.96×10^{-5}	0.993	6.58×10^{-6}	0.998
PEDOT/PSS/LuPc$_2$	1.23×10^{-6}	0.999	1.03×10^{-6}	0.999
PEDOT/PSS/CuPc	2.99×10^{-6}	0.992	1.81×10^{-6}	0.996
PEDOT/PSS/AuNP	2.18×10^{-6}	0.998	0.87×10^{-6}	0.999
Hydroquinone				
PEDOT/PSS	2.85×10^{-5}	0.971	1.61×10^{-5}	0.970
PEDOT/PSSLuPc$_2$	1.80×10^{-5}	0.996	9.41×10^{-6}	0.997
PEDOT/PSS/CuPc	2.69×10^{-5}	0.972	1.51×10^{-5}	0.998
PEDOT/PSS/AuNP	1.97×10^{-5}	0.993	7.55×10^{-6}	0.996

6×10^{-5} mol·L^{-1}. Then, a change in the slope occurred and a new linear range was observed from 9.0×10^{-5} to 1.5×10^{-4} mol·L^{-1}. The limit of detection calculated in both linear ranges is shown in Table 6. A LD in the low concentration range was attained (10^{-7} mol·L^{-1}), that is, one order of magnitude lower than the LD obtained in the nonenzymatic sensor. The LD in the high concentration scale were similar to those obtained for the nonenzymatic sensors and were in the μmol·L^{-1} range. The obtained LDs were similar to values reported in recent works using tyrosinase and graphene as electron mediator [38,39]. The LD obtained for hydroquinone was also one order of magnitude lower than in nonenzymatic sensors and attained values of 10^{-6} mol·L^{-1}.

Conclusion

Owing to their unique electrochemical properties, layered composite PEDOT/PSS/EM and PEDOT/PSS/EM-Enz electrodes offer efficient electron transfer, low detection limit, high sensitivity and good reproducibility toward the oxidation of catechol and hydroquinone.

Furthermore, the combination of PEDOT with AuNPs or phthalocyanines can promote electron transfer in the active centers of biological molecules, increasing the relative activity of the enzymes. The linear relationship between the peak current and the square root of the scan rate for both PEDOT/PSS/EM sensors and PEDOT/PSS/EM-Enz biosensors in catechol

Table 6: Limit of detection and regression coefficients obtained for catechol and hydroquinone using the cathodic (LD$_C$) peaks using biosensors.

Sensor	LD$_C$ (mol·L^{-1})		R^2 (cathode)	
Conc. range	4×10^{6}–6×10^{-5}	9×10^{-5}–1.5×10^{-4}	4×10^{-6}–6×10^{-5}	9×10^{-5}–1.5×10^{-4}
Catechol				
PEDOT/PSS-Tyr	6.62×10^{-7}	6.55×10^{-6}	0.9862	0.987
PEDOT/PSS/LuPc$_2$-Tyr	4.62×10^{-7}	2.53×10^{-6}	0.9925	0.999
PEDOT/PSS/CuPc-Tyr	4.37×10^{-7}	3.16×10^{-6}	0.9971	0.936
PEDOT/PSS/AuNP-Tyr	3.88×10^{-7}	2.80×10^{-6}	0.9889	0.971
Hydroquinone				
PEDOT/PSS-Lac	6.00×10^{-6}		0.997	
PEDOT/PSS/LuPc$_2$-Lac	1.11×10^{-6}		0.996	
PEDOT/PSS/CuPc-Lac	1.52×10^{-6}		0.999	
PEDOT/PSS/AuNP-Lac	2.51×10^{-6}		0.998	

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and hydroquinone indicate a diffusion-controlled analyte electrocatalytic oxidation, and Tafel slopes confirmed the improved electrocatalytic activity. The modified electrodes designed here show excellent limits of detection that are comparable with those found in literature. The reported sensors could be used to analyze the phenolic content of foods and beverages such as wine, must, coffee, saffron, etc.

Experimental
Reagents and solutions
Poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate) (PEDOT/PSS) aqueous solution (3.0–4.0% in H_2O, high conductivity grade) and copper(II) phthalocyanine tetrasulfonic acid tetrasodium salt (CuPc, 0.05 g/L) were purchased from Sigma-Aldrich. Gold nanoparticle (AuNPs, 30–40 nm) colloids were synthesized according to a modification of the procedure proposed by Slot and Geuze [12,40]. Using this procedure, a red colloid with a UV absorbance maximum at $\lambda = 540$ nm was obtained. The lutetium(III) bisphthalocyaninate (LuPc$_2$, 0.05 g/L) was synthesized following a previously published procedure [41].

Phenol oxidase enzymes were purchased from Sigma-Aldrich: tyrosinase (from mushroom, activity ≥ 1000 U·mg^{-1}) and laccase (from *Trametes versicolor*, activity ≥ 10 U·mg^{-1}). 5 mg·L^{-1} solutions of tyrosinase and laccase were prepared in buffered phosphate 0.01 mol·L^{-1} (pH 7.0).

Preparation of the electrochemical sensors and biosensors
Sensors were prepared using a spin coater, model 1H-D7 (Micasa Co., Tokyo, Japan). ITO glass substrates (1 cm^2 surface area) were used as the substrate. Prior to the film deposition, the substrates were washed in an ultrasonic bath with acetone and rinsed twice with deionized water (MilliQ).

PEDOT/PSS was diluted 1:10 in deionized water and stirred in an ultrasonic bath for 10 min. Then, 100 µL of the solution was dropped onto the ITO glass and spin-coated at 2000 rpm for 120 s (slope of 120 s). The PEDOT/PSS films obtained were annealed at 150 °C for 15 min.

PEDOT/PSS/EM layered sensors were prepared on the surface of PEDOT/PSS films by spin coating 200 µL of CuPc and AuNPs, or 100 µL of LuPc$_2$ at 2000 rpm for 120 s (slope of 120 s), followed by annealing at 150 °C for 15 min.

PEDOT/PSS/EM enzymatic biosensors were prepared by depositing laccase or tyrosinase onto the PEDOT/PSS/EM sensors. For this purpose, 50 µL of 0.01 mol·L^{-1} phosphate buffer (pH 7.0) containing 5 mg·mL^{-1} of enzyme were

deposited onto the PEDOT/PSS/EM electrode. After drying at room temperature for approximately 45 min., the biosensors were immersed in glutaraldehyde (2.5% v/v, buffer solution) for 5 min and dried in air for 15 min at room temperature. The biosensors were then rinsed with phosphate buffer to remove any unbound enzyme and stored at 4 °C.

Characterization of the sensors
Scanning electron microscopy (SEM) (FEI, QUANTA 200F) was used to record the images of the electrode surfaces. A square resistance was measured using a four-point tester (HAAMEG, HM 8040-2).

Electrochemical impedance spectroscopy (EIS) experiments were performed using a Solartron impedance analyzer. The measurements were carried out by applying a signal amplitude of 10 mV, at a working potential of -0.5 V with frequencies varied logarithmically from 0.1 Hz to 100 kHz. The measurements were made by immersing the electrode in 10^{-3} mol·L^{-1} catechol in 0.01 buffer phosphate (pH 7). The impedance spectra were fitted with the aid of the Zview2 software.

Voltammetric measurements were obtained using a potentiostat/galvanostat PGSTAT128 (Autolab Metrohm, Utrecht, Netherlands). The electrochemical cell was a three-electrode system using the corresponding PEDOT/PSS, PEDOT/PSS/EM or PEDOT/PSS/EM-Enz modified electrode as the working electrode; the reference electrode was Ag|AgCl/KCl 3 mol/L and the counter electrode was a platinum sheet with a surface area of 1 cm^2.

Cyclic voltammetry was carried out from -0.6 V to $+1.2$ V (vs Ag/AgCl) with a scan rate of 0.1 V·s^{-1}, except when indicated otherwise. A phosphate buffer solution (0.01 mol·L^{-1}; pH 7.0) was employed as the electrolytic medium in electroanalysis experiments. The influence of the potential sweep rate was studied in 10^{-3} mol·L^{-1} catechol or 10^{-3} mol·L^{-1} hydroquinone in 0.01 phosphate buffer (pH 7), while varying the scan rates from 0.025 to 1.0 V·s^{-1}.

The limits of detection (LD) was calculated from peak current responses taken from voltammograms recorded at different concentrations from 4·10^{-6}–1.5·10^{-4} mol/L, following the "3sd/m" criterion, where "m" is the slope of the calibration graph and "sd" was estimated as the standard deviation ($n = 5$) of the voltammetric signal at the concentration level corresponding to the lowest concentration of the calibration plot.

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PEDOT:PSS/phthalocyanines as electron mediators for sensors and biosensors.*

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3.8. Paper VII

Impedimetric electronic tongue based on nanocomposites for the analysis of red wines. Improving the variable selection method.

By Celia García-Hernández, Coral Salvo-Comino, Fernando Martín-Pedrosa, María Luz Rodríguez-Méndez and Cristina García-Cabezón.

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*Paper VII: Impedimetric electronic tongue based on nanocomposites for
the analysis of red wines. Improving the variable selection method.*

3.8.1. Motivation and objective

Electrochemical sensors implemented in the technology of electronic tongues are usually potentiometric, voltammetric or ampererometric. However, research works focused on impedimetric sensors applied in ET are scarce compared to the other electrochemical sensors. Moreover, impedimetric electronic tongues implemented for food authenticity have not been widely applied and only Riul et al. have reported research work in which they used an impedimetric ET to analyze different brands of red wines. Furthermore, Riul and co-workers have actively implemented these devices, being the first to develop an array of sensors based on LB films containing conducting polymers to assess brands of mineral water, tea and coffee.

Impedimetric sensors measure the impedance using impedance spectroscopy at a constant frequency or a full frequency spectrum. The use of these sensors can provide some advantages with respect to the most commonly used electrochemical sensors. For instance, in terms of taste sensors for liquid media, potentiometric sensors have limited sensitivity in non-electrolyte compounds, whereas voltammetric devices need redox species in the media and the responses are quite complex and require advanced statistical treatments. Moreover, impedimetric devices can be used in any kind of solution, even without electrical conductivity, and do not need a reference electrode to carry out the measurements, thus facilitating the data acquisition because a reliable reference electrode is a very important factor in many practical applications.

Basically, impedimetric electronic tongues have used to test different mineral waters, to study water contaminated with heavy metals, to detect trace levels of phenolic compounds in water, to analyze organophosphate pesticides and discriminate between different wines, to detect tetracycline residue in milk samples and discriminate the five classic tastes.

In a previous research work, we developed an array of sensors based on the conducting polymer PEDOT:PSS modified with different sensing materials that improved the electron transfer. PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNP composite sensors were identified as the most reproducible and sensitive sensors. For this reason, in this work, we have selected these sensors along with the non-modified sensor of PEDOT:PSS to form an impedimetric electronic tongue. The multisensor system has been applied to discriminate red wines with similar characteristics (same region, vintage and ageing method) but different variety. The novelty of this work consists in developing an improved impedimetric e-tongue applied to the analysis of red

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wines. For this purpose, we have carried out different strategies to find the most suitable method that will offer a better discrimination capability, including the classical method of selecting resistance and capacitance data at a fixed frequency, selecting parameters of the Nyquist and Bode plots obtained in electrochemical impedance spectroscopy (EIS) analysis, and finally, by fitting the results obtained in EIS expriments to an equivalent electric circuit.

The objective of this work was to develop an improved impedimetric e-tongue for the analysis of red wines, for which two strategies were followed. On the one hand, to develop new impedimetric sensors based on combinations of well-known electrocatalytic materials, including PEDOT:PSS, gold nanoparticles and phthalocyanines. On the other hand, the impedance spectroscopy measurements used for impedimetric e-tongues are diverse, in some cases measurements of electrical resistance and/or capacitance have been used at a fixed frequency value. Here, different possibilities were tested, including the fitting by means of an equivalent circuit to determine which method offers the best discrimination capability.

3.8.2. Summary of the research work

The sensors PEDOT:PSS, PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNPs were prepared on an ITO surface, as already described, by using the spin-coating technique.

The red wines analyzed were elaborated by the Oenological Centre of Rueda (Valladolid, Castilla y León) and they also provided the chemical parameters of total polyphenolic index (TPI), acidity, SO$_2$ content, reducing sugars, glucose and fructose content, alcoholic degree and pH. In addition, the Folin-Ciocalteu method was carried out to evaluate the phenolic content by means of another methodology different from TPI. Wines were harvested in different cellars of the same Denomination of Origin (D.O.), vinified with the same method and aged for 6 months in oak barrel and 18 months in bottle. The red wine samples were: three samples from the variety of grape Tempranillo, one from the variety Tinta de Toro (a clone of Tempranillo), and the third from a mixture of Tempranillo (90%) and Garnacha (10%).

Firstly, and before analyzing the red wines, the sensors were characterized by testing the conductivity and capacitance and carrying out electrochemical impedance spectroscopy measurements (EIS).

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Conductivity results evidenced that it increased for the sensors based on modified PEDOT:PSS sensors and, therefore, the values were higher for PEDOT:PSS/LuPc$_2$ followed by PEDOT:PSS/AuNPs. These results were related to the intimate contact between the conducting polymer chains with the charge carriers of the intrinsic semiconductor LuPc$_2$ and due to the enlargement of the effective surface area caused by the AuNPs, along with the existence of mixed valence states on their surface. Capacitance *vs.* voltage plots showed a characteristic p-doped behavior with a slight decrease at low potentials and then a steep rise at intermediate potentials. Further, carrier concentrations (N$_D$) and flat band potentials (V$_{FB}$) of the samples were **obtained by Mott−Schottky analysis and** these confirmed the results obtained by measuring the conductivity and capacitance. The carrier concentrations were higher in modified PEDOT:PSS sensors, confirming the improvement of the conductivity when implementing a secondary electrocatalytic material. Moreover, the flat band potentials were lower for PEDOT:PSS/AuNPs and PEDOT:PSS/ LuPc$_2$ than for PEDOT:PSS, again demonstrating the important effect of AuNPs and LuPc$_2$ as doping agents.

Nyquist and Bode plots from EIS experiments confirmed that modified PEDOT:PSS sensors showed lower charge-transfer resistance (Rct), a reduction in the impedance module and also more negative values in the phase angle. These results are in accordance with those mentioned before and evidence the efficient electrocatality properties when combining PEDOT:PSS with LuPc$_2$ or AuNPs in layered films.

The responses of the impedimetric electronic tongue for the red wine samples under study showed a Nyquist plot (Zimaginary vs Zreal) where the three wines elaborated with Tempranillo grapes had the highest values of Rct, whereas the other two wines, Tinta de Toro and the Tempranillo/Garnacha mixture, had lower values of Rct. This result might be correlated with the wine composition. A higher content of redox species, such as antioxidants, sugars or SO$_2$, as well as a higher acidity and alcoholic degree, will provide higher values of Rct.

Further, the discrimination of red wines was performed by PCA following different strategies of data extraction methods. Firstly, we used a classical method of selecting resistance and capacitance data at a fixed frequency. However, using this method, the red wines were not discriminated in PCA analysis. Therefore, the feature extraction method was improved following two other ways: in the first, we selected parameters of the Nyquist and Bode plots obtained in EIS analysis in wines at a constant frequency (Zimaginary, Zreal and maximum phase angle) and, in

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the second, we obtained parameters from an equivalent electric circuit resulting from simulating the EIS results with different equivalent circuits in order to understand the electrochemical behavior of an electrode coated with a semiconductor polymer material. In the last method, the number of variables was increased to seven.

In the first improved feature extraction method, the results of the PCA analysis showed well-defined and separate clusters for each variety of wine. A 2D PCA score plot showed the wine elaborated with two different varieties (Tempranillo and Garnacha) clearly separated from the rest of the samples. In the second analysis, PCA analysis was also capable of discriminating between the wine samples; however, observing the 3D PCA score plot, we can conclude that the degree of discrimination was higher than when using variables from the Nyquist and Bode plots: red wines elaborated from Tempranillo grape and with similar chemical parameters appeared on the left side of the diagram, whereas wines elaborated with other varieties appeared separated from them and in between.

Finally, PLS-1 analysis was performed to obtain regression models able to predict chemical parameters in the wines from the data acquired by means of EIS experiments and from the equivalent circuit parameters. Both methods established good correlations with the chemical parameters provided by the oenological center, with high correlation coefficients and a low number of latent variables (in the range from 1 to 3). The values of the root mean square errors (RMSE) of calibration and prediction analysis were also low. However, in general, regression models obtained using the equivalent circuit parameters provided the best correlations with the chemical parameters, confirming that this method improves the quality of the feature extraction.

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Impedimetric electronic tongue based on nanocomposites for the analysis of red wines. Improving the variable selection method

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ABSTRACT

An impedimetric electronic tongue to discriminate red wines with similar characteristics (same region, vintage and ageing method) is developed. The excellent performance achieved is due to the remarkable sensing properties of the nanocomposites forming the array and to the methodology developed to extract the input variables used in the statistical analysis. The multisensor system is formed by one PEDOT:PSS sensor and two nanocomposites formed by layers of PEDOT:PSS and gold nanoparticles (PEDOT:PSS/AuNP) or layers of PEDOT:PSS and lutetium bisphthalocyanine (PEDOT:PSS/LuPc$_2$). The well-known electrocatalytic activity of the sensing materials is promoted in the layered composites and enhances the cross-selectivity of the sensors. Besides, a feature extraction method that uses an equivalent electric circuit model to obtain the input variables used in the statistical analysis has been developed. By using the statistical procedure of Principal Component Analysis, the improved electronic tongue is able to discriminate three wines from the variety Tempranillo, one wine of the variety Tinta de Toro (a clon of Tempranillo) and one wine elaborated with a coupage of 90% Tempranillo and 10% Garnacha variety. The combination of layered nanomaterials and the new feature extraction method also enhances the capability of the impedimetric electronic tongue to predict chemical variables using Partial Least Squares regression.

1. Introduction

Electronic tongues (e-tongue) are an excellent alternative to traditional methods of analysis for the control of products and processes in the agrifood sector. Through a system of multisensors combined with chemometric tools, these systems are able to classify samples and quantify their physicochemical parameters [1,2]. They have many advantages over traditional analysis techniques. The main one is that the measures suppose an important saving of time because they are not individual tests, do not require preprocessing of the sample or specialized technicians. They are therefore an excellent tool in the quality control of food, especially in situations where only qualitative or semiquantitative information is required [3,4].

There are many types of e-tongues depending on the variety of sensitive materials and transduction methods [5]. Most of the works in the field make use of sensors based on electrochemical techniques including potentiometric, voltammetric or amperometric sensors [6–9]. In contrast, e-tongues based on impedimetric sensors have been studied in a much lesser extent.

E-tongues using impedance spectroscopy (IS) technique were initially developed by Riul et al. [10]. They are based on interdigitated electrodes modified with different chemosensitive materials deposited by LB (Langmuir-Blodgett) or LBL (Layer by Layer) techniques [11]. These e-tongues are based on the application of a sinusoidal signal in a range of frequencies with a constant signal amplitude and the measurement of the impedance at a fixed frequency [12,13]. For instance, applying sinusoidal signals (from 20 Hz to 100 kHz) to interdigitated electrodes coated with different polymeric materials, it was possible to detect and discriminate sucrose, sodium chloride, potassium chloride and hydrochloric acid [11]. Quantification of ions (sodium, potassium and ammonium) in solution could also be achieved under static conditions [14] or under a continuous flow system [15]. Pioggia et al. successfully developed a polymer membrane electrode system to measure standard solutions that simulated the five basic flavors (sweetness, sourness, bitterness, saltiness and umami or savory taste) [16,17]. Masot developed an electrochemical impedimetric spectroscopy (EIS) e-tongue to determine physicochemical parameters such as salt and moisture content in meats [18], to detect the level of curing in cheeses [19], to study the freshness of fish [20], to

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determine the botanical origin of honey [21] or to detect contaminants of microbiological origin [22]. The impedimetric e-tongues have also been used for the recognition of beverages as mineral waters [23,24] or black teas [25]. There are few works using impedimetric e-tongues applied to discrimination of wines [13,26]. In fact, most of the works in the oenological field use potentiometric [27,28] or voltammetric [29–32] sensors.

Many efforts have been made to improve the performance of e-tongues. In many cases such works were related to the use of new sensing materials that could improve the sensitivity and selectivity of the sensors forming the array. Phtalocyanines has been successfully used in LBL films for dopamine detection using EIS [33]. Moreover, it has been demonstrated that the combination of electrocatalytic materials can produce synergistic effects improving the performance of voltammetric sensors in the detection of phenols of interest in the food industry [34].An example of this in EIS sensors has been published on pesticide detection in real samples using nanocomposites modified with graphene and gold nanoparticles using the electrical resistance response in the medium frequency region [35].

The objective of this work was to develop an improved impedimetric e-tongue for the analysis of red wines. For this purpose, two strategies were followed. On one hand, to develop new impedimetric sensors based on combinations of well-known electrocatalytic materials including PEDOT:PSS [34,36,37], gold nanoparticles [38] and phthalocyanines [39]. On the other hand, the impedance spectroscopy measurements used for impedimetric e-tongues are diverse, in some cases measurements of electrical resistance and/or capacitance have been used at a fixed frequency value [26,35,40]. In this work, different possibilities were tested, including the fitting by means of an equivalent circuit, in order to determine which method will offer the best discrimination capability.

The performance of the improved system will be tested by evaluating its capability to discriminate red wines with similar characteristics but with different chemical composition and to establish correlations between the results obtained with the e-tongue and the traditional chemical analysis including Total Polyphenol Index (TPI), Folin Index, Total acidity, SO_2 content, sugars content, alcoholic degree and pH.

2. Materials and methods

2.1. Reagents and solutions

PEDOT:PSS aqueous solution (3.0–4.0% in H_2O, high conductivity grade) was purchased from Sigma-Aldrich. Gold nanoparticles (AuNPs, 30–40 nm) were synthesized according to a modification of the procedure proposed by Slot and Geuze [41,42]. Using this procedure, a red colloid with a UV absorbance maximum at $\lambda = 540\,nm$ was obtained. The lutetium(III) bisphthalocyaninate (LuPc$_2$, 0.05 g L^{-1}) was synthesized using a method developed by our group [43].

2.2. Preparation of the electrochemical sensors and biosensors

Sensors were prepared using a spin coater model 1H-D7 (Micasa Co., Tokyo, Japan). ITO glass substrates (1 cm² of surface) were used as the substrate. Prior to the film deposition, substrates were washed in an ultrasonic bath with acetone and rinsed twice with deionized water (MilliQ).

PEDOT:PSS was diluted 1:10 in deionized water and stirred in an ultrasonic bath for 10 min. Then, 100 μL of the solution was dropped onto the ITO glass (Indium Tin Oxide glass) and spin-coated with a ramp of 120 s from low speed to 2000 rpm and then, it was stayed during 120 s. The PEDOT:PSS films thus obtained were annealed at 150 °C for 15 min.

Electrochemical sensors based on layered composites were formed by alternate layers of PEDOT:PSS and a secondary electrocatalytic material (EM) (PEDOT:PSS/EM). They were prepared on the surface of PEDOT:PSS films by spin-coating 200 μL of AuNPs or 100 μL of LuPc$_2$ at

2000 rpm for 120 s (ramp of 120 s) followed by annealing at 150 °C for 15 min to obtain PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNPs sensors, respectively.

2.3. Characterization of the sensors

A SEM-FEI (QUANTA 200 F) (Hillsboro, USA) was used to record the images of the electrode surfaces. Square resistance was measured using a four-point tester (HAAMEG HM 8040-2).

Voltammetric measurements were obtained using a potentiostat/galvanostat PGSTAT128 (Autolab Metrohm, Utrecht, The Netherlands). The electrochemical cell was a three- electrode system using the corresponding PEDOT:PSS or PEDOT:PSS/EM modified electrode as the working electrode, a platinum foil with a size of 2 cm × 1 cm as the counter electrode and an Ag/AgCl/KCl 3 M electrode as the reference electrode.

Mott-Shottky analysis and EIS experiments were performed using a SOLARTRON Impedance Analyzer, SI 1260, (Hampshire, England). The Mott-Shottky measurements were performed at a fixed frequency of 100 Hz in KCl (0.1 mol L^{-1}). The impedance spectroscopy was carried out by applying a signal amplitude of 10 mV, with frequencies that varied logarithmically from 0.1 Hz to 10 kHz at different working potentials. Measurements were made in three electrolytes: KCl (0.1 mol L^{-1}), [Fe(CN)$_6$]$^{4-/3-}$ (5 mmol L^{-1}) in KCl (0.1 mol·L^{-1}) and diluted wines 1:1 in KCl (0.1 mol L^{-1})). Impedance spectra were fitted with aid of the Zview2 software, Scribner Associates, Inc. Four replicas per sample were carried out.

2.4. Wine samples

Wines were elaborated purposely for this work in the "Estación Enológica de Rueda" located in Castilla y León area (Valladolid, Spain). They included five red wines elaborated from grapes harvested in five cellars of the same Denomination of Origin (DO). They were vinified using the same method and aged for 6 months in oak barrel and 18 months in bottle. From the five wines, three of them (W3, W4 and W5) were prepared form the same variety of grape (Tempranillo). W1 was elaborated using grapes of the variety Tinta de Toro which is a clon of the variety Tempranillo and W2 was elaborated using a mixture of Tempranillo (90%) and Garnacha (10%) grapes. Total Polyphenol Index (TPI), Folin Index, Total acidity, SO_2 content, sugars content, alcoholic degree and pH were analyzed following international regulations [44]. Results of the chemical analysis are collected in Table 1.

2.5. Chemometrics

EIS measurements were normalized using Matlab v2014b (The Mathworks Inc., Natick, MA, USA). A non-supervised multivariate method, Principal Component Analysis (PCA) was used to evaluate the discrimination capability of the impedimetric e-tongue. The impedance curves used as data source for statistical analysis were first pre-processed. Then, they were adjusted to equivalent circuit using Zview2 software. Partial Least Square Regression-1 (PLS-1) was used to establish correlations between the results obtained from the impedimetric e-tongue and the chemical parameters.

3. Results and discussion

3.1. Structural characterization

The surface structure of PEDOT:PSS, PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNPs sensors was examined using SEM. PEDOT:PSS films showed a homogeneous smooth surface (Fig. 1a). The structure was not altered upon modification with LuPc$_2$ (Fig. 1b). The surface of the PEDOT:PSS/AuNPs showed the expected smooth surface of the PEDOT:PSS films where nanoparticles were well-distributed on the film

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Table 1
Average chemical parameters measured in wines.

WINE	W1	W2	W3	W4	W5
D.O. (Origin)	Toro	Cigales	Ribera Duero	Ribera Duero	Ribera Duero
Variety	Tinta Toro	Tempranillo (90%) Garnacha(10%)	Tempranillo	Tempranillo	Tempranillo
Year	2014	2014	2014	2014	2014
Folin	0.475	0.650	0.651	0.658	0.720
TPI	0.684	0.727	0.618	0.715	0.720
Total Acidity (g/l)	4.94	4.83	4.69	4.64	4.54
SO_2 (mg/l)	77	49	92	89	97
Reducing Sugar (g/l)	1.2	1.3	1.6	1.4	1.4
Glucose + Fructose (g/l)	0.56	0.11	0.35	0.22	0.19
Alcoholic Degree	14.35	13.97	14.78	14.41	14.50
pH	3.78	3.79	3.79	3.74	3.78

surface and only few nanoparticle clusters were observed, (Figure 1c and 1 d).

3.2. Conductivity and capacitance measurements

The conductivity of the electrodes was measured using the four-point probe test. The conductivity of PEDOT:PSS ($3.6 \times 10^4 \Omega^{-1} m^{-1}$) increased when films of lutetium phthalocyanine (PEDOT:PSS/LuPc$_2$: $6.4 \times 10^4 \Omega^{-1} m^{-1}$) or AuNPs (PEDOT:PSS/AuNPs: $5.1 \times 10^4 \Omega^{-1} m^{-1}$) were deposited on the top of the PEDOT:PSS film. This enhancement was similar to the increase observed when dopants were added to an aqueous solution of PEDOT:PSS [45]. The intensification of the conductivity in the PEDOT:PSS/LuPc$_2$ film can be associated with the increase in charge carriers caused by the interaction between PEDOT chains and the charge carriers of the intrinsic semiconductor LuPc$_2$

[46]. The increase in PEDOT:PSS/AuNPs can be correlated with the enlargement of the effective surface area caused by the AuNPs and to the existence of mixed valence states on their surface.

In order to further investigate the effect of the secondary electro-catalytic materials deposited on PEDOT:PSS films, capacitance versus voltage (C–V) measurements were performed from -0.5 V to 0.5 V. The capacitance C was calculated by using the relation $C = (-Z''2\pi w)^{-1}$ where Z'' is the imaginary part of the impedance and w is de frequency. As shown in Fig. 2a, at low potentials C slightly decreased and suddenly raised at intermediate potentials. This behavior is similar to that observed in ITO/PEDOT:PSS photoelectrochemical cells and is characteristic for p-doped systems [47].

With the aim of establishing the number of charge carriers, the Mott-Schottky equation (Eq. (1)) was applied [48].
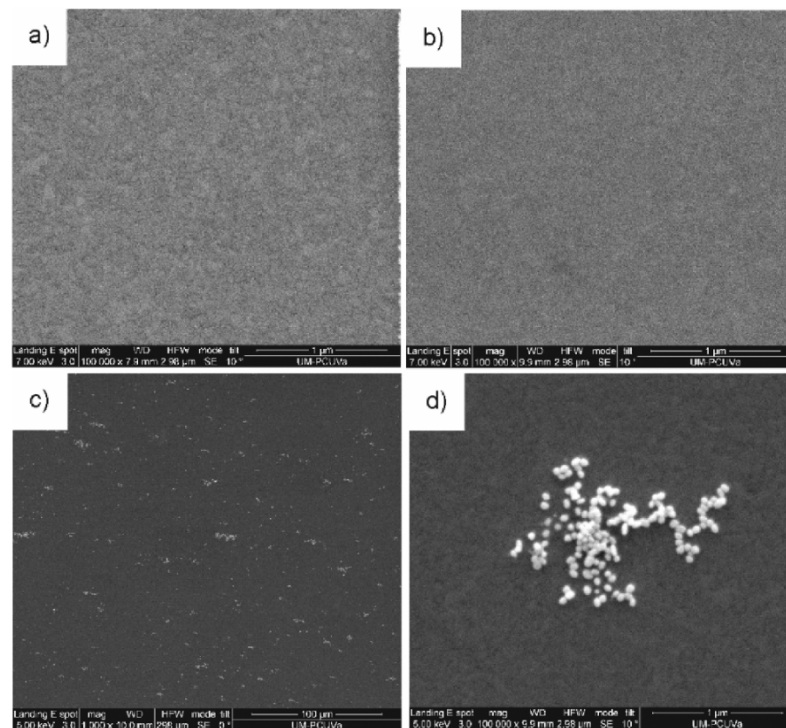

Fig. 1. SEM images of ITO electrodes modified with (a) PEDOT:PSS, (b) PEDOT:PSS/LuPc$_2$, (c) PEDOT:PSS/AuNPs, and (d) PEDOT:PSS/AuNPs at a higher magnification.
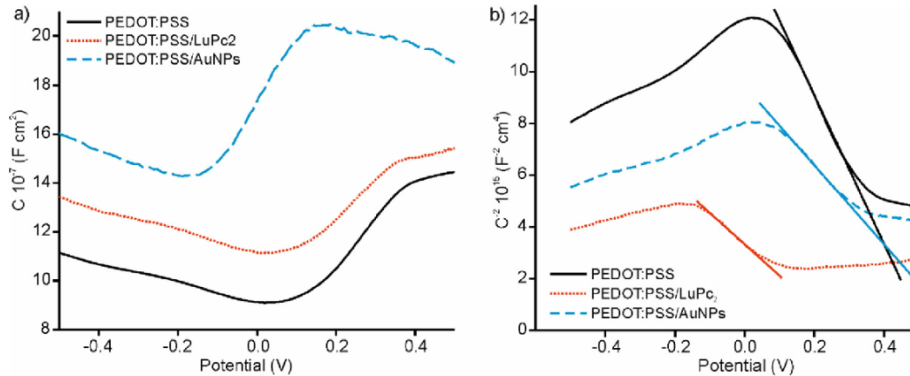
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Fig. 2. (a) Capacitance-voltage response and (b) Mott-Schottky analysis.

$$\frac{1}{C^2} = \frac{2}{\varepsilon\varepsilon_0 e N_D}\left(V - V_{FB} - \frac{KT}{e}\right) \tag{1}$$

Where, C is capacitance, N_D is the donor/acceptor density, ε is the dielectric constant of the nanocomposite, ε_0 is the dielectric constant under vacuum, e is the electron charge, V_{FB} is the flat band potential, T is the absolute temperature and K is the Boltzmann constant.

Mott-Schottky plots were obtained using a voltage sweep from -0.5 to 0.5 V and an input signal amplitude of 50 mV peak to peak at 100 Hz in 0.1 mol.L^{-1} KCl (Fig. 2b). Two important parameters were read directly from $1/C^2$–V plots: V_{FB} which is defined by the intercept of the straight line with the potential axis and N_D that can be determined from the slope of the Mott–Schottky plot. As observed in the Fig. 2b, V_{FB} was lower for PEDOT:PSS/AuNPs nanocomposite confirming the important effect of AuNPs as doping agents. The dopant density N_D was clearly higher in layered nanocomposites (0.98 10^{15} cm^{-3} for PEDOT:PSS, 2.9 10^{15} cm^{-3} for PEDOT:PSS/LuPc$_2$ and 3.1 10^{15} cm^{-3} for PEDOT:PSS/AuNPs) and this suggests an increase in the surface charge density which is in good correlation with the improvement of conductivity observed for PEDOT:PSS/AuNPs and PEDOT:PSS/LuPc$_2$. The higher conductivity and the higher surface charge density could induce an improvement on electrocatalytic properties. This will be analyzed in the next section.

3.3. EIS characterization

The interfacial properties of the layered composites PEDOT:PSS/EM electrodes were investigated by EIS using [Fe (CN)$_6$]$^{4-/3-}$ as a redox probe. The impedance measurements were performed at various potentials (-1 V, -0.5 V, 0 V, 0.5 V and 1 V).

The Nyquist plots (Z$_{REAL}$ vs. Z$_{IMAGINARY}$) registered at -1 V, -0.5 V, 0 V, 0.5 V and 1 V showed a semicircular shape in the high-frequency region. This region corresponds to electron-transfer limited processes and the diameter of the semicircle is equivalent to the electron transfer resistance (R$_{ct}$). In experiments performed at -1 V, -0.5 V, 0 V, 0.5 V and 1 V, it was observed that the diameter changed with the applied potential (Fig. 3.a shows the responses at 0.5 V. Figure S1 illustrates the curves obtained at 0 V). For all three sensors, the smallest R$_{ct}$ values were obtained at 0.5 V, near to the oxidation peak of the [Fe(CN)$_6$]$^{4-/3-}$ couple. Layered composites showed lower R$_{ct}$ values than PEDOT:PSS indicating that charge transfer rates were higher due to the efficient electrocatalytic properties of the nanocomposites. Bode diagrams (Fig. 3b), showed that the impedance module was also considerably reduced and the phase angle reached less negative values for layered composites. This agrees well with the aforementioned conductivity measurements. The smaller R$_{ct}$ values revealed a low
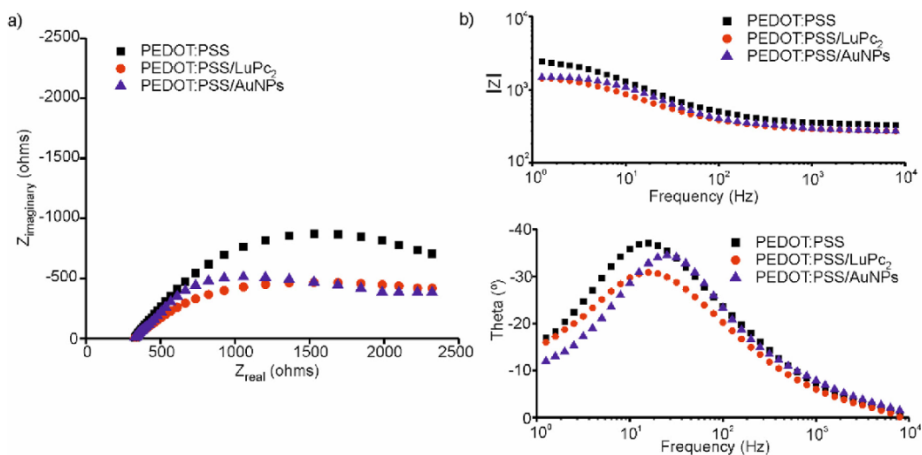

Fig. 3. (a) Nyquist plots and (b) Bode plots recorded in 0.1 mol L^{-1} KCl containing 5 mmol L^{-1} [Fe(CN)$_6$]$^{4-/3-}$ registered at 0.5 V.

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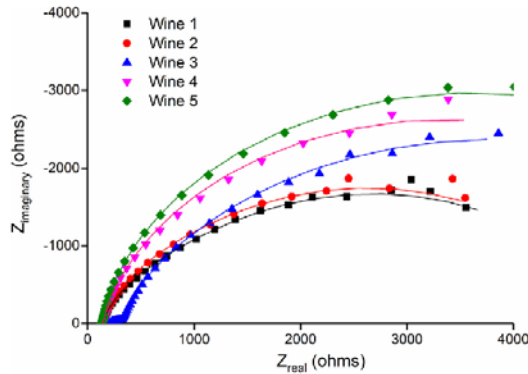
Fig. 4. EIS spectra of PEDOT:PSS/AuNPs electrode obtained for different varieties of wines from 10^4 to 0.1 Hz registered at -0.5 V. Equivalent circuit modeling are also included (solid lines).

electron-transfer resistance which might be caused by the enhanced electrocatalytic effect caused by the interaction between PEDOT:PSS and the AuNPs and LuPc$_2$ deposited films. The improvement of the observed performance can be attributed to the enhanced electron transfer rate provided by the composites due to the interactions between two electrocatalytic components.

3.4. Electronic tongue dedicated to wines

3.4.1. The response of the array of sensors to wines

The sensors responses to red wines are illustrated in Fig. 4. Nyquist plots registered at different potentials (1 V, -0.5 V, 0 V, 0.5 V and 1 V) were characterized by an incomplete semicircle. In the wine matrix, the lowest R_{ct} values were always obtained at -0.5 V (Figure S2 shows the data of the three sensors at 0 V and at -0.5 V for the wine W2). Fig. 4 shows the impedimetric responses (at -0.5 V) of the PEDOT:PSS/AuNPs sensor immersed in red wines. It was observed that the three wines elaborated with Tempranillo grapes (W3, W4 and W5) showed the highest R_{ct} values. Wines W1 and W2 elaborated with different grapes showed lowest R_{ct} values. This trend can be correlated to the higher content of W3, W4 and W5 in redox species (antioxidants measured by Folin, sugars and SO$_2$) and their higher acidity and alcoholic degree.

Therefore a higher R_{ct} is related with a higher content of redox species. To explain this two issues must be considered. First, wine is a complex mixture. Second, there is not an inverse correlation between concentration and R_{ct} as it would be intuitively thought. In some studies, a higher R_{ct} was correlated with a lower content of glucose [49] or cathecol [50], while on others works the opposite tendency was observed, a higher R_{ct} for higher concentration of redox species (chorpyrifos [51], cathecol [52], urea [53], thrombin [54], etc) were observed. Therefore, the correlation is a function of the interaction between the sensor and the analyte.

Another important observation was that the semicircle diameter increased along the sequence PEDOT:PSS/AuNPs < PEDOT:PSS/ LuPc$_2$ < PEDOT:PSS. The small R_{ct} values revealed a low electron-transfer resistance which might be caused by the synergetic enhanced effect between PEDOT:PSS and the secondary electrocatalytic materials. Moreover, this result indicates that sensors show different sensitivity and can be used as the sensing units of an impedimetric electronic tongue.

3.4.2. Impedimetric electronic tongue dedicated to wines. Improvement of the discrimination capability by developing new feature extraction methods

The outputs of the array of sensors were treated by chemometric techniques. In order to improve the quality of the feature extraction, impedance curves were pre-processed following different methods. First, we used the classical method that selects resistance/capacitance data at a fixed frequency [26,35]. Using this feature extraction method, the PCA analysis could not discriminate the similar wine samples (Supplementary Figure S3). Therefore, this method could be applied to interdigitated electrodes by itself or coated with several chemosentitive materials but it is not recommended when these materials are deposited on ITO. The feature extraction method was improved following two other ways: (1) Selection of other parameters of the impedance spectrum and (2) obtain parameters from an equivalent electric circuit.

In the method (1), the impedance parameters selected were: the maximum phase angle (θ_{MAX}) obtained from the Bode diagram and the real (Z_{REAL}) and the imaginary ($Z_{IMAGYNARY}$) impedance data at 1.25 Hz taken from the Nyquist diagram. This frequency was selected because for frequencies lower than 1 Hz, an oscillation of the impedance (real and imaginary) that could be related to the uptake-desorption of chemical species on the electrode was observed, (Supplementary Figure S4). The value of the impedance module was discarded since it was very similar for all wine samples at any frequency value.

In method (2), different strategy to improve the quality of the variable extraction, an equivalent electric circuit was established. After several attempts to simulate the EIS results with different equivalent circuits, the quality of the fittings and the possibility assigning a physical meaning to the circuit components led to simulate the electrochemical behavior of an electrode coated with a semiconductor polymer material [51] (Fig. 5).

In the proposed model, the high frequency region, 10 kHz–100 Hz, is dominated by the resistance of the electrolyte (R_s). In the mid-frequency region, 100 Hz – 0.1 Hz, the process is related to the formation of an electrical double layer at the film/solution interface. The charge transfer resistance (R_{ct}) is related with two effects: the energy potential associated with oxidation/reduction processes and with the energy barrier of the redox species reaching the electrode. The whole interface impedance has been modelled by a constant phase element (CPE) (which represents a non-ideal capacitive response of the interface material-solution), the depressedcircle is an indication of this non-pure capacitive behavior.

The impedance of a CPE element is defined as:

$$Z_{CPE} = \frac{1}{C(j\omega)^n} \tag{1}$$

where C is a parameter numerically equal to the admittance at $\omega = 1$ rad s^{-1}, j is the imaginary unit, ω is the frequency and n is a number that moves between 0 and 1. Finally, at low frequency region, the Warburg impedance (W) represents the delay arising from diffusion of the electroactive species to the electrode.

The Generalized Finite Warburg is defined by:

$$Z_{W_S} = \frac{W_R \, \tanh\left[(j\omega W_C)^{W_n}\right]}{(j\omega W_C)^{W_n}} \tag{2}$$

where W_R, W_N and W_C are the Warburg parameters. As an example, Table 2 shows the average values obtained with this fitting for the three sensors in wine W2.

The results of the PCA analysis performed with the parameters obtained from the impedance curves (Z_{REAL} and $Z_{IMAGINARY}$ at 1.25 Hz and θ_{MAX}) are shown in Fig. 6. PCA scores plots showed well-defined and separated clusters for each variety of wine. The captured information was 97% (PC1 = 63%; PC2 = 25%; PC3 = 9%). Clusters were
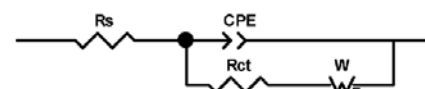

Fig. 5. Equivalent electric circuit to interpret EIS measurements.

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Table 2
EIS equivalent circuit parameters measured in wine W2 at −0.5 V.

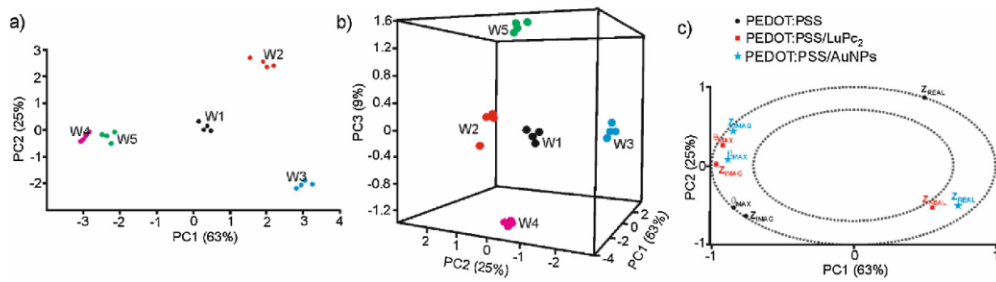| Sensors	R_S (Ω cm^2)	CPE_C (s^n Ω^{-1} cm^{-2})	CPE_n	R_{ct} (Ω cm^2)	W_R (Ω cm^2)	W_n	W_C (s^n Ω^{-1} cm^{-2})	χ^2 (10^{-5})
PEDOT-PSS	159.3	2.74 10^{-5}	0.89	4951	4646	0.55	0.55	6.4
PEDOT-PSS/LuPc$_2$	159.7	2.73 10^{-5}	0.88	6301	570.5	0.73	0.53	9.9
PEDOT-PSS/AuNPs	150.5	2.91 10^{-5}	0.84	2151	2691.6	0.46	0.56	5.9

Fig. 6. PCA corresponding to the classification of the wines (a) 2D scores plots, (b) 3D scores plots, and (c) correlation loadings plot using Z_{REAL} and $Z_{IMAGINARY}$ and θ_{MAX} obtained for PEDOT:PSS, PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNP.

distributed according to the variety of grape and the chemical composition. The loadings provided by each sensor, Fig. 6c, appeared in different regions of the diagram confirming that the secondary electrocatalytic materials played an important role in the selectivity of the sensors.

A different PCA analysis was carried out based on the parameters obtained from the fitting to an equivalent electric circuit showed in Fig. 5. Fig. 7 shows the final PCA analysis and the correlation loadings plots.

In the case of PCA using equivalent circuit parameters, a good separation of the clusters was also obtained. Due to the increase in the numbers of variables the three principal components bring an important amount of information. The PC1, PC2 and PC3 explain the 60%, 25% and 6% of the information, respectively.

Multivariate inspection of impedimetric data using both methods revealed the high capability of discrimination of this impedimetric e-tongue. It could be applied to the recognition of wines showing excellent sensitivity similar to the obtained with our voltammetric e-tongues [29,32] comprised of voltammetric electrodes chemically modified with different sensitive materials. Moreover, there is no requirement of active species in the measuring system and no need of a standard reference electrode. Although both feature extraction systems provide noticeable information about the samples, a better correlation with the composition was obtained using the equivalent circuit

parameters. In the 3D PCA it could be observed that samples were located according to their variety and their composition. Wines W3, W4 and W5 elaborated with the variety Tempranillo and with similar Folin, sugar content and acidity appear on the left side of the diagram, whereas wines elaborated with other varieties, W1 and W2 with lower Folin, sugar content, sugar and acidity appeared on the right side.

3.4.3. Correlations with chemical parameters

By means of regression techniques, the results obtained using the impedimetric e-tongue can be correlated with the chemical composition of the samples. The statistical parameters obtained from the Partial Least Squares (PLS) regression models, correlating the impedance spectrum parameters (θ_{MAX}, Z_{REAL}, $Z_{IMAGYNARY}$) or the equivalent circuit parameters (CPE_C, CPE_n, R_{ct}, W_R, W_C, W_n) with chemical parameters are shown in Tables 3 and 4 respectively. Using both methods, the squared correlation coefficients (R^2) obtained for calibration and prediction were high and the number of latent variables (LV) was low. The values of the root mean square error (RMSE) of calibration/prediction which are an estimate of the absolute error were also low.

The regression using the equivalent circuit parameters provided the best correlations and lower residual errors with a lower number of latent variables, confirming that this method improves the quality of the features extraction.

The prediction ability of the polyphenol index, alcoholic degree and
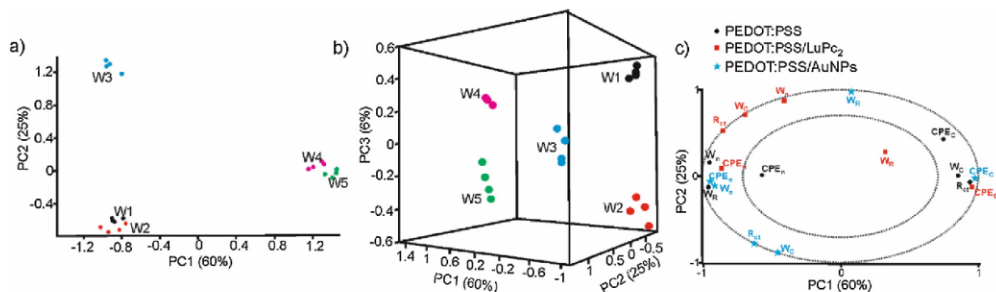

Fig. 7. PCA corresponding to the classification of the wines using equivalent circuit parameters. (a) 2D scores plot, (b) 3D scores plot, and (c) correlation loadings plot obtained for PEDOT:PSS, PEDOT:PSS/LuPc$_2$ and PEDOT:PSS/AuNP.

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Table 3
Results of the PLS-1 analysis using impedance spectrum parameters.

Parameters	$R_C^{2 1}$	$RMSE_C^{2}$	$R_P^{2 3}$	$RMSE_P^{4}$	LV
FOLIN	0.8288	0.0336	0.8219	0.0361	3
TPI	0.9554	0.0139	0.9381	0.0173	3
Acidity	0.9815	0.0231	0.9815	0.0263	3
SO_2	0.9919	1.5478	0.9876	2.0110	2
Reducig Sugars	0.9420	0.0430	0.9666	0.0575	3
Glucose + Fructose	0.8902	0.0430	0.8308	0.0681	3
°Alcoholic	0.9815	0.0355	0.9763	0.0422	2
pH	0.8288	0.0336	0.8219	0.0361	3

LV latent variables.
[1] Squared correlation coefficient in calibration.
[2] Root mean square error of calibration.
[3] Squared correlation coefficient in prediction.
[4] Root mean square error of prediction.

Table 4
Results of the PLS-1 analysis using equivalent circuit parameters.

Parameters	$R_C^{2 1}$	$RMSE_C^{2}$	$R_P^{2 3}$	$RMSE_P^{4}$	LV
FOLIN	0.9913	0.0593	0.9886	0.0674	2
TPI	0.9974	0.0363	0.9965	0.0423	1
Acidity	0.9977	0.2247	0.9963	0.2857	2
SO_2	0.9894	8.4480	0.9859	9.7800	2
Reducig Sugar	0.9894	0.1549	0.9858	0.1794	2
Glucose + Fructose	0.9837	0.0415	0.9706	0.0559	3
°Alcoholic	0.9982	0.6110	0.9970	0.7800	2
pH	0.9980	0.1653	0.9980	0.2129	2

LV latent variables.
[1] Squared correlation coefficient in calibration.
[2] Root mean square error of calibration.
[3] Squared correlation coefficient in prediction.
[4] Root mean square error of prediction.

acidity using circuit equivalent parameters were particularly remarkable. This array provides a correlating and predicting errors similar to the obtained with voltammetric e-tongue formed by modified carbon paste electrode [55] or by different families of sensitive materials [29] with which the authors have also worked.

4. Conclusions

The performance of an impedimetric e-tongue dedicated to the analysis of red wines has been improved using two different strategies. On one hand, sensors have been constructed using PEDOT-PSS layered nanocomposites with improved electrocatalytic properties thanks to the application of nanolayers of lutetium bisphthalocyanine (LuPc$_2$) and gold nanoparticles (AuNPs). On the other hand, the information extracted from the impedimetric curves has been upgraded by extracting EIS parameters from an equivalent circuit model. This improved e-tongue is able to discriminate five red wines with very similar characteristics. In addition, using PLS-1, excellent correlations with chemical data such as Total Polyphenol Index (TPI), Folin Index, Total acidity, SO_2 content, sugars, alcoholic degree and pH are obtained.

Author contributions

M.L. Rodriguez-Mendez and C. García Cabezón, conceived the experiments, search for funds and wrote the paper. F. Martin-Pedrosa, performed chemometrics. C. García-Hernandez and C. Salvo performed the experiments, analyzed the data with chemometrics and contributed to the manuscript writing.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2018.09.023.

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3.9. Paper VIII

An electrochemical quartz crystal microbalance multisensor system based on phthalocyanine nanostructured films: Discrimination of musts.

By Celia García-Hernández, Cristina Medina-Plaza, Cristina García-Cabezón, Fernando Martín-Pedrosa, Isabel del Valle, José Antonio de Saja and María Luz Rodríguez-Méndez.

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3.9.1. Motivation and objective

Quartz crystal microbalance (QCM) is a simple, high-resolution gravimetric technique with no specific selectivity. Combining electrochemistry and QCM is denoted as electrochemical quartz crystal microbalance (EQCM). In EQCM assays one can simultaneously register the electrochemical response and mass changes that accompany the oxidation/reduction of electroactive species. Therefore, electrochemical quartz crystal microbalance (EQCM) sensors can be an alternative or complement to the traditional transducers implemented in electronic tongues (mainly potentiometric, voltammetric and amprerometric).

Most applications for QCM sensors are in electronic noses for the analysis of volatile organic compounds (VOCs). The sensor group of Prof. Di Natale and Prof. Paolesse is one of the most active in the field of QCM and they have designed a commercial electronic nose based on QCM sensors coated with porphyrins for the analysis of organic gases and vapors. QCM sensors have also been applied in several applications, such as piezoelectric immunosensors, for the recognition of biomolecular interactions. In addition, EQCM has been used as a gravimetric sensor in many types of electrochemical studies, including: metal deposition; corrosion studies; oxide formation; dissolution studies (e.g., phenol oxidation); surfactant adsorption/desorption; enzyme adsorption/desorption; changes in the conductivity of polymer films during redox processes; electropolymerization studies; ion insertion studies; or load transfer studies during redox processes (e.g., phthalocyanines). However, there are no works reporting the use of an EQCM multisensor system implemented as an electronic tongue in the analysis of food.

When no sensing element is added, QCM works just like a transducer. Therefore, in several works, QCM electrode surfaces have been modified with various chemicals and biomaterials to obtain selectivity and specificity. A careful choice of the sensing material is essential for achieving high performance sensor arrays, such as those used in nanostructured electronic tongues. As already mentioned, phtaholcyanines are good candidates due to their excellent electrocatalytic properties. Moreover, the use of nanostructured films has played an important role in increasing sensitivity as of the number of active sites and the intimate contact with the analyte are greatly improved by the use of thin films.

Herein, we have developed an electronic tongue based on EQCM sensors, modified with metallophthalocyanines, using the LbL technique. The multisensor system as-prepared was able

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to discriminate musts with different phenolic and sugar contents. The response of the array of sensors for must samples was studied by simultaneously recording cyclic voltammetry and mass changes. The capability of the system to discriminate between grapes of different varieties was evaluated by means of parallel factor analysis (PARAFAC). The relationship between the voltammetric or mass signals with the results obtained by chemical analysis was studied by means of multi-way partial least squares (N-PLS).

3.9.2. Summary of the research work

Sensors were prepared by depositing 20 thin film bilayers onto a quartz crystal coated with platinum by means of the LbL technique. For this purpose, we used metalphthalocyanines as the anionic layers and polyallylamine hydrochloride (PAH) as the polycation. Iron(III) phthalocyanine tetrasulfonic acid monosodium salt (FePc^{SO3-}), copper(II) phthalocyanine tetrasulfonic acid tetrasodium salt (CuPc^{SO3-}) and nickel(II) phthalocyanine tetrasulfonic acid tetrasodium salt (NiPc^{SO3-}) were used to achieve an array of partially selective sensors with overlapping specificities. The quality of the LbL films was monitored by UV-Vis following the characteristic Q band of the metallophthalocyanie at 620-640 nm, which confirmed a linear relationship between the number of layers and the absorbance of the Q band and, therefore, a uniform growth of the LbL films and good quality in the deposition.

The red grape samples analyzed, harvested in September 2013, were provided by the the vineyards Bodega Cooperativa de Cigales and the Instituto Tecnológico Agrario de Castilla y León (ITACYL). The chemical parameters of the corresponding red grapes, analyzed according to international regulations, including sugar content, ºBrix, total polyphenolic content (TPI) and degree 16.8, were provided by the Oenological Centre of Castilla y León. In addition, the Folin-Ciocalteu method was carried out to evaluate the phenolic content by means of another methodology different from TPI.

The electrochemical experiments were carried out with a quartz crystal microbalance and working electrodes were quartz crystals coated with platinum (5 MHz, AT-cut planoconvex) with the LbL films onto the surface as sensitive and electrocatalytic material. Before performing the experiments, the quartz crystal microbalance was calibrated according to the corresponding procedure. For this purpose, a galvanostatic deposition of copper onto the surface of the crystals

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was applied in order to determine the Sauerbrey constant of the quartz crystals coated with platinum. Then, cyclic voltammetry experiments were carried out and mass variations were simultaneously registered. In order to know the electrochemical responses and register mass variations for phenols and sugars, the EQCM sensors were tested in catechol, which is an antioxidant usually found in grapes and wines, and glucose, one of the major sugars.

Voltammograms registered in catechol showed two redox processes corresponding to catechol and the decomposition of water. Depending on the metallophthalocyanine used, the peak position and intensities were different for each one, although the shapes of the voltammograms were similar. The intensities of the as-prepared EQCM sensors were higher than those observed in the bare platinum quartz crystal, confirming the electrocatalytic effect of the phthalocyanines. It was observed that the intensities of the oxidation peaks were higher when the concentration of catechol increased; whereas the intensities in the reduction peaks decreased. The position of the peaks demonstrated that the electrocatalytic effect of the sensors decreased when advancing in the transition metal series ($FePc^{SO3}$ < $NiPc^{SO3}$ < $CuPc^{SO3}$). Mass variation signals showed that mass increased during the oxidation of catechol and decreased during the reduction. Voltammograms registered in glucose were poor and did not show any redox peak, except for the peaks associated to protons at negative potentials, whereas the massograms evidenced a mass increase that was attributed to the adhesion of the sugar onto the surface. The mass deposited onto the EQCM sensors increased when the concentration of glucose increased.

Due to the grapes having different phenolic and sugar compositions, depending on the variety of grape, the as-prepared EQCM sensors were good candidates to analyze different grape musts, so they were immersed in six different Spanish grape musts (Juan García, Prieto Picudo, Mencía Regadío, Cabernet Sauvignon, Garnacha and Tempranillo). The voltammograms and massograms registered in must samples were different from one grape to another, but in general, the voltammograms showed a broad anodic peak that could be associated to the polyphenolic content in grapes and wines and the massograms showed a progressive increase in mass which was associated with the adhesion of sugars onto the crystal surface. The richness and cross-selectivity of the responses were successfully used to discriminate between the grape samples by means of PARAFAC (parallel factor analysis), a non-supervised multivariate technique. The PARAFAC score plot obtained from the voltammogram analysis showed that samples can be discriminated according to the Total Polyphenolic Index (TPI). When analyzing the mass outputs

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for grape musts, the PARAFAC score plot showed that samples were located according to the Brix degree (a sugar content parameter).

With the purpose of establishing correlations between the chemical parameters provided by the Oenological Centre of Castilla y León and the data obtained with the EQCM array in must samples, N-PLS (multi-way partial least squares) were carried out. The prediction models obtained confirmed that the voltammetric data presented a better correlation with the parameters related to the phenolic content (TPI and Folin-Ciocalteau index), whereas the mass data had good correlations with the sugar parameters (sugar content and ºBrix), so the results obtained with the PARAFAC analysis were confirmed.

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Article

An Electrochemical Quartz Crystal Microbalance Multisensor System Based on Phthalocyanine Nanostructured Films: Discrimination of Musts

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Abstract: An array of electrochemical quartz crystal electrodes (EQCM) modified with nanostructured films based on phthalocyanines was developed and used to discriminate musts prepared from different varieties of grapes. Nanostructured films of iron, nickel and copper phthalocyanines were deposited on Pt/quartz crystals through the Layer by Layer technique by alternating layers of the corresponding phthalocyanine and poly-allylamine hydrochloride. Simultaneous electrochemical and mass measurements were used to study the mass changes accompanying the oxidation of electroactive species present in must samples obtained from six Spanish varieties of grapes (Juan García, Prieto Picudo, Mencía Regadío, Cabernet Sauvignon, Garnacha and Tempranillo). The mass and voltammetric outputs were processed using three-way models. Parallel Factor Analysis (PARAFAC) was successfully used to discriminate the must samples according to their

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variety. Multi-way partial least squares (N-PLS) evidenced the correlations existing between the voltammetric data and the polyphenolic content measured by chemical methods. Similarly, N-PLS showed a correlation between mass outputs and parameters related to the sugar content. These results demonstrated that electronic tongues based on arrays of EQCM sensors can offer advantages over arrays of mass or voltammetric sensors used separately.

Keywords: LbL; EQCM; phthalocyanine; must; grapes; electronic tongue

1. Introduction

The combination of an array of partially selective sensors with overlapping specificities with advanced mathematical signal-processing methods has yielded a new sensing technology for chemical analysis in liquid media, the so called electronic tongue (ET) [1,2]. ETs are holistic systems that provide global and qualitative information about samples instead of quantitative data about specific compounds. However, if the data matrix obtained by such multisensor systems is analyzed with adequate chemometric processing tools, descriptive or predictive information of particular parameters can be extracted [3,4].

Arrays of sensors dedicated to the analysis of liquids are usually based on electrochemical sensors including potentiometric [5], amperometric [6], voltammetric [7–9] or impedimetric ones [10]. Many efforts have been dedicated to the discrimination of wines and musts using electronic tongues [2,3,8,9,11–14]. Voltammetric electrodes chemically modified with electrocatalytic materials are particularly suitable for the analysis of wines because voltammograms contain information related to the redox and ionic species present in wines [15]. For this reason, e-tongues based on voltammetry have been able to discriminate red wines with different antioxidant capability [16], wines elaborated with different varieties of grapes [17], to detect adulterations [8] or to follow the ageing of red wines using different methods [18].

The intrinsic complexity, richness and cross-selectivity of the signals generated by voltammetric sensor arrays are an advantage because the datasets contain meaningful information about the samples. Using partial least squares regression analysis (PLS), correlations could be established between the voltammetric curves (not only specific peaks) and the polyphenol content, or the acidity of red wines [12,16,19]. Finally, multi-transduction systems are more and more popular, since they combine different classes of sensors that provide complementary information [13,20–24].

Quartz Crystal Microbalances (QCM) are a different approach where the sensing element is a coated resonator. The interaction with the sample results in a change in the mass of the crystal which affects the frequency at which the crystal oscillates. QCM sensors modified with a variety of materials (porphyrins, CNTs, calixarenes and biomolecules, among others) have been widely used in electronic noses to analyze vapors with sub-nanogram sensitivity [25–28]. QCMs can also work in an aqueous environment and they have been able to detect a variety of substances in solution [29,30]. However, only few examples of electronic tongues based on QCM sensors for the analysis of liquids have been reported [31–33].

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There are numerous methods to functionalize QCM surfaces. They include hydrophobic bonds, ionic bonds, hydrogen bonds or electrostatic interactions, among others [34]. Nanostructured films show enhanced surface to volume ratios that can increase the sensitivity of the sensors [35]. In fact, QCM sensors have been prepared using Langmuir-Blodgett (LB) [36], Layer by Layer (LbL) [37], Self-Assembling Monolayer (SAM) [38], Electrodeposition (EDP) [39,40], Plasma-Polymerized Films (PPF) [41] or Molecular Imprint (MIP) [42] techniques.

Apart from their sub-nanogram sensitivity, another possible advantage of QCM sensors for the analysis of liquids, is that the same device can be used to obtain simultaneously mass changes and electrochemical signals. For this reason, the Electrochemical Quartz Crystal Microbalance (EQCM) has become a well-established technique for the investigation of mass changes associated with electrochemical surface processes such as adsorption of enzymes [43], electrodeposition [44], electropolymerization [45], ion insertion [46] or redox processes [33]. In this way, EQCM can provide higher amounts of information about a sample than electrochemistry or mass measurements performed separately [33,47,48].

EQCM sensors covered with phthalocyanines have been successfully used to analyze phenols present in wines [33]. The reason for their success is that phthalocyanines show electrocatalytic properties towards a variety of analytes including phenols [33,49,50]. In addition, nanostructured electrodes based on phthalocyanines can be prepared by electrodeposition [40], the Langmuir-Blodgett (LB) [35,51] or the Layer by Layer (LbL) technique [52–54] on different substrates such as indium tin oxide (ITO) or platinum.

The objective of this work was to combine EQCM sensors to form an array able to discriminate musts with different phenolic content, obtained from different varieties of Spanish grapes. For this purpose, QCM substrates were modified with three different metallophthalocyanines using the LbL technique. The response of the array of sensors towards the must samples was studied by recording cyclic voltammetry and mass changes synchronously. The capability of the system to discriminate grapes of different varieties was evaluated by means of parallel factor analysis (PARAFAC). The relationship between the voltammetric or the mass signals with the results obtained by chemical analysis was studied by means of multi-way partial least squares (N-PLS). The complementarity of the information provided by the electrochemical and mass outputs was discussed.

2. Experimental Section

2.1. Chemicals and Grape Samples

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. The solutions were obtained by dissolving substances in deionized water (resistivity of 18.2 $M\Omega \cdot cm^{-1}$) acquired from a Milli-Q system (Millipore, Billerica, MA, USA).

Three water soluble tetrasulfonate acid metallophthalocyanines (MPc^{SO3}) were used as the anions in the LbL films. They included iron(III) phthalocyanine tetrasulfonic acid monosodium salt, copper(II) phthalocyanine tetrasulfonic acid tetrasodium salt, and nickel(II) phthalocyanine tetrasulfonic acid tetrasodium salt; (denoted as $FePc^{SO3}$, $NiPc^{SO3}$ and $CuPc^{SO3}$ respectively). Polyallylamine hydrochloride (PAH), was used as the polycation in LbL films.

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Six varieties of red grapes were included in the study (Juan García, Tempranillo, Prieto Picudo, Mencía Regadío, Cabernet, Garnacha). They were harvested in September 2013 from the vineyards "Bodega Cooperativa de Cigales" and "Instituto Tecnológico Agrario de Castilla y León (ITACYL)", both located in the Valladolid area of Castilla y León in Spain. To obtain the musts, 200 berries were introduced in a plastic bag and crushed for one minute. The Oenological Centre of Castilla y León carried out the chemical analysis following international regulations [55]. Parameters analyzed included the typical indicators of the glucose content: sugar content (g/L), degree 16.8 and Brix degree. The classical indicator of the polyphenolic content, the Total Polyphenol Index (TPI) was also analyzed. The polyphenolic content was also evaluated using the Folin-Ciocalteau method. The results are collected in Table 1.

Table 1. Results of the chemical analysis carried out by traditional chemical methods.

Grape Variety	Sugar Content (g/L)	Brix Degree	Total Polyphenol Index. TPI	Degree 16.8	Polyphenol Content. Folin-Ciocalteau Method (g/L)
Prieto Picudo	224.1	22.89	19	13.31	0.46
Garnacha	187.4	19.68	15	11.13	0.38
Cabernet-Sauvignon	246.4	24.75	28	14.64	0.62
Tempranillo	209.1	21.53	28	12.42	0.52
Juan García	216.0	22.18	29	12.83	0.69
Mencía Regadio	203.3	21.05	23	12.08	0.54

2.2. Electrochemical Measurements

Electrochemical quartz crystal microbalance experiments were carried out with a quartz crystal microbalance (QCM200 + QCM25 Crystal Oscillator, purchased from Standford Research Systems, Sunnyvale, CA, USA) connected to a Parstat 2273 potentiostat/galvanostat (EG&G, Oak Ridge, TN, USA). The mass-sensitive oscillators were 2.54 cm diameter, 5 MHz AT-cut planoconvex quartz crystals coated with platinum.

The oscillators were used simultaneously to register mass changes and as the working electrode of the electrochemical measurements. PC software displays the relative frequency changes in synchronicity with the electrochemical data.

Cyclic voltammetry was carried out using a conventional three-electrode cell. The reference electrode was Ag|AgCl/KCl 3 mol·L^{-1} and the counter electrode was a platinum sheet with a surface of 1 cm^2. Cyclic voltammograms were registered at a sweep rate of 0.1 V·s^{-1}. The variations of mass taking place simultaneously to the cyclic voltammetry experiment were registered. All the samples were measured seven times with each sensor to check the robustness of the experiments.

2.3. Calibration of the Quartz Crystal

A calibration of the quartz crystal was carried out to determine the sensitivity factor (also called Sauerbrey constant) by galvanostatic deposition of copper on the platinum surface. Sauerbrey constants have been used to calculate mass shifts during the experiment (Equation (1)):

$$\Delta f = -C_f \cdot \Delta m$$

(1)

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where Δf is the observed frequency change (Hz), C_f is the Sauerbrey constant (56.6 Hz·cm^2·µg^{-1} for a 5 MHz At-cut quartz crystal at room temperature) and Δm is the change in mass per unit area (µg·cm^{-2}).

A 0.5 mol·L^{-1} solution of CuSO$_4$ in 0.1 mol·L^{-1} H$_2$SO$_4$ (pH = 1.37) was used to deposit copper on the platinum electrode using chronopotentiometry (-2 mA, 70 s). The plot of the frequency shift *vs.* charge showed excellent linearity ($y = 7 \times 10^{-5}x - 0.0632$; $R^2 = 0.9996$). According to the linear plot obtained in the calibration process, the Sauerbrey constant of the quartz crystal obtained was 59.4 Hz·cm^2·µg^{-1}, which is consistent with the theoretical value of 56.6 Hz·cm^2·µg^{-1}.

2.4. Sensor Preparation

The platinum-coated quartz crystal substrates were cleaned using a mixture of H$_2$SO$_4$ and H$_2$O$_2$ (piranha solution; 3:1 mixture of sulfuric acid and 30% hydrogen peroxide) and thoroughly rinsed in deionized water (resistivity of 18.2 MΩ·cm^{-1}) before use.

The LbL films were grown using PAH as the positive layer and iron, nickel or copper tetrasulfonate phthalocyanines (FePc^{SO3}, NiPc^{SO3} or CuPc^{SO3}) as the negative layer using a previously published procedure [56]. Water solutions of PAH (0.5 g·L^{-1}) and of the corresponding phthalocyanine (0.05 g·L^{-1}) were used to build the films. LbL films were fabricated by successive immersions of the QCM substrate in the PAH and phthalocyanine solutions. The following sequence of immersions was followed: (1) PAH solution (5 min); (2) deionized water gently stirred to remove excess of non-adsorbed PAH (1 min); (3) MPc^{SO3} solution (5 min); (4) deionized water gently stirred to remove excess and non-adsorbed MPc^{SO3} (1 min). After these four steps a bilayer was formed and more layers are grown by repeating the sequence. After each electrochemical measurement, QCM substrates were cleaned by immersing the Pt/quartz device in HNO$_3$ 60% (sonication during 1 min) to remove LbL films. QCM devices were then rinsed in deionized water and dried with nitrogen gas.

2.5. UV-Visible Characterization

The growth of LbL films was monitored with UV-Vis absorption spectroscopy (UV-2600 model, purchased from Shimadzu, Kyoto, Japan) by testing the linearity between the number of layers deposited and the absorbance.

2.6. Multisensor System Statistical Analysis

A multisensor system was constructed using the signals obtained from four sensors: the already described FePc^{SO3}/PAH, NiPc^{SO3}/PAH and CuPc^{SO3}/PAH LbL films and a bare Pt quartz crystal microbalance. The multivariate data analysis was performed by using Matlab v2014b (The Mathworks Inc., Natick, MA, USA) and The Unscrambler (CAMO Software AS, Oslo, Norway). Voltammograms and massograms were pre-processed by using an adaptation of a data reduction technique based on predefined response "bell-shaped-windowing" curves called "kernels" [15,57]. Using this method, 10 variables were obtained from each voltammogram and from each massogram. These data were used as the input variable in statistical analysis. A non-supervised multivariate method, the Parallel Factor Analysis (PARAFAC) was used to analyze the curves and to evaluate the capability of discrimination

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of the array of sensors. Multi-way partial least squares (N-PLS) was used to evidence the correlations between the voltammetric and mass outputs and the chemical indicators.

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3.1. UV-Visible characterization

LbL films were prepared using the four-step sequence described above. The procedure was repeated 20 times to obtain 20 bilayer-thick films. The growth of bilayers was monitored every four bilayers by UV-vis absorption spectroscopy. The characteristic B band at shorter wavelengths and Q band at longer wavelengths, which are attributed to HOMO→LUMO electronic transitions of the π electrons of the Pc ring, could be clearly seen. The features observed in the UV-Vis absorption spectra are assigned to the MPc^{SO3} since PAH does not absorb within this wavelength range.

The absorbance of the Q band increased linearly with the number of bilayers. This is illustrated in Figure 1 for the $CuPc^{SO3}$/PAH film where the absorbance at 620 nm *vs.* the number of deposited bilayers is represented, confirming the good quality of the deposition. This shows that a similar amount of material is transferred onto the substrate per deposited layer confirming a uniform growth of the LbL films. The quality of the layers (and hence the linearity regression coefficient) decreased when more than 20 bilayers were deposited. For this reason, further studies were carried out with 20 bilayers.

Similar results were obtained with the three phthalocyanines tested and the only difference was the value of the Q band position (640 nm for $FePc^{SO3}$/PAH, 620 nm for $NiPc^{SO3}$/PAH and 620 for $CuPc^{SO3}$/PAH), which are in good agreement with previous results [53,54].

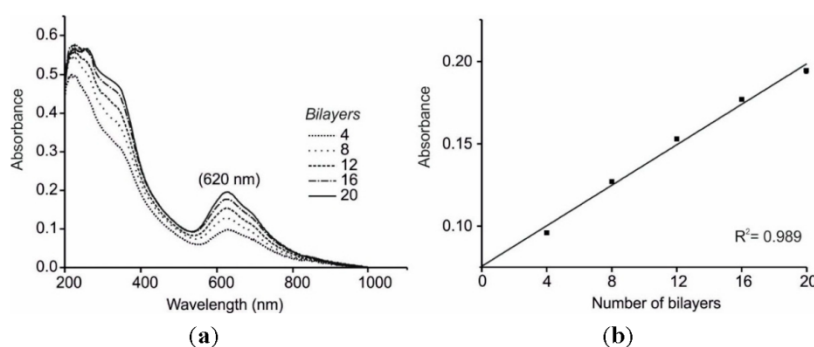**Figure 1.** UV-Vis characterization of 4–20 $CuPc^{SO3}$/PAH LbL bilayers. (**a**) UV-Vis absorption spectra; (**b**) Linear correlation between absorbance *vs.* number of bilayers.

In addition, the absorbance values registered increased when advancing in the transition metal series ($FePc^{SO3}$/PAH < $NiPc^{SO3}$/PAH < $CuPc^{SO3}$/PAH films) (for instance, the values of Q band absorbance registered for 20 bilayers were $FePc^{SO3}$/PAH: 0.031, $NiPc^{SO3}$/PAH: 0.158 and $CuPc^{SO3}$/PAH: 0.189). Taking into account that the molar extinction coefficients of the three phthalocyanines are of the same order of magnitude, it could be concluded that the $CuPc^{SO3}$/PAH films were more closely packed than $NiPc^{SO3}$/PAH films or $FePc^{SO3}$/PAH films. The preparation method was highly reproducible and coefficients of variation calculated from the maximum absorbance of 20 bilayer films, were lower than 2%.

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3.2. EQCM Measurements in Glucose and Catechol

In a first approach and in order to test the sensing performance of the EQCM LbL films, they were immersed in catechol (an antioxidant usually found in grape juices) and glucose, one of the major sugars. Cyclic voltammograms (potential range from −1.0 to +1.0 V *vs.* Ag|AgCl) and massograms were recorded simultaneously.

The responses towards catechol are illustrated in Figure 2 for NiPc[SO3]/PAH films. It is important to remark that in all cases, the first scan was always different from the subsequent cycles. After the second cycle, scans were highly reproducible with a coefficient of variation (%CV) of the highest peak were lower than 2%.
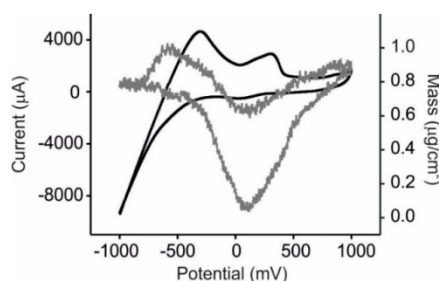

Figure 2. Response of the array of sensors towards catechol 10^{-3} mol·L^{-1} in KCl 0.1 mol·L^{-1}. Voltammetric output (black line) and mass output (grey line) for the NiPc[SO3]/PAH sensor.

The voltammetric responses were characterized by two redox process, one corresponding to the oxidation/reduction of catechol (at +0.30 V and +0.05 V for the anodic and cathodic waves respectively). The decomposition of water occurring at negative potentials was accompanied by the oxidation of hydrogen that was observed as an anodic wave at −0.45 V. The four electrodes forming the array showed similar trends but the peak positions and their intensities differ from one electrode to another. For instance, the oxidation of catechol occurs at +0.25 V for FePc[SO3]/PAH, +0.30 V for NiPc[SO3]/PAH and +0.42 V for CuPc[SO3]/PAH sensor, indicating that the electrocatalytic effect of the phthalocyanine decreased when advancing in the transition metal series. The intensity of the peaks increased with respect to the values observed in the bare Pt electrode (2780 µA), FePc[SO3]/PAH (2800 µA), NiPc[SO3]/PAH (2930 µA) and CuPc[SO3]/PAH (3260 µA).

Hydrogen was formed during the water decomposition that occurred at ca. −0.45 V according to the next reaction:

$$2H_2O + 2e^- \longrightarrow H_2 + 2OH^- \tag{2}$$

Also in this case, the peak position shifted to more negative values when advancing in the transition metal series (−0.25 V for FePc[SO3]/PAH, −0.35 V for NiPc[SO3]/PAH and −0.42 for CuPc[SO3]/PAH sensor and −0.45 for the Pt bare electrode), confirming the electrocatalytic effect of the nanostructured films.

Massograms showed a mass increase during the oxidation of catechol and a large decrease in mass during the reduction. Massograms were highly stable, however, a net increase in the mass at the end of

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the experiment was observed, indicating that catechol was adhering to the surface of the electrode. This result is in good accordance with previously published results that demonstrated the passivation of the electrodes during cycling due to polymerization of phenols [58]. In fact, when sensors used to analyze catechol were immersed in a KCl 0.1 mol·L^{-1} solution, the presence of catechol was still observed. According to this, sensors were considered as single use devices and a brand new sensor must be prepared to measure each sample.

As expected, the glucose solution did not produce redox peaks in the studied range (except peaks associated to protons at negative potentials). A progressive increase in mass was observed under consecutive cycling that could be attributed to the adhesion of sugars to the sensing surface. The effect of the concentration in the responses of the sensors was analyzed by exposing the EQCM devices to different catechol and glucose concentrations. The results are illustrated in Figure 3 for CuPc^{SO3}/PAH. When increasing the concentration of catechol, the voltammetric peak at −0.4 V decreased in intensity, while peak at ca. +0.4 increased in intensity and simultaneously shifts to lower potentials. Similarly, the shape of massograms was different depending on the concentration. This behavior impedes the construction of calibration curves and justifies the need of an array of electrodes, where the whole curve provides information about the sample.
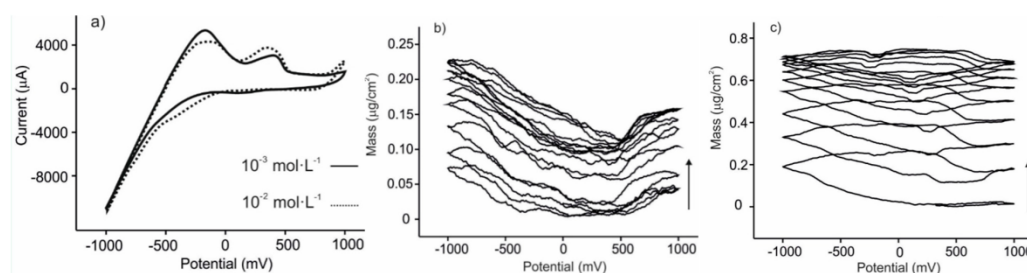

Figure 3. (**a**) Voltammetric response towards catechol in KCl 0.1 mol·L^{-1} for the CuPc^{SO3}/PAH sensor; (**b**) Mass response of the CuPc^{SO3}/PAH sensor towards glucose 10^{-3} mol·L^{-1} in 0.01 mol·L^{-1} phosphate buffer (pH 7.0); (**c**) Mass response of the CuPc^{SO3}/PAH sensor towards glucose 10^{-2} mol·L^{-1} in 0.01 mol·L^{-1} phosphate buffer (pH 7.0).

3.3. Analysis of Grape Juices

The array of sensors was immersed in must samples prepared from different varieties of grape and EQCM analysis was performed. It has to be pointed out that preliminary experiments were carried out in undiluted must and the signals demonstrated a poor reproducibility. This can be attributed to the large amount of suspended materials (*i.e.* proteins) present in must samples that can adhere to the sensor surface. The reproducibility was clearly improved when musts were diluted 1:2 in KCl 0.05 mol·L^{-1}. For this reason, further experiments were carried out in diluted musts. Cyclic voltammetry was carried out at a potential range from −0.6 to +1.0 V *vs.* Ag|AgCl and voltammograms/massograms were simultaneously registered. As an example of the responses obtained Figure 4 shows the voltammetric and mass outputs of NiPc^{SO3}/PAH sensor towards a must obtained from the Juan García grape variety.

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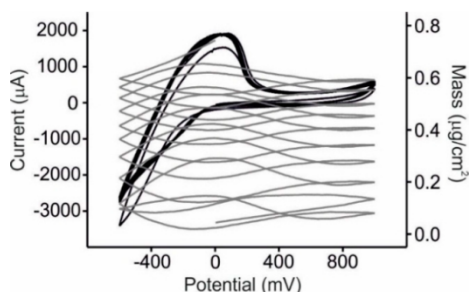
Figure 4. Voltammetric (black line) and mass (grey line) response of the NiPc^{SO3}/PAH sensor towards a must obtained from Juan García grapes.

As usual, the first cycle was different from the rest, but subsequent responses were stable and highly reproducible with coefficients of variation (%CV) within the range of 0.4%–2.8%. Voltammograms were dominated by a broad anodic peak that could be associated to the polyphenolic content of wines [8,59]. On the other hand, the massograms showed a progressive increase in mass. In a complex mixture such as must, it is difficult to establish the process by which the mass increases. Taken into account the results obtained in the presence of catechol and glucose, it can be assumed that polymerization of phenols can play a key role in the observed mass changes. In addition, the adhesion of other main components of musts without redox activity in the studied range such as sugars cannot be neglected.

The results obtained were similar for all the phthalocyanines tested, but the peak positions and their intensities depend on the phthalocyanine used to form the LbL film. Figure 5 illustrates the voltammetric response of the array of sensors towards musts. The responses obtained towards the Mencía Regadío and the Juan García varieties have been chosen as examples. In good accordance with the results obtained in catechol, the peak positions shifted to higher values when advancing in the transition metal series, confirming that these anodic peaks are associated to the polyphenolic content of grapes.

Because the polyphenolic content varied from one must to another (see Table 1), the positions, broadness and intensities of the peaks were different.
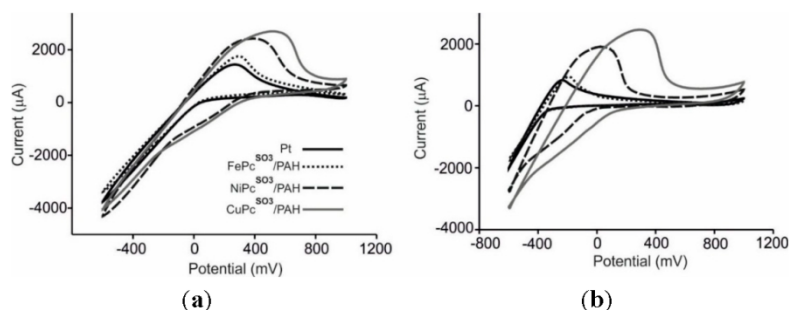

Figure 5. Voltammetric outputs of the EQCM sensors immersed in (**a**) Mencía Regadío; (**b**) Juan García grapes.

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The massograms registered simultaneously to the cyclic voltammetry are illustrated in Figure 6 for different sensors/musts (due to the complexity of the signals it is not possible to superimpose signals for comparison purposes). As expected, all massograms showed a progressive increase in mass, but also in this case, each must produce a different signature. The change in mass could be attributed to the adhesion of sugars and/or to other complex processes and polymerizations. It has to be noticed that in some cases, the adhesion of suspended material caused spikes or a sudden increase in mass. In those cases (15% of the measures), the results were discarded. In the absence of such processes, the experiments were highly reproducible.
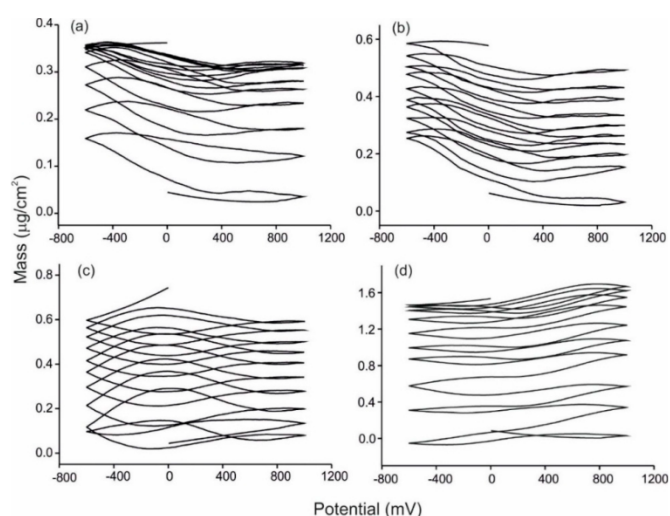

Figure 6. Mass outputs of (**a**) Pt bare sensor immersed in Prieto Picudo; (**b**) FePc^{SO3}/PAH sensor immersed in Tempranillo; (**c**) NiPc^{SO3}/PAH sensor immersed in Juan García; (**d**) CuPc^{SO3}/PAH sensor immersed in Cabernet.

3.4. Statistical Analysis

The intrinsic complexity, richness and cross-selectivity of the signals generated by voltammetric sensor arrays are an advantage because the dataset contains meaningful information about the sample. As the array of EQCM sensors showed a characteristic voltammetric and mass response depending on the must analyzed, these data could be used to discriminate musts according to their chemical nature. In order to evaluate the capability of discrimination of the system, a non-supervised multivariate technique, the multi-way decomposition PARAFAC method was used.

Prior to statistical analysis, voltammograms and massograms were preprocessed by means of a data reduction technique using "kernels" to obtain 10 variables from each voltammogram and 10 variables from each massogram. The input array for computing the PARAFAC model was a three-way data matrix ("6 must samples with 3 replicas" × "10 kernels" × "4 sensors"), therefore the size was ($18 \times 10 \times 4$).

Figure 7 represents the tridimensional PARAFAC scores plot for the multisensor system using voltammetric outputs. As observed in the figure, the clusters corresponding to the six studied musts

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(three replicas per must) were clearly separated and were located according to the Total Polyphenol Index (TPI): musts with high TPI (Mencía Regadío, Cabernet Sauvignon, Tempranillo and Juan García variety) appeared in the region corresponding to a positive C3, while musts with low TPI (Prieto Picudo and Garnacha variety) appeared in the negative C3 values region. This good discrimination can be explained taking into account that voltammograms reflect the redox activity of the phenols. The error in terms of root-mean-square error (RMSE) was 0.4551.
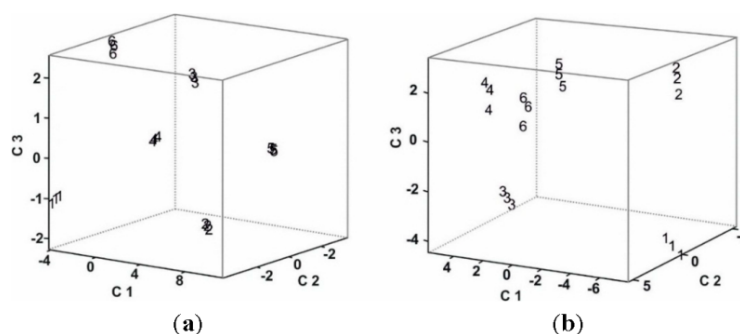

Figure 7. PARAFAC scores plot of the array obtained from (**a**) The voltammetric responses; (**b**) The mass responses. Must samples are 1: Prieto Picudo; 2: Garnacha; 3: Cabernet Saugvinon; 4: Tempranillo; 5: Juan García; and 6: Mencía Regadío.

Figure 7 also shows the tridimensional PARAFAC scores plot when analyzing the mass output. In this case RMSE was 0.6472. Massograms also allowed a good-quality discrimination. However, the positions of the clusters were not related to the Total Polyphenol Index. Instead, their positions were located according to the sugar content.

Musts with the highest Brix degree (Cabernet Sauvignon and Prieto Picudo) were located at negative values of C3, whereas those with lower sugar content at positive values of C3 (Tempranillo, Mencía Regadío, Juan García and Garnacha). This result confirms that changes in mass observed from one massogram to the subsequent one are due to the adhesion of sugars.

Prediction models were established by N-PLS. The classification models were subjected to full cross-validation by means of the "leave-one-out" method. N-PLS was carried out to establish correlations between the EQCM signals obtained from the array and chemical parameters. N-PLS regression builds a calibration model incorporating a relationship between the sets of predictors and responses based on the multiway structure of the arrays. Calibration fits the model to the available data, while validation checks the model for new data. Results are shown in Tables 2 and 3.

Correlations were obtained with the voltammetric data, which showed higher coefficients of correlations and lower errors. Results indicate that the voltammetric signals are better correlated with the TPI with only three components (Table 2).

The mass outputs present a better correlation with those parameters related to the sugar content (sugar content, degree 16.8 and Brix degrees) (Table 3) than with those related to the polyphenolic content. The correlations calculated between mass data and chemical parameters showed lower correlation coefficients and higher errors than the correlations obtained with voltammetric data.

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Table 2. Statistical parameters obtained for the N-PLS regression model established between the chemical parameters and the voltammetric responses of the sensors towards musts.

Voltammetric Outputs					
Parameters	R^2_C [a]	RMSEC [b]	R^2_P [c]	RMSEP [d]	Number of Components
Sugar content	0.997	0.99187	0.945	4.24917	4
Brix degree	0.996	0.09242	0.935	0.40019	4
Degree 16.8	0.997	0.05894	0.946	0.25147	4
TPI	0.992	0.46538	0.983	0.68089	3
Polyphenolic content Folin-Ciocalteau method	0.998	0.33442	0.989	1.11841	3

[a] Squared correlation coefficient in calibration; [b] Root Mean Square Error of Calibration; [c] Squared correlation coefficient in prediction; [d] Root Mean Square Error of Prediction.

Table 3. Statistical parameters obtained for the N-PLS regression model established between the chemical parameters and the mass responses of the sensors towards musts.

Mass Outputs					
Parameters	R^2_C [a]	RMSEC [b]	R^2_P [c]	RMSEP [d]	Number of Components
Sugar content	0.941	4.45005	0.839	7.31293	4
Brix degree	0.972	0.00176	0.865	0.00291	4
Degree 16.8	0.941	0.26438	0.840	0.43420	4
TPI	0.961	1.02511	0.845	2.04940	5
Polyphenolic content Folin-Ciocalteau method	0.965	1.91428	0.921	3.0353	5

[a] Squared correlation coefficient in calibration; [b] Root Mean Square Error of Calibration; [c] Squared correlation coefficient in prediction; [d] Root Mean Square Error of Prediction.

As voltammetric and mass data are registered simultaneously, EQCM can improve the discrimination of must samples while providing good correlations with both sugar and polyhenolic content.

4. Conclusions

A multisensor system formed by EQCM sensors modified with LbL films containing phthalocyanines have been successfully fabricated and used to discriminate musts obtained from different varieties of grapes. The capability of the sensor array to discriminate grapes according to their chemical parameters has been evidenced using the multivariate decomposition method PARAFAC. Voltammograms showed anodic peaks that were related to the phenolic content in musts and massograms showed an increase of mass that was related to the sugar content of the musts. Prediction models performed by multi-way partial least squares (N-PLS) have showed correlations between the voltammetric and mass outputs with the chemical parameters. EQCM has demonstrated to be advantageous because voltammetric and mass data can be registered simultaneously, providing good correlations with the sugar and the polyhenolic content, which are the most important indicators of quality and degree of ripeness in grapes.

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Acknowledgments

Author Contributions

The manuscript was written by Maria Luz Rodríguez-Méndez and Celia Garcia-Hernandez. In addition, the concept of the experimental setup was also proposed by Maria Luz Rodríguez-Méndez and implemented by Celia Garcia-Hernandez. For the experimental works, the sensing tests and material analyses were done by Celia Garcia-Hernandez, Cristina Medina-Plaza, Cristina Garcia-Cabezon, Isabel del Valle and Jose Antonio de Saja. Finally, data analyses were accomplished by Celia Garcia-Hernandez and Fernando Martin-Pedrosa. The final manuscript has been read and approved by all authors.

Conflicts of Interest

The authors declare no conflict of interest.

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3.10. Paper IX

In situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological applications.

By Celia García-Hernández, Allison Freese, María L. Rodríguez-Méndez and Adam K. Wanekaya.

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gold nanoparticles for biological applications.

3.10.1. Motivation and objective

This work was carried out in the Missouri State University, in the Chemistry Department, under the supervision of Prof. Wanekaya during an international research stay. It has a different topic, not related to electronic tongue devices, but the LbL technique has also been used in this work to encapsulate the protein lysozyme for drug delivery purposes. It has, therefore, been included in this compilation of papers as it can be of interest for the developed bioelectronic tongues, where enzymes have improved the specificity of the electrochemical sensors. In previous works, the enzyme material was deposited onto the electrode surfaces by means of the drop-casting technique and, later, cross-linked with glutaraldehyde. Hence, the present work envisions a new methodology in which an enzyme can be bonded to AuNPs using stronger interactions, concretely, covalent bonds.

Nanomaterials applied to the field of delivering proteins and drugs into cells provide important advances for biomedical purposes. Most proposed methods for delivering proteins are based on mechanical methods, covalent protein modification and supramolecular delivery systems. All these approaches present different strengths and challenges. The Layer by Layer (LbL) technique has gained great interest in protein delivery and biosensor applications. LbL designs to immobilize or encapsulate enzymes provide a huge versatility and potential in the pharmaceutical and biopharmaceutical fields. Besides the advantages provided by LbL, such as ultrathin films, easy preparation, possibility to perform on supports of any shape and dimension, high versatility to surface modification with different polyelectrolytes, etc., in the field of protein immobilization, the use of mild conditions (e.g., aqueous solutions) is particularly advantageous in order to avoid the protein denaturation and protect the activity in comparison with the use of organic solvents used in other encapsulation systems (microparticles, microcapsules, microemulsions and liposomes). The most suitable polymers used for LbL designs are the positive charged polymers poly(ethyleneimine) (PEI) and poly(allylamine hydrochloride) (PAH) and the negative charged polymers poly(sodium 4-styrenesulfonate) (PSS) and poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA). However, LbL multilayers have been successfully grown by means of polymers with a low charge density, so it has been demonstrated that the buildup of LbL films is not restricted to materials with a minimum charge density, since other driving interactions play an important role during the assembly. Moreover, when using purely electrostatic interactions, variations in the pH, ionic strength, electrical field, light,

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temperature, mechanical stress, addition of biological compounds (e.g., proteins), or ionic surfactants might affect the properties of the adsorbed layers or even induce their desorption.

Gold nanoparticles (AuNPs) have attracted considerable interest because of their unique optical, electronic and catalytic properties. They have been widely used in chemical and biological applications due to their biocompatibility, dimension, easy characterization and the versatility offered by its surface chemistry. Gold nanoparticles can be synthesized using naturally occurring biomacromolecules instead of traditional methods that use reducing agents like borohydride and/or sodium citrate. However, some of these alternative methods use additional activators to initiate the reducing properties of the biomacromolecules. Amino acids, such as cysteine, are fine reducing agents and the biomaterials synthesized by using proteins are far more chemically pure and easy to characterize than by using different reducing and stabilizing agents in the presence of proteins.

In this work, we have developed a carrier system based on lysozyme and gold nanoparticles using the LbL technology. Lysozyme (Lys) is a natural enzyme abundantly found in secretions, such as tears, saliva, and mucus of the mammalian. The major function of Lys *in vivo* is against infection due to its antibacterial properties. Lysozyme has also been reported as an effective enzyme against many food spoilage and pathogenic microorganisms. However, the practical application of free lysozyme is limited because it is unstable and easily inactivated. Its relatively small size, low molecular weight (14,370 kDa) and high isoelectric point (pI 11.35) allows an easier characterization and manipulation. In addition, lysozyme is stable over wide temperature and pH ranges, making it suitable for a variety of synthetic approaches. These properties also make this protein attractive to use as a model protein to develop specific carriers for small drug delivery.

3.10.2. Summary of the research work

The carrier systems based on Lys and AuNPs were prepared by following two different approaches. First, we envisioned a new strategy based on the use of the lysozyme as reducing agent to make AuNPs covalently bond with lysozyme, followed by the deposition of the PSS and PEI layers by means of LbL (PEI-PSS-Lys-AuNPs). PEI was used as the cationic layer and it was also deposited as the outer layer, since it plays an important role as the transfection agent and

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avoids the degradation of the protein. Secondly, colloidal citrate-stabilized AuNPs were functionalized with MUA (11-mercapto-undecanoic acid) and LbL was subsequently performed to deposit the PEI and PSS layers. In this case, the negatively charged polymer PSS was used to electrostatically immobilize the positive lysozyme in between two PSS layers. The resulting bioconjugate as-prepared was PEI-PSS-Lys-PSS-PEI-MUA-AuNPs.

The deposition of each layer was characterized following three important parameters: particle size, zeta potential, UV-Vis absorbance. In addition, the enzymatic activity was tested using the bacteria *Micrococcus lysodeikticus* as substrate.

In the case of the bioconjugate PEI-PSS-Lys-AuNPs, the lysozyme was mixed with a solution of $HAuCl_4$ for 6 hours at 45ºC. Then, Lys acted as the reducing and capping agent of the AuNPs. During the reaction, the UV-Vis spectra showed a progressive increase in the Au Plasmon resonance peak after 4 hours of reaction. This peak reached its maximum at approximately 12 hours. The hydrodynamic diameters by DLS were found to be about 60 nm for Lys-AuNPs and PSS-Lys-AuNPs. The diameter increased to 90 nm after PEI deposition. The Zeta potential results evidenced the successful deposition of layers on the particle surface. Initially, before the coating, the zeta potential value of Lys-AuNPs was +24 mV. The charge reversed to a negative value of -51 mV after the deposition of the negatively charged PSS polyelectrolyte and back to a positive charge of +18 mV after the deposition of the positively charged PEI polyelectrolyte. The deposition of the layers was also monitored by UV-Vis in the wavelength range 200-800 nm. The Plasmon resonance peak positions ranged from 535 to 545 and were in accordance with the results obtained by DLS measurements. The typical lysozyme absorbance due to the amino acids was also observed at 280 nm. Amide bands of PEI-PSS-Lys-AuNPs were analyzed by FTIR spectroscopy to evaluate the possibility of thermal denaturation of the lysozyme during the reaction. Amide bands can provide useful information about the **secondary structure of proteins such as α-helix, β**-sheets, turns or unordered structures. The second derivative spectra of the native lysozyme and Lys–AuNP samples showed both amide I (1700–1600 cm^{-1}) and amide II (1580–1480 cm^{-1}) bands. After FTIR analysis, it was observed that the activity of the protein was not compromised during the reaction processes. In addition, TEM images confirmed the formation of gold nanoparticles using lysozyme as a reducing agent with an average size of 35 ± 10 nm ranging between 10–45 nm.

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In the case of the synthesis of AuNPs bioconjugate via MUA-AuNPs, the hydrodynamic diameter of the gold nanoparticles increased during the sequential coating procedures from about 30-40 nm to just below 140 nm for PEI-PSS-Lys-PSS-PEI-MUA-AuNPs. The deposition of each layer was evaluated by zeta potential values. A reversal of the zeta-potential after each deposition was observed, confirming the negative and positive charge of each compound. The UV-Vis spectra confirmed an increase in the position of the Plasmon resonance peak associated with AuNPs when increasing the size of the nanoparticles in accordance with the results obtained by DLS measurements. Lysozyme absorbance was also observed at 280 nm.

Finally, enzymatic assays were carried out to verify the activity of the enzyme in addition to its observable presence, confirmed by UV-Vis. The residual activities of free lysozyme, encapsulated lysozyme via MUA-AuNPs and bioconjugates based on Lys-AuNPs were monitored for 24 minutes. After 6 minutes the enzymatic activity of the free lysozyme decreased significantly to reach zero activity after 12 minutes due to the degradation of the bacteria walls, which occurred immediately in the presence of native lysozyme. The PEI-PSS-Lys-PSS-PEI-MUA-AuNP and PEI-PSS-Lys-AuNP bioconjugates showed an increase during the first 6 minutes; then the PEI-PSS-Lys-PSS-PEI-MUA-AuNPs digested the bacteria walls, decreasing the activity linearly to become zero after 24 minutes; whereas the activity of PEI-PSS-Lys-AuNPs continued to increase after 6 minutes until it reached a plateau. This result shows that the interaction between lysozyme and AuNPs affects the activity of the enzyme retaining it for longer in bioconjugates made via *in situ* synthesis than those made via citrate reduction prior to stabilization by MUA. In fact, the means of interaction between lysozyme and AuNPs in the as-prepared bioconjugates is different: For PEI-PSS-Lys-PSS-PEI-MUA-AuNPs, the interaction of different layers is entirely electrostatic in nature; whereas for Lys-AuNPs, the simultaneous reduction of gold ions into nucleating centers provides a greater probability of synergistic interactions between Lys and NPs. The Au nucleating centers produced upon the reduction of $AuCl_4^-$ ions are expected to have a greater potential to break the disulfide groups and form covalent bonds.

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Biomaterials Science

PAPER

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In situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological applications

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Herein, we demonstrate the use of lysozyme (Lys) as a model to fabricate a protein carrier system based on gold nanoparticles (AuNPs) *via* the Layer-by-Layer (LbL) technology. Poly(ethyleneimine) (PEI) and poly (sodium 4-styrenesulfonate) (PSS) were used as cationic and anionic polymers respectively to grow oppositely charged layers. Mild aqueous conditions were utilized to avoid protein denaturation and activity instead of organic solvents that have been used in other encapsulation systems. Two different strategies were used: (A) lysozyme acting as a reducing and stabilizing agent in the formation of AuNPs at a temperature of 45 ± 2 °C followed by only two subsequent polymeric layers deposited by LbL, and (B) citrate acting as a reducing agent prior to stabilization of the AuNPs by mercaptoundecanoic acid. Dynamic light scattering, UV-vis spectroscopy, IR spectroscopy and transmission electron microscopy were used to characterize the nanoconjugates. Furthermore, the enzymatic activity of the resulting protein/nanoparticle conjugates was evaluated using the bacteria *Micrococcus lysodeikticus* as a substrate.

1. Introduction

Many researchers, including ourselves, have demonstrated that nanomaterials can be used to protect, stabilize and preserve the activity of proteins in cells[1-3] and in various other materials and media.[1,4,5] In particular, protein stabilization and delivery have important applications in the manipulation of signaling pathways,[3] stimulation of potent antitumor immune cells[6,7] and tissue engineering and wound healing.[8] Among the known nanomaterials, gold nanoparticles have additional unique properties such as size- and shape-dependent optical and electronic features, easily modifiable surface, biocompatibility and non-toxicity.[9]

For many decades, gold nanoparticles have been typically synthesized using sodium borohydride and/or sodium citrate reducing agents.[10,11] Recently, there has been growing interest directed towards synthesizing and stabilizing gold nanoparticles using naturally occurring biomacromolecules.[12-19] However, some of these methods use additional activators such as superoxide anions,[18] light,[19] *etc.* to initiate the reducing properties of the biomacromolecules. Amino acids such as cysteine are fine reducing agents and the biomaterials synthesized by using proteins are far more chemically pure and easy to characterize than by using different reducing and stabilizing agents in the presence of proteins.[20]

Herein, we report the *in situ* synthesis and stabilization of protein-modified gold nanoparticles at a temperature of 45 °C without the use of activators under mild, near ambient conditions. The protein acted not only as a reducing agent of the gold salt precursor but also as a stabilizing agent of the resulting gold nanoparticles. The protein/nanoparticle conjugates were coated with poly(ethyleneimine) (PEI), a well-known cationic polymer used for transfection purposes.[21] We evaluated the enzymatic activity of the resulting PEI-coated protein/nanoparticle conjugates. We also compared the activity of the nanoparticle conjugates with those prepared from the well-known method of synthesizing gold nanoparticles *via* the reduction of the gold salt precursor by citrate. The citrate-reduced gold nanoparticles were stabilized with mercaptoundecanoic acid (MUA) prior to coating with protein and PEI to form PEI–protein/MUA nanoparticle conjugates.

Proteins have been used to synthesize bioconjugate nanomaterials in the aqueous phase within a temperature range of 40–80 °C. Lead sulphide (PbS) nanocrystals have been synthesized using bovine serum albumen (BSA), an important blood protein, as the capping/stabilizing agent in the range of 40–80 °C.[22] The same protein was utilized to develop BSA-conjugated AuNPs to explore their applications as drug delivery vehicles. The BSA-conjugated AuNPs were fabricated by react-

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ing BSA with $HAuCl_4$ at a temperature of 70 °C for 6 h under static conditions.[23] Cytochrome c protein has also been used to synthesize gold nanoparticles under mild conditions in a temperature range of 40–80 °C.[20]

Lysozyme was selected as the model protein for various reasons. Lysozyme is a well-characterized protein with a high isoelectric point (pI 11.35).[24] In addition, lysozyme is stable over wide temperature and pH ranges, making it suitable for a variety of synthetic approaches. These properties also make this protein attractive for use as a model protein for various applications. Therefore, a large number of attempts have emerged to investigate different ways for lysozyme immobilization, or encapsulation, many of which include Layer-by-Layer (LbL) steps during the process.[25–27] Therefore, LbL structures have been constructed to immobilize proteins such as catalase, α-chymotrypsin, cholesterol oxidase, glucose isomerase, β-glucosidase, urease, peroxidase and glucose oxidase.[28–35]

2. Experimental

2.1. Materials

All chemicals and solvents were of reagent grade and used without further purification. Chloroauric acid ($HAuCl_4 \cdot 3H_2O$), poly(ethyleneimine) (branched, 25 kDa) (PEI), poly(sodium 4-styrenesulfonate) (70 kDa) (PSS), 11-mercapto-undecanoic acid (MUA), lysozyme (Lys) from chicken egg white, the bacteria *Micrococcus lysodeikticus* and Corning® syringe filter membrane (with diameter 15 mm and pore size 0.2 μm) were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium citrate dihydrate and sodium phosphate monobasic were purchased from Spectrum Chemical Manufacturing Group (Gardena, CA, USA). Sodium phosphate dibasic was purchased from Mallinckrodt Chemical Works (St Louis, MO, USA). Deionized water with a resistivity of 18.2 MΩ cm was used to prepare all the solutions.

2.2. Instrumentation and characterization

Purification of colloidal gold nanoparticle suspensions was performed using a micro-centrifuge D3024 from Scilogex (Rocky Hill, CT, USA). Dynamic light scattering (DLS) and zeta-potential measurements were carried out using a NanoBrook Omni from Brookhaven Instruments (Holtsville, NY, USA). A Cary 60 UV-vis spectrophotometer from Agilent Technologies (Santa Clara, CA, USA) was used for recording UV-vis spectra and for the enzymatic activity assays. Absorbance spectra were recorded from 200 to 800 nm. For particle size measurements using DLS, the samples were re-suspended in 1 mM NaCl and filtered with 0.2 μm filters. Infrared spectra were recorded on a Jasco Model FT/IR-6600 Spectrometer (Jasco, Tokyo, Japan) with a diamond ATR crystal accessory. The software used for FTIR data collection was Spectra Manager II (Jasco, Tokyo, Japan). Before the analysis the instrument was purged with nitrogen for 10 min. As a reference, the background spectrum of air (100 BKG) was recorded before the acquisition of the sample spectrum. Spectra were recorded at 25 °C with a resolu-

tion of 2 cm^{-1} and 300 scans were averaged for each spectrum (scan 1800–1300 cm^{-1}). Transmission electron microscopy (TEM) analysis of Lys–AuNPs was carried out using a TEM JEOL JEM-1011 HR (JEOL Ltd, Tokyo, Japan) operating at 100 kV. ImageJ software was used to calculate particle size distribution from transmission electron micrographs.

2.3. Synthesis of lysozyme–gold nanoparticle conjugates (Scheme 1Aa)
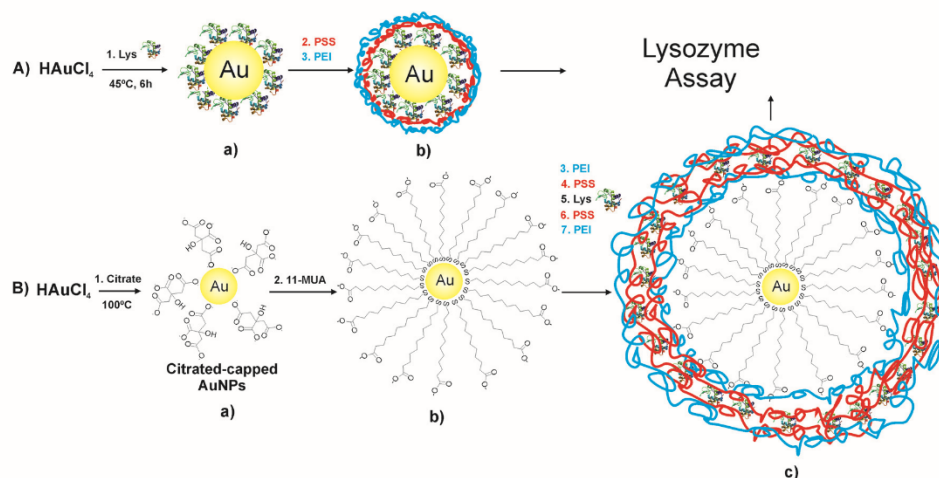
Gold nanoparticles were synthesized using lysozyme as a reducing agent. The synthesis of Lys–AuNPs was done *via* modification of a previous protocol.[20] Briefly, 25 mL of 0.25 mM $HAuCl_4$ and 250 μL of 700 μM lysozyme were mixed in an Erlenmeyer flask at room temperature (final concentration ratio $HAuCl_4$/Lys = 36). After the components were mixed, the reaction mixture was kept in a water bath at 45 ± 2 °C for 6 hours under static conditions. The reaction mixture was then cooled down to room temperature and kept overnight under ambient conditions. The Lys–AuNPs were purified by centrifugation at 2380*g* for 5 minutes followed by washing in deionized water. The Lys–AuNPs were re-suspended in 1 mM NaCl for subsequent deposition with layers of poly(sodium 4-styrenesulfonate) (PSS) and poly(ethyleneimine) (PEI).

2.4. Coating of Lys–AuNPs (Scheme 1Ab)

The Layer-by-Layer technique was used for further deposition following the coating protocols previously described in the litterature[36,37] with some modifications. A colloidal solution of Lys–AuNPs in 1 mM NaCl was added dropwise to a stirred solution of PSS under ambient conditions. The concentration of PSS in the final reaction solution was 5 mg mL^{-1}. Stirring was continued over 1 h. The resulting PSS–Lys–AuNPs were purified by centrifugation as before (2380*g* for 5 min at room temperature followed by washing with deionized water), re-suspended in 1 mM NaCl and added dropwise to a stirred solution of PEI under ambient conditions. The concentration of PEI in the final reaction solution was 5 mg mL^{-1}. Stirring was continued over 1 h. The resulting PEI–PSS–Lys–AuNPs were purified by centrifugation and re-suspended in 1 mM NaCl as before.

2.5. Synthesis of gold nanoparticles (Scheme 1Bb)

A colloidal solution of citrate-stabilized AuNPs was synthesized according to the method introduced by Frens[11] based on the Turkevich method[10] using sodium citrate as a reducing and stabilizing agent. Briefly, 500 mL of 0.01% w/v $HAuCl_4$ solution was heated to 100 °C and then 3.6 mL of 1% w/v sodium citrate solution was added dropwise with stirring. The reaction mixture was stirred and heated for 20 minutes. The color of the solution turned from light yellow to dark red. AuNP size can be adjusted by varying the $HAuCl_4$/citrate ratio. The AuNPs obtained from this synthesis resulted in a size of about 30–40 nm and were stabilized by a cover of citrate ions preventing the aggregation of the particles (Scheme 1Ba). The AuNPs were further stabilized using 11-mercapto-undecanoic acid (MUA) as a dispersant. Briefly, the pH of the AuNP suspension

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Scheme 1 Overview of the experimental schematic showing the synthesis of Lys–AuNPs (Aa), PEI–PSS–Lys–AuNPs (Ab), citrate–AuNPs (Ba), MUA–AuNPs (Bb) and PEI–PSS–Lys–PSS–PEI–MUA–AuNPs (Bc). (Not to scale).

was adjusted to 11.00 with NaOH. After this, MUA (20 mg mL^{-1} in 10 mM NaOH) was added dropwise to the AuNP suspension under stirring to give a final concentration of 0.1 mg mL^{-1} of MUA. Stirring was continued under dark conditions over 3 days to ensure a stable surface modification. After 3 days, MUA–AuNPs were purified by centrifugation to remove the excess MUA. Centrifugation conditions depend strongly on the particle size. Purification was done by centrifuging the MUA–AuNPs at 2380g for 5 min at room temperature followed by washing with deionized water. The nanoparticles were re-suspended in 1 mM NaCl. Unpurified MUA–AuNP nanoparticles can be stored over weeks. They were tested every single week to verify the size.

2.6. Coating of MUA–AuNPs (Scheme 1Bc)

The coating of MUA–AuNPs was done using the LbL technique as was previously described by sequential layering with poly(ethyleneimine) (PEI) and poly(sodium 4-styrenesulfonate) (PSS) respectively. MUA–AuNPs were resuspended in 1 mM NaCl and were added dropwise to a solution of PEI under stirring at room temperature. The final concentration of the PEI solution was 5 mg mL^{-1}. Stirring was continued for 1 h. Afterwards, PEI–MUA–AuNPs were purified as before (2380g for 5 min at room temperature followed by washing with deionized water). After purification the nanoparticles were resuspended in 1 mM NaCl and the PSS layer was deposited by LbL. PEI–MUA–AuNPs were added dropwise to a PSS solution under stirring at room temperature up to a final PSS concentration of 5 mg mL^{-1}. After 1 h the PSS–PEI–MUA–AuNP suspension was purified as before and resuspended in 1 mM NaCl. The resulting PSS–PEI–MUA–AuNPs were coated with lysozyme as follows: 10 mg mL^{-1} lysozyme in phosphate buffer solution (pH 7.00) was added dropwise to a stirred colloidal solution of

PSS–PEI–MUA–AuNPs under ambient conditions. The final concentration of lysozyme in the reaction mixture was 120 μM. Stirring was continued over 3 h. The resulting Lys–PSS–PEI–MUA–AuNPs were purified as described before. They were then sequentially coated with layers of PSS and PEI as described previously forming PEI–PSS–Lys–PSS–PEI–MUA–AuNPs (Scheme 1Bc).

2.7. Lysozyme activity assay

The bacteria *Micrococcus lysodeikticus* was used as a substrate for the enzymatic action of lysozyme. In order to compare the protein gold nanoparticles with free lysozyme, an estimated concentration of encapsulated lysozyme was determined spectrophotometrically using an extinction coefficient of 2.63 mL mg^{-1} cm^{-1} when measuring the absorbance at 280 nm after lysozyme deposition.[38,39] *Micrococcus lysodeikticus* solution was prepared by dissolving 50 mg of bacteria in 100 mL of 50 mM buffer phosphate (pH 6.25). The AuNP samples and free lysozyme were dissolved in 50 mM buffer phosphate (pH 6.25). 600 μL of the sample was added to 3 mL of bacteria solution at room temperature and mixed. The decrease in the absorbance was monitored at 600 nm for 30 minutes at room temperature. The enzymatic activities were determined from the slope of the fitted line when representing the absorbance decay plots and using eqn (1).

$$\frac{\text{Units}}{\text{mL}_{\text{enzyme sample}}} = \frac{(\Delta\text{Abs}_{600}/\min \text{ test} - \Delta\text{Abs}_{600}/\min \text{ blank})}{0.001 \cdot (0.6)}$$

(1)

where 0.001 is the change in the absorbance per minute for lysozyme and 0.6 is the added volume of the sample to the bac-

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teria solution during the tests in mL. The blank used for the assays was 50 mM phosphate buffer.

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3.1. Synthesis and coating of Lys–AuNPs

Fig. 1 shows the visible absorption spectra of the synthesis of Lys–AuNPs from the beginning to the end of the reaction process. Lysozyme acted as the reducing and capping agent of the AuNPs. There was a noticeable and progressive increase in the Au plasmon resonance peak after 4 h. This peak reached its maximum at approximately 12 h. The Lys–AuNPs were purified by sequential centrifugation and rinsing.

Fig. 2A and Table 1 shows DLS spectroscopy results for the hydrodynamic diameter and polydispersity indexes of Lys–AuNPs before and after PSS and PEI deposition to form PSS–Lys–AuNPs and PEI–PSS–Lys–AuNPs, respectively. The hydrodynamic diameters of Lys–AuNPs and PSS–Lys–AuNPs were about 60 nm. The diameter increased to 90 nm after PEI deposition. In most cases, the polydispersity indexes were approximately 30% or lower.

In addition to the hydrodynamic diameter, the deposition of each layer was monitored by measuring the zeta potential values of the nanoconjugates (Fig. 2B and Table 1). Zeta potentials not only provide quantitative values of the overall surface charges of the nanoparticles, but also predict colloidal stability. A large value of the zeta potential, regardless of the charge, is a prediction of colloidal stability due to extra electrostatic repulsion between similarly charged particles. Therefore, zeta potentials also confirm the successful deposition of polyelectrolytes on the particle surface.

Initially before the coating the zeta potential value of Lys–AuNPs was +24 mV. This is expected due to the overall positive charge of lysozyme. The charge reversed to a negative value of
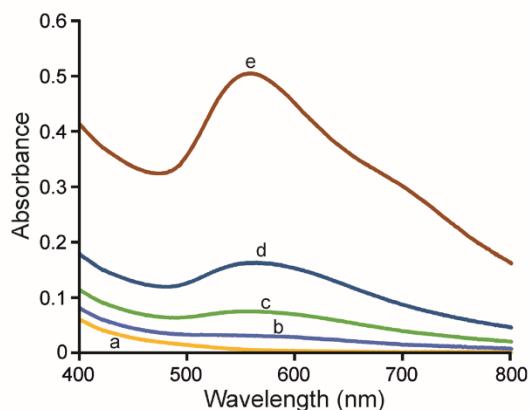
Fig. 2 (A) Hydrodynamic diameter and polydispersity index of PEI–PSS–Lys–AuNPs. (B) Zeta-potential values before and after sequential coating steps of the Lys–AuNPs.

Table 1 Hydrodynamic diameter, polydispersity index and zeta potential values of Lys–AuNPs and Lys–AuNPs coated with PSS and PEI

	DLS measurements		ELS measurements
	Size (nm)	Polydispersity	ZP (mV)
Lys–AuNPs	63.24 ± 0.86	0.294 ± 0.004	23.96 ± 2.56
PSS–Lys–AuNPs	59.07 ± 0.46	0.320 ± 0.004	−51.16 ± 5.77
PEI–PSS–Lys–AuNPs	92.79 ± 3.81	0.284 ± 0.005	18.47 ± 2.25

−51 mV after deposition of the negatively charged PSS polyelectrolyte and back to a positive charge of +18 mV after deposition of the positively charged PEI polyelectrolyte.

The deposition of the layers was monitored by UV-vis in the wavelength range of 200 to 800 nm (Fig. 3). The Au plasmon resonance peaks were in the 535 to 545 nm range. The shifts observed in the plasmon resonance peak position showed a similar trend to that of the results obtained by DLS measurements. The typical lysozyme absorbance due to the amino acids was also observed at 280 nm. The characteristic absorbance peak of PSS at 260 nm was present after the deposition of the polymer.

In order to evaluate the possibility of thermal denaturation of lysozyme during the reaction with $HAuCl_4$ at 45 ± 2 °C, FTIR spectroscopy was used to monitor the secondary structural nature of lysozyme. Lysozyme has been one of the most common protein models used in thermal denaturation assays.[40–42] Amide bands can provide useful information

Fig. 1 Time-dependent absorbance spectra of AuNPs by reducing $HAuCl_4$ with lysozyme at 45 ± 2 °C. (a) Immediately after the start of the reaction and after (b) 4 h, (c) 5 h, (d) 6 h and (e) 12 h.
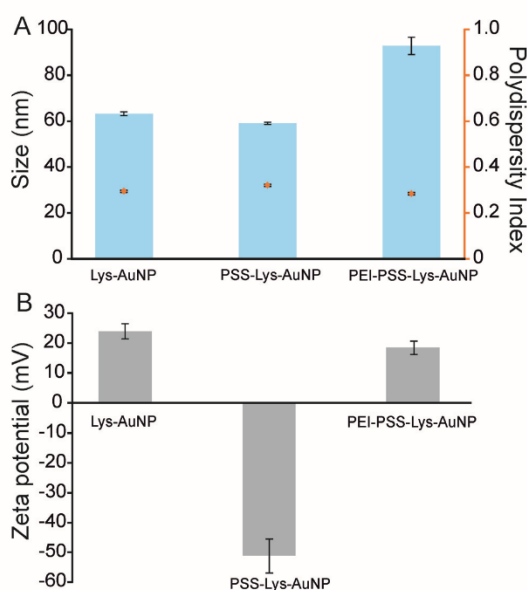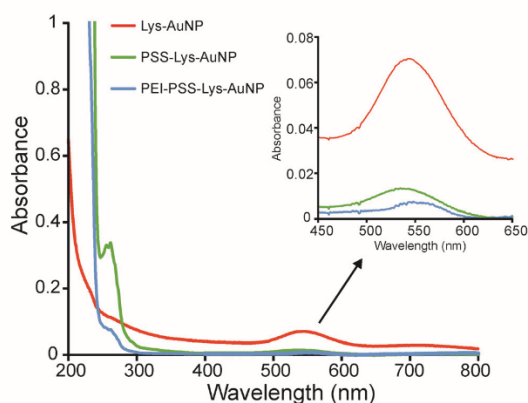
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Fig. 3 Absorbance spectra of PEI–PSS–Lys–AuNPs at different layers. Inset: Plasmon peak position shift.
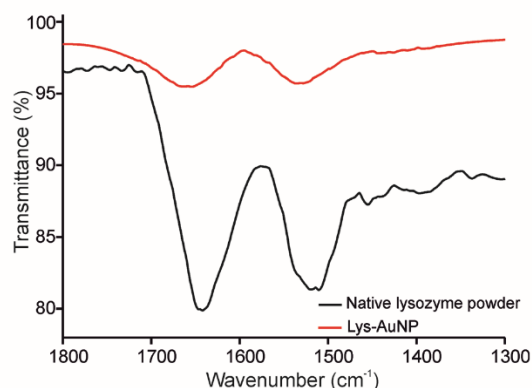

Fig. 4 ATR–FTIR spectra of lysozyme stabilized AuNPs and native lysozyme.

about the secondary structure of proteins such as α-helix, β-sheets, turns or unordered structures. Amides I and II are the major bands in the protein IR spectrum. Amide I (1700–1600 cm^{-1}) is the most intense absorption band in proteins and is due to C=O stretching vibrations of the peptide bonds. The exact band position is determined by the backbone conformation and the hydrogen bonding pattern. Amide II (1580–1480 cm^{-1}) derives mainly from in-plane N–H bending and the rest of the potential energy arises from the C–N and C–C stretching vibrations. Amide III and V are very complex bands dependent on the details of the force field, the nature of side chains and hydrogen bonding. Due to the technical and theoretical limitations, only amide I and amide III bands are used for investigating the secondary protein structure.[43–46] Amide I modes, however, are the most extensively used in such studies.[43–45]

The relationship between the amide I band position and the type of secondary structure is best recognized by analyzing the IR spectra of simple homopolypeptides that fold into well-defined structures (purely alpha-helical or beta-sheet). The amide I band consists of many overlapping component bands that represent different structural elements (*e.g.* alpha helices, beta sheets, turns, and non-ordered or irregular structures). Extraction of single subcomponent bands can be achieved using curve-fitting approaches to assign each subcomponent to a particular protein secondary structure (*e.g.* Gaussian curve-fitting).[43]

The second derivative spectra of native lysozyme and Lys–AuNP samples showed both amide I (1700–1600 cm^{-1}) and amide II (1580–1480 cm^{-1}) bands (Fig. 4). Moreover, Gaussian curve-fitting of the amide I band showed typical components already reported for lysozyme (Fig. 5).[40,41,45,47] For Lys–AuNPs, the assignment of bands were β-sheet secondary structure at *ca.* 1628 cm^{-1} and 1681 cm^{-1}; α-helical structure at 1652 cm^{-1}; β-turns at *ca.* 1667 cm^{-1}; and random coil structures at *ca.* 1638 cm^{-1} (Table 2). It has been reported that thermal dena-
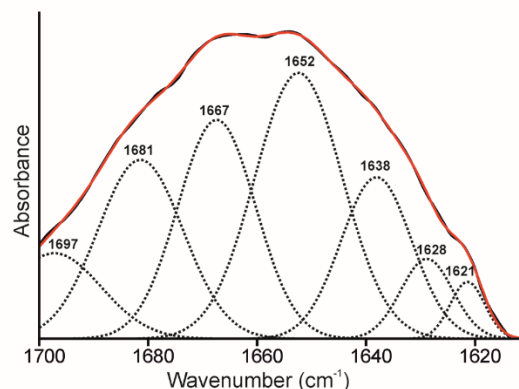

Fig. 5 Gaussian curve-fitting of amide I FTIR spectra of lysozyme stabilized AuNPs (black curve represents the original spectrum and red curve represents the overall fit).

Table 2 Secondary structure assignment of lysozyme in the amide I region

Frequency (cm^{-1})	Assignment
1628, 1681	β-Sheet
1638	Random coil
1652	α-Helix
1667	Turns

turation causes an increase in β content in amide I accompanied by a decrease in the α-helix content.[40] Such "helix to sheet" transitions have been observed in the irreversible thermal denaturation of many proteins.[40] Therefore, it reasonable to conclude that the integrity of the protein was not compromised during the reaction processes.

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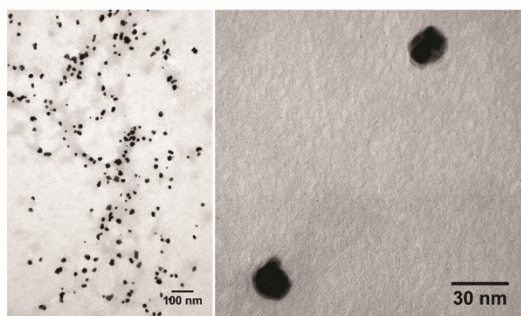gold nanoparticles for biological applications.*

Fig. 6 TEM images of the synthesized Lys–AuNPs.

Moreover, TEM images also confirmed the formation of gold nanoparticles using lysozyme as a reducing agent. Lysozyme–gold nanoparticles were generally uniformly dispersed and mainly spherical in morphology (Fig. 6). The average size of Lys–AuNPs was 35 ± 10 nm ranging between 10–45 nm. Particle size results from TEM images were smaller than the ones obtained from DLS analysis (~60 nm). The differences in particle size from DLS analysis and TEM imaging is not unusual. In general, particle sizes from DLS spectra are larger than those obtained from TEM images. DLS measures the hydrodynamic diameter of particles in liquids while TEM results are from the direct imaging of dry particles.

3.2. Synthesis and coating of MUA–AuNPs

Fig. 7A and Table 3 show the hydrodynamic diameter of the gold nanoparticles, including the polydispersity index as measured by DLS spectroscopy for PEI–PSS–Lys–PSS–PEI–MUA–AuNPs. The size of AuNPs increased during the sequential coating procedures from about 30–40 nm to just below 140 nm for PEI–PSS–Lys–PSS–PEI–MUA–AuNPs. We have found that different molecular weights of PEI and PSS did not appear to influence the final particle size. However, the molecular weight and the degree of branching of PEI has been reported to influence the efficacy of PEI based vectors in delivering plasmid DNA and siRNAs.[48] The most suitable PEI molecular weight ranges between 5 and 25 kDa. Higher molecular weights are associated with an increase in the cytotoxicity due to aggregation of huge clusters of the cationic polymer on the outer cell membrane and therefore inducing necrosis.[48]

The deposition of each layer was confirmed by zeta potential values (Fig. 7B and Table 3). A reversal of the zeta-potential after each deposition was observed.

Unpurified MUA–AuNPs were stored at room temperature for weeks. The hydrodynamic diameter was periodically tested to confirm that no aggregation had occurred (Fig. 8). The size of MUA–AuNPs ranged between 39–45 nm over weeks.

The deposition of the layers was also monitored by UV-vis in the wavelength range of 200 to 800 nm (Fig. 9). There was a gradual shift of the Au plasmon resonance peak from 525 nm to 565 nm as the layers were deposited on the particles.
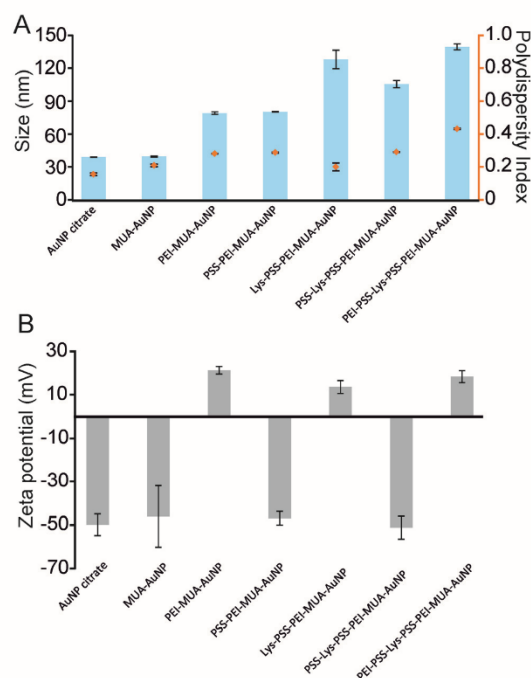

Fig. 7 (A) Hydrodynamic diameter and polydispersity index of PEI–PSS–Lys–PSS–PEI–MUA–AuNPs. (B) The effect of the zeta-potential of the subsequent coating steps.

Lysozyme exhibited the typical protein absorbance at 280 nm. The characteristic absorbance peak of PSS at 260 nm was also observed after the deposition of the polymer.
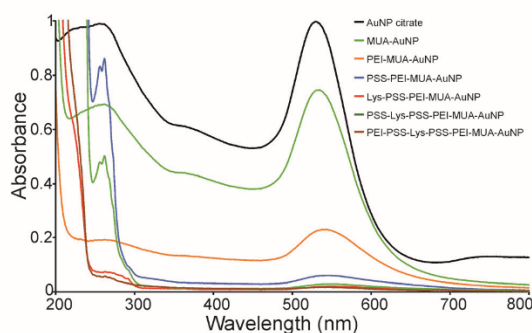
3.3. Lysozyme activity assay

Finally, enzymatic assays were carried out to verify the activity of the enzyme in addition to its observable presence confirmed by UV-vis. Using an extinction coefficient of 2.63 mL mg^{-1} cm^{-1}, the lysozyme concentration was estimated to be 3 μM for PEI–PSS–Lys–PSS–PEI–MUA–AuNPs and 2.7 μM for PEI–PSS–Lys–AuNPs. The free lysozyme concentration used for the enzymatic assay was 3 μM in order to compare enzymatic activities. The enzymatic activity of free lysozyme, encapsulated lysozyme *via* MUA–AuNPs and bioconjugates based on Lys–AuNPs was monitored for 24 minutes. Fig. 10 shows the enzymatic activities of free lysozyme and the lysozyme–gold nanoparticles. Within 6 minutes the enzymatic activity of free lysozyme decreased significantly and it became almost zero in 12 minutes. Bacteria wall degradation occurred immediately in the presence of pure native lysozyme such that within 12 minutes the lysozyme had digested all the bacteria used in the assay.

On the other hand, PEI–PSS–Lys–PSS–PEI–MUA–AuNPs and PEI–PSS–Lys–AuNP bioconjugates exhibited a different behaviour. The enzymatic activity increased in the first 6 minutes

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Table 3 Hydrodynamic diameter, polydispersity index and zeta potential values of PEI–PSS–Lys–PSS–PEI–MUA–AuNPs at different layers
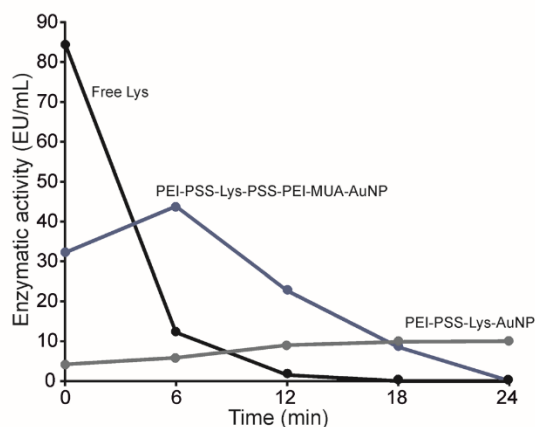
| | DLS measurements | | ELS measurements |
	Size (nm)	Polydispersity	ZP (mV)
AuNP citrate	38.82 ± 0.19	0.154 ± 0.008	−49.90 ± 4.96
MUA–AuNP	39.44 ± 0.48	0.208 ± 0.008	−46.10 ± 14.16
PEI–MUA–AuNP	79.18 ± 1.12	0.280 ± 0.002	21.25 ± 1.79
PSS–PEI–MUA–AuNP	80.38 ± 0.28	0.287 ± 0.003	−46.90 ± 3.16
Lys–PSS–PEI–MUA–AuNP	127.95 ± 8.50	0.199 ± 0.023	13.57 ± 2.93
PSS–Lys–PSS–PEI–MUA–AuNP	105.72 ± 3.33	0.290 ± 0.003	−51.24 ± 5.39
PEI–PSS–Lys–PSS–PEI–MUA–AuNP	139.51 ± 2.70	0.431 ± 0.004	18.36 ± 2.77
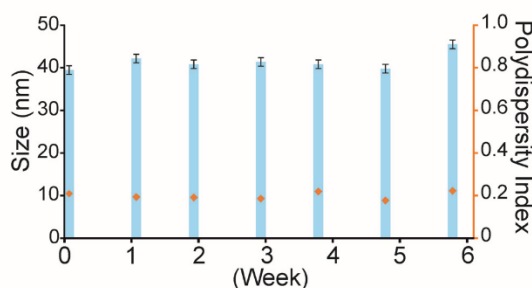
Fig. 8 Hydrodynamic diameter of MUA–AuNPs up to 6 weeks.

Fig. 9 Absorbance spectra of PEI–PSS–Lys–PSS–PEI–MUA–AuNPs at different layers.

Fig. 10 Enzymatic activities of free lysozyme and the lysozyme–gold nanoparticles expressed as EU per mL$_{enzyme\ sample}$.

due to protection from the polymeric layers. After that time PEI–PSS–Lys–PSS–PEI–MUA–AuNPs exhibited a decrease in activity to zero in 24 minutes. Surprisingly, the activity of PEI–PSS–Lys–AuNPs continued increasing after 6 minutes to reach a plateau. This implies that the interaction between lysozyme and AuNPs occurs in a different manner that modifies the activity of the enzyme.

It is therefore evident that the reaction conditions (high temperature for a long time) in the formation of Lys–AuNPs did not appear to affect the activity of the protein.

Moreover, the *in situ* synthesis and stabilization of Lys–AuNPs by lysozyme has considerable advantages compared to

the AuNPs fabricated *via* citrate reduction prior to stabilization by MUA. The former procedure is much shorter and involves less deposition. Moreover, the simultaneous reduction of gold ions into nucleating centers provides synergistic interactions between Lys and the nanoparticles. The Au nucleating centers produced upon the reduction of AuCl$_4^-$ ions are expected to have greater potential to break the disulfide groups and form covalent bonds.[20] On the other hand, the interaction of different layers in PEI–PSS–Lys–PSS–PEI–MUA–AuNPs is entirely electrostatic in nature. Electrostatic interactions are much weaker, thus exposing the enzyme to conditions that may impact its activity.

4. Conclusions

In summary, we have demonstrated the *in situ* synthesis and stabilization of protein modified gold nanoparticles without the use of activators under mild and near ambient conditions. The protein/nanoparticle conjugates were coated with poly (ethyleneimine). The enzymatic activity of the resulting protein/nanoparticle conjugates was evaluated. The enzymatic activity was compared with those prepared by the well-known

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citrate reduction of chloroauric acid followed by the layer-by-layer coating procedure. The activity of the protein was much longer for the protein/nanoparticle conjugates than for the native protein. Also, the protein/nanoparticle conjugates synthesized from protein reduction of chloroauric acid seem to retain the activity for a much longer time than those synthesized from citrate reduction. Further standardized experiments will be conducted to confirm this. While the utility of the technique was demonstrated for stabilization and activity of lysozyme, it could easily be adapted and optimized for other proteins. However, as the isoelectric point (pI) of most proteins is in the pH range of 4 to 7 it is very important to consider the pH of the solution in which the LbL-based layers will be performed as well as the charge of the polymeric materials used to encapsulate the protein. Other proteins, with a pI greater than 7, to be used as positive layer following the protocol described in this work include histone, cytochrome c, ribonuclease A, hemoglobin and myoglobin among others. It, therefore, has great potential in *in vivo* cytosolic protein delivery.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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CHAPTER 4

CONCLUSIONS

This chapter provides the conclusions extracted from the experimental results that support the achievement of the research objectives proposed for this dissertation relative to the electronic tongue technology applied to the analysis of grapes and wines.

1. Multisensor systems based on voltammetric sensors modified with phthalocyanines are a good alternative to analyze grapes and wines. Carbon paste electrodes (CPEs) based on phthalocyanines can be considered ideal for industrial applications because they are cheap and can be easily recovered by cleaning the surface with a filter paper. Sensors prepared by means of nanostructured films of LB or LbL are more sophisticated, but imply a high cost; however, they do improve the sensitivity of the sensors. Finally, biosensors can introduce a degree of specificity very useful for analyzing specific compounds of interest. In the case of the wine sector, this can be very interesting for monitoring the quality of grapes and wines.

2. An electronic tongue based on CPEs modified with metal oxide nanoparticles has been successfully used to monitor the phenolic maturity of grapes. Although oenologists tend to evaluate the sugar content and acidity to estimate the optimal ripening date, the phenolic content is a very important parameter that must be considered at the same level because it can strongly affect the final organoleptic properties of wines. The electrochemical sensors showed an interesting cross-selectivity and improved the limits of detection due to the different electrocatalytic properties provided by metal oxide nanoparticles. The electronic tongue has been shown to be very useful for establishing a better accuracy of the optimal harvesting date and it can therefore be applied as a supplementary tool to classical analytical techniques.

3. A sensor system based on CPEs modified with the skin of grapes has been successfully used to evaluate the phenolic maturity of grapes. The use of grape skin as the sensitive layer provides an easy and direct method to assess their phenolic maturity. However, the correlations between the chemical parameters obtained by traditional methods and the data obtained with the skin sensors were not so good as expected. In this sense, such factors as the skin properties of each variety, as well as environmental factors, can affect the degree of correlation.

   This study is considered an original approach for monitoring the phenolic maturity, although some issues must be highlighted: 1) The phenolic maturation process of the vintage and the structural and mechanical skin grape properties are essential factors in finding good

correlations between the responses provided by skin sensors and the chemical data measured by classical methods; and 2) the preparation of the sensors is a task that must be carried out carefully when peeling the skin off the grapes.

4. Sensors based on Ppy and Ppy/AuNP films evidenced that many factors can influence their conductivity and electrochemical responses, such as the electrodeposition technique used to prepare Ppy films, the way of incorporating AuNPs into the polymeric matrix, the importance of the substrate, as well as the electrolyte solution. The incorporation of AuNP films increased the conductivity of the films and provided electrochemical responses with higher intensities that are more reversible. The method used to introduce the AuNPs did not have a strong influence. However, the concentration of AuNPs was slightly higher when using the cogeneration method, as the SEM images showed. The electrocatalytic activity of the films was improved when using platinum substrate and chronopoentiometry. However, the limits of detection obtained with stainless steel substrate were similar to those obtained with films deposited onto Pt. Therefore, SS is a good substrate in the absence of KCl or similar, for implementation as a low priced electrochemical sensor. Based on this research, a voltammetric bioET, based on Ppy and Ppy/AuNP films, that is able to predict wine quality values of interest from the data obtained in musts has successfully been developed. The electrochemical sensors are stable during data acquisition, but they are of a single use, as must and wines are complex solutions with components (sugars, proteins and others) that adhere to the sensor surface. Additionally, the bioET was able to discriminate musts and wines in accordance with their chemical parameters: the Brix degree and TPI for musts and the alcoholic degree and TPI for wines.

    Phenolic compounds are important components of wines as they can strongly influence their final organoleptic properties. As time is a critical factor in the food industry, the development of this kind of system is clearly necessary to develop new analytical tools that can determine quality features in wines from the beginning of the vinification process, through analyses of the selected grapes for the elaboration of wine.

5. Sensors and biosensors based on PEDOT:PSS films have been shown to have good qualities for being used in the technology of electronic tongues. Moreover, the combination of the conducting polymer with a secondary electrocatalytic component improved the electrochemical responses and limits of detection for di-phenols and, furthermore, the enzymatic sensors provided higher intensities in the responses, lower limits of detection and

a better charge transfer between the analyte and the electrode surface than non-biosensors. In addition, the spin-coating technique is an easy and simple way to manufacture sensors based on conducting polymers in a short time.

An impedimetric electronic tongue based on the conducting polymer PEDOT:PSS has shown different responses in the EIS experiments carried out on red wines, providing a cross-selectivity. They were successfully used as sensing units in an impedimetric electronic tongue. The new feature extraction strategies of selecting parameters from the Nyquist and Bode plots, as well as using the parameters from the equivalent electric circuit, have improved the discrimination of red wines, which could not be achieved with the classical methodology of selecting the resistance and capacitance data at a fixed frequency. The excellent capability of discrimination was similar to those obtained with voltammetric and/or potentiometric tongues. Moreover, the data obtained with the impedimetric electronic tongue can be used to create regression models able to predict the quality parameters of interest in wines.

6. EQCM sensor arrays based on LbL films of metal phthalocyanines have successfully been applied to the discrimination of grape samples of different varieties. The EQCM sensors have provided different information related to the phenolic and sugar content, mainly redox processes, which can be observed in voltammetric measurements; whilst massograms provided information concerning the sugar content. Therefore, this kind of devices can be of interest in the analysis of grape juices and wines, since, simultaneously and in one single experiment, interesting information can be obtained about compounds with different natures that affect the quality of grapes and wines. Moreover, results based on the N-PLS analysis evidenced that it might be possible to establish prediction models able to determine the phenolic content and sugar parameters from the data obtained with the EQCM sensor array in grape samples, which is also very important for monitoring the quality of the grapes.

7. Finally, a protocol has been established to obtain the *in situ* synthesis and stabilization of lysozyme modified gold nanoparticles where interaction between them is a covalent bond. This protocol does not need the use of activators and could be extended and optimized to create strong interactions between other enzymes and the metal nanoparticles for electrochemical sensing purposes. Therefore, this work is of interest for improving the immobilization of the enzymes tyrosinase, laccase and glucose oxidase, which are usually implemented in our sensors to improve their specificity for phenols and sugars.

Although several research works have achieved the encapsulation of lysozyme by different strategies, not all of them verify that the lysozyme is still active after its assembly by means of an enzymatic and/or antimicrobial assay. Some of these works only report the presence of the enzyme by means of spectroscopy techniques. Here, we have demonstrated that the enzymatic activity of lysozyme remained intact after the encapsulation of the protein.

As final conclusions, the research work developed during this dissertation demonstrates that:

a) Electronic and bioelectronics tongues developed here are valid and reliable alternatives to analyze grapes and wines for quality purposes, allowing us to analyze such parameters of interest as the phenolic content, sugar or alcoholic degree, among others, which are very important for the organoleptic characteristics of wines.

b) Many electrocatalitic materials have been used and combined, such as AuNPs, metallophthalocyanines, conducting polymers and metal oxide nanoparticles, to modify electrochemical sensors, improving their performance with high reproducibitity, low limits of detection and high specificity, etc.

c) Biosensors based on different enzymes of interest have been developed to study the phenolic and sugar content in grapes and wines and we have implemented these biosensors in bioelectronics tongues. Moreover, biosensors that include specific enzymes have been shown to significantly improve the electrochemical responses of the non-enzymatic sensors.

c) A great variety of methodologies for electrochemical sensor preparation have been implemented, including simple (carbon paste, spin-coating, electrodeposition) and sophisticated (Layer by Layer) techniques.

d) Electronic tongue technology has allowed us to discriminate between grapes of different varieties and wines with different characteristics, as well as wines with different Denomination of Origin, etc.

e) The phenolic ripening of grapes can be monitored using multisensor systems to predict the optimal harvesting time, which is of great importance for wine growers. In the near future, these electronic devices could be applied *in situ* instead of carrying out lengthy and more expensive experiments in the laboratory.

f) The electrochemical responses obtained when analyzing grapes can be used to predict the quality parameters of future wines using appropriate chemometric tools for data processing.

g) A new strategy to covalently bond enzymes to gold nanoparticles has been explored that was easy to obtain from the lysozyme protein and that can be applied to other enzymes of interest for sensing applications in the food industry.

ANNEXES

Annex A: Resumen en castellano

1.- Introducción

La Tesis Doctoral titulada "Tecnología de la lengua electrónica aplicada al análisis de uvas y vinos" ha sido desarrollada en el grupo de sensores UVaSens bajo la dirección de las Profesoras María Luz Rodríguez Méndez, del Departamento de Química Inorgánica y Química Física de la Escuela de Ingenieros Industriales, y Ana Cristina García Cabezón, del Departamento de Ciencia de Materiales e Ingeniería Metalúrgica de la Escuela de Ingenieros Industriales, ambas de la Universidad de Valladolid (España). Además, la autora de esta tesis ha realizado una estancia de investigación de 4 meses en la Universidad Estatal de Misuri (Missouri State University, Springfield, EE.UU.) bajo la supervisión del Profesor Adam Wanekaya para optar a la Mención Internacional de doctorado.

Esta disertación contiene trabajos experimentales que se han publicado en revistas internacionales donde se ha estudiado el uso de sensores y biosensores implementados como redes de electrodos en lenguas electrónicas y bioelectrónicas para analizar parámetros relacionados con la calidad de vinos y uvas. Para la construcción de estas redes de sensores se han utilizado y/o combinado diferentes y novedosos materiales electrocatalíticos. Las muestras de vino y uva utilizadas en este estudio han sido proporcionadas por las bodegas "Cooperativa de Cigales" e "Instituto Tecnológico Agrario de Castilla y León (ITACyL)", ambas situadas en la región de Castilla y León (Valladolid, España), y por el "Centro Enológico de Haro" situado en La Rioja (Haro, España). Los parámetros químicos de las muestras fueron analizados en el laboratorio del grupo UVaSens y en el ITACyL siguiendo la normativa internacional (OIV, The International Organisation of Vine and Wine).

El desarrollo de nuevos métodos de análisis para caracterizar los alimentos es de vital importancia para mejorar los actuales sistemas de control de calidad de los productos alimenticios. Dentro de este campo, el concepto de lengua electrónica (ETs o e-tongues) ha crecido rápidamente en los últimos años debido a su gran potencial. Estos dispositivos se basan en sensores electroquímicos combinados con análisis de datos multivariantes. De acuerdo con la IUPAC (Unión Internacional de Química Pura y Aplicada), una lengua electrónica es un sistema multisensor, que consiste en un número de sensores de baja selectividad y utiliza procedimientos matemáticos avanzados para el procesamiento de señales basados en el reconocimiento de patrones (PARC) y/o análisis multivariante [redes neuronales artificiales (RNA), análisis de componentes principales (PCA), etc.]. Por lo tanto, las ETs son sistemas holísticos que proporcionan información global y cualitativa acerca de la muestra en lugar de datos cuantitativos

acerca de compuestos específicos. Sin embargo, si la matriz de datos obtenida por estos sistemas se analiza con herramientas de procesamiento quimiométrico adecuadas, se podría extraer información descriptiva o predictiva de parámetros específicos. Existe un término más reciente en el campo de las lenguas electrónicas, ampliamente denominado lengua bioelectrónica (bioET), que incluye el uso de uno o varios biosensores implementados en las ETs.

Durante esta investigación se han aplicado ETs y bioETs para estudiar las uvas tintas y los vinos con el fin de predecir mejor el momento óptimo de la vendimia de uvas, así como los parámetros de calidad de interés en los vinos.

El vino es la bebida alcohólica obtenida de la fermentación de la uva y es uno de los alimentos más antiguo conocido desde hace más de 8000 años. El origen de la viticultura se remonta a los años 6000-5000 a.C. en Georgia e Irán. Desde entonces, la cultura vitivinícola sigue siendo prácticamente la misma: el vino moderno es muy similar al que elaboraban nuestros antepasados, y las prácticas modernas de viticultura y enología todavía se refieren a las practicadas por los antiguos griegos, que fueron conservadas y luego evolucionadas durante el Imperio Romano.

La matriz del vino presenta una de las composiciones químicas más complejas con más de 800-1000 compuestos volátiles que van desde alcoholes, ésteres, ácidos, cetonas, éteres, aldehídos, terpenos, lactonas, compuestos fenólicos, etc., procedentes de la uva y/o que se forman durante el proceso de fermentación, con concentraciones muy variadas. La abundancia de estos compuestos depende de muchos factores: variedad y origen de la uva, prácticas vitivinícolas (mezcla, envejecimiento, levadura utilizada durante la fermentación), tratamiento de la producción, almacenamiento y exposición al oxígeno, etc.

Los análisis rutinarios que se llevan a cabo en los laboratorios enológicos, desde la vendimia de la uva hasta el producto final embotellado, incluyen una serie de cromatografías, así como técnicas más tradicionales (espectroscopía, fotometría, ensayos enzimáticos, etc.). Estas técnicas analíticas se combinan para obtener un estudio profundo de la composición de los vinos, lo que da lugar a análisis muy costosos, que consumen mucho tiempo y exigen personal altamente cualificado. Por lo tanto, es necesario desarrollar nuevas tecnologías que nos permitan obtener una información global de las muestras en lugar de información de componentes específicos. Desde el punto de vista analítico, el análisis del vino es una tarea difícil debido a la complejidad de la mezcla y, además, pequeñas diferencias en la concentración de ciertos

compuestos dan lugar a vinos con características organolépticas completamente diferentes. Además, la sinergia entre grupos de compuestos tiene a menudo una mayor influencia en las características organolépticas que los compuestos individuales.

Tradicionalmente, los viticultores han estimado la fecha óptima de vendimia evaluando el contenido de azúcar y la acidez de las uvas, pero otros compuestos como los fenoles son tan importantes como los azúcares y ácidos presentes en las uvas debido a que la composición fenólica afecta directamente a las características organolépticas (sabor, color, textura, olor) de los vinos.

Los sistemas ETs pueden convertirse en una alternativa o complemento a los métodos tradicionales de análisis de vino y uva. Además, estos sistemas pueden ser utilizados no sólo en los laboratorios analíticos sino también *in situ* por los agricultores y viticultores en los viñedos como una herramienta de control de calidad.

Los sensores utilizados en lenguas electrónicas son principalmente ópticos, electroquímicos y gravimétricos. En aplicaciones para el análisis de alimentos, los más utilizados son los sensores electroquímicos (potenciométricos, amperométricos, voltamperométricos e impedimétricos) debido a su sencilla instrumentación, alta sensibilidad, bajo tiempo de respuesta y facilidad de operación.

Dentro de la investigación que aplica lenguas electrónicas, existe un especial interés en implementar materiales electrocatalíticos en los sensores electroquímicos que mejore las prestaciones de estos dispositivos, especialmente su sensibilidad y especificidad. En este sentido, los materiales utilizados para los sensores electroquímicos se pueden clasificar como: 1) materiales para el electrodo y el sustrato de soporte, 2) materiales para mejorar las prestaciones electroanalíticas, 3) materiales para la inmovilización de elementos de reconocimiento biológico y 4) elementos biológicos, siendo estos dos últimos aplicables a los biosensores electroquímicos. La elección de estos materiales sensibles para su aplicación en lenguas electrónicas o bioelectrónicas es crucial para obtener un alto rendimiento. En función de sus propiedades, estos materiales pueden actuar de forma diferente en un sistema electroquímico aunque las funciones básicas de los mismos pueden clasificarse principalmente como: 1) mejora de la transferencia de electrones, 2) catálisis de reacciones electroquímicas, 3) inmovilización de biomoléculas, 4) etiquetado de biomoléculas y 5) actuar como reactante. En los trabajos de investigación desarrollados durante la presente Tesis Doctoral se han utilizado diferentes materiales sensibles como son ftalocianinas, nanopartículas metálicas, materiales a base de carbono y polímeros

conductores para mejorar la sensibilidad de los sensores y, también, se ha trabajado con enzimas de interés para la detección de fenoles y azúcares con el fin de mejorar la especificidad de los sensores.

Por otro lado, los sensores se pueden modificar químicamente con materiales electrocatalíticos mediante un gran variedad de técnicas, abarcando la preparación de sensores clásicos, preparados mediante técnicas sencillas (electrodos de pasta de carbono, *casting*, electrodeposición, etc.), hasta la implementación de técnicas más sofisticadas y costosas para fabricar sensores nanoestructurados mediante técnicas como SAM (*Self-assembled Monolayer*, SAM), *Layer by Layer* (LbL) o Langmuir-Blodgett (LB).

2.- Objetivos

El objetivo principal de esta investigación ha sido el desarrollo de nuevos sensores electroquímicos utilizando materiales electrocatalíticos que mejoren las prestaciones en cuanto a la sensibilidad de los sensores. Estos sensores fueron después implementados en lenguas electrónicas y bioelectrónicas para el estudio, principalmente, de antioxidantes y polifenoles presentes en la uva y el vino.

Los sensores que se han desarrollado en este trabajo de investigación han sido tanto de tipo no-nanoestructurados como nanoestructurados, para lo cual la nanotecnología ha jugado un papel muy importante en su fabricación mediante el uso de diferentes técnicas de modificación de superficies. Además, también se han combinado algunos de los materiales electrocatalíticos utilizados para así estudiar el efecto sinérgico entre ellos y la mejora en la respuesta de los sensores. Los dispositivos de ETs y/o bioETs se han utilizado para analizar muestras reales de vino y uva. Mediante los análisis estadísticos apropiados, aplicados los datos multivariantes obtenidos de los análisis, se ha discriminado entre las muestras analizadas y se han establecido correlaciones entre los datos obtenidos con los dispositivos ETs y bioETs y los parámetros químicos obtenidos por el análisis tradicional.

Los objetivos específicos del trabajo de investigación han sido:

1. Diseñar y construir lenguas electrónicas y bioelectrónicas específicamente dedicadas al análisis de calidad de vinos y uvas mediante sensores electroquímicos modificados químicamente.

2. Mejorar el rendimiento de los sensores mediante: (a) nuevos materiales electrocatalíticos, (b) sensores basados en películas nanoestructuradas, (c) combinación de diferentes materiales electrocatalíticos para aumentar la capacidad electroquímica de los sensores y (c) la introducción de enzimas para mejorar la especificidad de los sensores para detectar compuestos fenólicos y/o azúcares.

3. Implementar diferentes técnicas de modificación de superficie como, por ejemplo, electrodeposición, *spin-coating* y/o *Layer by Layer* con el fin de mejorar el rendimiento de los sensores, tales como su sensibilidad, reproducibilidad y/o vida útil.

4. Aplicar diferentes técnicas electroquímicas de análisis (voltametría cíclica, espectroscopía de impedancias, microbalanza electroquímica de cristal de cuarzo, etc.) para evaluar su versatilidad.

5. Estudiar nuevos métodos para unir enzimas con nanomateriales (ej. nanopartículas metálicas) mediante interacciones fuertes de tipo covalente.

6. Validar la lengua electrónica como herramienta de control durante la maduración fenólica de la uva, desde el momento de la maduración hasta la fecha de óptima de maduración.

7. Evaluar la capacidad de las lenguas electrónicas en la autenticación de muestras de uva y vino.

8. Establecer modelos de correlación válidos entre los resultados obtenidos con la lengua electrónica y/o bioelectrónica en muestras reales de vino y uva con los parámetros químicos medidos con métodos analíticos tradicionales.

9. Evaluar la capacidad del sistema para predecir parámetros de calidad en vinos a partir de los datos obtenidos del análisis de uvas utilizadas para elaborar los correspondientes vinos.

3.- Metodología y resultados

Para llevar a cabo los objetivos propuestos se han realizado diferentes trabajos de investigación en el grupo UVaSens donde se han utilizado diferentes materiales electrocatalíticos, técnicas de preparación de sensores y técnicas electroquímicas de análisis. Además, se han llevado a cabo diferentes análisis en muestras de uva y vino para validar los sistemas de ETs y bioETs.

UVaSens es un grupo multidisciplinar experto en el desarrollo de sensores electroquímicos, principalmente voltamperométricos, para su aplicación en lenguas electrónicas. El trabajo de investigación del grupo incluye la fabricación y caracterización de sensores clásicos basados en CPE, *spin-coating* o electrodeposición, pero también la preparación de películas delgadas nanoestructuradas utilizando técnicas como *Layer by Layer* o Langmuir-Blodgett. Además, para la construcción de los sensores, el grupo ha utilizado ampliamente materiales electrocatalíticos como ftalocianinas y bisftalocianinas, tanto en sensores nanoestructurados como no nanoestructurados. El grupo también ha implementado polímeros conductores en los sensores cuando comenzaron a ser aplicados en sensores electroquímicos. Por otro lado, el grupo UVaSens fue uno de los primeros en desarrollar lenguas electrónicas voltamperométricas modificadas basadas en CPE y ftalocianinas para mejorar la respuesta electroquímica. Así, el artículo I que se presenta en esta Tesis, *"Multisensor systems based on phthalocyanines for monitoring the quality of grapes"*, es un *review* que ofrece una visión general de las lenguas electrónicas basadas en ftalocianinas para el análisis de uvas en el que se discute el uso de sensores y biosensores no nanoestructurados y nanoestructurados.

Los sistemas multisensor basados en sensores voltamperométricos modificados con ftalocianinas son una buena alternativa para analizar uvas y vinos. Los electrodos de pasta de carbono (CPEs) basados en ftalocianinas pueden considerarse ideales para aplicaciones industriales porque son baratos y pueden recuperarse fácilmente limpiando la superficie con un papel de filtro. Los sensores preparados mediante películas nanoestructuradas LB o LbL son más sofisticados implicando un alto coste, sin embargo, mejoran la sensibilidad de los sensores ya que ofrecen una mayor relación superficie-volumen. Finalmente, los biosensores basados en sensores modificados con enzimas pueden introducir un grado de especificidad muy útil para analizar compuestos específicos de interés, como pueden ser los azúcares y polifenoles en el caso del vino y la uva. Por lo tanto, los biosensores pueden ser muy interesantes para el control de la calidad de las uvas y los vinos.

Los artículos II a VII se centran en sensores electroquímicos preparados mediante técnicas clásicas como son CPEs, electrodeposición y *spin-coating.* En el artículo II, *"Monitoring the phenolic ripening of red grapes using a multisensor system based on metal-oxide nanoparticles"*, el grafito se mezcló con diferentes nanopartículas de óxidos metálicos para construir una red de sensores químicamente modificados. Esta red de sensores se ha utilizado por primera vez como lengua electrónica voltamperométrica para monitorizar la madurez de las uvas en función de su madurez fenólica. Además, las respuestas voltamétricas obtenidas con

esta lengua electrónica mostraron una buena correlación con el contenido fenólico de las uvas. Con estos sensores se consiguió una interesante selectividad cruzada en las respuestas y límites de detección en compuestos fenólicos del mismo orden que los obtenidos con otros sensores más sofisticados y nanoestructurados, todo ello gracias a las propiedades electrocatalíticas que proporcionan las nanopartículas de óxidos metálicos. Estos sensores tienen la ventaja de que pueden recuperarse fácilmente limpiando la superficie con un papel de filtro, lo que los hace muy apropiados para aplicaciones industriales.

En el artículo III, "*A different approach for the analysis of grapes: Using the skin as sensing element*", se ha desarrollado un trabajo original, nunca antes propuesto, en el que se ha sustituido el uso de un material electrocatalítico para modificar el electrodo CPE por la propia piel de la uva. Para ello, la piel de la uva se depositó sobre la superficie de un electrodo CPE con un contacto íntimo entre el grafito y la cara interna de la piel. Teniendo en cuenta que los hollejos de la uva contienen fenoles que serán liberados a la pulpa durante el proceso de maduración e influirán en el color, sabor y calidad de las uvas y los vinos, se pensó en estudiar la madurez de la uva directamente mediante el análisis de la piel. Las respuestas voltamétricas de los sensores durante la maduración de las uvas mostraron cambios en las intensidades y los picos asociados a los compuestos polifenólicos y se ha podido monitorizar la madurez fenólica de las uvas. Sin embargo, algunos aspectos deben ser mejorados y tomados en cuenta para obtener mejores correlaciones entre las respuestas de los sensores y los parámetros químicos obtenidos por los métodos tradicionales. En este sentido, factores como las propiedades de la piel de cada variedad y los factores ambientales pueden afectar el grado de correlación. Además, en este caso, estos sensores son de un solo uso, ya que la limpieza de la superficie con un papel de filtro destruiría la piel despositada sobre la superficie del electrodo.

En los artículos IV a VII se han utilizado polímeros conductores en la preparación de los sensores y/o biosensores. Así, en el artículo IV, "*Electrochemical behavior of polypyrrol/AuNP composites deposited by different electrochemical methods: sensing properties towards catechol*", se ha electrodepositado una película de polipirrol sobre sustratos de platino y acero inoxidable y, también, se han incorporado AuNPs en las películas para mejorar el efecto electrocatalítico de los sensores. Los sensores preparados se caracterizaron utilizando SEM, EIS y voltamperometría cíclica. Posteriormente, se aplicaron para analizar el polifenol catecol, que es un grupo fenólico de interés en el proceso de oxidación del vino. Los resultados obtenidos con los sensores basados en películas de Ppy y Ppy/AuNPs evidenciaron que muchos factores pueden influir en su conductividad y respuesta electroquímica,

tales como la técnica de electrodeposición utilizada para preparar las películas de Ppy (cronopotenciometría o cronoamperometría), el método de incorporación de AuNPs en la matriz polimérica, la importancia del sustrato, así como el medio electrolito utilizado para realizar las medidas electroquímicas. La incorporación AuNPs en la matriz polimérica aumenta la conductividad de las películas y proporciona respuestas electroquímicas con intensidades más altas y más reversibles. La influencia del método utilizado para introducir las AuNPs, ya sea previamente sintetizadas y mezcladas con el precursor del polímero antes de realizar la electrodeposición (atrapamiento) o mediante la síntesis *in situ* durante la electrodeposición a partir de la sal precursora (cogeneración), no influye significativamente en la respuesta electroquímica. Sin embargo, la concentración de AuNPs es ligeramente superior cuando se utiliza el método de cogeneración, como han demostrado las imágenes SEM. La actividad electrocatalítica de las películas mejora cuando se utiliza un sustrato de platino y la técnica de cronopoentiometría. Los límites de detección obtenidos con sustrato de acero inoxidable son similares a los obtenidos con películas depositadas sobre platino y, por tanto, supone una alternativa de bajo coste a los sustratos basados en metales nobles utilizados en sensores electroquímicos.

Los sensores desarrollados en el artículo IV se han utilizado en el trabajo de investigación que se expone el artículo V, "*Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator*". En este trabajo se ha implementado, por primera vez, una lengua bioelectrónica voltamétrica para el análisis de mostos y los vinos elaborados a partir de ellos. La lengua bioelectrónica consistió en sensores de Ppy/AuNPs donde se ha depositado una enzima sobre el sustrato mediante la téncia de *drop-casting* y entrecruzamiento con glutaraldehído. De este modo, se desarrollaron biosensores a partir de los sensores de Ppy/AuNPs que mejor respuesta electroquímica ofrecieron en el trabajo de investigación previo. Las enzimas que se han utilizado en este estudio fueron tirosinasa y glucosa oxidasa, para mejorar la especificidad de los sensores a los fenoles y azúcares, respectivamente. La novedad de este trabajo radica en el hecho de que, nunca antes, se habían utilizado los datos voltamétricos obtenidos en uvas para predecir parámetros de calidad de los vinos elaborados con dichas uvas. Los sensores electroquímicos ofrecieron respuestas estables en mostos y vinos, pero son de un solo uso debido a que las muestras son mezclas muy complejas con componentes que se adhieren a la superficie del sensor (azúcares, proteínas y otros). En este trabajo se han utilizado las técnicas multivariantes de PARAFAC y PCA para discriminar entre

las muestras analizadas a partir de los datos obtenidos con la lengua bioelectrónica. Se han discriminado los mostos de diferente variedad de uva de acuerdo con su contenido en azúcares y contenido polifenólcio y, también, se discriminaron vinos con distinto grado alcohólico y contenido polifenólico. Además, el análisis con PLS-1 permitió establecer modelos de correlación aceptables con los parámetros químicos de azúcar, grado alcohólico y contenido polifenólico y, finalmente, se ha podido predecir el contenido polifenólico y el grado alcohólico de muestras de vino a partir de los datos voltamétricos obtenidos en los mostos de uva que se utilizaron para elaborar dichos vinos. Este resultado es muy interesante, ya que los viticultores podrían conocer cuál será la calidad de sus vinos antes de su elaboración, simplemente a partir de los datos obtenidos en las uvas de vendimia.

Los artículos VI y VII se basan en el polímero conductor PEDOT:PSS. Así, en el artículo VI, "*Layered composites of PEDOT/PSS/nanoparticles and PEDOT/PSS/phthalocyanines as electron mediators for sensors and biosensors*", se prepararon sensores modificados con PEDOT:PSS mediante la técnica de *spin-coating* sobre sustrato de ITO. En una segunda etapa, se han modificado los sensores de PEDOT:PSS incorporado otros materiales electrocatalíticos como AuNPs, ftalocianina de cobre y bisftalocianina de lutecio, con el fin de mejorar el rendimiento electroquímico de los sensores. Además, las enzimas tirosinasa y lacasa se depositaron en la superficie de los electrodos utilizando también la técnica de *spin-coating*. Estos sensores y biosensores han mostrado excelentes límites de detección y reproducibilidad hacia el polifenol catecol y su isómero hidroquinona. Los sensores enzimáticos proporcionaron mayores intensidades en las respuestas, mejores límites de detección y una mejor transferencia de carga entre el analito y la superficie del electrodo que los no-enzimáticos.

Tras los resultados obtenidos con los sensores basados en PEDOT:PSS, en el artículo VII, "*Impedimetric electronic tongue based on nanocomposites for the analysis of red wines. Improving the variable selection method*", se han aplicado estos sensores en lengua electrónica impedimétrica para la autentificación de vinos elaborados con diferentes variedades de uva y de diferentes Denominaciones de Origen. Esta aplicación es novedosa ya que sólo existe un trabajo de investigación que reporte el uso de una lengua impedimétrica para el análisis de vinos. Además, el uso de lenguas electrónicas impedimétricas aplicadas al análisis de alimentos es bastante escaso. Los sensores basados en PEDOT:PSS han mostrado diferentes respuestas en los experimentos EIS hacia los vinos tintos proporcionando una selectividad cruzada y, por lo tanto, se ha obtenido una buena discriminación entre los diferentes vinos analizados.

En este trabajo, además, se ha llevado a cabo una nueva estrategia de extracción de variables a partir de parámetros obtenidos de los gráficos de Nyquist y Bode, así como el uso de parámetros del circuito eléctrico equivalente, que han mejorado notablemente la discriminación de los vinos tintos, resultado que no se ha obtenido mediante la metodología clásica que utiliza los datos de resistencia y capacitancia a una frecuencia fija. La excelente capacidad de discriminación fue similar a la obtenida con lenguas voltamperométricas y/o potenciométricas. Además, los datos obtenidos con la lengua electrónica impedimétrica se han utilizado para crear modelos de regresión capaces de predecir los parámetros de calidad de interés en los vinos.

En los artículos VIII y IX se ha aplicado la nanociencia y la nanotecnología, concretamente, la tecnología de *Layer by Layer* (LbL), aunque con dos propósitos diferentes. En el artículo VIII, "***An electrochemical quartz crystal microbalance multisensor system based on phthalocyanine nanostructured films: Discrimination of musts***", se depositaron películas LbL nanoestructuradas de ftalocianinas metálicas en sensores de microbalanza de cristal de cuarzo (QCM) para su uso como lengua electrónica voltamétrica y másica. Estos cristales están recubiertos por un electrodo soporte que consiste en una lámina de platino. En concreto, se utilizaron diferentes ftalocianinas sulfónicas para obtener una mayor selectividad cruzada en las respuestas voltamétricas. Esta lengua electrónica se ha utilizado por primera vez en el análisis de alimentos para registrar de manera simultáneamente datos voltamperométricos y cambios de masa que ocurren durante los procesos de oxidación y reducción en mostos de uva. Se analizaron y discriminaron mostos de uvas de diferentes variedades en función de su contenido en azúcares y fenoles. Los procesos redox observados en las medidas voltamétricas aportan información sobre el contenido de polifenoles, mientras que los masogramas proporcionan información sobre el contenido de azúcar. La principal ventaja de los sensores basados en QCM sobre los sensores electroquímicos tradicionales es que proporcionan más información (electroquímica y másica) en un solo experimento. Además, en comparación con los trabajos mencionados anteriormente, los sensores basados en nanoestructuras LbL son más sofisticados y proporcionan una mayor relación superficie/volumen, pero siguen siendo fáciles de preparar y económicos. Por lo tanto, este tipo de dispositivos puede ser de gran interés para utilizarse en el análisis de alimentos ya que se puede obtener, simultáneamente y en un solo experimento, información interesante sobre compuestos de diferente naturaleza. Además, mediante el análisis con N-PLS se establecieron modelos de predicción capaces de determinar el contenido fenólico y los parámetros de azúcar a partir de los datos obtenidos con la lengua electrónica basada en sensores QCM, lo que puede ser una alternativa para controlar la calidad de las uvas.

En el último trabajo, "*In situ synthesis, stabilization and activity of protein-modified gold nanoparticles for biological applications*", también se ha utilizado la técnica LbL, si bien en este trabajo se ha utilizado para preparar una nanoestructura que contiene AuNPs y la enzima lisozima. Este trabajo ha sido desarrollado en la Universidad Estatal de Misuri (Springfield, USA) durante una estancia de investigación de 4 meses. Aunque este trabajo no se basa en el desarrollo de sensores para su aplicación en lenguas electrónicas, o incluso en la industria alimentaria, puede extenderse y optimizarse a este campo ya que el ensamblaje que se ha desarrolado se puede aplicar al caso de otras enzimas que sean de interés para la industria de la alimentación como la tirosinasa, lacasa o glucosa oxidasa, que ya han sido utilizados en los trabajos mencionados anteriormente. Aunque en otros trabajos de investigación se ha logrado la encapsulación de la lisozima mediante diferentes estrategias, no todos ellos verifican que la lisozima tenga actividad enzimática después de su ensamblaje mediante un ensayo enzimático y/o antimicrobiano. Algunos de estos trabajos sólo informan de la presencia de la enzima mediante técnicas de espectroscopía. En este trabajo se ha demostrado que la actividad enzimática de la lisozima permanece intacta después de su encapsulación.

4.- Conclusiones

A continuación, se presentan unas conclusiones generales que apoyan la consecución de los objetivos de investigación propuestos en esta disertación sobre la tecnología de la lengua electrónica aplicada al análisis de uvas y vinos.

a) Las lenguas electrónicas y bioelectrónicas que se han desarrollado son alternativas válidas y fiables para analizar la calidad de uvas y vinos que permite evaluar parámetros de interés tales como el contenido fenólico, azúcar o grado alcohólico, entre otros, muy importantes para las características organolépticas de los vinos.

b) Se han utilizado y combinado muchos materiales electrocatalíticos como AuNPs, ftalocianinas metálicas, polímeros conductores y nanopartículas de óxido metálicos para modificar los sensores electroquímicos y mejorar el rendimiento de los sensores en términos de alta reproducibilidad, bajos límites de detección, alta especificidad, etc.

c) Se han desarrollado biosensores basados en diferentes enzimas de interés para el estudio del contenido fenólico y en azúcares en uvas y vinos y se han implementado estos biosensores en lenguas bioelectrónicas. Además, los biosensores que incluyen enzimas

específicas han demostrado que mejoran significativamente las respuestas electroquímicas con respecto a los sensores no-enzimáticos.

c) Se han aplicado una gran variedad de metodologías para la preparación de los sensores electroquímicos que incluyen técnicas simples (pasta de carbono, *spin-coating*, electrodeposición) y otras más sofisticadas (*Layer by Layer*).

d) Los sistemas de lengua electrónica han permitido discriminar entre uvas y vinos de diferentes variedades y características, vinos con diferentes Denominaciones de Origen, etc.

e) La maduración fenólica de las uvas puede ser monitorizada mediante sistemas multisensor con el fin de predecir el momento óptimo de la vendimia, lo que es de gran importancia para los viticultores que, en un futuro próximo, podrían aplicar estos dispositivos electrónicos *in situ* en lugar de llevar a cabo experimentos en los laboratorios que requieren de mucho tiempo, personal cualificado y son más costosos.

f) Las respuestas electroquímicas obtenidas en el análisis de las uvas pueden ser utilizadas para predecir los parámetros de calidad de los futuros vinos mediante el uso de un análisis quimiométrico de datos adecuado.

g) Se ha explorado una nueva estrategia para unir covalentemente la enzima lisozima a nanopartículas de oro, fácil de obtener y que puede aplicarse a otras enzimas de interés específicas para evaluar el contenido fenólico y en azúcares.

Dado que el tiempo es un factor crítico en la industria del vino, el desarrollo de este tipo de sistemas basados en lenguas electrónicas puede ser novedoso y de gran interés para desarrollar nuevas herramientas analíticas que permitan determinar la calidad de los vinos desde el inicio del proceso de vinificación, analizando las uvas seleccionadas para la elaboración del vino. De esta forma, los enólogos podrán conocer las características organolépticas y la calidad de los vinos desde la etapa más temprana.

Annex B: List of publications

The co-authored list of papers published during the Doctoral studies is attached below:

- C. García-Hernández, A. Freese, M.L. Rodríguez-Méndez, A.K. Wanekaya. *In situ* synthesis, stabilization and activity of protein modified gold nanoparticles for biological applications. *Biomaterials Science* 7 (2019) 2511-2519.
  doi: 10.1039/c9bm00129h
  Impact Factor (2018 JCR): 5.251; Quartil (2018 JCR): Q1, Category: Materials Science, Biomaterials.

- C. García-Cabezón, C. García-Hernández, M.L. Rodríguez-Méndez, F. Martín-Pedrosa. A new strategy for corrosion protection of porous stainless steel using polypyrrole films. *Journal of Materials Science and Technology* (2019) **[In Press, Journal Pre-proof]**.
  doi: 10.1016/j.jmst.2019.05.071
  Impact Factor (2018 JCR): 5.040; Quartil (2018 JCR): Q1, Category: Metallurgy and Metallurgical Engineering; Materials Science, Multidisciplinary.

- C. García-Hernández, C. García-Cabezón, F. Martín-Pedrosa, M.L. Rodríguez-Méndez. Analysis of musts and wines by means of a bio-electronic tongue based on tyrosinase and glucose oxidase using polypyrrole/gold nanoparticles as the electron mediator. *Food Chemistry* 289 (2019) 751–756.
  doi: 10.1016/j.foodchem.2019.03.107
  Impact Factor (2018 JCR): 5.399; Quartil (2018 JCR): Q1, Categories: Food Science and Technology; Chemistry, Applied; Nutrition and Dietetics.

- C. García-Hernández, C. Salvo Comino, F. Martín-Pedrosa, M.L. Rodríguez-Méndez, C. García-Cabezón. Impedimetric electronic tongue based on nanocomposites for the analysis of red wines. Improving the variable selection method. *Sensors and Actuators B* 277 (2018) 365–372.
  doi: 10.1016/j.snb.2018.09.023
  Impact Factor (2018 JCR): 6.393; Quartil (2018 JCR): Q1, Categories: Electrochemistry; Chemistry, Analytical; Instruments and Instrumentation.

➕ R. Muñoz, <u>C. García-Hernández</u>, C. Medina-Plaza, C. García-Cabezón, J.A. Fernández-Escudero, E. Barajas, G. Medrano, M.L. Rodríguez-Méndez. A different approach for the analysis of grapes: Using the skin as sensing element. *Food Research International* 107 (2018) 544–550.
doi: 10.1016/j.foodres.2018.02.060
Impact Factor (2018 JCR): 3.579; Quartil (2018 JCR): Q1, Category: Food Science and Technology.

➕ C. Salvo-Comino, <u>C. García-Hernández</u>, C. García-Cabezón, M.L. Rodríguez-Méndez. Discrimination of milks with a multisensor system based on layer-by-layer films. *Sensors* 18 (2018) 2716.
doi: 10.3390/s18082716.
Impact Factor (2018 JCR): 3.031; Quartil (2018 JCR): Q1, Categories: Instruments and Instrumentation.

➕ <u>C. García-Hernández</u>, C. Medina-Plaza, C. García-Cabezón, Y. Blanco, J.A. Fernández-Escudero, E. Barajas-Tola, M.A. Rodríguez-Pérez, F. Martín-Pedrosa, M.L. Rodríguez-Méndez. Monitoring the phenolic ripening of red grapes using a multisensor system based on metal-oxide nanoparticles. *Frontiers in Chemistry* 6 (2018) 131.
doi: 10.3389/fchem.2018.00131
Impact Factor (2018 JCR): 3.782; Quartil (2018 JCR): Q2, Categories: Chemistry, Multidisciplinary.

➕ R. González-Antón, M.M. Osipova, <u>C. García-Hernández</u>, T.V. Dubinina, L.G. Tomilova, C. García-Cabezón, M.L. Rodríguez-Méndez. Subphthalocyanines as electron mediators in biosensors based on phenol oxidases: Application to the analysis of red wines. *Electrochimica Acta* 255 (2017) 239–247.
doi: 10.1016/j.electacta.2017.09.168
Impact Factor (2017 JCR): 5.116; Quartil (2017 JCR): Q1, Category: Electrochemistry.

- M.L. Rodríguez-Méndez, J.A. de Saja, R. González-Antón, <u>C. García-Hernández</u>, C. Medina-Plaza, C. García-Cabezón, F. Martín-Pedrosa. Electronic noses and tongues in wine industry. *Frontiers in Bioengineering and Biotechnology* 4 (2016) 1-12.
  doi: 10.3389/fbioe.2016.00081
  Impact Factor (2018 JCR): 5.122; Quartil (2018 JCR): Q1, Category: Multidisciplinary Sciences.

- M.L. Rodríguez-Méndez, <u>C. García- Hernández</u>, C. Medina-Plaza, C. García-Cabezón, D. Paniagua-Albillos, S. Rodríguez-Sánchez, J.A. de Saja. Electrochemical sensors for the detection of antioxidants. In Frontiers in Bioactive Compounds (Vol. I). Natural sources, physicochemical characterization and applications, Ed. C. Apetrei. Benthan Science Publishers. Sharjah, UAB, 2016, pp. 127-146.
  ISBN: 978-1-68108-342-1

- M.L. Rodríguez-Méndez, J.A. de Saja, C. Medina-Plaza, <u>C. García- Hernández</u>. Electronic tongues for the organoleptic characterization of wines. In Electronic Noses and Tongues in Food Science, Ed. M.L. Rodríguez-Méndez. Elsevier: Amsterdam, The Netherlands, 2016, pp. 265-273.
  ISBN: 978-0-12-800243-8

- M. Sliwinska, <u>C. García-Hernández</u>, M. Koscinski, T. Dymerski, W. Wardencki, J. Namiesnik, M. Sliwinska-Bartkowiak, S. Jurga, C. García-Cabezón, M.L. Rodríguez-Méndez. Discrimination of apple liqueurs (Nalewka) using a voltammetric electronic tongue, UV-Vis and Raman spectroscopy. *Sensors* 16 (2016) 1654.
  doi: 10.3390/s16101654
  Impact Factor (2016 JCR): 2.677; Quartil (2016 JCR): Q1, Category: Instruments and Instrumentation.

🞣 C. García-Hernández, C. García-Cabezón, F. Martín-Pedrosa, J.A. de Saja, M.L. Rodríguez-Méndez. Layered composites of PEDOT/PSS/nanoparticles and PEDOT/PSS/phthalocyanines as electron mediators for sensors and biosensors. *Beilstein Journal of Nanotechnology* 7 (2016) 1948–1959.
doi: 10.3762/bjnano.7.186
Impact Factor (2016 JCR): 3.127; Quartil (2016 JCR): Q1, Category: Materials Science, Multidisciplinary; Physics, Applied.

🞣 M.L. Rodríguez-Méndez, C. García-Hernandez, C. Medina-Plaza, C. García-Cabezón, J.A. de Saja. Multisensor systems based on phthalocyanines for monitoring the quality of grapes. *Journal of Porphyrins and Phthalocyanines* 20 (2016) 889–894.
doi: 10.1142/S1088424616500796
Impact Factor (2016 JCR): 1.043; Quartil (2016 JCR): Q3, Category: Chemistry, Multidiscipliary.

🞣 M.L. Rodríguez-Méndez, C. Medina-Plaza, C. García-Hernández, S. Rodríguez, C. García-Cabezón, D. Paniagua, M.A. Rodríguez-Pérez, J.A. de Saja. Improvement of electrocatalytic effect in voltammetric sensors based on phthalocyanines. *Journal of Porphyrins and Phthalocyanines* 20 (2016) 413-420.
doi: 10.1142/S1088424616500218
Impact Factor (2016 JCR): 1.043; Quartil (2016 JCR): Q3, Category: Chemistry, Multidiscipliary.

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  doi: 10.1016/j.lwt.2015.02.027
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  doi: 10.1016/j.aca.2014.10.046
  Impact Factor (2015 JCR): 4.712; Quartil (2015 JCR): Q1, Category: Chemistry, Analytical.

- M.L. Rodríguez-Méndez, C. Medina-Plaza, C. García-Hernández, J.A. de Saja, J.A. Fernández- Escudero, E. Barajas-Tola, G. Medrano. Analysis of grapes and wines using a voltammetric bioelectronic tongue. Correlation with the phenolic and sugar content. *IEEE Sensors Journal* IEEE Sensors 2014 Proceedings (2014) 2139-2142.
  doi: 10.1109/ICSENS.2014.6985461
  Impact Factor (2014 JCR): 1.762; Quartil (2014 JCR): Q2, Category: Engineering, Electrical and Electronic; Instruments and Instrumentation; Physics, Applied.

Annex C: International research stay attestation letter

The attestation letter from Prof. Wanekaya that certifies an international research stay is attached below:

February 06, 2018

To whom it may concern

<u>RE: Celia Garcia Hernandez</u>

The purpose of this letter is to confirm that Celia Hernandez performed research in my lab at Missouri State University in Springfield, Missouri from August 2017 to December 2017. During that time she worked on a project entitled "In situ Formation, Stabilization and Activity of Protein Modified Gold Nanoparticles for Biological Applicatons" Lysozyme was used as a model protein to demonstate the in-situ reduction and stabilization of the gold nanoparticles prior to their modification by the layer-by-layer technique. The nanoconjugates were characterized by Dynamic Light Scattering Spectroscopy (particle sizing), zeta potential (surface charge analysis) and Ultraviolet-Visible Spectroscopy. Dynamic Light Scattering Spectroscopy confirmed that the sizes of the resulting nanoconjugates were below 100 nm after the layer-by-layer deposition procedures. Zeta potentials measurements also confirmed the altermation depostion of the oppositely charged layers. The enzymatic activity of the lysozyme was evaluated and confirmed using the bacteria *Micrococcus lysodeikticus* as a substrate. The success of this project has potential implications in the biomedical science field especially in protein delivery, drug delivery, nucleic acid delivery and biosensing applicatons.

Celia did all these work in less than 4 months including the completion of a draft manuscript. I am still reviewing the manuscript before I submit it for publication in a couple of weeks. In summary, Celia is a very brilliant and dedicated researcher. Her ability to design, execute, trouble shoot and optimize experiments is truly commendable. Her impressive experimental skills give me all reasons to believe that she will continue her remarkable efforts in the scientific field in future. If you have any questions regarding this letter, please do not hesitate to contact me.

Sincerely,

Adam K. Wanekaya, Ph. D
Professor of Chemistry
wanekaya@missouristate.edu

Annex D: Assessment reports

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Form 2T

ASSESSMENT REPORT OF A PhD THESIS PRIOR TO ITS DEFENSE
(As required by Section 2.1c. of the Regulation concerning doctoral thesis defense at UVa)

Full name: Carlos José Leopoldo Constantino
Department: Physics
University or Research Institution: **University of São Paulo State (UNESP) – campus of Presidente Prudente**
Regarding the thesis entitled: Electronic Tongue Technology Applied to the Analysis of Grapes and Wines.
Written by Mr./Mrs.: Celia García Hernández

Please, report your arguments and critical opinion on the following issues concerning the PhD thesis, writing as much as necessary:

1. Is the topic relevant? Are the research objectives well defined?

   Yes, the topic is relevant. It is within the context of developing analytical methods to improve the food quality control.

   Yes, the objectives are well defined. The main objective of this PhD thesis was to develop new electrochemical sensors with electrocatalytic materials to improve their performance (sensors) in terms of sensitivity. These sensors were implemented in electronic and bioelectronic tongues to study, mainly, antioxidants and polyphenols present in grapes and wines.

2. Is the selected methodology sound and suitable for the topic and the objectives pursued in the thesis?

   Yes, the methodology is suitable. Different phthalocyanines, metal nanoparticles, carbon-based materials, and conducting polymers were applied in this PhD thesis to improve the sensitivity of the sensors, and enzymes were applied to improve the selectivity (specificity) of the sensors regarding the detection of phenols.

   The developed sensors are based either on non-nanostructured or nanostructured materials, for which the nanotechnology played an important role in its preparation by using different surface modification techniques. In addition, some of the electrocatalytic materials were applied in a combined way to study the possible synergistic effect between them, targeting to improve the sensor responses.

   The electronic and bioelectronic tongues were applied to analyze real wine and grape samples. Statistical analyses (multivariate data) were carried out to discriminate these samples and to establish correlations between the data obtained with the electrochemical sensors and the chemical parameters obtained by traditional analysis.

3. Is the body of reviewed literature up to date and complete? Have all relevant sources been considered and cited?

   Yes, the review is appropriate and relevant sources were considered/quoted. For instance, the article I presented in this PhD thesis, entitled "Multisensor systems based on phthalocyanines for monitoring the quality of grapes", is a review that provides an overview of phthalocyanine-based electronic tongues for grape analysis, in which the use of non-nanostructured and nanostructured sensors and biosensors are considered. Besides, the chapter II of this PhD thesis is totally dedicated to literature review. This chapter II quoted 195 references.

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4. Does the thesis make original contributions that expand the current knowledge on the subject? Are these contributions relevant?

   Yes, this PhD thesis brings original and relevant contributions to the field of sensor and biosensors. For instance, this PhD thesis is composed by 9 articles accepted for publication in international and important journals.

5. Is the thesis structure adequate to explain the research carried out and the results achieved? Is language used properly? Are formal elements, like figures or tables, well laid out and helpful to understand the research and results?

   The answer is Yes for all the questions above. Considering the PhD thesis is a compilation of articles, the evaluation process of these articles by several referees helped with aspects such as explanation, language and formal elements.

6. Only if this is a compilation thesis (written in the format of a collection of articles): Is there a clear and coherent connection among the topics and methodology of the different articles that comprise the thesis? Do the introduction and conclusions of the thesis provide a unifying picture of the whole research?

   Yes for all the questions above. The PhD thesis brings a resume with the main aspects related to each article, which makes easier to the reader the role comprehension of the PhD thesis.

7. Please mention three strengths and three weaknesses of this thesis.

   Please, this PhD thesis is a compilation on 9 articles, published in international and important journals, as previous mentioned, between 2015 and 2019. Besides, the candidate Celia Garcia Hernández is the 1st author in 7 of these 9 articles, and the 2nd author in the other 2 articles (one of them is the review, in which the advisor Maria Luz Rodríguez Mendez is the 1st author). Therefore, I do not see weaknesses that deserve to be mentioned here.

8. If you think the thesis should NOT be defended in its current form, please mention the changes that you consider MUST be done before it can proceed to defense.

   In my opinion, the PhD thesis can be defended in its present form.

9. Please mention other changes that MAY be done in order to improve the thesis quality, but that you do not consider strictly necessary to authorize its defense.

   Nothing to declare.

10. Any other comments:

    I would like to congratulate the candidate and the advisor for the quality of this PhD thesis, besides such productive period.

11. Please provide your recommendation to the Academic Board of the PhD Program:

    X   This thesis should be ADMITTED for defense, either in its current form or after taking into account the suggestions made in point 9 of this report.

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☐ This thesis should be MODIFIED before its admission for defense in order to make the changes requested in point 8 of this report.

☐ This thesis should be REJECTED for defense, due to the arguments given in this report.

Place and date: Presidente Prudente, Brazil, September 30, 2019.

Signature:

Notes: The length of this report is not restricted. Please remember to sign it (digital signatures are accepted).

Form 2T

ASSESSMENT REPORT OF A PhD THESIS PRIOR TO ITS DEFENSE
(As required by Section 2.1c. of the Regulation concerning doctoral thesis defense at UVa)

Full name: **LUÍS AVELINO GUIMARÃES DIAS**
Department: **Environment and Natural Resources**
University or Research Institution: **Polytechnic Institute of Bragança, Portugal**
Regarding the thesis entitled: **Electronic tongue technology applied to the analysis of grapes and wines**
Written by Mrs.: **Celia García Hernández**

Please, report your arguments and critical opinion on the following issues concerning the PhD thesis, writing as much as necessary:

1. Is the topic relevant? Are the research objectives well defined?
    The subject of the thesis is current and relevant, focusing on the construction of multi-sensor systems that allow the development of new analytical technical applications relevant to the research area of viticulture and oenology, with possible future applications in the wine industry.
    The objectives of the research work are well defined, showing that the research work involved the construction of several electronic tongues, testing the response performance of working electrodes using various electrochemical materials, applying various electrochemical techniques and evaluating the responsiveness of the analytical systems built in the analysis of real samples, grapes, musts and wines.

2. Is the selected methodology sound and suitable for the topic and the objectives pursued in the thesis?
    The doctoral work involved the application of electronic tongues in the analysis of grapes, musts and wines, being an added value building multi-sensor systems specific to these matrices, which would not happen if commercial systems were used. Therefore, the study of the application of different materials and construction techniques for electrode preparation in the electronic tongue's development made research work complex and demanding, as expected from a doctoral work, but very suitable to the topic since the work has the three steps referred in the work objectives: construction, performance evaluation and application.

3. Is the body of reviewed literature up to date and complete? Have all relevant sources been considered and cited?
    Regarding the literature review (chapter 2), the organization of the text made by the author allows any reader to have a clear understanding of the importance of electronic tongues, referred to as a new sensory technology developed to evaluate the taste of food and beverages. It begins by referring the importance of the sensory panel in the food industry and its disadvantages, for which an electronic tongue can be an analytical solution. It makes a comparison with the gustatory system of the human tongue showing that the electronic tongue is an analytical tool that mimics it. Provides a background on early work with the electronic tongues, developments and applications. Later, presents the most applied principles of detection, the materials used in the construction of electrochemical sensors and techniques used in its construction, as well as the multivariate statistical methodologies for the data analysis. Finally, the paper presents examples of application in various scientific areas and commercial systems of electronic languages, focusing later on applications in the wine industry.
    In the reviewed literature, the references are current and from relevant sources. There was no need to include any more particular references, so I think there is enough information for any reader to understand the contents explained.

4. Does the thesis make original contributions that expand the current knowledge on the subject? Are these contributions relevant?

Yes, the relevance of the work is expressed in the 9 articles selected for this PhD thesis that were published, which are from journals with good scientific impact: 1) Journal of Porphyrins and Phthalocyanines; 2) Frontiers in Chemistry; 3) Food Research International; 4) Beilstein Journal of Nanotechnology; 5) Food Chemistry; 6) Beilstein Journal of Nanotechnology; 7) Sensors and Actuators; 8) Sensors; 9) Biomaterials Science. The journals are on food, sensors and materials scientific areas, showing that the doctoral research work allowed to expand the knowledge, in general, through the use of materials and recent techniques of electrodes construction, with applications in the analysis of grape, must and wine.

5. Is the thesis structure adequate to explain the research carried out and the results achieved? Is language used properly? Are formal elements, like figures or tables, well laid out and helpful to understand the research and results?

The doctoral dissertation has a suitable structure. It introduces the subject of electronic language allowing the reader to have a comprehensive understanding of how it works and the possible applications. In chapter 3, the initial introduction of the 9 selected articles allows an overview of the work done and the presentation of the motivations and objectives, as well as the summary of each research work allows to understand why the study was carried out under the PhD thesis.

The language present in the text is adequate, presenting the contents and results of the research work objectively, precisely and clearly.

The figures and tables presented are adequate and allowed to highlight explained concepts and schematize information. Globally, they allowed to summarize information that would be complicated to explain in the thesis text.

6. Only if this is a compilation thesis (written in the format of a collection of articles): Is there a clear and coherent connection among the topics and methodology of the different articles that comprise the thesis? Do the introduction and conclusions of the thesis provide a unifying picture of the whole research?

Yes, as I mentioned in the previous point to indicate that the structure of the thesis is adequate to explain the research work done. Globally, the different articles are based on electrodes construction using different materials and techniques and in their performance evaluation study as in electrode arrays, which can be applied in analysis of grape, must or wine samples. The electrode arrays were applied as an electronic tongue and, therefore, there is a connection between the topics and the methodology of the articles presented in the thesis.

The introduction, objectives and conclusions of the thesis are coherent with each other, showing that the thesis has a careful structure in order to allow the unification of the research work presented.

7. Please mention three strengths and three weaknesses of this thesis.

Strengths:

The work focused on building and testing several electronic tongues architectures that showed effective analytical potentialities in the analysis of vineyard products; The works show the potential application of voltammetric electronic languages in food analysis with advantages inherent in the methodology, such as the speed and simplicity of analysis; The electrodes built and tested in this work have great analytical application potential in other matrices, which should be considered as a major scientific contribution.

Weaknesses:

The weaknesses do not invalidate the research line followed in the thesis of developing, studying and applying sensor lines in the analysis of vineyard products. In fact, these weaknesses can be considered as future work objectives to develop based on the work presented in the thesis. Thus, it would be important to develop a single electronic tongue solution that enables applications across the wine production line by doing: a comparison of the analytical performance of the different electrodes in order to select them; developing methodologies and procedures that allow any

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technician to use them, especially in in-situ analysis environments in the vineyard sector should be considered; including the development of a control platform to simplify the use of the analytical system and data treatment by any technician.

8. If you think the thesis should NOT be defended in its current form, please mention the changes that you consider MUST be done before it can proceed to defense.

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....
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9. Please mention other changes that MAY be done in order to improve the thesis quality, but that you do not consider strictly necessary to authorize its defense.
There are no corrections that MAY be done. The thesis presented has quality to be evaluated.

10. Any other comments:
No.

11. Please provide your recommendation to the Academic Board of the PhD Program:

☒ This thesis should be ADMITTED for defense, either in its current form or after taking into account the suggestions made in point 9 of this report.

☐ This thesis should be MODIFIED before its admission for defense in order to make the changes requested in point 8 of this report.

☐ This thesis should be REJECTED for defense, due to the arguments given in this report.

Portugal, October 4, 2019