# Electrochemical Oxidation Pathways of Hydroxycoumarins on Carbon Electrodes Examined by LSCV and LC–MS/MS

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**Abstract**

It is reported the voltammetric behavior and the identification of the oxidation products of four different hydroxycoumarins, namely, 3-, 4-, 6- and 7-hydroxycoumarin (3HC, 4HC, 6HC and 7HC), on carbon electrodes by performing bulk electrolyses in buffered solutions. LC–MS/MS in high resolution mode is used for the product identification. It was found that the voltammetric behaviours of the four compounds are quite different, as well as the corresponding oxidation products. For 3HC the end product was 2-hydroxymandelic acid [2-hydroxy-2-(2-hydroxyphenyl)acetic acid]: for 4HC, salicylic acid [2-hydroxybenzoic acid]; for 6HC, 2H-1-benzopyran-2,6(8aH)-dione and for 7HC no low-molecular weight products were identified. Based on these products and on the voltammetric results, the overall oxidation pathways are formulated as four-electron processes for 3HC and 4HC, and two-electron processes for 6HC. 7HC oxidation is proposed to be an electropolymerization reaction.

**Keywords:** Hydroxycoumarins; antioxidants; electrolysis; products identification; liquid chromatography–mass spectrometry

## Introduction

Coumarins are compounds commonly found in many classes of plants. Natural and synthetized coumarins can produce beneficial effects for the organism as antitumoral[1,2], anti-inflammatory[2-4] and neuroprotective[5,6]. Coumarins can also originate oxidative damage in the living organisms via free radical interaction and scavenging of reactive oxygen species (ROS)[7, 8]. In addition, the antioxidant capacity of coumarin derivatives has been proved[7, 9-13], these compounds acting as radical scavengers and enzymes modulators. This antioxidant activity is associated to the presence of at least one hydroxyl group in the coumarin molecule [7, 9, 11], being these hydroxylated derivatives nontoxic antioxidants of natural origin[11]. Due to their involvement in redox reactions, hydroxy- coumarins have been determined by electrochemical techniques as alternatives to other analytical techniques[14-17].

The involvement of antioxidants in electrochemical reactions makes relevant the investigation of their redox behavior to relate the oxidation-reduction mechanisms with the antioxidant activity[18-20]. To establish a redox pathway, the rate determining step must be identified and the reaction orders with respect to the reactant species (that is, the antioxidant and $H^+$ or $OH^-$ ions) must be determined. This can be accomplished by voltammetric techniques. However, it is very important to determine the end products of the redox reaction, which are decisive in the knowledge of the rupture and/or formation of bonds in the curse of the redox reaction and also informs on the overall electrons involved in the global reaction. Though some data about the peak potentials and peak currents of the 4- and 7-hydroxycoumarin, have been previously reported[16], as far as the authors know, no studies can be found in the literature dealing with the identification of the electro-oxidation products of hydroxycoumarines in aqueous media, though it has been reported that the anodic oxidation of 4-hydroxycoumarin in methanol originates a methoxy- derivative[21]. The oxidative spectroelectrochemistry of 4-hydroxycoumarin and 7-hydroxycoumarin (umbelliferone) has been reported, and evidences on the formation of an insulating polymer film at the electrode surface in the electrooxidation of umbelliferone were given[22]. Such evidences were based in the results obtained by in situ long-path-length thin-layer UV-vis spectroelectrochemistry and ex situ ATRFTIR spectrometry.

The aim of this work was to identify the oxidation products of 3-, 4-, 6- and 7-hydroxycoumarin (3HC, 4HC, 6HC and 7HC), on carbon electrodes and, based on these products and on voltammetric measurements, to formulate the overall oxidation pathways, as a first step in the interpretation of the antioxidant activity of these derivatives. To achieve this goal, bulk electrolyses are made in buffered solutions and LC– MS/MS in high resolution mode is used for the product identification.

## Experimental

*Reagents*

All chemicals, of analytical grade, were used without further purification. LC–MS grade acetonitrile and formic acid (Fisher Scientific, UK) and deionized water (18 MΩ.cm) from a Millipore Milli-Q water purification system (Bedford, MA) were used to prepare the mobile chromatographic phases and solutions. Hydroxycoumarins were from Sigma-Aldrich, and the rest of reactants were from Merck.

*Electrochemical measurements*

For the voltammetric measurements a 0.1 M Britton-Robinson Buffer solution (equimolar acetic, phosphoric and boric acid solution) was used as supporting electrolyte. At very low pH values, sulfuric acid solutions were used. For the electrolyses, a 0.05 M of ammonium acetate buffer solution at pH 5.00 (obtained by adding the appropriate amount of acetic acid), was used as supporting electrolyte. Stock solutions of coumarins were prepared in absolute ethanol. The final ethanol content in the reaction medium was 1.5% (v/v). The aqueous solutions were prepared using type I ultrapure water (resistivity 18.2 MΩ.cm at 298 K) obtained from an ultrapure water Millipore system. The *pH* was adjusted with solid NaOH. Stock solutions were stored in the dark at 277 K to avoid decomposition.

All electrochemical measurements were made with an Autolab PGSTAT302N potentiostat using the software package NOVA 1.7. A three-electrode cell equipped with a Pt wire counter electrode, and a BAS MF-2079 Ag/AgCl 3 M KCl reference electrode was employed. All tests were performed at 298 K.

The working electrodes used for electrochemical measurements were:

– Voltammetric measurements: a glassy carbon electrode (GCE) from IJCambria with 7.5 $mm^2$ area. The electrode was polished with a silicon carbide paper, followed by diamond slurry (0.25 μm) and alumina (0.3 and 0.05 μm) slurries, to regenerate the electrode surface. Residual polishing material was removed from the surface by sonication in a water bath for 10 minutes.

– Electrolysis: high density graphite rods (GRE) of 99.995% purity form of 6 mm diameter protected with a silicone tube Aldrich (CAS number 7782-42-5 C). The electrode was polished with a silicon carbide paper, followed by alumina (0.3 μm) slurry. Residual polishing material was removed from the surface by sonication of the electrode in a water bath for 10 minutes. Electrolyses were made under continuous stirring and the decrease in concentration with time was monitored by cyclic voltammetry.

*LC–QTOF MS/MS analysis*

Chromatographic separation was performed by using an Agilent 1200 series LC furnished with an Inertsil ODS-2 C18 analytical column (250×4.6 mm i.d., 5 mm particle) from GL Science (Tokyo, Japan). The chromatograph was coupled to a 6540 quadrupole–time-of-flight detector (QTOF MS/MS; Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization source for detection. The injection volume was 5 μL, and the mobile phases were 0.1% formic acid in deionized water (phase A) and acetonitrile (phase B) at a constant flow rate of 0.75 mL $min^{-1}$. The gradient was as follows: 4% to 10% B in 5 min, change from 10% to 25% B in 30 min, from 25% to 100% B in 15 min and constant at 100% B for 5 min. After analysis, the column was equilibrated to the initial conditions for 5 min. The dual ESI source operated in both positive and negative ionization modes under the following conditions: nebulizer gas at 40 psi, drying gas flow rate and temperature at 12 $L \cdot min^{-1}$ and 325°C, respectively. The capillary voltage was set at 3500 V, while the Q1, skimmer, and octapole voltages were fixed at 130, 65, and 750 V, respectively. The data were

acquired in centroid mode in the extended dynamic range (2 GHz). Full scan was carried out at 6 spectra $s^{-1}$ within the *m/z* range of 40–1700, with subsequent activation of the three most intense precursor ions (allowed charge: single or double) by MS/MS using a collision energy of 20 eV and 40 eV at 3 spectra $s^{-1}$ within the *m/z* range 30–1700. An active exclusion window was programmed after the first spectrum and released after 0.75 min, to avoid repetitive fragmentation of the most intense precursor ions, thus increasing the detection coverage. To assure the desired mass accuracy of the recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and m/z 922.0098 (protonated hexakis-1H,1H,3H-tetrafluoropropoxy)phosphazine or HP-921) in positive ion mode; and *m/z* 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in negative ion mode.

*Identification of reaction products*

MassHunter Qualitative Analysis software (version B7.00; Agilent Technologies, Santa Clara, CA) was used to extract the molecular features and process the data obtained by LC–QTOF in auto-MS/MS mode. The target parameters for feature extraction included a threshold of 500 counts and a maximum charge state of 2. In addition, the isotopic distribution for a valid feature had to be defined by two or more ions with a peak spacing tolerance of 0.0025 m/z, plus 10.0 ppm. The minimum absolute height required for feature extraction was set at 500 counts.

The proposed fragmentation patterns of the electrochemical reaction products were achieved by the analysis of MS/MS information (identification of precursor ion and main fragments) by the formula calculator tool in Qualitative Analysis software, which provides the theoretical molecular formula. Special emphasis was paid to those entities that led to fragments at *m/z* 93.0345 ($C_6H_6O$) or 77.0396 ($C_6H_6$) for phenol and benzene, respectively, which are representative of the HC family.

Additionally, the polarizability index of the extracted molecular features was calculated by Chemsketch software (ACD/Labs, Toronto, Ca.) to confirm the elution order.

**Results and Discussion**

The voltammetric behaviour of the four compounds studied is very different, as can be appreciated in figure 1. The cyclic voltammograms of 3HC showed only one oxidation peak in the direct scan of the first cycle, except for very alkaline solutions, probably because the reaction with the $OH^-$ ions present in high concentrations in these media promotes the progress of the oxidation to a more advanced stage. In addition, at these pH values the intensity increases with respect to that observed in acidic and neutral media. On the contrary, the cyclic voltammograms of 4HC showed two oxidation peaks in the entire pH range. The same increase of the intensity and the appearance of additional oxidation peaks is observed in basic media. In the cases of 6HC and 7HC, the cyclic voltammograms showed one or two oxidation peaks in the first cycle, depending on the medium pH, but for 6HC reduction peaks were observed in the reverse scans, appearing at less positive potentials than the oxidation

peaks. For these two compounds, in very basic media the voltammograms did not show the increase in intensity observed for the other two hydroxycoumarin isomers.

-- Figure 1 –

The reduction peak of 6HC was investigated by recording additional voltammetric cycles. Figure 2 shows that a new oxidation peak appears in the second cycle at intermediate potentials between the reduction and the main oxidation peaks.

Since in the first cycle this new peak is not observed, it is evident that the oxidation signal must correspond to the oxidation of a species that is not initially present, but is near the electrode after this first scan. The cathodic peak observed in the first scan must corresponds to the reduction of the product of the main oxidation peak. If the species originated in this reduction process was 6HC, a second scan should give the same couple of oxidation-reduction peaks that appear in the first scan. The appearance of an additional oxidation peak at potentials lower than the main oxidation peak indicates that the species originated in the reduction peak is not 6HC and that this product is oxidized at the potentials corresponding to the new oxidation peak.

By recording the cyclic voltammograms in a potential window above the reduction peak, the intermediate oxidation peak does not appear in the second scan (see figure 2), this confirming the above assumption. Moreover, one can discard that the adsorption of 6HC (or the product) on the electrode is responsible for the appearance of the additional oxidation peak. The redox couple appears in the second cycle at low and high pH values, but not in weakly acid or basic media.

-- Figure 2 –

Figure 3 shows the variations with pH of the peak potentials of the oxidation peaks of the four investigated hydroxycoumarines. In the case of 6HC, only the results corresponding to the main oxidation peak are shown, because the other oxidation peak, which appears above pH 8, is very close to the main peak and the $E_P$ values are subjected to a great uncertainty.

-- Figure 3 –

For all HC derivatives, the peak potentials of the main oxidation peaks shifted towards less positive values as the pH was increased, with a slope near to –59 mV, and remains unchanged after a given pH, which depends on the compound. The intercepts of these two dependencies are close to the dissociation pK of each compound[23, 24], namely 7.1,

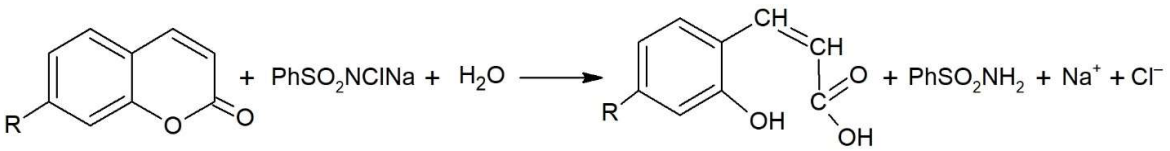
for 3HC, 4.2, for 4HC, 9.1, for 6HC, and 8.0, for 7HC. These pK values correspond to the following generic equilibrium:

$$HC \rightleftharpoons C^- + H^+$$

At pH values higher than the pK, the species in solution are the dissociated anionic forms oh the hydroxycoumarines, $C^-$, whereas at pH<pK the species in solution are the neutral forms, HC. Due to the negative charges of the anions $C^-$, their oxidation potentials are lower than those of the neutral undissociated forms HC. If the oxidation of both forms occurs by two independent pathways, two anodic peaks should be observed in the pH range pK–2<pH<pK+2, which intensities should change as a dissociation curve, that is, the intensity of the peak corresponding to the oxidation of HC, appearing at more positive potentials, should decrease until disappear in that pH range, whereas the intensity of the peak corresponding to the oxidation of $H^-$, appearing at less positive potentials, should parallelly increase. Since only one peak is observed, the electroactive species must be the same at any pH. At high pH values, the anionic form $C^-$ is directly oxidized, the $H^+$ ion being not involved in the process, at least prior or in the rate-determining step. This conclusion is inferred from the independence of the peak potential with the pH. At pH values lower than the pK, the $C^-$ must be formed from the neutral form by dissociation prior the oxidation process, that is, one $H^+$ ion must be released from the HC molecule, this originating the pH-dependence of the oxidation potential as occurs in many other cases[18,25].

The differences in the voltammetric behaviour between the four compounds must be originated by the differences in the oxidation mechanisms. Controlled-potential electrolyses were made in the conditions given in the experimental section and at potentials above the main voltammetric peak, in all cases. The electrolysis products were identified as follows.

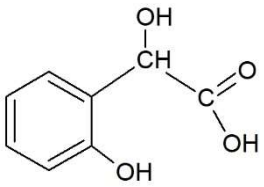
For coumarin, and some derivatives as 7HC, it was claimed out that the chemical oxidation using chloramine-B gives the corresponding o-hydroxycinnamic acid, the observed stoichiometry being[26]:




But it is known since long time ago that the o-hydroxycinnamic (or coumaric) acid is obtained by simple non-oxidative hydrolysis of coumarins[27]. So, the products of the electrooxidation of the coumarin derivatives here investigated cannot be the corresponding coumaric acids.

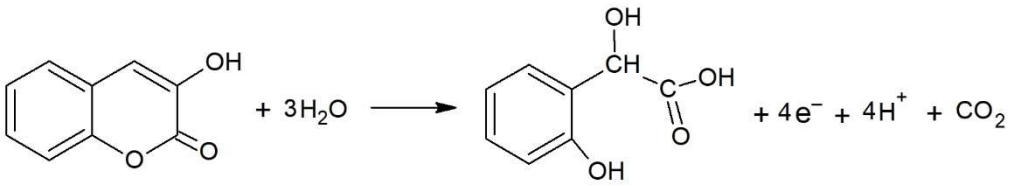
The products of the electrooxidation of the studied HC were analyzed by LC─QTOF MS/MS in high resolution mode. The oxidation product of 3HC was preferentially detected in negative ionization as $[M-H]^-$ ion at *m/z* 167.0349 that fits with a theoretical molecular formula of $C_8H_8O_4$ (1.09 ppm). This product should be formed by opening the benzopyrone cycle, probably due to the loss of $CO_2$ in presence of water during the reaction. The

identification of this oxidation product was supported on the main fragments detected in the MS/MS spectrum. Thus, the fragment detected at *m/z* 121.0291 ($C_7H_6O_2^{\bullet}$) should correspond to the precursor ion after the neutral mass loss of $CO_2$. Complementarily, the fragments at *m/z* 93.0345 ($C_6H_6O^{\bullet}$) and 77.0396 ($C_6H_6^{\bullet}$) clearly fit the phenol and the benzene ring, respectively. It worth mentioning that 3-HC was not detected in the oxidation product, which means that electrooxidation was completed. So, the end product of the oxidation of 3HC identified was the following:

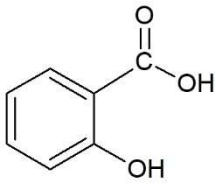



The MS/MS spectrum and assignation of the main product ions for the 3HC oxidation product are shown in Supplementary figure 1.

For 3HC, the voltammograms showed on unique peak and, based on the identified oxidation product, the overall reaction of the electrooxidation process can be formulated as:

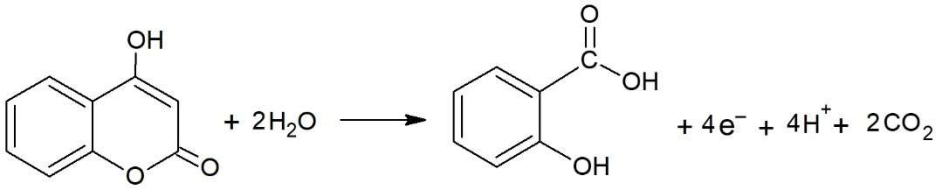



The electrooxidation of 4-HC led to a more predictable reaction product, which was also preferentially detected in negative ionization mode. The precursor ion for this compound was detected as $[M\text{-}H]^-$ at *m/z* 137.0244, which can be associated in high resolution mode to $C_7H_6O_3$ as molecular formula (0.13 ppm). The opening of the benzopyrone ring in this case led to the formation of salicylic acid due to the position of the hydroxyl in the 4-HC. MS/MS fragmentation in this oxidation product led to a main product ion detected at *m/z* 93.0345 characteristic of the phenol structure by the neutral mass loss of the carboxylic group. As in the case of 3-HC, no 4HC was detected in the oxidation product, the end product of the oxidation of 4HC identified being:

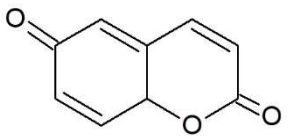



The MS/MS spectrum and assignation of the main product ions for the 4HC oxidation product are shown in Supplementary figure 2. As can be seen, oxalic acid was not detected. This is important because this could be a two-electron route to the end product, and small fragments as $CO_2$ and methanol must be released from 4HC, this representing a four-electron oxidation.

In the case of 4HC, the value of the intensity of the main oxidation peak is close to that of the main oxidation peak of 3HC (though somewhat higher), this meaning that the same electrons must be involved in the overall processes of both compounds, that is, four. In addition, the peak intensity of the oxidation peak of 4HC appearing at less positive potentials is roughly one half of the intensity of the main peak, which is explained by the formation of a stable intermediate after a two-electron transfer. This intermediate product has been not identified because the proximity between the two peaks. So, only the overall reaction for the four-electron process can be formulated as:

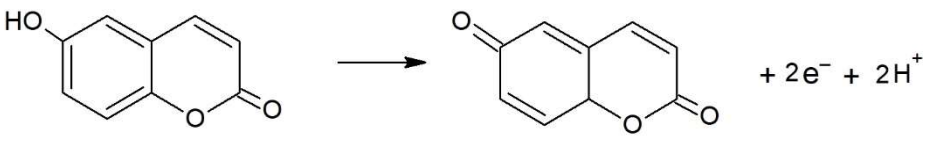



A minimal structural modification was proposed for the electrooxidation of 6-HC according to the obtained results due to the similarity between the MS/MS spectra of 6-HC and its oxidation product. The oxidation product was preferentially detected in positive ionization mode as $[M+H]^+$ at *m/z* 163.0390, which fits exactly with the precursor ion detected for 6-HC. However, two significant peaks were detected in the chromatographic analysis. The first peak, eluting at 17.5 min, fits 6-HC, which means that electrooxidation was not complete. In fact, by comparing quantitative responses, the conversion efficiency should be around 20%. The second peak eluted at 23.7 min, which is indicative of a polarity decrease as compared to 6-HC. As previously mentioned, the MS/MS spectra for 6-HC and the oxidation product were dominated by the same product ions. Therefore, a minimum structural variation was occurred. With these premises, the proposed structure should be formed by a simple oxidation of the hydroxyl group at position 6 of the coumarin structure and, therefore, the benzopyrone ring was not altered:




The MS/MS spectra and assignation of the main product ions for 6HC and its oxidation product are shown in Supplementary figure 3.

The oxidation of 6HC is more simple tan the rest, and a hydroquinone-like compound is formed. In this case, the reduction of the product can be observed in the reverse scan of the cyclic voltammograms at less positive potentials, as occurs for hydroquinone derivatives[20]. The overall reaction is:




Finally, the analysis of reaction media corresponding to 7-HC revealed a drastic reduction in the concentration of this isomer, but no oxidation products were detected. In this

case, the hypothesis was that 7HC is polymerized on the electrode surface and, for this reason, no oxidation products are detected in the reaction media[22]. Supplementary figure 4 shows the chromatograms provided by analysis of 7HC standard and the reaction media that should contain the potential oxidation product.

## Conclusions

The first cycle of the cyclic voltammograms of 3HC and 4HC increased in intensity and additional oxidation peaks appeared in very basic media, this not occurring in the cases of 6HC and 7HC. For 6HC reduction peaks were observed in the reverse scans, appearing at less positive potentials than the oxidation peaks. This peak is due to the reduction of the product of the main oxidation peak, the product of such reduction being oxidized at the potentials corresponding to the new oxidation peak.

The peak potentials of the main oxidation peaks shifted towards less positive values as the pH was increased, remaining unchanged after a given pH, which depends on the dissociation pK of each compound. For pH<pK, one $H^+$ ion is released from the HC molecule prior to the oxidation process. At pH>pK, the anionic form of the HC is directly oxidized, the $H^+$ ion being not involved in the process, at least prior or in the rate-determining step.

The oxidation products of the electrolyses, identified by LC– MS/MS in high resolution mode, were: 2-hydroxymandelic acid [2-hydroxy-2-(2-hydroxyphenyl)acetic acid], for 3HC; salicylic acid [2-hydroxybenzoic acid], for 4HC; 2H-1-benzopyran-2,6(8aH)-dione, for 6HC, and no low-molecular weight products were identified for 7HC. Based on these products and on the voltammetric results, the overall oxidation pathways are formulated: four-electron processes for 3HC and 4HC, and a two-electron process for 6HC. 7HC oxidation is proposed to be an electropolymerization reaction.

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**Figure Captions**

**Figure 1.** Cyclic voltammograms (first cycle) of the hydroxycoumarines on the GCE electrode at concentration of 1 mM and a scan rate of 0.1 $V \cdot s^{-1}$. pH values are given in the figures.

**Figure 2.** Cyclic voltammograms of 1 mM 6HC on the GCE electrode at pH 1.33, and a scan rate of 0.1 $V \cdot s^{-1}$. Continuous lines: first cycle. Dotted lines: second cycle.

**Figure 3.** Dependence of the peak potentials of the oxidation peaks of the hydroxycoumarines. Hollow symbols correspond to the second oxidation peak.

Figure 1

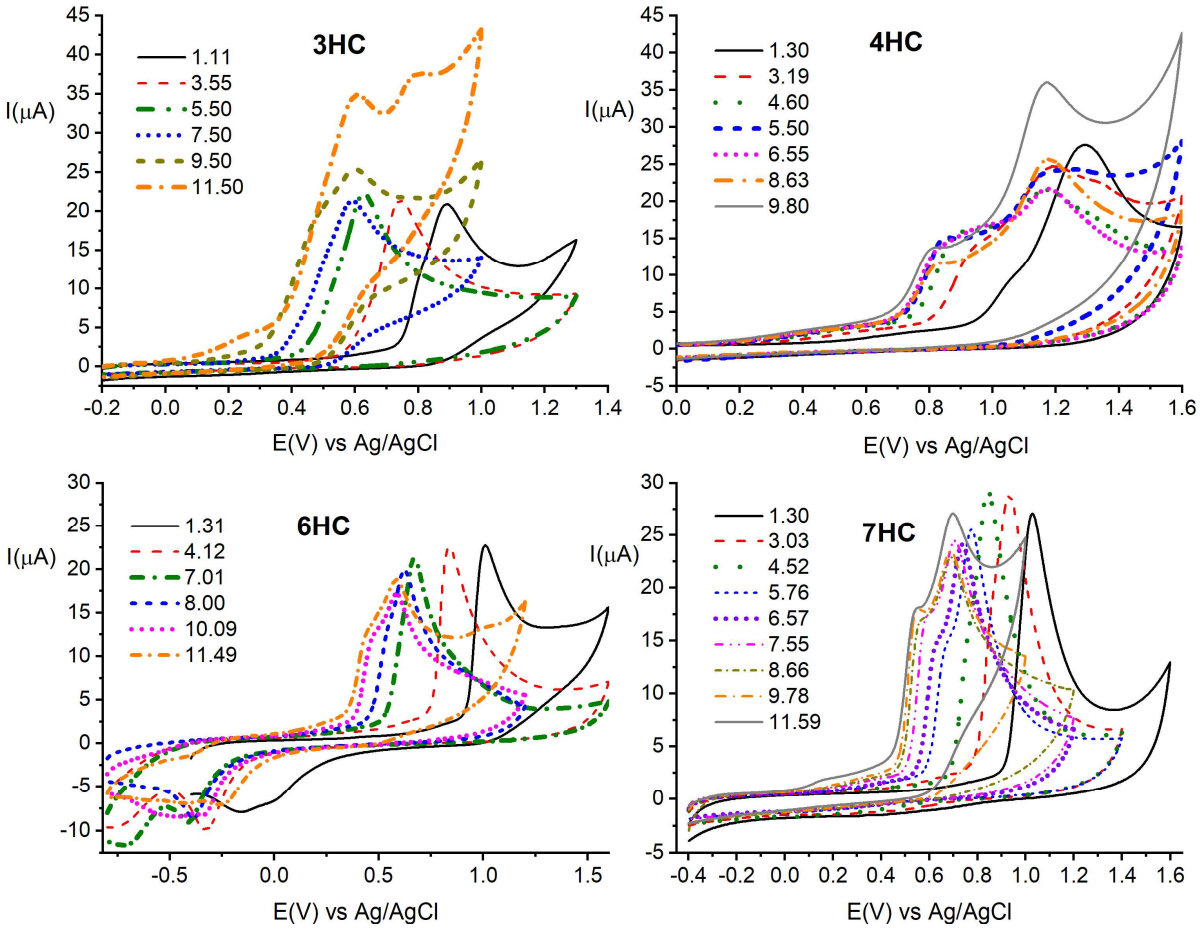
3HC
1.11
3.55
5.50
7.50
9.50
11.50
I(μA)
E(V) vs Ag/AgCl
4HC
1.30
3.19
4.60
5.50
6.55
8.63
9.80
I(μA)
E(V) vs Ag/AgCl
6HC
1.31
4.12
7.01
8.00
10.09
11.49
I(μA)
E(V) vs Ag/AgCl
7HC
1.30
3.03
4.52
5.76
6.57
7.55
8.66
9.78
11.59
I(μA)
E(V) vs Ag/AgCl

Figure 2

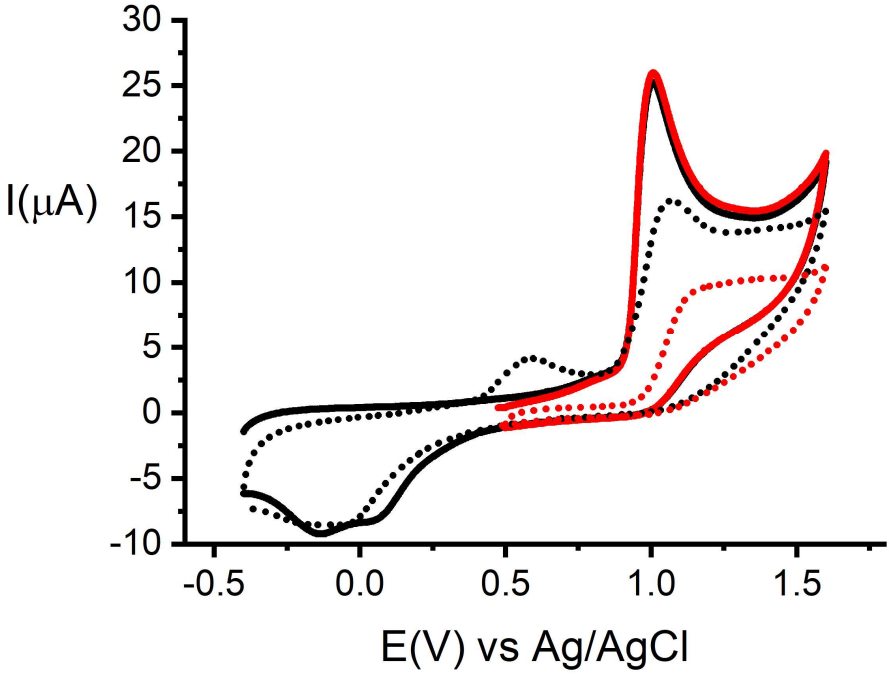


Figure 3