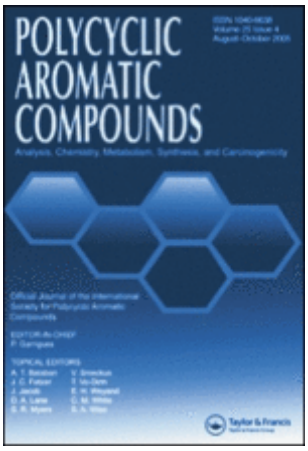

Evaluation of the oxidative potential of pyrenequinone isomers by the dithiothreitol (DTT) assay

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Evaluation of the oxidative potential of pyrenequinone isomers by the dithiothreitol (DTT) assay

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Evaluation of oxidative potential of pyrenequinone isomers by the dithiothreitol (DTT) assay

Abstract

Atmospheric quinoid polycyclic aromatic hydrocarbons (PAHQs) have adverse health effects as redox-active species in particulate matter (PM). Several PAHQs are known to be very reactive in the dithiothreitol (DTT) assay; however, it is unclear if pyrenequinones, their parent pyrene is one of the most abundant polycyclic aromatic hydrocarbons (PAHs) in the atmosphere, contribute to the loss of DTT in PM extracts. Herein, by employing the DTT assay, we evaluated oxidative potentials of three pyrenequinone isomers (4,5-pyrenequinone [4,5-PyQ] and a mixture of 1,6-/1,8-pyrenequinones [1,6-/1,8-PyQ]), along with 9,10-phenanthrenequinone (PQN), 1,2-naphthoquinone (1,2-NQ), and 1,4-naphthoquinone (1,4-NQ), of which DTT loss rates were examined previously. Our results demonstrate that the DTT consumption by ortho-type PAHQs is fast, particularly by 4,5-PyQ. The order of DTT loss rate by the PAHQs tested in this study was as follows: 4,5-PyQ ~ PQN > 1,2-NQ > 1,4-NQ ~ 1,6-/1,8-PyQ. 4,5-PyQ was confirmed to be active in the DTT assay for the first time in this study. The DTT consumption rate by 4,5-PyQ is 14.6 ± 0.8 mol/min/PAHQ-mol, which is comparable to that of PQN (14.4 ± 0.1 mol/min/PAHQ-mol) known as the most active PAHQs by the DTT assay so far.

Keywords: DTT assay; particulate matter; oxidation potential, pyrenequinone isomers

1 Introduction

Exposure to airborne particulate matter (PM), one of the air pollutants, can have adverse health effects on humans, regardless of the duration of exposure.[1-4] The harmful health effects of PM on humans include those caused by a single component of PM[5] and those caused by the synergistic effects of multiple components.[6] They are regarded as a multifaceted phenomenon, involving many chemical components, such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and metals.[7] In recent years, there has been much interest and research on the health effects of oxidative stress due to the generation of reactive oxygen species (ROS) caused by PM.[8, 9] ROS are reactive chemical species based on oxygen atoms, such as superoxide radical (O_2^-), hydroxy radical ($OH\cdot$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). It is known that

excessive production of ROS, such as superoxide radicals, or the lack of an antioxidant's ability to eliminate them *in vivo,* leads to the oxidization and damage of biomolecules like fats, proteins/enzymes, and DNA molecules responsible for genetic information. This results in various conditions like inflammation, cancer, cardiovascular diseases, and neurodegenerative diseases.

The dithiothreitol (DTT; $HSCH_2(CH(OH))_2CH_2SH$) assay is a method used to quantitatively evaluate the oxidative ability of PM without the use of cells. Many studies on the DTT assay method have been reported.[10–13] In this assay, compounds contained in PM that have the ability to oxidize DTT to a disulfide structure when mixed with DTT are analyzed, and the rate of oxidation is considered an indicator of the oxidative ability of the compound. Studies using the DTT assay have been reported for the analysis of metals, such as copper and manganese, and substances, such as some quinoid PAHs (PAHQs), which contribute to the oxidative ability of PM.[12] In particular, PAHQs, which have a ketone group at the ortho position, have been reported to catalyze a redox reaction by a redox cycle and generate excessive ROS (Fig. 1).[13]

Insert Fig. 1 here

However, there are many PAHQs that are known to exist in PM, whose oxidative ability has not yet been investigated. For example, pyrenequinones are compounds derived from pyrene which is one of the major PAHs present in atmospheric PM, yet their oxidative ability has not been investigated so far. Therefore, we evaluated the oxidative ability of two pyrenequinone isomers, 4,5-pyrenequinone (4,5-PyQ) and 1,6-/1,8-pyrenequinone (1,6-/1,8-PyQ), by DTT assay along with those of 9,10-phenanthrenequinone (PQN), 1,2-naphthoquinone (1,2-NQ), and 1,4-naphthoquinone (1,4-NQ), which have already been evaluated by the DTT assay.

2 Materials and methods

2.1 Chemicals and reagents

PQN, 1,2-NQ, and 4,5-PyQ were purchased from Sigma-Aldrich. 1,4-NQ was purchased from Tokyo Chemical Industry Co., Ltd. 1,6-/1,8-PyQ (a mixture of 1,6-PyQ and 1,8-PyQ) was provided by Kanto Chemical Co., Inc. DTT, Tris-HCl buffer (pH 9.0), and dimethyl sulfoxide (DMSO) were supplied by FUJIFILM Wako Pure Chemical Co. Dithiobisnitrobenzoic acid (DTNB) was obtained from DOJINDO MOLECULAR TECHNOLOGIES, Inc. Acetonitrile, potassium dihydrogen phosphate (KH_2PO_4), and dipotassium hydrogen phosphate (K_2HPO_4) were procured from NACALAI TESQUE, Inc.

2.2 Preparation of reagents for the DTT assay

Solutions of PQN, 1,2-NQ, and 4,5-PyQ at 5 μM concentrations, and those of 1,4-NQ and 1,6-/1,8-PyQ at 25 μM concentrations, were prepared in DMSO. The concentrations of the reagents used in this study were determined within the range where a linear relationship between the concentration and the DTT consumption rate was observed.

2.3 DTT assay

Among different methods of measurement, we followed the method reported by Li et al.[14] In a conical tube immersed in a bath maintained at 37 ℃ (DIGITAL WATER BATH SB-1000: EYELA), 1425 μL of a 79 μM DTT solution in a 0.05 M potassium phosphate buffer (15 mM K_2HPO_4 and 35 mM KH_2PO_4; pH 7.2) and 75 μL of each test sample were mixed well. PQN, 1,2-NQN, and 4,5-PyQ were reacted for 10 min, while 1,4-NQN and 1,6-/1,8-PyQ were reacted for 20 min. In the blank test, DMSO which was used as a solvent for the preparation of test samples was used. 100 μL of the reacted mixture was collected after a predetermined reaction time and mixed with 20 μL of a 3 mM DTNB solution in a 0.05 M potassium phosphate buffer (pH 7.2) in a disposable spectrophotometer cell. 5-Mercapto-2-nitrobenzoic acid (TNB) was produced by the reaction of DTT and DTNB. The generated TNB was quantified using a UV-Vis spectrophotometer (UVmini-1240: SHIMADZU) at a wavelength of 412 nm. A mixture of the 0.05 M potassium phosphate buffer (pH 7.2) and the DTNB solution was used as a reference for absorbance measurement. The corresponding TNB reduction was calculated as the DTT consumption by each PAHQ after each reaction time. Each sample was analyzed thrice and the results of the three measurements were averaged and divided by reaction time and sample concentration to calculate the DTT consumption rate per unit sample amount (mol/min/PAHQ-mol).

2.4 HPLC analysis

The reaction mixtures of PyQs with DTT were analyzed by HPLC. A 6 mM DTT solution was prepared by diluting the 1 M DTT solution with water. Then, 4,5-PyQ and 1,6-/1,8-PyQ were dissolved in DMSO at prescribed concentrations, followed by the dissolution of these solutions in 250 mM Tris-HCl to a final concentration of 15 μM PyQ (the DMSO concentration in 4,5-PyQ and 1,6-/1,8-PyQ was 5.9 and 7.5%, respectively). Then, 200 μL of the 6 mM DTT solution and 200 μL of each test sample (15 μM 4,5-PyQ or 1,6-/1,8-PyQ solution) were mixed and reacted. After 15 min of reaction, the solution was analyzed using an HPLC system with a HITACHI L-4200 UV-Vis detector. The PyQs were separately eluted from an ODS-P column (4.6 × 250 mm i.d., 5 μm particle size, GL Science) using H_2O/CH_3CN (1/1, v/v) as the mobile phase at a flow rate of 1.0 mL/min, and the compounds were detected at the wavelength of 254 nm. For reference, each sample solution was also analyzed by HPLC before its reaction with DTT.

3 Results and Discussion

3.1 DTT consumption rate by individual quinone compounds

Insert Fig. 2 here

As shown in Fig. 2, the order of the DTT consumption rate of the five quinones analyzed in this study is 4,5-PyQ ~ PQN > 1,2-NQ > 1,4-NQ ~ 1,6-/1,8-PyQ. The activities of three of these quinones, viz., PQN, 1,2-NQ, and 1,4-NQ, on DTT have already been reported by Xiong et al.[10], and this order is consistent with their results.

Charrier et al. also studied the activities of PQN, 1,2-NQ, and 1,4-NQ[12], and reported a DTT consumption rate ratio of 111: 32: 4. Meanwhile, 4,5-PyQ was confirmed to be active in the DTT assay for the first time in this study. The DTT consumption rate by 4,5-PyQ is 14.6 ± 0.8 mol/min/PAHQ-mol, which is comparable to that of PQN (14.4 ± 0.1 mol/min/PAHQ-mol) known as the most active PAHQs[10, 12] by the DTT assay so far.

3.2 HPLC analysis of 4,5-PyQ and 1,6-/1,8-PyQ

The DTT assay indicated that the oxidation abilities of 1,6-/1,8-PyQ and 4,5-PyQ are remarkably different, although they are positional isomers having similar structures. In order to verify the key factor that affects the DTT consumption rate, the residual amount of the PyQs in the DTT assay samples were analyzed by HPLC.

Insert Figs. 3 and 4 here

After the reaction with DTT, 58% of the initial amount of 4,5-PyQ remained (Fig. 3), whereas only 1% of the initial amount of 1,6-/1,8-PyQ remained at the retention time of ~8 min (Fig. 4), which is much lower than the residual amount of 4,5-PyQ. In addition, after the reaction of 1,6-/1,8-PyQ with DTT, two peaks different from those of 1,6-/1,8-PyQ were observed at the retention time of ~5 min.

Motoyama et al. reported that ortho-PAHQs have higher oxidative ability than para-PAHQs because the oxidation–reduction reaction of PAHQs occurs cyclically, leading to the generation of an excessive amount of ROS, such as H_2O_2, while they oxidize and consume DTT.[13] It is assumed that the two-electron reduced form of 4,5-PyQ is extremely unstable, and is rapidly oxidized after its formation. It is then converted to 4,5-PyQ via the one-electron reduced form, a semiquinone radical, which continues to catalytically consume DTT. After the reaction with DTT, 58% of 4,5-PyQ remained, although the amount of DTT consumed in the reaction was the largest among the five PAHQs examined in this study. This result indicates that the redox reaction is actively cycled and ROS is generated excessively in the reaction of DTT with 4,5-PyQ. By contrast, 1,6-/1,8-PyQ is considered to have been consumed immediately by the reaction with DTT, because the two-electron reductant is relatively stable, and no redox cycle is formed. The peaks observed in Fig. 4 at retention times of 4.9 and 5.3 min are presumed to be derived from dihydroxypyrenes, the two-electron reduced forms of 1,6-/1,8-PyQ. These results support the hypothesis that the high ROS-generation ability of PAHQs is related to the stability of the two-electron reduced form of the PAHQ compounds.[15]

3.3 Atmospheric implications

Table 1 shows the concentrations of various PAHQs present in four reference PMs: NIST SRM1648a (urban PM), SRM1649b (urban dust), SRM2786 (fine PM), and NIES CRM No. 28 (urban aerosol), as reported by Toriba et al.[16]

We estimated the oxidative ability of each PAHQ in the reference materials by multiplying the molar concentration of each PAHQ per particle weight by the DTT consumption rate obtained in this study (Table 2). 1,6-PyQ and 1,8-PyQ were assumed

to be present at equal proportions in the standard sample solution analyzed by the DTT assay.

The oxidative ability of 4,5-PyQ in the reference materials is equivalent to that of PQN. The contribution of the oxidative ability of PQN in PM samples was reported by Charrier et al. to be 17%,[12] but 4,5-PyQ was not considered as a contributing factor. Our result suggests that 4,5-PyQ is also an important substance in terms of the oxidative ability exhibited by PM as well as PQN.

4 Conclusion

We evaluated the oxidative abilities of five PAHQs by the DTT assay. As a result, in addition to the well-known oxidants, PQN, 1,2-NQN, and 1,4-NQN, another, 4,5-PyQ, was found to have oxidative ability. The DTT consumption rate by 4,5-PyQ was found to be comparable to that of PQN. On the other hand, the DTT consumption rate of 1,6-/1,8-PyQ was the lowest of the five PAHQs evaluated in this study. In order to examine the cause of the difference in DTT activity between 4,5-PyQ and 1,6-/1,8-PyQ, we used HPLC to analyze the solutions of 4,5-PyQ and 1,6-/1,8-PyQ reacted with DTT. The results revealed that 58% of the initial amount of 4,5-PyQ remained after the reaction with DTT, whereas only 1% of the initial amount of 1,6-/1,8-PyQ remained after the reaction. The result suggests that a redox cycle was formed during the reaction of DTT with 4,5-PyQ, and the ROS were excessively produced catalytically. It is speculated that 1,6-/1,8-PyQ was transformed to a more stable two-electron reductant by the reaction. We speculate that the activity of PAHQs in the DTT assay is related to the stability of the two-electron reduced form of the PAHQs. Based on the results of the DTT assay in this study, and the concentration of PAHQs in the reference materials of airborne particulates, we found that 4,5-PyQ makes a large contribution to the ROS production ability of PM, similar to PQN. However, the information obtained from the DTT assay is not sufficient to evaluate the biological effect of 4,5-PyQ. To clarify the oxidative potential of the substances tested in this study, not only other acellular assays such as ascorbic acid (AA) assay [17] and Glutathione (GSH) assay [18], although they usually measure the capability of metals present in PM to deplete antioxidants [19-22], but also cell-based assays such as dichloro-dihydro-fluorescein diacetate (DCFH-DA) [23] should be used.

Acknowledgments

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Declaration of Interest

There are no conflicts of interest to declare.

Word Count: 2193 words

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Table 1. PAHQ concentrations in the reference materials of airborne particulates (unit: μmol/kg)[16]

	SRM1649b	SRM1648a	SRM2786	CRM No.28
4,5-PyQ	4.6	3.7	2.7	3.5
1,6-PyQ	0.47	0.44	0.76	0.67
1,8-PyQ	0.57	0.31	0.92	0.65
PQN	3.7	3.8	2.7	3.4
1,2-NQ	0.49	0.79	1.4	1.0
1,4-NQ	0.53	0.35	0.80	0.43

Table 2. Estimated oxidative ability of PAHQs in the reference materials of airborne particulates (unit: DTT-μmol/min/kg)

	SRM1649b	SRM1648a	SRM2786	CRM No.28
4,5-PyQ	67	54	40	51
1,6-PyQ	0.70	0.65	1.1	0.99
1,8-PyQ	0.67	0.36	1.1	0.76
PQN	53	54	39	49
1,2-NQ	1.2	2.0	3.6	2.6
1,4-NQ	0.77	0.52	1.2	0.63

Figures

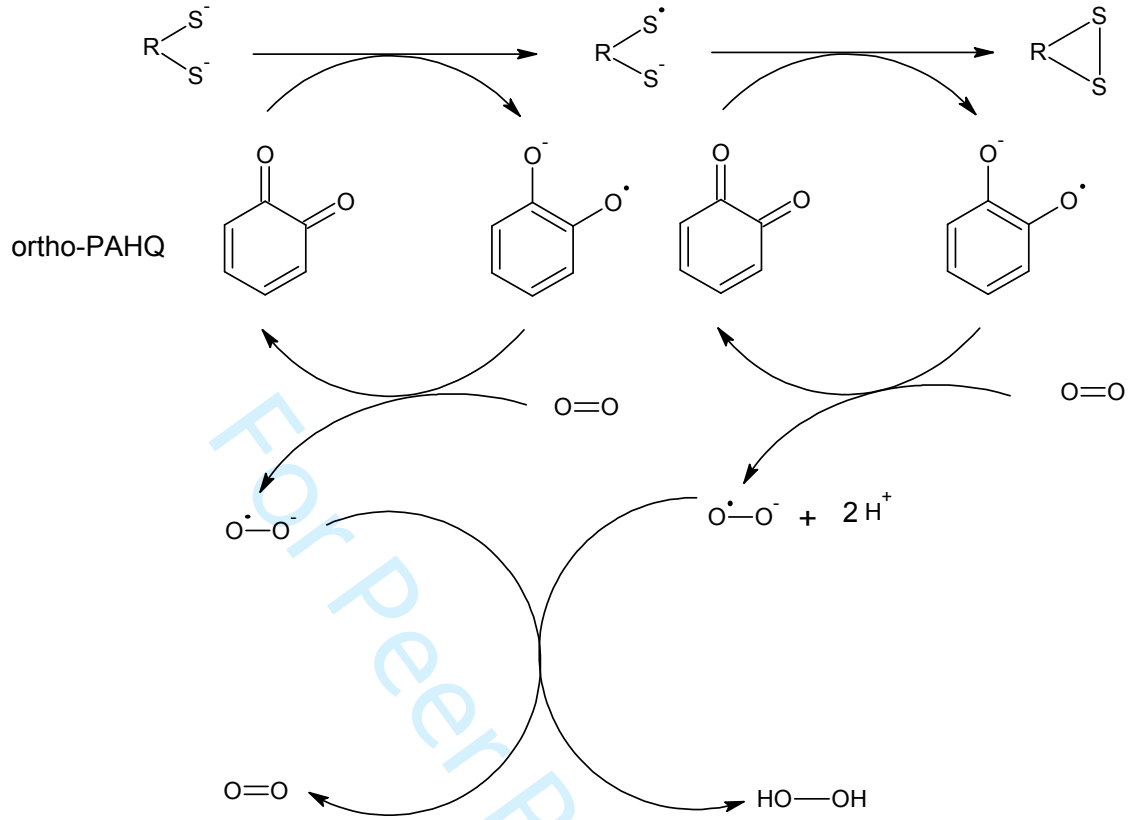


Fig. 1

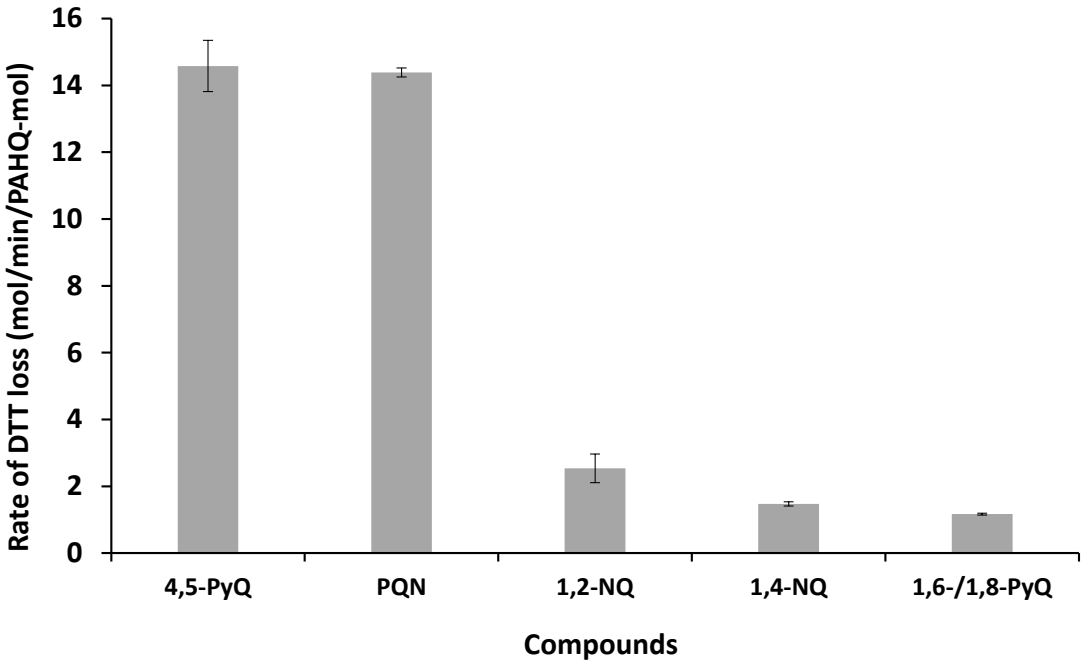
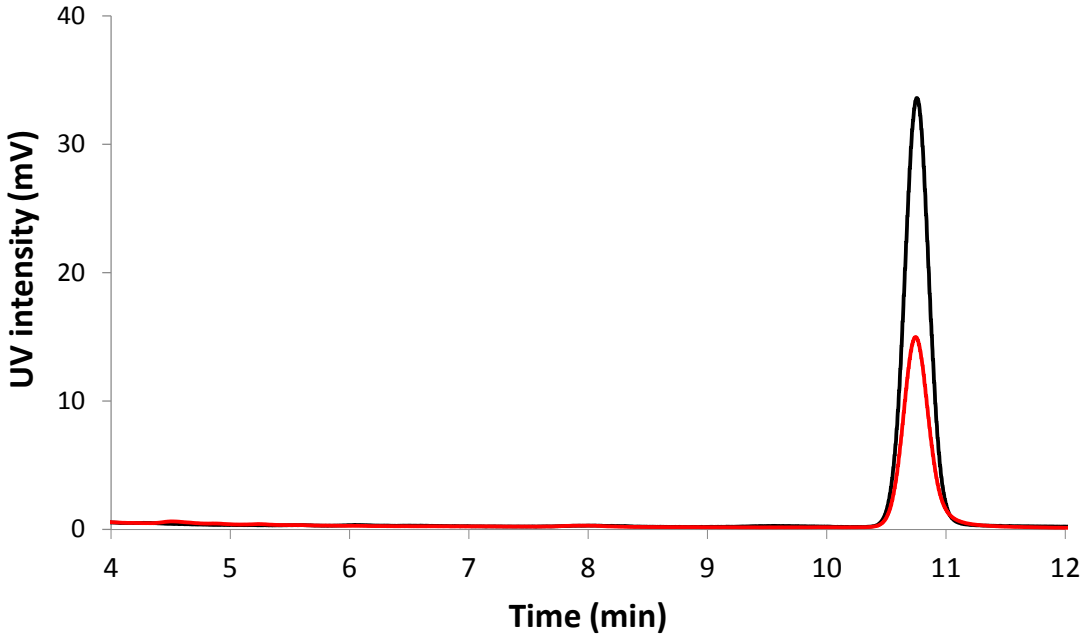


Fig. 2



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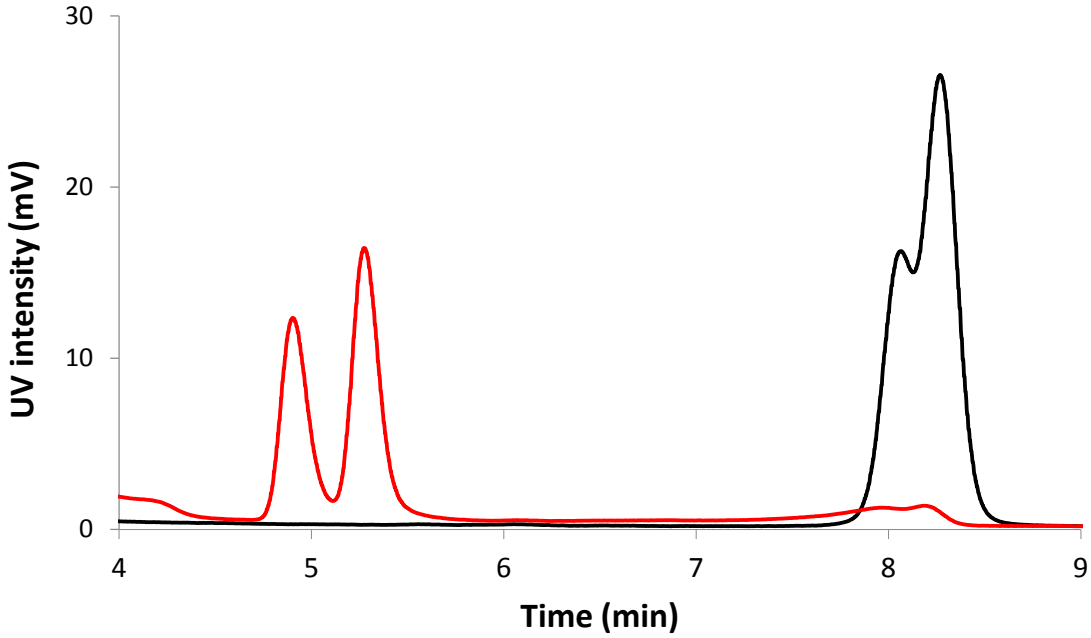

Fig. 4

Figure captions

Figure 1. Redox cycle causing the overproduction of H_2O_2 by ortho-PAHQ.

Figure 2. Blank-corrected rate of DTT consumption by five PAHQs: 4,5-PyQ, PQN, 1,2-NQ, 1,4-NQ and 1,6-/1,8-PyQ.

Figure 3. HPLC analysis of 4,5-PyQ in the test solution, before DTT assay (black line) and after DTT assay (red line).

Figure 4. HPLC analysis of 1,6-/1,8-PyQ in the test solution, before DTT assay (black line) and after DTT assay (red line).