Analysis of the interaction of radical scavengers with ROS electrogenerated from hydrogen peroxide

by

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KEYWORDS: Hydroperoxide radical; superoxide anion radical; electrode kinetics; radical scavengers; antioxidant activity; ROS

ABSTRACT

The polarographic (direct current, dc, and differential pulse, DP) and voltammetric oxidation of hydrogen peroxide on mercury electrodes has been examined. The electrooxidation of H_2O_2 involves hydroperoxide radical and superoxide anion radical able to interact with radical scavengers (antioxidants), namely 3-hydroxycoumarin, carvacrol, vanillin and gallic acid. For such interaction, theoretical equations are derived using the convective diffusion approximation and the steady-state conditions, as well as solving the differential equations of the Nernst diffusion-layer approximation by the method of dimensionless variables. The experimental results agree with the theoretical predictions and a kinetic parameter is proposed to evaluate the scavenging activity of antioxidants.

INTRODUCTION

An antioxidant is defined as a substance that may delay or prevent the oxidation of a substrate, at relatively low concentrations[1]. Primary antioxidants (prooxidants) prevent the formation of free radicals, particularly reactive oxygen species (ROS). These primary antioxidants include vitamin E[2], polyphenols[3], antioxidant enzymes and membranes[4-5]. Secondary antioxidants (antioxidants with radical scavenging activity) operate either interrupting the propagation of free radicals or inhibiting the generation of ROS, as well as preventing the metabolic activation of carcinogens[6-7]. Tertiary antioxidants repair the damage from free radicals or eliminate damaged molecules[8-9].

Although different assays have been used to evaluate the antioxidant activity of natural products, there is not a universal method to cover this task. Direct methods have been proposed, based on the determination of the concentration of antioxidant compounds[10,11], and indirect methods, as the measure of glutathione levels[12] and the reduction of Fe(III) or Cu(II) ions[13,14]. In addition, enzymatic methods measure the activity of antioxidant enzymes[15]. A good review detailing these methods can be found in reference[16].

The electrochemical measurements have advantages for the determination of antioxidant activity such as their use as a rapid proof of the antioxidant capacity of many organic molecules[17,18] and the capacity to study the interaction between the antioxidant and ROS, as was observed for dihydropyridines and electrogenerated superoxide radical[19-21]. The oxidation potentials measured by cyclic voltammetry have been used to compare the antioxidant strength of compounds as phenolic acids, flavonoids, cinnamic acids etc.[22-24], being the glassy carbon electrode, GCE, the most frequently used electrode. Low oxidation potentials are associated with a greater facility of a given molecule for the electrodonation and, thus, to act as an antioxidant, with good correlation with other techniques such as the scavenging of the DPPH$^{\bullet}$ radical[25]. The results show that the DPPH$^{\bullet}$ assay can assess radical scavenging for substances

that are thermodynamically capable of reacting with agents with a redox potential below a given value, related to the reduction potential of $DPPH^{\bullet}$. However, other less stable radicals of biological interest, as $ROO^{\bullet}$ and $OH^{\bullet}$, exhibit much higher formal potentials than $DPPH^{\bullet}$. These radicals can react with species with high oxidation potentials for which it is not possible to determine the antioxidant activity by the DPPH assay.

The anodic oxidation reaction of hydrogen peroxide has also been used to determine the antioxidant character of fenolic compounds, wines, strong alcoholic beverages or beers[26-30]. This peroxide has the advantage of being sufficiently stable in cells, compared with other ROS that need to be produced "in vivo". The polarographic oxidation of hydrogen peroxide, from alkaline solutions, was used as the source of the peroxy- radical. The decrease of the oxidation current of hydrogen peroxide caused by an addition of antioxidant is used to determine the antioxidant capacity. These authors used the peak intensity of a polarographic maximum to measure the oxidation current. The origin of the maximum is often due to the increase in the transport of the electroactive species towards the electrode by movements in the solution, so that the peak intensity is dependent on the hydrodynamic conditions of the solution. The result is that the amount of electroactive species that reaches the electrode is greater than that transported by diffusion. Unfortunately, the intensity of the maximum is modified by the presence of surface active species such as certain organic solvents like ethanol. In fact, the Hg electrode is very sensitive to changes in the concentration of organic solvents and these are used in many cases to remove the polarographic maxima due to processes occurring in the adsorbed phase.

This leads us to propose an electrochemical method for determining the antioxidant activity based on the scavenging of radicals produced in the electrochemical oxidation of hydrogen peroxide which avoids the measurement of the intensity in a polarographic maximum. The differential pulse voltammetry on mercury electrode, DPV, is used. This technique, faster

than DC polarography, leads to well defined peaks whose current is directly proportional to the concentration of hydrogen peroxide in the cell.

To achieve this goal, the interaction between radical scavengers and the radicals formed in the electrochemical oxidation of H_2O_2 must be investigated in depth, this being the aim of this work.

EXPERIMENTAL

3-hydroxycoumarin, carvacrol and vanillin were purchased from Aldrich, and gallic acid from Sigma-Aldrich and the rest of chemicals were Merck analytical grade reagents. All the reactants were used without further purification.

Solutions of 0.1M in both sodium carbonate and phosphoric acid at pH = 10.5 were used as supporting electrolytes. The aqueous solutions were prepared with ultrapure water type I (resistivity 18.2 MΩ.cm at 25° C) obtained from a Millipore Simplicity® system. The ionic strength was adjusted to 0.5 M with solid KNO_3 and the *pH* was adjusted with solid NaOH. Antioxidants were dissolved in ethanol and the stock solution concentrations were $5x10^{-3}$ M. These solutions were stored in darkness at 277 K to avoid decomposition.

The concentration of hydrogen peroxide in the cell was $5x10^{-4}$ M and the percentage of ethanol in the cell was 30%. Solutions were prepared with a fixed amount of supporting electrolyte and with variable amounts of the stock solution of antioxidant and ethanol completing the total volume. It was therefore necessary to make a separate solution for each concentration of antioxidant. The H_2O_2 was added after the above solutions were purged with purified nitrogen.

Measurements were made on a CHI650A electrochemical workstation from IJCambria coupled to an EF-1400 controlled growth mercury electrode from BAS instruments. The Hg drop area was 6.70×10^{-3} cm^2. The temperature was kept at 298±0.1 K. All potentials were measured

against an Ag|AgCl|KCl_{sat} electrode (BAS MF-2052). A platinum counter electrode BAS MW-1034 was used. The parameters selected in the differential pulse voltammetry (DPV) were: pulse amplitude 0.05 V, pulse width 0.05 s and pulse period 0.2 s.

The reproducibility of measurements was ensured by repeating the experiments and the deviations of the data were less than 5%.

RESULTS AND DISCUSSION

In the oxidation of hydrogen peroxide on electrodes as platinum[31], glassy carbon[32] and mercury[33,34], the superoxide anion is formed as an intermediate. The pH dependence of the oxidation potential indicates that an acid dissociation reaction must occur prior to the rate-determining step, r.d.s., or in the r.d.s. itself. A simplified pathway for the electrooxidation of H_2O_2 that is in agreement with the experimental data is:

$$H_2O_2 \rightleftharpoons HO_2^- + H^+ \quad (1)$$

$$HO_2^- \rightleftharpoons HO_2^{\bullet} + e^- \quad (2)$$

$$HO_2^{\bullet} \rightleftharpoons O_2^{-\bullet} + H^+ \quad (3)$$

$$O_2^{-\bullet} \rightleftharpoons O_2 + e^- \quad (4)$$

Above pH 11.7 (the dissociation pK of hydrogen peroxide[35]) reaction (1) does not occur. Moreover, in basic media reaction 3 must be written:

$$HO_2^{\bullet} + HO^- \rightleftharpoons O_2^{-\bullet} + H_2O$$

It was shown[33] that mercury participates in the oxidation process as a catalyst, being essential the adsorption of the radicals on the electrode surface. For this reason, the polarograms of the H_2O_2 oxidation showed a strong maximum[24-28]. The intensity of the polarographic maximum

cannot be strictly proportional to the H_2O_2 concentration due to the adsorptive origin of the maximum.

Moreover, if solvents different from water are added in variable quantities to the aqueous solution, their effect on the intensity must be taken into account. To illustrate this affirmation, let one consider the results shown in figure 1.

– – – FIGURE 1 – – –

The addition of ethanol to the reaction medium causes a decrease in the intensity of the polarographic maximum. The solubility of the antioxidants in water is lower than the concentration required for the experiments, and so, the stock solutions are prepared in ethanol. Thus, if one adds different amounts of the antioxidant to a given H_2O_2 solution, the final ethanol contents changes, and the decrease of intensity observed is integrated by two contributions, namely the effect of the antioxidant and the effect due to the increase in organic solvent content. This implies that the measurements of intensity relative to an original H_2O_2 solution must be made at a constant ethanol content, high enough to allow a significant range of concentrations of antioxidant.

The decrease of the intensity of the DP voltammogram after the addition of ethanol is mainly due to the decrease of the effective area of the electrode, which is partially covered by the ethanol molecules (the variation due to the change in the medium viscosity can be neglected). So, the interaction between the scavenger and the electrogenerated ROS takes place not on the electrode surface, but in the diffusion layer, due to the presence of the ethanol. It seems reasonable to think that this "homogeneous" reaction takes place without surface complications and the measured interaction corresponds effectively to the reactions studied.

On the other hand, though the presence of non-aqueous solvent decreases the adsorption effects on the polarogram, such effects are still present. To minimize these effects, the differential pulse

voltammetry, DPV, was chosen instead of the dc polarography, because the lower influence of adsorption associated to differential pulse techniques[36]. Moreover, the quantity of mercury used in a DPV experiment is much lower than the quantity of mercury used in polarography, making the DPV technique safer, more eco-friendly and less expensive than polarography. In addition the measuring time is much lower than that required in polarography. To test the technique, measurements of the H_2O_2 oxidation were made at different concentrations and, as can be seen in figure 2, the area of the DPV peak is proportional to the peroxide concentration.

– – – FIGURE 2 – – –

This proportionality is relevant because a rigorous quantification of the interaction between the antioxidants and the radicals generated in reactions 2 and 3 implies the knowledge of the evolution of the H_2O_2 concentration.

The analysis of the DP voltammograms was performed by using the following equation corresponding to first-order processes[37]:

$$I = 4I_P \frac{L}{(1+L)^2} \qquad (5)$$

where $L=\exp[-(E-E_P)/b]$, I_P and E_P being the peak intensity and the peak potential, respectively, and b is a parameter which has the same value and meaning as the slope of the dc logarithmic analysis[37-38]. In dc polarography, the I-E relationship for a first-order process reads:

$$E = E_{1/2} + b \ln \frac{I_L - I}{I} \qquad (6)$$

where b is the logarithmic analysis slope that is related to the kinetics of the electrode process.

Equation 5 corresponds to a symmetrical peak having an area proportional to the concentration of the reactant and to the overall number of electrons involved in the electrochemical process. The DPV peaks of H_2O_2 shown in figure 2 were well described by equation 4 until the peak

potential and even to potentials above this value. Nevertheless, the oxidation of the mercury itself causes a distortion for potentials more positive than the peak potential and the curve is distorted. The peaks recorded at lower concentrations of H_2O_2 are more intensely affected by this distortion. In any case, the first half of the curves, that is, the part of the curves appearing at more negative potentials than E_P, is not affected by the mercury oxidation. The b parameter was obtained from the fitting of the experimental results to equation 5 at these potentials. The b values so obtained were of 38-42 mV, corresponding to a two-electron process in which the rate-determining step, r.d.s., is the second electron transfer[37-38].

Let us consider that an antioxidant, AO, acts as a radical scavenger and is able to react with the radicals generated in reactions 2 (hydroperoxide radical) and 3 (superoxide anion radical) to give the reaction products P and P'. In this case, the reaction scheme can be represented as:

$$H_2O_2 \rightleftharpoons HO_2^- + H^+ \quad (1)$$

$$HO_2^- \rightleftharpoons HO_2^{\bullet} + e^- \quad (2)$$

$$HO_2^{\bullet} \xrightarrow[+\,AO]{k_1} P \quad (3')$$

$$HO_2^{\bullet} \xrightarrow{k_2} O_2^{-\bullet} + H^+ \quad (3)$$

$$O_2^{-\bullet} + AO \longrightarrow P' \quad (3'')$$

$$O_2^{-\bullet} \rightleftharpoons O_2 + e^- \quad (4)$$

The I-E relationships for these processes can be obtained by using the convective diffusion approximation and the steady-state conditions. Moreover, the differential equations of the Nernst diffusion-layer approximation can be solved by the method of dimensionless variables applied as shown in the literature[39-44]. Using this approximation, the following equation can be obtained:

$$\frac{A^o/2}{A - A^o/2} = \left(\frac{k_1}{k_2}[AO] + 1\right) + k_3[AO] \qquad (7)$$

equivalent to:

$$\frac{A^o - A}{A - A^o/2} = \left(\frac{k_1}{k_2} + k_3\right)[AO] + \frac{k_1 k_3}{k_2}[AO]^2 \qquad (8)$$

where [AO] is the antioxidant concentration, A is the actual area of the DPV peak and A^o the area of this peak in the absence of antioxidant.

From equation 7 it easily follows that when the rate of reaction 3' is very high, reactions 3, 3" and 4 cannot take place, and the minimum value of the peak area of the DPV must be one half of the area in the absence of antioxidant. Lower values of area cannot be found because reaction 2 must always take place to produce the hydroperoxide radical, and the minimum value of the peak area must be that corresponding to a one-electron process instead of the original two-electron oxidation. This situation can be originated by an intrinsically very high value of k_1, high AO concentrations, or both.

Figure 3 shows the DP voltammograms of the H_2O_2 oxidation after the addition of increasing amounts of several antioxidants. The experiments were made at a constant ethanol content of 30%, both in the absence and in the presence of antioxidant, to avoid the above mentioned effect of this variable on the DPV peak and to ensure the proportionality between the peak area and the H_2O_2 concentration.

– – – FIGURE 3 – – –

From figure 3 it follows that the oxidation peak decreases as the antioxidant concentration increases. This decrease was never higher than 50% of the original area for any of the

investigated compounds, as expected. The percentage of the area diminution is shown in figure 4.

– – – FIGURE 4 – – –

The decrease of the DPV peak is different for the different antioxidants essayed, due to differences in k_1 and k_3 values, i.e., due to the different scavenging activity. So, to decrease the peak area in e.g. a 25%, lower concentrations of gallic acid than of 3-hydroxycoumarin, carvacrol or vainillin (in this order) are needed.

At AO concentration values high enough, the rate of reaction 3' is faster than those of reactions 3, 3" and 4 and the process consists essentially of an one-electron oxidation followed by a chemical reaction (namely, 3'). This happens when the peak area is close to the minimum area value, the oxidation process of H_2O_2 thus corresponding to a one-electron EC type, and the b value being close to 60 mV[37,38]. This value must be reached from the original value of c.a. 40-43 mV in the absence of antioxidant, increasing with the antioxidant concentration. This tendency is also shown in figure 4. These facts support the reaction scheme.

Finally, figure 5 shows the dependence of the area function, AF, with the added antioxidant amount, AF being given by:

$$AF = \frac{A^o - A}{A - A^o/2} \qquad (9)$$

– – – FIGURE 5 – – –

The shapes of the plots are curves having zero intercepts, as expected from equation 8. For 3-hydroxycoumarin, vainillin, and carvacrol, the data are well represented by a second-order polynomial function, whereas for gallic acid, the curvature is most pronounced. This can be due

to the possibility of reaction between one antioxidant molecule and two or more radicals of the each class (hydroperoxide radical or superoxide anion radical) and reactions 3' and 3" would be of the third order or higher, instead of the second order. So, as the antioxidant concentration increases, the rate of reactions 3' and 3" increase more markedly and deviations from the expected behavior are produced. In any case, at low antioxidant concentrations the plots can be approximated to straight lines having slopes (see equation 8):

$$m = \frac{k_1}{k_2} + k_3 \qquad (10)$$

This parameter contains information about the scavenging activity of the antioxidant through k_1 and k_3 (if the experiments are made at the same pH and temperature, k_2 is a constant value, because it corresponds to the oxidation of the hydrogen peroxide itself). So, a greater value of slope (m) implies a greater scavenging activity.

Table 1 shows the values of m (equation 10) obtained by plotting AF vs. [AO] in $mmol \cdot L^{-1}$, at low concentration values where the graphs showed linear variations. These m values are compared with ARP values obtained by the DPPH method[25]. This involves the measuring of $DPPH^{\bullet}$ absorbance at 515 nm as a function of time, at a given concentration, with a variable concentration of antioxidant. The reverse of the amount of antioxidant required to decrease the initial concentration of $DPPH^{\bullet}$ to 50% is named anti-radical power, ARP, being higher as the antioxidant is more efficient. Finally, in the table the peak potentials of the voltammograms of the AO oxidation on a glassy carbon electrode[25] are also given.

– – – TABLE 1 – – –

The results obtained by both methods are quite different. The ARP value for 3-hydroxycoumarin is very low, which indicates that the antioxidant capacity of this compound is also very low. This conclusion is supported by the high oxidation potential. Nevertheless, the

corresponding m value is high, this indicating a high scavenging activity. In addition, the ARP values of carvacrol and vainillin are very similar (and also the oxidation potentials) but the m value of carvacrol is c.a. three times the m for vainillin.

These facts can be explained taking into account that ARP (and also the oxidation potentials[25]) measures the capacity of a given compound to react with the DPPH$^{\bullet}$ radical, whereas m is a measurement of the interaction of the antioxidant with the ROS (hydroperoxide and superoxide radicals). Thus, the measurement here proposed is more significant than DPPH or the voltammetric methods to assess and quantify the radical scavenging capacity of a given antioxidant interacting with ROS.

Acknowledgements

Financial support from Junta de Andalucía (Research Groups FQM-0198 and RNM-371) is gratefully acknowledged.

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Table headings

Table 1. Values of m (antioxidant concentrations in mmol·L^{-1}), ARP and oxidation potentials vs. Ag/AgCl/KCl (3M) electrode for the antioxidants studied.

Figure Captions

Figure 1. dc polarograms of $5x10^{-3}$ M H_2O_2 at different percentages of ethanol in the medium at pH = 10.51. SE: Supporting electrolyte.

Figure 2. DP voltammograms of H_2O_2 at different concentration values. pH = 10.50, 30% ethanol. Iinset: area of DPV peak vs. H_2O_2 concentration

Figure 3. DPV voltammograms of $5x10^{-3}$ M H_2O_2 at pH = 10.50, 30% ethanol, and different amounts of $5x10^{-3}$ M antioxidant (AO) solution. AO: gallic acid, 3-hydroxicoumarin, carvacrol and vainillin.

Figure 4. Decrease of the DPV peak area (solid squares, continuous line) and variation of b parameter value (hollow circles, dotted line) with the added amounts of $5x10^{-3}$ M antioxidant (AO) solution. AO: gallic acid, 3-hydroxicoumarin, carvacrol and vainillin.

Figure 5. Dependencies of the area function, AF, with the added amount of $5x10^{-3}$ M antioxidant (AO) solution. AO: gallic acid, 3-hydroxicoumarin, carvacrol and vainillin.

	Slope/mM^{-1}	ARP [25]	$E_{p,a}$/mV [25]
Gallic acid	23.0	8.5	274
3-hydroxycoumarin	18.5	$<10^{-3}$	763
Carvacrol	7.5	0.12	552
Vainillin	2.6	0.11	584

Table 1

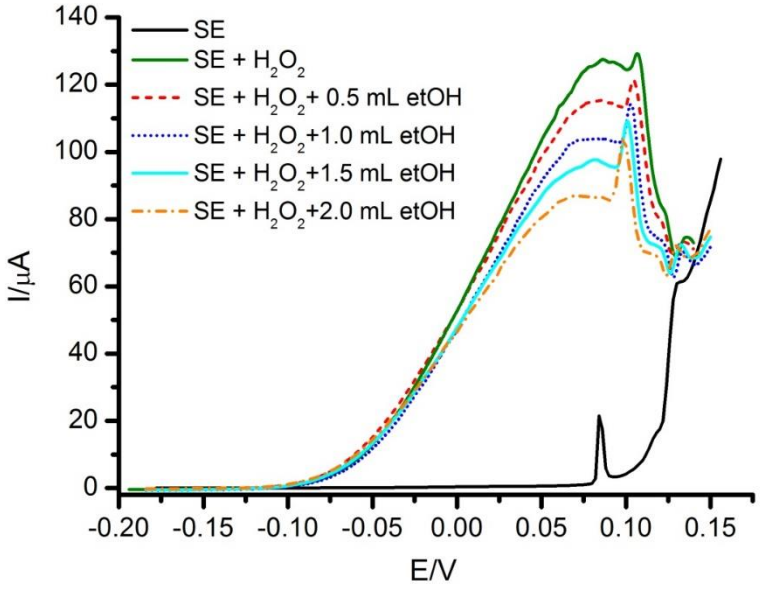


Figure 1

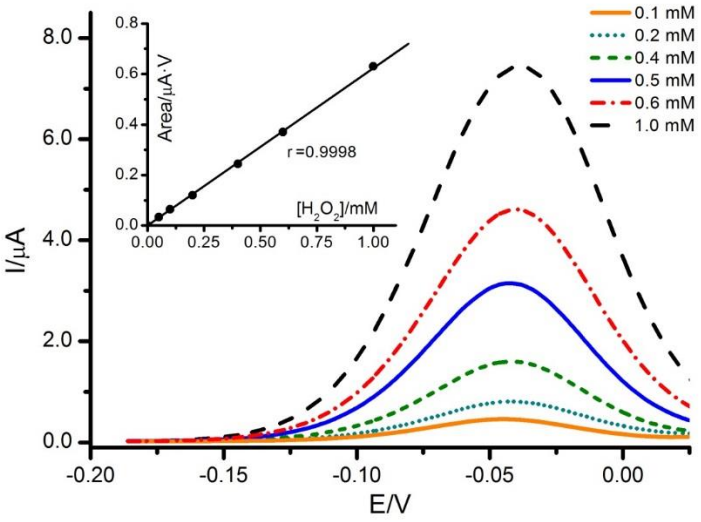
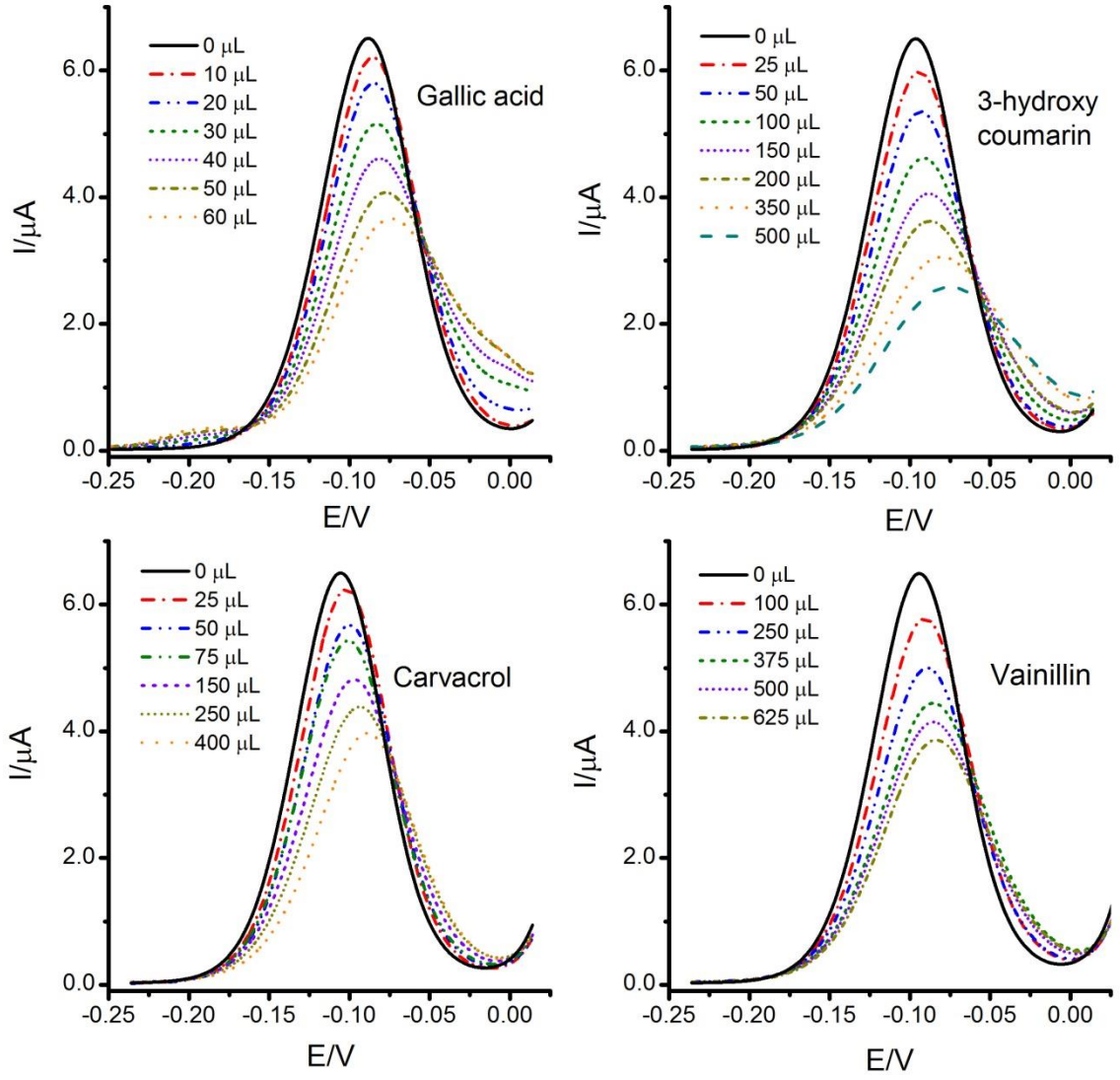


Figure 2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50 µL
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150 µL
250 µL
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Figure 3

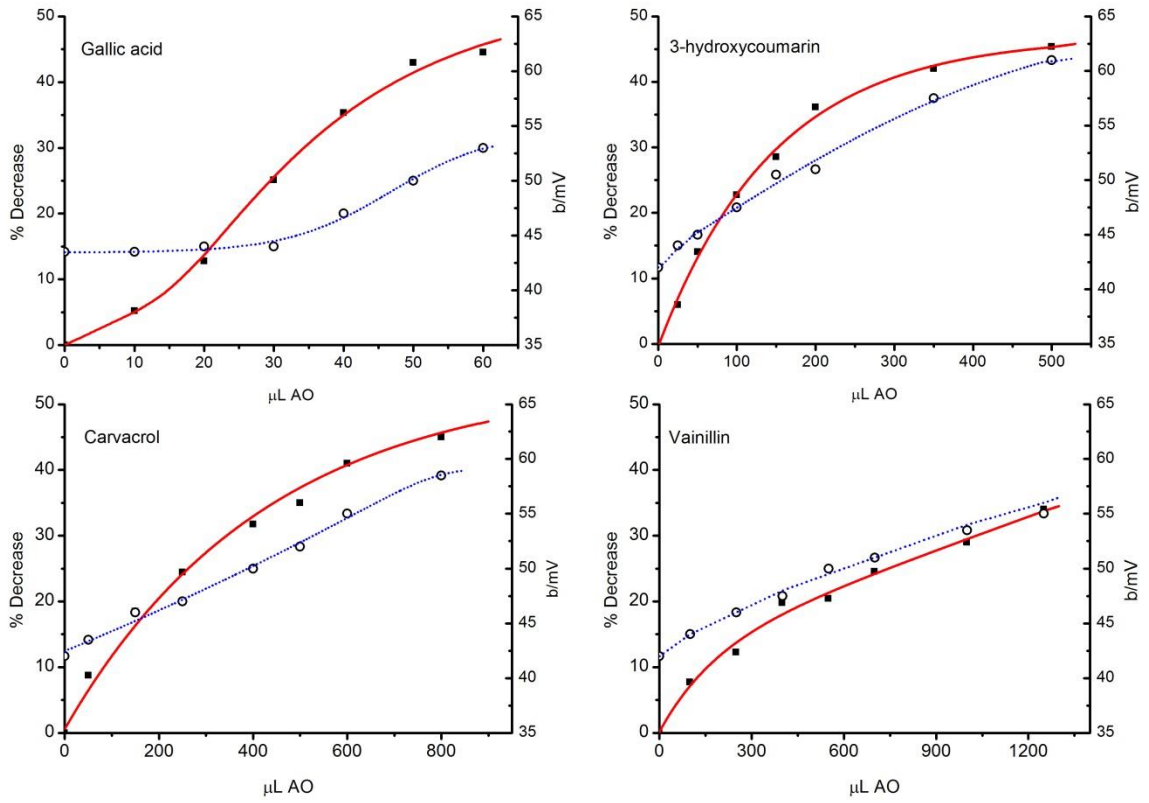

Figure 4

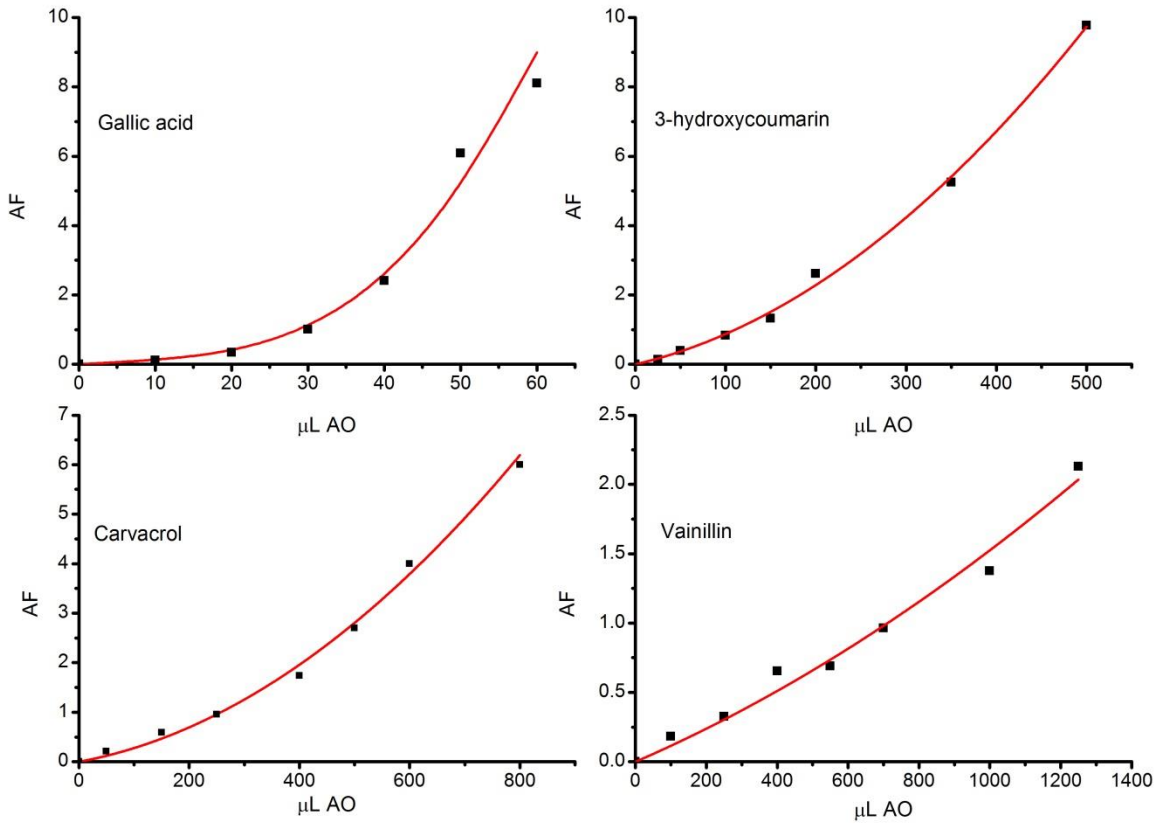

Figure 5