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Determination of the hydroxyl radical by its adduct formation with phenol and liquid chromatography/electrochemical detection

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Abstract

An HPLC-ECD method is described for the indirect determination of the hydroxyl (OH) radical. Fenton's reaction is used to produce •OH, which simultaneously attacks phenols (phenol or pyrocatechol) to form the adducts, pyrocatechol or pyrogallic acid. Thus, [•OH] quantification is based on the separation and detection of pyrogallic acid and/or pyrocatechol by an isocratic HPLC-ECD method. The quantification of •OH is also performed alternatively by a chronoamperometric detection in an electrochemical cell, where simultaneously formed Fe^{III} (Fenton's reaction) combines $[Fe^{II}(CN)_6]^{4-}$ to produce the Prussian blue (PB) molecules ($Fe_4{}^{III}[Fe^{II}(CN)_6]_3$). Newly formed PB molecules are then immediately converted to colorless Everitts salt ($K_4Fe_4{}^{II}[Fe^{II}(CN)_6]_3$) with the reduction of the high-spin Fe^{III} to Fe^{II} at the surface of a glassy carbon electrode at +0.150 V (versus Ag/AgCl). The calculated concentration of •OH during incubation (0.626 ppm) can be detected with negative errors by the HPLC-ECD (0.595 and 0.615 ppm with the errors −5.2 and −1.8%, respectively) and by the chronoamperometric method (0.552 and 0.607 ppm with the errors −11.8 and −3.0%, respectively). For the comparison of the two sets of data, HPLC-ECD method is much more promising.

Keywords: Hydroxyl radical; ROS scavenger; HPLC-ECD; Amperometry

1. Introduction

Today, it is known that virtually every disease state involves free radicals, particularly the most reactive of all, the hydroxyl (OH) radical, a well-known molecule of great physiological and pathophysiological importance [1]. It is now also recognized that to help maintain homeostasis in the cellular level •OH is well regulated, which is formed via the Fenton reaction in vivo [2]. However, when •OH is generated in excess with the cellular antioxidant defense being deficient, some free radical chain reactions may take place by the interaction with proteins, lipids and nucleic acids causing cellular damage. The in vivo half-life of the •OH, though difficult to measure directly by conventional analytical techniques, is estimated to be around 10^{-9} s [1].

Therefore, a confident analytical approach is needed to ascertain the importance of the quantification of the OH radical. Although the spin trap coupled with electron spin resonance (ESR) has been proved to be promising for the measurement of •OH, it is restricted in sensitivity in vivo. In addition, chemiluminescence, gas chromatography MS (GC–MS) and capillary electrophoresis (CE) techniques have also been developed for the determination of the •OH adducts [1].

Ever since the importance of OH radicals in the troposphere has been known, some measurement systems, that have high accuracy and sensitivity, have been developed in the 1990s also for atmospheric applications. Among those, laser-induced fluorescence (LIF) method, laser long-path absorption technique, chemical ionization mass spectroscopy (CIMS) and chemical amplification method are the most commonly used techniques for the measurement of atmospheric OH radicals [3]. In LIF method, OH radicals in the

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ground state are excited to their first excited state by a laser beam whose wavelength is resonant with the rovibronic A-X band and then the excited OH radicals release their energy as fluorescence. Usually, the A-X band at around 282 nm is utilized for excitation and the fluorescence at around 308 nm is detected. The LIF method has two main advantages. First, high selectivity is expected without interference by other species at the wavelength of detection signal, since this technique is a direct measurement. Second, this method is an in situ measurement, so that good spatial resolution is expected. The disadvantage is that calibrations with known amounts of •OH are needed, since the LIF method cannot determine absolute •OH concentrations.

The laser long-path absorption technique, used for •OH quantification, utilizes the A-X (0,0) band, which has a characteristic structured pattern constituted by sharp rovibronic lines. The advantage of this technique is that calibrations are not needed by using the accurate effective absorption cross-section of •OH due to absolute measurement of •OH concentrations. As a disadvantage, the improvement of spatial resolution is limited due to the necessity of the long optical length.

In CIMS method, atmospheric •OH is converted to isotopically labeled sulfuric acid, which in turn produces cluster anions after various sub-reactions. The concentration of OH radicals is determined by measuring the ratios of molar concentrations of different cluster ions. The method requires calibration as an indirect technique, its accuracy may be less than other direct laser spectroscopy techniques while it has an especially high sensitivity.

The chemical amplification method, also termed as the PERCA method, is based on the peroxy radical measurements by chemical amplification. The experimental set-up of the method is suited to field observations due to its compact system. But it is necessary to perform background measurements because of the large fluctuation of NO_2 and O_3 concentrations.

For HPLC applications, because the formation of the highly reactive •OH in vivo and in vitro is extremely difficult to detect, indirect methods also have been used to monitor the molecule. Some •OH adducts such as benzoic acid derivatives have been measured by HPLC with ultraviolet (UV), electrochemical detection (ECD) and mass spectrometry (MS) [4–10].

In 1984, Floyd et al. developed a sensitive method for the detection of •OH. Their methodology employed HPLC-ECD for the quantification and identification of the hydroxylation products from the reaction of •OH with salicylate. The detection limit was less than 1 pmol [11].

HPLC-ECD then became an important method for the in vivo detection of the •OH, and is based on the ability of the •OH to attack the benzene rings of aromatic molecules and being trapped by them. The sensitivity of electrochemical detection is reported to be about 1000 times higher than optical detection [12,13], ESR [14] or HPLC with UV–vis detection [15,16].

In 1992, M.Y. Ye introduced a HPLC method for the quantification of the hydroxylation products of phenol by the OH radical [17]. This method focused on quantification of the products of the addition rather than on the quantification of OH radical itself. The separation and quantification was performed with electrochemical and UV–vis detectors, under isocratic conditions with a mobile phase of 2% acetonitrile and 0.2% acetic acid in H_2O, through an RP-18 analytical column. In the methodology described, OH radicals were produced in the gamma radiolysis of N_2O-saturated aqueous solution. During the radiolysis process, simultaneously formed OH radicals attacked phenol to form the •OH adducts: hydroxylated hydroxycyclohexadienyl radicals. The molecule $K_3Fe(CN)_6$ was used to oxidize hydroxycyclohexadienyl radicals to dihydroxybenzenes. In the presence of 1 mM $K_3Fe(CN)_6$, at pH 5.2, the peaks of hydroxylation products of phenol (hydroquinone, catechol and resorcinol) increased about 2–10 times. The additions of OH radicals to the ortho and para sites were found to be the main processes in the oxidation of phenol by •OH. Thus, the probabilities for the attachment of •OH to the para, ortho and meta positions were 11, 8 and 1, respectively. The OH radical was seen to exhibit a strong preference for addition at the positions activated by the •OH group. This preference resulted from the electrophilic character of •OH.

In the present paper, an HPLC-ECD method on •OH trapping with phenols is described. The indirect determination of the OH radical, formed simultaneously with Fenton's reagent [18], is based on the quantification of the hydroxylation products: phenol and pyrocatechol. Alternatively, the indirect quantification of •OH is also performed simultaneously in a standard electrochemical cell by chronoamperometry with a different methodology through the reduction of Prussian blue formed in the cell. Thus, a comparison of the two different sets of data on the same •OH samples is provided.

2. Experimental

2.1. Chemicals

Phenol, pyrocatechol and pyrogallic acid were obtained as standards from Sigma Chemical Company. Solvents used for chromatography were methanol and phosphoric acid (HPLC ultragradient grade) supplied by J.T. Baker and Riedel-de Haen AG, respectively. Membranes (0.45 μm pore size) used for filtration of the samples were obtained from Sartorius AG (16555 Minisart©). Distilled water used in the mobile-phase fractions was of HPLC grade.

2.2. Apparatus

The liquid chromatographic system (Agilent 1100 series) supplied by SEM Company (Izmir, Turkey) was equipped with an electrochemical detector (HP 1049A programmable electrochemical detector), a pump (HP 1100 series G1310A

isocratic pump), a thermostatted column unit (HP 1100 series G1316A thermostatted column compartment), a manual injector (HP 1100 series G1328A Rheodyne 7725İ) with 20-μl loop and a chromatographic data processing software (HP ChemStation for LC Rev. A. 06. 03 [509]). The separation was performed using an octadecyl (C18) analytical column (Hichrom 5 C18, 7.75 mm × 300 mm, 5 μm particle size).

Additional electrochemical analyzer used was the Cypress Systems OMNI-101 Microprocessor Controlled Potentiostat with the electrochemical data processing software (Cypress Systems Acquire-101SER, version 1.3.1). Electrochemical experiments were carried out in a 10-mL voltammetry cell (BAS VC-2, part # MF-1052) The cell was hermetically sealed with PTFE adapters. The latter is important so as to ensure that there is no gas leakage to or from the cell as the solution in cell was deoxygenized by pure N_2 bubbling before (0.5 h) and during data acquisition. Ag/AgCl reference electrode (BAS RE-5B with flexible connector, part # MF-2052) and a platinum wire auxiliary (BAS, 6 cm with gold-plated connector, part # MW-4130) joined through the holes in its teflon cover. Working electrode was a glassy carbon (BAS GCE, 3.0 mm diameter, part # MF-2012) and a magnetic stirrer at 400 rpm achieved the convective transport to the surface of the GCE during chronoamperometric detection.

3. Procedure

3.1. Chromatographic conditions and detection

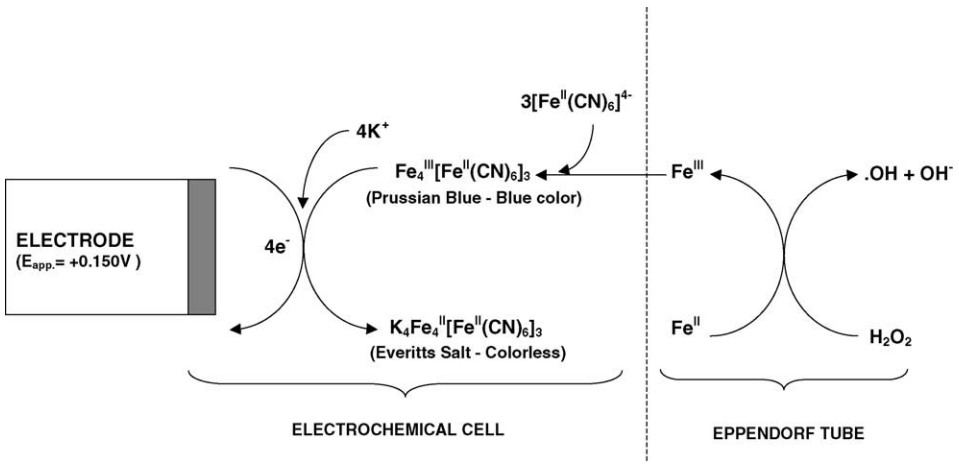
For the chromatographic detection, $^{\bullet}OH$ is formed in a 1.5-mL Eppendorf tube with a reaction taking place by the addition of 50 μL of 100 mM Fe^{II} ($FeSO_4$), 150 μL of 1.8 ppm H_2O_2 and 200 μL of 5 mM scavenger (phenol or pyrocatechol). The OH molecules being formed are simultaneously trapped by the scavenger and the adduct(s) is formed. An optimum incubation time of 5 min is found experimentally. A portion (≈100 μL) of this mixture was then injected into HPLC-ECD system through the 20-μL sample loop at 21.005 °C. Separation of phenolic adduct(s) was performed with a flow rate of 1 mL/min for 80 min. Amperometric detection was carried out at +1.20 V (versus Ag/AgCl, 0.5 μA detector fullscale) in the electrochemical flow cell. The solvents used and their proportions are as follows: methanol/0.01 M phosphoric acid (30/70 v/v). This isocratic mobile-phase combination was shown to be very suitable for the separation of various antioxidant benzoic acid derivatives of very similar stucture [19,20]. Both solvents were degassed (ELMA LC 30/H ultrasonic bath) for 1 h before use. Each compound was tentatively identified by its unique retention time under the same conditions. Quantitative determinations were carried out by the external standard method based on peak heights.

3.2. Chronoamperometric detection (CAD)

The OH radical formed in the Eppendorf tube (for chromatographic detection) is also used here. When the OH radical is formed, Fe^{II} is converted to Fe^{III} in return. When 5 min of incubation time is reached, immediately 50 μL portion of this mixture is injected into the electrochemical cell (BAS VC-2) where the chronoamperometric detection is carried out at an operating potential of +150 mV (versus Ag/AgCl). The electrochemical cell contained 10 mL of 1 mM $K_4Fe(CN)_6$ and 1 mM KCl. After the injection, $K_4Fe(CN)_6$ meets Fe^{III} and Prussian blue (PB) is formed. The formation of PB can also be checked by the appearance of a dark blue color. Simultaneously formed PB molecules are converted to Everitts salt molecules by the electrochemical reduction of the high-spin iron atoms Fe^{III} to Fe^{II} at +150 mV (versus Ag/AgCl):

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + OH + OH^-$$

$$4Fe^{III} + 3[Fe^{II}(CN)_6]^{4-} \rightarrow Fe_4^{III}[Fe^{II}(CN)_6]_3 \quad \text{(prussian blue)}$$



Scheme 1. The overall redox reactions during incubation (Eppendorf tube) and on the surface of the electrode (in the electrochemical cell).

$$Fe_4^{III}[Fe^{II}(CN)_6]_3 + 4e^- + 4K^+ \xrightarrow{E_{app.}=+0.150\,V} K_4Fe_4^{II}[Fe^{II}(CN)_6]_3 \text{ (Everitts salt-colorless)}$$

The overall electrode reactions taking place during chronoamperometric detection are summarized in Scheme 1.

4. Results and discussion

The overall redox reactions taking place in the Eppendorf tube (during incubation) and on the surface of the electrode (in the electrochemical cell) are summarized in Scheme 1. During incubation described as the Fenton's reaction, when H_2O_2 is added, Fe^{II} species are oxidized to Fe^{III}, while simultaneously OH radical and hydroxyl anion (OH^-) molecules are formed. At this point two different experimental approaches are applicable. Either the newly formed OH molecule can be trapped with spin traps (phenol or pyrocatechol) and analyzed through their adducts by the HLC-ECD method or another indirect detection can be performed through the newly formed Fe^{III} species. When a portion of the mixture is injected into the electrochemical cell containing $[Fe^{II}(CN)_6]^{4-}$, Fe^{III} molecules combine with $[Fe^{II}(CN)_6]^{4-}$ molecules and form $Fe_4^{III}[Fe^{II}(CN)_6]_3$, which is known as Prussian blue. PB molecules then in turn are reduced to colorless Everitts salt molecules as $K_4Fe_4^{II}[Fe^{II}(CN)_6]_3$ at +0.150 V (versus Ag/AgCl). This potential has been described as the redox potential for the high-spin iron, Fe^{III}/Fe^{II} couple in the PB structure [21,22]. The paper by M.Y. Ye [17] focuses more on the quantification of the products of the hydroxylation than the quantification of the OH radical itself. The methodology described by M.Y. Ye, though has similar sides, has unique differences. First, •OH is produced in the gamma radiolysis of N_2O saturated aqueous solution instead of Fenton's reaction. Second, OH radicals attacked phenols to form the •OH adducts: hydroxylated hydroxycyclohexadienyl radicals. $K_3Fe(CN)_6$ was used to oxidize these hydroxycyclohexadienyl radicals to dihydroxybenzenes. In the present paper, $K_4Fe(CN)_6$ is used to produce PB in the electrochemical cell, not to oxidize any reactant or adduct. The main difference in the methodology of the two papers is the alternative chronoamperometric approach for •OH determination, which is not present in M.Y. Ye's paper. Thus, a comparison of the data of two different electrochemical approaches (HPLC-ECD and batch analysis) on the same •OH samples is a contribution to the literature.

Typical HPLC-ECD chromatograms of the •OH adducts formed, with 5 mM phenol (A) and pyrocatechol (B) as the spin trapping agents, are displayed in Fig. 1. Separation of the adducts are performed isocratically in MeOH/0.01 M orthophosphoric acid (30/70 v/v) as the mobile phase. Each peak was tentatively identified by its unique retention time (t_R) of the standards under the same conditions. The t_R values assigned for pyrogallic acid and pyrocatechol are 13.38 and

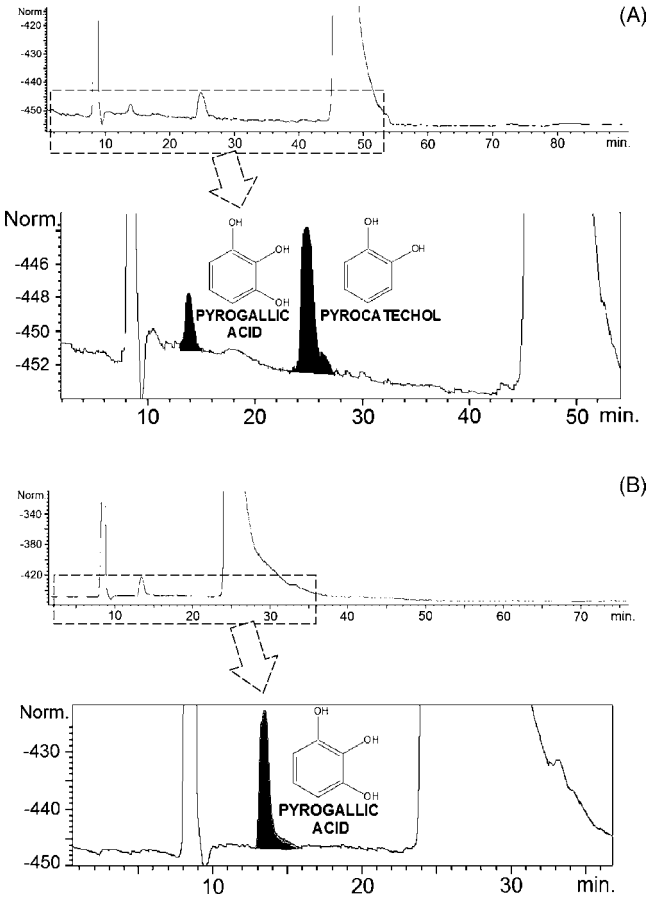


Fig. 1. Typical HPLC-ECD chromatograms of the •OH adducts formed with 5 mM phenol (A) and pyrocatechol (B) as the spin-trapping agents. Amperometric mode, +1.2 V (vs. Ag/AgCl), 0.5 μA full scale, flow rate 1.0 mL/min, isocratic separation in MeOH/0.01 M orthophosphoric acid (30/70 v/v) through an octadecyl (C18) analytical column (Hichrom 5 C18, 7.75 mm × 300 mm, 5 μm particle size) at 21.0 ± 0.5 °C column temperature.

24.63 min, respectively. Additionally, two well-defined but overloading peaks are also identified in both chromatograms to be the excess Fe^{II} (t_R = 8.94 min) and excess phenol (A) (t_R = 47.32 min) and pyrocatechol (B) (t_R = 24.63 min). As can be seen in both chromatograms, when phenol is used (as the spin trap) pyrocatechol and pyrogallic acid are formed, whereas when pyrocatechol is used pyrogallic acid is formed. When phenol is used, in accordance to the initial calibration plots obtained, the peak heights of pyrogallic acid and pyrocatechol correspond to 0.155 and 0.440 ppm, respectively, which makes 0.595 ppm of total adduct (Fig. 1A). When pyrocatechol is used, 0.615 ppm pyrogallic acid is detected (Fig. 1B). For both chromatograms an incubation mixture was prepared as described in Section 2, where the final concentration of H_2O_2 added is calculated to be 0.626 ppm, which directly corresponds to the OH radical concentration stoichiometrically through the Fenton's reaction. This calculated •OH concentration is found experimentally to be 0.595 and 0.615 ppm when phenol (Fig. 1A) or pyrocatechol (Fig. 1B) is used as the spin traps, respectively. Thus, it can be con-

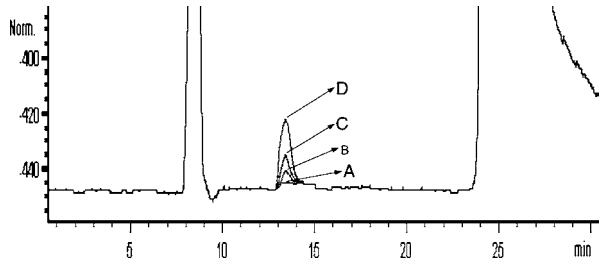


Fig. 2. The effect of incubation time t = 0 min (A), 1 min (B), 3 min (C) and 5 min (D) on peak height of pyrogallic acid in the chromatogram of the incubation mixture. Experimental conditions as in Fig. 1.

cluded that the calculated concentration values can be detected with relatively small errors −5.2 (Fig. 1A) and −1.8% (Fig. 1B) experimentally. The chromatograms in M.Y. Ye's paper were obtained with a totally different mobile phase of 2% acetonitrile–0.2% acetic acid in H_2O, displaying peaks for catechol, hydroquinone, resorcinol and excess phenol. The t_R value assigned for catechol is around 10 min which we found to be 24.63 min. There is no peak for pyrogallic acid. Phenol (10 mM) is used to trap $^{\bullet}$OH, which doubles the concentration used in the present paper (5 mM). Two different operating potentials are used as +650 and −500 mV which are both quite low in comparison to the potential in this paper: +1200 mV (versus Ag/AgCl). Using such a high potential value helps to detect $^{\bullet}$OH concentrations as low as 0.626 ppm through the oxidation of the adducts (pyrocatechol and pyrogallic acid).

Fig. 2 displays the effect of incubation time on the chromatogram of the incubation mixture where pyrocatechol is used as the spin trap. Among the various incubation periods used, the optimum time interval is found to be 5 min (Fig. 2D) with a peak height of 20.37 nA for pyrogallic acid. With shorter time intervals smaller responses are observed, such as 8.96 nA for t = 3 min (Fig. 2C) and 4.07 nA for t = 1 min (Fig. 2B), where Fig. 2A stands for t = 0 min. The detector response is also checked versus incubation time in a graphic (not shown), and a trend line displaying the linearity has an equation: $y = 3.9085x - 0.441$ ($R^2 = 0.9675$). It is found to be useless to try longer incubation periods, as the response does not increase linearly; in fact, it reaches a plateau and remains constant. This might be explained with the absence of H_2O_2, as it is used up completely in the incubation process. The remaining reactants are Fe^{II} (12.5 mM final concentration) and pyrocatechol (2.5 mM final concentration, as the spin trap) and they are extremely rich in concentration, so the only key molecule here could be H_2O_2 which is relatively too diluted (0.626 ppm final concentration). The situation may be hypothesized such as that when H_2O_2 is depleted after t = 5 min, the linear increase in response versus incubation time is not observed anymore. It is also obvious that if H_2O_2 final concentration is increased, the response raise will again be observed for longer incubation periods.

In Fig. 3, typical chronoamperometric detection of OH radical adducts: pyrogallic acid + pyrocatechol (Fig. 3A) and pyrogallic acid (Fig. 3B) is displayed. Detection is performed at +0.150 V (versus Ag/AgCl) in the electrochemical cell with the glassy carbon electrode, under 400 rpm convective transport (sensitivity 100 nA/V, filter 1 ms). The incubation mixtures used here are the very same samples used in HPLC-ECD detections in Fig. 1. When a 50 μL of incubation mixture of phenol (as the spin trap) is injected, a response current of 0.431 μA is observed (Fig. 3A) which corresponds to 0.552 ppm PB, which in turn finally corresponds to 0.552 ppm H_2O_2 (according to the electrode reactions described in Scheme 1). When the other incubation mixture (pyrocatechol as the spin trap) is injected (Fig. 3B), a response current of 0.474 μA is detected which corresponds to 0.607 ppm PB, which in turn finally corresponds to 0.607 ppm H_2O_2. These experimentally determined H_2O_2 concentrations were expected to be 0.626 ppm, which is the calculated concentration, also mentioned in Fig. 1. Therefore, it can be concluded that the calculated concentration values can be estimated relatively higher (in comparison to Fig. 1), errors

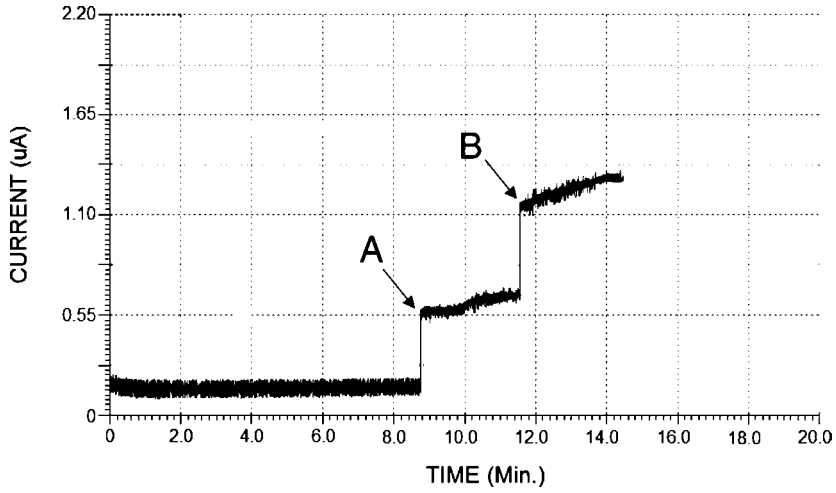


Fig. 3. Typical chronoamperometric detection of $^{\bullet}$OH radical adducts: pyrogallic acid + pyrocatechol (A) and pyrogallic acid (B). E_{app} + 0.150 V (vs. Ag/AgCl) GCE, 400 rpm (sensitivity 100 nA/V, filter 1 ms).

−11.82 (Fig. 3A) and −3.04 (Fig. 3B), then observed experimentally. A calibration plot for PB, where again 50 μL of each Fe^{III} calibration solution was injected into the 10 mL cell containing 1 mM $K_4Fe(CN)_6$ and 1 mM KCl, was also obtained (not shown). Such a calibration plot is linear up to 3 ppm, with a linear trend line of $y = 0.7248x + 0.0096$ ($R^2 = 0.9999$).

5. Conclusion

As a conclusion, an HPLC-ECD method is described for the detection of pyrogallic acid and pyrocatechol as the adducts of phenol with hydroxyl (OH) radical. Thus, an indirect quantification of •OH is performed; alternatively, an indirect chronoamperometric detection is also described in a conventional electrochemical cell for batch analysis. The calculated concentration values of •OH during incubation period are closely related by the HPLC-ECD and chronoamperometric methods as mentioned in the Section 4. For the comparison of the two sets of data, HPLC-ECD method is much more promising. Both methods (HPLC-ECD and chronoamperometric batch analysis) are discussed in comparison to previous papers and M.Y. Ye's approach [17]. Regarding further studies on the subject, a detailed method validation process for the HPLC-ECD technique described is currently proceeding.

Acknowledgements

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