Voltammetric electronic tongue to identify Brett character in wines. On-site quantification of its ethylphenol metabolites

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Abstract

This work reports the applicability of a voltammetric sensor array able to evaluate the content of 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol in spiked wine samples using the electronic tongue (ET) principles. The ET uses cyclic voltammetry signals, obtained from an array of six graphite epoxy modified composite electrodes, compressed using Discret Wavelet transform with chemometric tools among these, artificial neural networks (ANNs) were employed to build the quantitative prediction model. In this manner, a set of standards based on a modified full factorial design and ranging from 0 to 25 mg·L^{-1} on each phenol, was prepared to build the model; afterwards, the model was validated with an external set of standards. The model successfully predicted the concentration of the three considered phenols with a normalized root mean square error of 0.02 and 0.05 for the training and test subsets respectively and correlation coefficient better than 0.958.

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1. Introduction

The wine sector is an important economic that still has some unsolved issues, such as the generation of undesired volatile phenols during the early stages of wine production, the alcoholic fermentation. Volatile phenols such as phenol, guaiacol, cresol, ethylphenol, vinylphenol, eugenol and vainilline are present in wine and are the main responsible for the wine aroma [1]. Depending on their concentration levels and their aromatic properties, some of them contribute positively to wine aroma, but others are responsible for undesired aromas and flavors. Among these compounds the 4-ethylphenols and vinylphenols are mainly the responsible for unpleasant aromas often described as "phenolic", "leather" or "barnyard", and are the origin of this defect in wine product.

Presence of 4-ethylphenols in wine is mainly due to the enzymatic side-processes during its fermentation, especially when the *Brettanomyces* and *Dekkera* yeast families are present [2, 3]. This yeasts are naturally present in the fruit skin so it is almost impossible to avoid the presence of this kind of microorganisms when harvesting; the problem arise when the activity of this yeasts is very high, producing a wine with a concentration of 4-ethylphenols that surpasses the human threshold, wich has been reported to be aprox. 0.5 mg·L^{-1} of 4-ethylphenols [3]. In the industry, there are two alternatives to minimize the presence of these compounds: the early detection of proliferating *Brettanomyces* yeasts via cell culture [4] or a gas chromatography analysis of their metabolites [5]; it has to be remarked that both methods are time consuming, require trained personnel and cannot be used on-site.

In this context, electrochemical sensors offer an opportunity to detect the *Brettanomyces* metabolites on site at the wine producer. Electrochemical systems present known advantages that include high sensivity and selectivity, a wide linear

range, and low-cost instrumentation. In addition, electrochemical measurement devices can be readily miniaturized and/or integrated to facilitate on-site testing. Unfortunately, there are some factors that hinder the applicability of such sensors in real samples, e.g. matrix effects, interferents, electrode fouling, etc. In this context a new sensor approach was proposed in the early 90s to overcome some of the limitations that a single sensory element presented, the use of sensor arrays [6]. However, the highly complex data generated by the sensor array needs to be treated in order to extract the meaningful information; the data is processed employing advanced mathematical tools such as Principal Component analysis (PCA), partial least squares (PLS) or artificial neural networks (ANNs) [7]. The resulting approach is known as electronic tongue (ET) [8], due to its similarities to the biological taste sense. ETs have been specifically employed in applications related to the wine field as the determination of the total polyphenolic content in wine, prediction of the sensory score or the detection of adulterations, among many others [9-12]. Hence, with this methodology, it is possible to achieve a simultaneous determination of a large number of different species, while diminishing any interference effect using these advanced mathematical tools [13].

The ET principles are reported now in an approach based on the coupling of cyclic voltammetry responses obtained from an array of modified epoxy graphite electrodes, compressed with Discrete Wavelet Transform [14], and processed with ANNs to build a predictive model able to quantify the content in 4-ethylphenol, 4-ethylguayacol and 4-ethylcatechol in wine, as its depicted in Figure 1, this approach attempt to obtain an alert tool, to detect the defect of volatile phenols in wine, applicable in the wineyard and directed to improve wine quality and prevent the appearance of this defect.
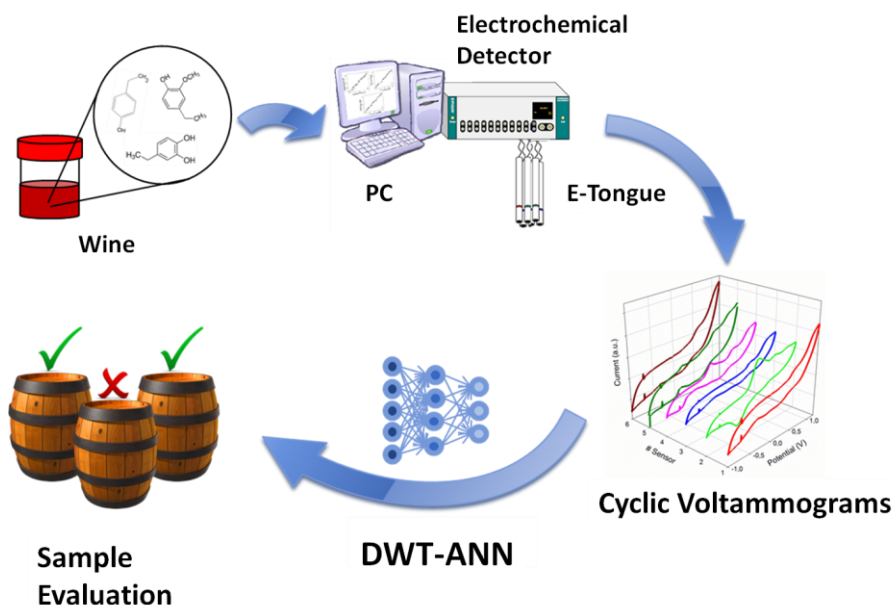
Figure 1. Scheme of the experimental setup used for the early detection of volatile phenol defects in wine.

2. Experimental

2.1 Reagents and chemicals

All reagents used in this work were analytical reagent grade. 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, Cu nanoparticles (particle size 50 nm), WO_3 nanoparticles (particle size <100 nm), 2% Bi_2O_3 nanoparticles (particle size 90-210 nm), polypyrrole and Co phtalocyanine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Graphite powder (particle size 50 µm) was received from BDH (BDH Laboratory Supplies, Poole, UK). Epotek H77 resin was purchased from Epoxy Technology (Billerica MA, USA). *Don Simon* wine was purchased at the local supermarket.

2.2 Electronic tongue

The voltammetric ET was formed by an array of 6 sensors, plus a combined Pt auxiliary and a Ag/AgCl reference electrode (Crison 5261, Barcelona, Spain). Working

electrodes were bulk modified graphite epoxy composites, which were prepared by mixing the resin, graphite powder and modifiers in the ratio 83:15:2 (w/w) [15]. Afterwards, resin was allowed to harden at 80 ºC for 24 h; and electrode surfaces were next polished with different sandpapers of decreasing grain size.

In this manner, the array of 6 voltammetric electrodes was prepared, consisting in one blank electrode plus five composite electrodes modified with Cu nanoparticles, WO_3 nanoparticles, Co phtalocyanine, Bi_2O_3 nanoparticles and polypyrrole. This choice was intended as to maximize the differences in the obtained voltammograms for the different sensors used as is demonstrated in section 3.1.

Electrochemical measurements were performed at room temperature (25ºC), using a 6-channel AUTOLAB PGSTAT20 (Ecochemie, Netherlands) controlled with GPES Multichannel 4.7 software package. A complete voltammogram was recorded for each sample by cycling the potential between -1.1 V and +1.2 V vs. Ag/AgCl with a step potential of 9 mV and a scan rate of 100 mV·s-1.

In order to get stable voltammetric responses and ensure reproducible signals from the array during the experiment, the electrodes were cycled in buffer solution after the sample measurements and an electrochemical cleaning step was performed between samples at +1.4 V during 40 s in a cell containing 20 ml of 100 mM saline solution at pH 10 [16].

2.3 Data pre-processing

The main objective of the pre-processing is to reduce the complexity of the input signal (6 sensors x 490 current values at different potential) while preserving the relevant information, this step allows a gain in training time, avoids redundancy in the input data and a obtained model with better generalization ability [7].

The compression of the voltammetric data was achieved by means of Discrete Wavelet Transform [14]: each voltamogram was compressed using *Daubechies* 3 and a 4th decomposition level. In this manner, the 2940 inputs per sample were reduced down to 132 coefficients, achieving a compression ratio of 93.5%.

The statistical treatment and analysis was performed using routines written by the authors through MATLAB 2016b (MathWorks, Natick, MA) programming environment and its Neural Network Toolbox; the graphical representation and analysis of the results was performed with Sigmaplot (Systat Software Inc., San Jose, CA).

3. Results and Discussion

3.1 Voltammetric array response

The voltammetric responses for each of the electrodes towards individual compounds were first evaluated, to assure that the generated signals are different enough and the obtained data is rich enough to be the departure point for a multivariate calibration model.

Therefore, under the described conditions in section 2.2, individual stock solutions of 25 ppm of 4-ethylphenol, 4-ethylguayacol and 4-ethylcatechol were analyzed (Figure 2). As a general trend, as it is already reported in the literature [17], two processes are observed for all the sensors corresponding to the oxidation of the corresponding phenol to its quinone form, and the reduction of the quinone to the phenolic form.

Moreover, it can also be seen that the copper nanoparticle modified electrode displays higher currents, a fact somehow explained by the fact that the main natural phenolic-degrading enzymes, like *tyrosinase* or *laccase*, are copper containing redox enzymes [18, 19]. Besides, slightly differentiated curves are obtained for each of the compounds, a necessary condition for any ET study.

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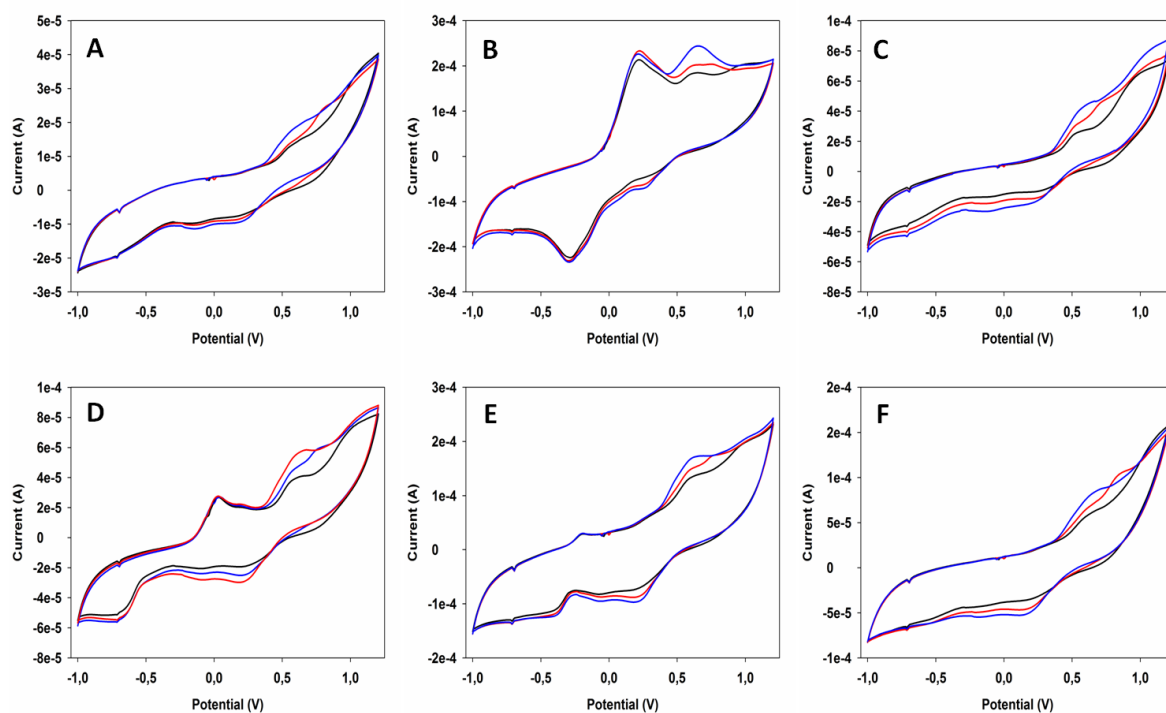
Figure 2. Example of the different voltammograms obtained for 25 ppm of 4-EP (black), 4-EG (red) and 4-EC (blue) in a wine matrix for a (A) Bare epoxy-graphite electrode, and electrodes modified with (B) Cu nanoparticles, (C) WO_3 nanoparticles, (D) Bi_2O_3 nanoparticles, (E) Polypyrrole and (F) Co(II) phthalocyanine.

However, if it is not enough to asses visually that each sensor in the array responded in a different manner towards 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol a chemometric assay was further done. In order to assess mathematically the complementarities between the voltammetric responses of Figure 2 a principal component analysis (PCA) was performed [20]. In Figure 3A are plotted the scores of samples correcponding to the two first principal components for the array response towards the 3 ethylphenols; in there, it can be seen that each compound sample is differentiated and clearly clustered. Figure 3B also plots the scores of the two first principal components but taking into account each sensor, as it can be seen each sensor provides a distinct signal for each analyte. Moreover, the different sensors appear in different coordinates in the scores plot meaning that they have different responses that

complement each other. Perhaps the two electrodes showing the closest response were these of Co (II) phthalocyanine and WO_3 that were kept given the very different nature of the catalysts involved.
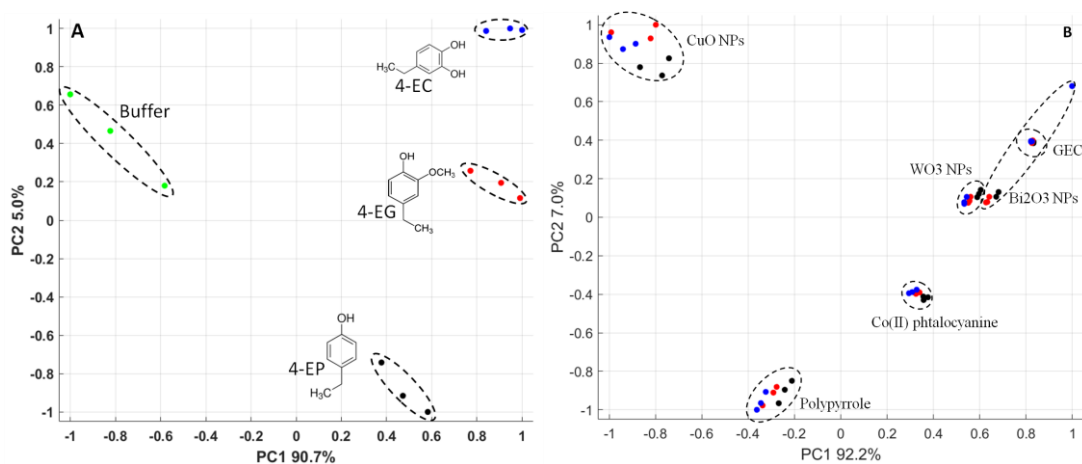
Figure 3. The scores plot of graphite-epoxy electrode (GEC) , CuO NPs electrode, WO_3 NPs electrode, Bi_2O_3 NPs electrode, polypyrrole electrode and Co(II) phthalocyanine electrode for 25 ppm of 4-EP (black), 25 ppm of 4-EG (red) and 25 ppm of 4-EC (blue).

The array response towards the compounds of interest, once confirmed the different behaviour of the employed electrodes, allowed the differentiation of the different ethylphenol compounds considered; the next step was to proceed with the design of the architecture of the ANN model capable to quantify these compounds in a complex matrix such as wine.

3.2 Building of the ANN model

The first step in the construction of the artificial neural network is the design of the training and test subsets. In this case the chosen experimental design for the train subset was a modified (tilted) 3^3 factorial design (27 samples) [21]; while the validation of the constructed model was done with the test set (10 samples), these were randomly distributed along the experimental domain (0 to 25 mg·mL^{-1} for each phenolic compound) on a *Don Simon* commercial wine matrix.

Once the data of the samples in the different subsets was collected the voltammograms were compressed by use of DWT, as result the obtained dataset is suited to be feed to the different ANN models. The next step is to optimize the appropriate ANN architecture, this is usually done by trial-and-error procedure due to the difficulties to predict the best configuration as there are several parameters involved (compression pretreatment, number of neurons in the hidden layer, transfer functions, etc.) [22].

As previously commented, the samples from the training subset were used to build the ANN model and the performance of the model was evaluated with the prediction of the analyte concentrations in the test subset samples. As mentioned, the test subset is an external set that has not been used in the modeling procedure; the goodness of fit for this subset is a good parameter to evaluate the modeling performance.

After the evaluation of different topologies, the final ANN architecture had 132 neurons (6 sensors $\times$ 22 DWT coeffs.) in the input layer, 3 neurons and *satlins* transfer function in the hidden layer and 3 neurons and *purelin* transfer function in the output layer, providing simultaneously the concentration of the three compounds considered. Comparison graphs of predicted vs. expected concentrations for training and testing subsets, for each of the compounds, were built to evaluate the prediction ability of the ANN model (Figure 4). A satisfactory trend is obtained for both subsets, with regression lines values very close to the theoretical ones, slope and intercept equal to 1 and 0 respectively. Nevertheless, the training subset showed better correlation coefficients (r $\geq$ 0.99) than the test subset (r $\geq$ 0.95) but this is expected to be as the train subset is used to optimize the architecture, therefore the model is tailored to fit this data, while the test subset is not used at all during the modeling. The detailed results, described in Table 1,

showing promising results for the test subset as the NRSME (normalized root mean square error) for the three compounds is 0.05. Additionally, PLS was employed to compare the ANN model with the usual chemometric methods, such as partial least squares model. The NRMSE obtained for the test subset with the PLS model was 0.159. However, the PLS models achieves comparable NRMSE values in quantification of 4-EG, the individual NRMSE errors were 0.059, 0.062 and 0.135 for 4-EP, 4-EG and 4EC respectively. The results of the PLS model can are detailed in the supporting information.
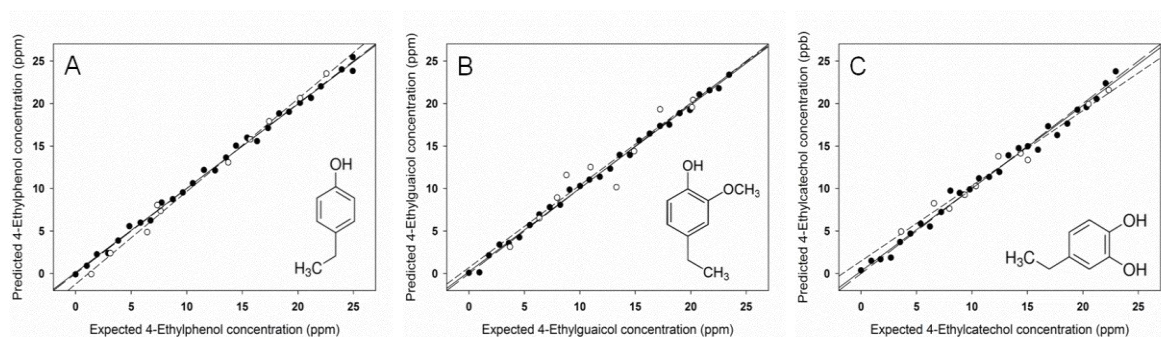
Figure 4. Modeling ability of the developed DWT-ANN. Adjustments of expected vs. predicted concentrations for (A) 4-EP, (B) 4-EG and (C) 4-EC, both for training (●, solid line) and testing subsets (○, dashed line). Dotted line corresponds to theoretical diagonal line.

Table 1. Results of the fitted regression lines for the comparison between obtained vs. expected values, both for the training and testing subsets of samples and the three considered species (intervals calculated at the 95% confidence level).

		r	*Slope*	*Intercept (mg·L⁻¹)*	*NRMSE*	*Total NRMSE*
Train	*4-Ethylphenol*	*0.998*	*0.990±0.0487*	*0.131±0.715*	*0.019*	
	4-Ethylguaiacol	*0.998*	*0.981±0.0486*	*0.213±0.667*	*0.018*	*0.022*
	4-Ethylcatechol	*0.995*	*0.974±0.0816*	*0.282±1.095*	*0.029*	
Test	*4-Ethylphenol*	*0.997*	*1.089±0.133*	*-1.243±1.798*	*0.037*	
	4-Ethylguaiacol	*0.958*	*0.969±0.474*	*0.691±6.400*	*0.069*	*0.050*
	4-Ethylcatechol	*0.988*	*0.886±0.227*	*1.461±3.069*	*0.043*	

NRMSE: Normalized Root Mean Square Error

Additionally, to double check the goodness of the fit joint confidence intervals (JCI) were calculated and plotted according to advanced linear regression methodology [23]. The use of JCI plots has been previously employed as a rapid visualization tool to detect if two methods have significant differences [24], allowing simultaneous evaluation of the slope and intercept. The plotting of the JCI takes into account the uncertainties from both axes to calculate the estimated covariance matrix based on a F distribution. In this manner the plots were constructed, for the plots shown in Figure 4, and the theoretical comparison point (0,1) was included for comparison purposes. As can be observed in Figure 5, the theoretical point is included in the confidence intervals for both for the training and testing subsets; confirming that statistically there are no significant differences for the ET predicted values and the expected ones. As before, the results obtained for the training subset are more precise, being close to the ideal point, than the ones in the test subset.

Again it can be seen that the test subset gives a bigger joint confidence ellipse, this fact can be easily explained; firstly, the test subset is not employed at all during the construction of the model. Moreover, the number of samples in the testing subset is much lower that the train subset, and consequently, the tabulated values of t and F are higher, resulting in higher dispersion and larger confidence intervals.

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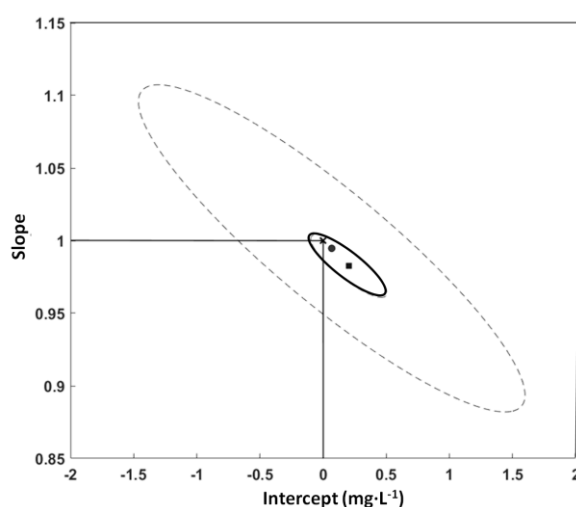
Figure 5. Joint confidence intervals for the train subset: (■, solid line) and the test subset (●,dashed line). Also ideal point (1,0) is plotted (x); intervals calculated at the 95% confidence level.

4. Conclusions

The approach presented here combines an array of six voltammetric sensors with artificial neural networks to simultaneously quantify the concentrations of 4-ethylphenol, 4-ethylguaiachol and 4-ethylcatechol, as important defect episode in wine samples.

The ET strategy allowed the resolution of signal overlapping and therefore the quantification of the individual species considered. This fact combined with the advantages of electrochemical sensors for on-field analysis results in a promising tool

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that substituted the classical expensive and time consuming methods to help winemakers in the early detection of the Brett defect.

Acknowledgments

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