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Communication

Determination of hydroperoxides in aqueous solutions containing surfactants by the ferrous oxidation-xylenol orange method★

Piotr Meisner and Jerzy L. Gębicki✉

Institute of Applied Radiation Chemistry, Faculty of Chemistry, Technical University of Łódź; Łódź, Poland

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Some surfactants widely used as additives in food, pharmaceuticals, and cosmetic formulations are susceptible to peroxidation resulting in accumulation of hydroperoxides (HP). Our investigation was aimed to study the possible influence of different surfactants on the proportionality and reproducibility of the ferrous oxidation-xylenol orange method developed originally for the determination of hydroperoxides. We also attempted to apply this method to determine hydroperoxides produced radiolytically in surfactant molecules. From our preliminary studies we conclude that the method can be applied for determination of hydroperoxides in systems containing non-ionic or anionic surfactants provided careful calibration is performed for each surfactant.

Keywords: FOX method, gamma-irradiation, hydroperoxides, surfactants

INTRODUCTION

Non-ionic as well as some anionic surfactants are additives widely used in food, pharmaceuticals, and cosmetic formulations. It is known that many of them, especially those containing polyether chain, are susceptible to peroxidation resulting in hydroperoxide (HP) accumulation (Ding, 1993; Jaeger *et al*., 1994). This is unfortunate as HP may influence the results of antioxidant tests or even may change the activity of biological materials. A wide range of methods have been developed for the detection and quantitation of HP. The ferrous oxidation-xylenol orange (FOX) method counts to the most useful ones due to its broad applicability and ease of use. The method has also drawbacks like narrow linear range and relatively low reproducibility. The FOX assay is based on the ability of hydroperoxides to oxidize Fe^{2+} into Fe^{3+} and the subsequent formation of a complex between ferric ions and xylenol orange (XO). The complex can be easily determined spectrophotometrically due to its high absorption coefficient in the range 550–600 nm. The FOX method was first proposed by Gupta (1973) to determine hydrogen peroxide in irradiated aqueous solutions. Wolff and collaborators (Jiang *et al*., 1990) applied this method to detect HP in liposomes, plasma, and lipoproteins. Gay *et al*. (1999a; 1999b) and Gebicki *et al*. (2000) showed the applicability of the FOX method for determinations of HP in proteins. The same authors proposed the use of perchloric acid instead of sulfuric acid used in typical assays in order to enhance the sensitivity and reproducibility of the method and named this version the perchloric acid-ferrous oxidation-xylenol orange (PCA-FOX) assay (Gay & Gebicki, 2002). Very recently an extensive review of the factors that influence the method's performance was presented by Bou *et al*. (2008). One of the conclusions of that review is that there is a need to further study the reproducibility of the method under different conditions.

In this short communication we present a study of PCA-FOX assay reproducibility in aqueous solutions containing some non-ionic and anionic

✉Corresponding author: Jerzy L. Gębicki, Institute of Applied Radiation Chemistry, Faculty of Chemistry, Technical University of Łódź, W. Wróblewskiego 15, 93-590 Łódź, Poland; phone: (048) 42 631 3165; fax: (048) 684 0043; e-mail: jlgebick@mitr.p.lodz.pl

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Abbreviations: AOT, sodium bis(2-ethylhexyl)sulfosuccinate; CMC, critical micelle concentration; FOX, ferrous oxidation-xylenol orange method; HP, hydroperoxides; IG720, Igepal CO-720; PCA-FOX method, perchloric acid ferrous oxidation-xylenol orange method; t-BuOOH, tert-butyl hydroperoxide; XO, xylenol orange.

surfactants, both below and above their critical micelle concentrations (CMC). We also show some preliminary results on the accumulation of HP in surfactants upon gamma-irradiation or upon exposure to daylight.

MATERIALS AND METHODS

All chemicals were of at least analytical grade and used without further purification. Xylenol orange [3,3′-bis(*N*,*N*-bis(carboxymethyl)aminomethyl)-*o*-cresolsulfonaphthalein tetrasodium salt] (Cat. No. 398187), ferrous sulfate [ammonium iron(II) sulfate hexahydrate] (Cat. No. F3754), AOT [sodium bis(2-ethylhexyl)sulfosuccinate] (Cat. No. D-4422), Igepal CO-720 [polyoxyethylene(12)nonylphenyl ether] (Cat. No. 23,865-1), Brij 35 [polyoxyethylene(23)lauryl ether] (Cat. No. 85,836-6), perchloric acid 70% (11.65 M) (Cat. No. 24,425-2), SDS (dodecyl sulfate sodium salt) (Cat. No. L-4509), and tert-butyl hydroperoxide 70% (7.3 M) (Cat. No. B-2633) were purchased from Sigma-Aldrich, and ferric chloride hexahydrate (Cat. No. 904180113) from POCh (Gliwice, Poland).

All measurements were done at ambient temperature 23±1°C. Water from MilliQ Plus (Millipore) was used throughout.

Stock standard solutions of ferric chloride, ferrous sulfate and xylenol orange were made up in 110 mM perchloric acid and were stored in the dark at room temp. Stock solutions of 100 mM Igepal CO-720, Brij 35, SDS, and stock solution of 20 mM AOT were also stored in the dark at room temp. From these stock solutions the FOX reagent was prepared according to the protocol suggested by Gay & Gebicki, (2002). The concentration of $HClO_4$ was checked with titration and was kept between 100 and 120 mM, which ensured the error of absorbance readings to be well below 1%. Stock solution of 50 μM tert-butyl hydroperoxide was stored at +5°C. At least three independent measurements were done for each experimental point.

Irradiation of surfactant solutions equilibrated with air or saturated with oxygen or nitrous oxide were done with a cobalt bomb BK-10000 installed at the Institute of Applied Radiation Chemistry, at the dose rate 0.01 Gy s^{-1}. Hydrogen peroxide formed upon radiolysis of water was removed with catalase prior to performing the PCA-FOX assay. Some solutions were exposed to daylight under conditions simulating the storage of chemicals on an open shelf.

Absorbance measurements in the range of 200–700 nm were done in Hewlett Packard 8452A or Specord S600 (Analytik Jena) diode array spectrophotometers using 1 mm cells.

RESULTS AND DISCUSSION

We determined the absorbance coefficient of the Fe–XO complex generated by ferric ions using freshly-made solutions of ferric chloride in 110 mM $HClO_4$ to avoid hydrolysis. Concentrations were calculated from the weight. The absorbance coefficient was found to be 18500±500 $M^{-1}s^{-1}$ in 110 mM $HClO_4$ at room temp. (23±1°C). This value is below that determined by Gay and Gebicki (2002) in 110 mM $HClO_4$ (25400±80 $M^{-1}s^{-1}$) or in 25 mM H_2SO_4 (20550±200 $M^{-1}s^{-1}$), both for XO from Sigma, but above the values published by Jiang *et al*. (1990) in 25 mM H_2SO_4 at room temp. (15000 $M^{-1}s^{-1}$) for XO from an unknown source and by Gay *et al*. (1999a) in 25 mM H_2SO_4 (14500±700 $M^{-1}s^{-1}$) for XO from Aldrich. In all our experiments we used XO from the same batch.

tert-Butyl hydroperoxide was chosen as a model hydroperoxide to check whether the PCA-FOX assay can be applied for the determination of hydroperoxides in the presence of surfactants. Two non-ionic surfactants, Igepal CO-720 and Brij 35, as well as two anionic surfactants, SDS and AOT, were used in this study. Both IG720 and Brij 35 contain polyoxyethylene and hydrocarbon chains, but in the former the chains are connected with a phenyl ring. The most important difference between SDS and AOT is that the molecule of the former has a single hydrocarbon chain, while that of the latter has two.

The inset in Fig. 1 shows the absorption spectra of samples containing different amounts of

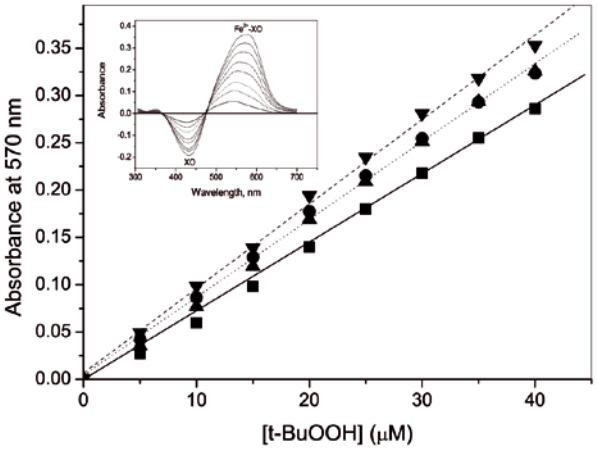

Figure 1. Absorbance of the Fe^{3+}–XO complex as a function of t-BuOOH concentration.
Samples without surfactant (■), samples containing Igepal CO-720 at concentrations of 0.5 mM (●), 1 mM (▲), and 5 mM (▼), respectively. The blank contained all the components except for t-BuOOH. The optical path was 1 mm. Inset: Absorption spectra of solution containing FOX reagent in the presence of 5 mM Igepal CO-720 and t-BuOOH at concentrations changing every 5 μM in the range 5–40 μM.

Table 1. Apparent absorbance coefficients of Fe^{3+}–XO complex formed in the presence of different surfactants.

Values of ε_{app} were determined from the linear dependencies of absorbance at 560, 570 and 580 nm on t-butyl hydroperoxide concentrations. Mean ±S.D. values were calculated from at least three independent measurements.

	No. surfactant	[Igepal CO-720] (mM)			[Brij 35] (mM)		[SDS] (mM)			[AOT] (mM)	
		0.5	1	5	0.5	5	0.5	5	20	0.5	5
ε_{app} ($M^{-1}cm^{-1}$) 560 nm	73500 ± 2000	82500 ± 1500	83000 ± 1600	90000 ± 1700	87500 ± 2100	94000 ± 3000	89500 ± 3400	76000 ± 2300	34800 ± 2100	86000 ± 2200	56000 ± 1000
ε_{app} ($M^{-1}cm^{-1}$) 570 nm	75000 ± 1000	84000 ± 1100	85000 ± 700	92000 ± 1300	90000 ± 1500	98000 ± 2300	92000 ± 3000	78000 ± 1500	32700 ± 1800	88000 ± 1400	53500 ± 1300
ε_{app} ($M^{-1}cm^{-1}$) 580 nm	74000 ± 1300	84000 ± 1500	85000 ± 1200	93000 ± 1400	90000 ± 1700	100000 ± 2200	92700 ± 2700	77500 ± 1100	30000 ± 1700	88000 ± 1400	50300 ± 1500

tert-BuOOH in the presence of a constant amount (5 mM) of the non-ionic surfactant IG720 analyzed with the PCA-FOX method. The blank contained all the components except for t-BuOOH. The decreasing absorption band around 430 nm represents the decay of the dye and the increasing absorbance band around 560 nm represents the formation of the Fe^{3+}-XO complex. Analogous spectra were obtained in the absence of any surfactant as well as in the presence of IG720, Brij 35, SDS and AOT at concentrations below, around, and above the respective CMC. From such spectra the absorbances at 560, 570, and 580 nm were taken as a measure of the concentration of the Fe^{3+}–XO complex.

For all the systems studied in this work a very good linearity of A = f([t-BuOOH]) was obtained up to [t-BuOOH] = 40 μM, however, for the absorbance measured at 570 nm the linearity was slightly better than that for 560 or 580 nm. Figure 1 shows the dependence of the absorbance at 570 nm on the concentration of t-BuOOH for the system containing IG720 at three different concentrations below (0.5 mM), around (1 mM), and above (5 mM) CMC of this surfactant. For comparison the absorbances measured in system without a surfactant are also shown. It can be seen that IG720 increases the response of the PCA-FOX assay. The absorbances for samples containing the surfactant below and around CMC are almost identical and the signal increases on going above CMC. Similar results were obtained for Brij 35. To exclude the possible influence of a contamination of the surfactant by transient metal ions we performed the PCA-FOX assay for solutions containing the surfactants without the addition of ferrous sulfate. The result was negative, i.e. no measurable absorbance around 570 nm was detected. The anionic surfactants SDS and AOT decreased the response of the PCA-FOX assay, but only when present at concentrations above their CMC. Below and around CMC of these anionic surfactants the response of the FOX method remained at the same level as in the surfactant-free solution.

From the slopes of the straight lines like those shown in Fig. 1 we calculated the apparent absorbance coefficients, ε_{app}, of the Fe^{3+}–XO complex at three wavelengths (560, 570 and 580 nm) and the obtained values are gathered in Table 1. The value of ε_{app} for Fe^{3+}–XO complex in the absence of surfactant obtained by us is half of that published by Gay and Gebicki (2002), however the stoichiometry of the oxidation of ferrous ions by tert-butyl peroxide is 4, which is close to the value of five given by Gay *et al*. (1999b). In the presence of non-ionic surfactants both below and above CMC as well as in the presence of anionic surfactants below CMC the apparent absorbance coefficient, ε_{app}, is higher than in their absence. This suggests that these surfactants may act as slight propagators of the ferrous oxidation step. The micelles formed of anionic surfactants, SDS and AOT, significantly lower the apparent absorbance coefficient of the Fe(III)–XO complex. This effect can be accounted for by the attraction of ferric ions by the negative micellar surface and repulsion of xylenol orange anions by the same surface preventing complex formation. The effect of AOT micelles is weaker because the charge density on their surface is lower than on the surface of SDS micelles due to the looser packing of the double-chain AOT molecules.

Next we attempted to apply the FOX method to determine hydroperoxides formed in surfactants. Here preliminary results for non-ionic surfactants are presented. Aqueous solution of IG720 at 5 mM (i.e. above CMC) saturated with molecular oxygen was gamma-irradiated with different doses at a dose rate of 0.01 $Gy\,s^{-1}$. After irradiation the samples were analyzed with the PCA-FOX method without or after catalase treatment. The catalase was used to remove hydrogen peroxide formed upon water radiolysis. To do so, catalase was added to the irradiated sample at the

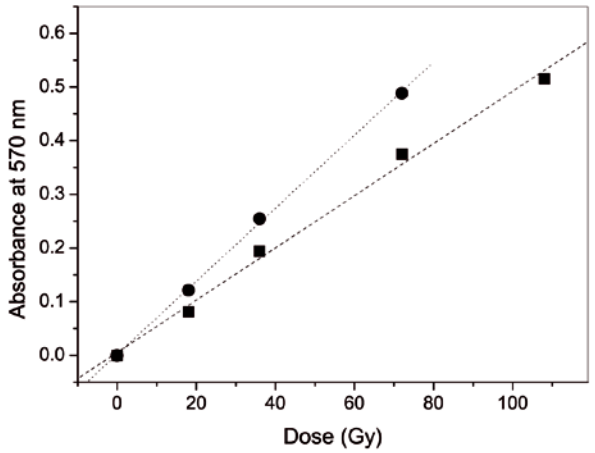

Figure 2. Effect of gamma-irradiation of O_2-saturated aqueous solutions of IG 720 (5 mM) on the absorbance of the Fe^{3+}–XO complex.
Unirradiated solution of IG 720 was used as blank. The optical path was 1 mm. The dose rate was 0.01 Gy s^{-1}. The irradiated samples were not incubated (●) or incubated (■) with catalase (4 nM) for 5 min to remove H_2O_2, the molecular product of water radiolysis, prior to analysis with FOX method.

concentration of 4 nM and after 5 min of incubation the sample was analyzed. It is seen in Fig. 2 that the absorbance at 570 nm is proportional to the dose for up to 72 Gy for samples not treated with catalase and up to 112 Gy for those treated with catalase. It is obvious that a component of the assay was exhausted by the high peroxide concentrations produced above those doses. The difference between the two lines shown in Fig. 2 is proportional to the dose and is attributable to H_2O_2 removed by catalase.

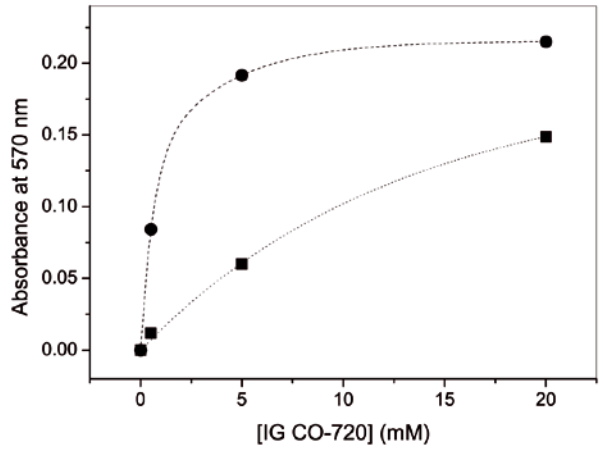

Figure 3. Absorbance of the Fe^{3+}–XO complex as a function of IG 720 concentration in gamma-irradiated aqueous solution equilibrated with air (■) or O_2-saturated (●).
The dose was 18 Gy. The blank contained all the components but surfactant solutions were non-irradiated. The optical path was 1 mm.

In the next experiment air-equilibrated, O_2-saturated, and N_2O-saturated aqueous solution of IG720 were gamma-irradiated with the dose of 18 Gy. The results are shown in Fig. 3. Each sample was treated with catalase prior to PCA-FOX determination. As expected, the yield of HP is much higher in the samples saturated with molecular oxygen and increases with the increase of surfactant concentration. In the samples saturated with N_2O no absorbance between 550–600 nm was detected. This confirms that hydroperoxides are indeed formed in the surfactant solutions gamma-irradiated in the presence of molecular oxygen.

Finally, we would like to mention the experiment in which aqueous solution of IG720 was exposed to daylight for 40 days and samples were taken for analysis every day. The amount of HP detected was proportional to the exposure time up to 10 days. Within this time period the concentration of HP approached the upper limit of linearity of the FOX method, i.e. around 40 μM. Similar result was published by the Wolff's group for other polyether surfactants, Tween 20 and Triton X-100 (Jaeger *et al.*, 1994). With this we have confirmed that polyether surfactants can easily undergo peroxidation when exposed to the daylight, which leads to the accumulation of hydroperoxides.

In conclusion one may say that the FOX method can be successfully applied for HP determination in systems containing non-ionic or anionic surfactants after, however, careful calibration performed individually for each surfactant. Our observations made with surfactant solutions exposed to the daylight show that one has carefully to store such solutions in the dark (and possibly at low temperature) to avoid hydroperoxide accumulation. Further investigations on the detection and quantitation of HP formed in surfactants, amino acids, and proteins using the PCA-FOX assay are in progress in our laboratory.

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