Determination of total plasma hydroperoxides using a diphenyl-1-pyrenylphosphine fluorescent probe

Short title: Determination of plasma hydroperoxides by DPPP

Jonathan Santas\textsuperscript{a,b}, Francesc Guardiola\textsuperscript{a}, Magda Rafecas\textsuperscript{a}, Ricard Bou\textsuperscript{a,c,*}

\textsuperscript{a} Department of Nutrition and Food Science, XaRTA-INSA, University of Barcelona, 08028, Barcelona, Spain.

\textsuperscript{b} AB-Biotics, S.A., Parc Tecnològic del Vallès, 08290, Cerdanyola del Vallès, Spain.

\textsuperscript{c} Institute of Food Science, Technology and Nutrition (ICTAN) formerly Instituto del Frio CSIC, C. Jose Antonio Novais, 10, 28040, Madrid, Spain

*Corresponding author: R. Bou, Department of Nutrition and Food Science, XaRTA–INSA, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII s/n, E-08028 Barcelona, Spain. Phone: (+34) 93 402 4508; Fax: (+34) 93 403 5931; Email: ricard_bou@ictan.csic.es.edu
Abstract

Plasma hydroperoxides (HP) are widely accepted to be good indicators of oxidative stress. By means of the method proposed herein, which uses diphenyl-1-pyrenylphosphine (DPPP) as a fluorescent probe, all types of plasma HP were determined. The detection and quantification limits of the method were 0.08 and 0.25 nmols of cumene hydroperoxide (CHP) equivalents in 40 µL of plasma, respectively. The method is satisfactory in terms of precision (5.3% for 14.5 µM CHP eq.; n=8) and the recoveries were 91% and 92% after standard additions of 26 and 52 µM of CHP, respectively. The selectivity of the proposed method is higher than 96%. Moreover, optimization of the reaction conditions and the addition of ethylenediaminetetraacetic acid (EDTA) disodium salt and 2,6-di-tert-butyl-4-methylphenol (BHT) prevented the formation of HP artifacts during the analysis. Therefore, the proposed method is useful for simple and quantitative determination of total plasma HP.

Keywords: oxidative stress, hydroperoxides, plasma, diphenyl-1-pyrenylphosphine.
Oxidative stress plays an important role in the development of many pathologies, including cancer [1, 2], cardiovascular [3, 4] and neurodegenerative diseases as well as other physiological processes such as ageing [5, 6]. Among the different methods used for the assessment of oxidative stress, the determination of lipid oxidation products in plasma has been widely accepted as a good indicator of oxidative imbalance [7]. However, there is growing evidence that proteins are also a major target of reactive oxygen species, and the resulting oxidative damage can lead to loss of their biological function. Furthermore, some protein oxidation products are good biomarkers to predict neurological disorders and age-related diseases [6, 8]. Therefore, due to the biological relevance of oxidative damage regardless of its lipid or protein origin, the overall measurement of oxidation products by means of accurate and simple methods is of interest.

Routine analysis techniques to estimate oxidative stress in biological samples include iodometric assays [9], spectrophotometric determination of conjugated dienes [7, 10], determination of thiobarbituric acid reactive substances values [11] and measurement of carbonyl content [12]. However, these methods are usually criticized for their lack of sensitivity and/or specificity. More sensitive methods, such as determination of hydroperoxides HP using luminal chemiluminescence [13] or HP activation of cyclooxygenase [14] have also been proposed. However, the application of these methods is sometimes limited because they are often complex and require sophisticated instrumentation [13]. Alternatively, plasma lipid and protein HP can be determined by the formation of colored metal complexes with thiocyanate or xylenol orange. These methods are satisfactory in terms of sensitivity and simplicity [15, 16], but the procedures are subject to interference caused by chelators, ferric iron, some redox compounds or the presence of other chromophores. The ferrous oxidation-xylenol orange (FOX) method is commonly used for all kinds of biological
sample, but plasma samples contain many compounds that can react with the dye and thus interfere with determination. Hence, it is common to use specific reducing agents for HP to discriminate the background signal from authentic HP [16, 17]. However, DPPP is a non-fluorescent molecule that specifically reacts with HP, forming DPPP oxide which then emits fluorescence at 380 nm (excitation wavelength 353 nm). In fact, the use of DPPP has been proven to allow selective and very sensitive determination of lipid HP in biological samples using flow injection and HPLC post-column methods [18, 19]. In addition, it has recently been reported that lipid and protein HP can be determined by this fluorescent probe using simple batch methods [20].

The aim of this study was to set up and validate a modified version of this method based on the DPPP fluorescent probe for simple, sensitive and selective determination of total lipid and protein HP in plasma due to their clinical relevance as biomarkers of oxidative stress.

Material and methods

Materials

EDTA disodium salt solution, BHT, phosphate-buffer saline (PBS; 0.01M, pH=7.4), guanidine hydrochloride (GdnHCl), 80% CHP and triphenylphosphine (TPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diphenyl-1-pyrenylphosphine was purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). Methanol and n-butanol were of HPLC grade from Panreac (Barcelona, Spain). Bidistilled water was obtained using a Milli-Q® Gradient System (Millipore Co., Billerica, MA, USA). Positive displacement pipettes were used throughout the study.

Final procedure for determination of total plasma hydroperoxides
The entire procedure was conducted under subdued light conditions and 1.5-mL microtubes with safe-lock were used to avoid evaporation. First, 40 µL of plasma was mixed with 160 µL of a solution which contained 0.125% EDTA and 6M GdnHCl in PBS. Then, 100 µL of 4 mM BHT in methanol was immediately added. After vortexing for 1 min, 100 µL of 400 µM DPPP and 4 mM BHT in butanol were added and samples were vortexed again for 1 min. These solutions were extemporaneously prepared. The final concentrations of EDTA, BHT and DPPP were 0.05%, 2 mM and 100 µM, respectively. The head-space of the microtubes was flushed with nitrogen, immediately closed and then samples were incubated at 40ºC for 3h under constant agitation. The reaction was stopped by placing the samples in an ice bath for 20 min. Then, 1 mL of 6M GdnHCl in PBS was added and samples were vortexed for 1 min. One hundred µL of the resulting solution was thoroughly mixed for 2 min with 1 mL of butanol and samples were then centrifuged at 1500 x g for 10 min at 4ºC. One hundred µL of the supernatant was immediately transferred to 96-microwell plates and fluorescence was determined in a Fluostar Optima fluorimeter (BMG Labtech, Ortenberg, Germany) at 30ºC using the 360 ± 10 nm and 380 ± 10 nm fluorescence filters for excitation and emission, respectively. The signal was consecutively measured at intervals of 2 min for 10 min. Since the signal was observed to be stable, the average of the measurements was used for calculations.

Sample collection

Blood samples were extracted by heart puncture from healthy Sprague-Dawley rats (6-8 months old) fed with 2014 Teklad Global 14% Protein Rodent Maintenance Diet (Teklan Harlan, Madison, WI, USA). Samples were collected in heparin tubes as anticoagulant and immediately centrifuged at 1500 x g for 15 min at 4ºC for plasma separation. Plasma samples used for method development were pooled and stored in aliquots at -80ºC until analysis to
avoid oxidation (unoxidized rat plasma, URP). Plasma samples used to determine the precision and recovery of the method were stored in the dark at 4°C and analyzed within 24h after extraction (fresh URP).

Hypercholesterolemic blood samples were obtained from eight Dunkin Harley guinea pigs (4 weeks old) from Harlan Interfauna Ibérica (Barcelona, Spain). Animals were fed with Teklad Global Diet for guinea pigs (Teklan Harlan, Madison, WI, USA) for one week of acclimation. Subsequently, blood samples from fasting animals were obtained from the saphenous vein and collected in heparin tubes. Animals were switched to a hypercholesterolemic experimental diet, the composition of which was as follows: protein 18.8%, fat 17.1%, carbohydrates (non-fiber) 45.6%, cellulose 12.0%, cholesterol 0.25%, mineral mix 5.5% and vitamin mix 1.0%. The fat mix content of the diet was olive oil/palm-kernel oil/safflower oil (1:2:1.8) and the carbohydrates were added as starch/sucrose (1:1.34). The mineral and vitamin mix was designed to meet all the nutritional requirements of guinea pigs [21]. After 4 weeks, blood samples from fasting animals were obtained from the saphenous vein and collected in heparin tubes. Plasma from initial and final blood samples was separated by centrifugation as described earlier and stored at -80°C until analysis. In order to assess the hypercholesterolemic effect of the diet, plasma LDL cholesterol was determined fluorimetrically using enzymatic kits from BioVision, Inc. (Millpitas, CA). All the procedures were approved by the University of Barcelona’s Animal Care and Use Committee.

Preparation of oxidized plasma samples

Oxidized rat plasma (ORP) samples were obtained by thermal oxidation of URP. A determined amount of URP was diluted with an equal volume of 6M GdnHCl in PBS and incubated at 80°C for 1h under continuous magnetic stirring (500 rpm). ORP samples were used immediately for analysis.
Reaction kinetics

Reaction time was studied by mixing 40 µL of diluted ORP samples (equivalent to 20 µL of plasma) with 160 µL of 6M GdnHCl in PBS containing 0.063% of EDTA (final concentration in the media of 0.025%), 100 µL of 2 mM BHT in methanol and 100 µL of 400 µM DPPP in 2 mM BHT in butanol. Then, samples were incubated at 40, 50 and 60ºC and plasma HP determined as described earlier. All sample kinetics studies were conducted in triplicate.

Effect of antioxidant addition

The influence of EDTA and BHT addition on the reaction was assessed by using a two-factor four-level (2x4) experimental design. First, 40 µL of ORP samples (equivalent to 20 µL of plasma) was diluted with 160 µL of 6M GdnHCl in PBS containing 0%, 0.0625%, 0.125% or 0.25% of EDTA (final concentration in the media of 0%, 0.025%, 0.05%, 0.1% respectively). Then, 100 µL of 0, 2, 4 or 8 mM of BHT in methanol was added (final concentration in the media of 0, 0.5, 1, and 2 mM respectively). Samples were incubated at 40ºC for 3h and plasma HP determined as described earlier. The experiment was replicated 4 times.

Effect of sample amount

The optimal amount of sample volume was studied by determining the HP of different amounts of ORP. Equivalent plasma volumes of 5, 10, 20, 30, 40 and 50 µL were made up to 100 µL with 6M GdnHCl in PBS. Then, 100 µL 6M GdnHCl in PBS containing 0.2% of EDTA and 100 µL of 4 mM BHT in methanol were added. Finally, 100 µL of 400 µM DPPP in butanol containing 4 mM BHT were added and samples were then incubated for 3h at 40ºC. Plasma HP was determined as described earlier. The final concentrations of EDTA,
BHT and DPPP were 0.05%, 2 mM and 100 µM respectively. Studies were conducted in triplicate.

Selectivity of the method

The selectivity of the method was assessed by adding TPP (reductant of organic HP) to plasma samples in a final concentration of 2 mM in the media. After mixing, samples were allowed to react for 1h and results were compared with the same plasma samples without added TPP.

Statistical analysis

Data were analyzed by SPSS v.17 for Windows. Two-way ANOVA was used to determine the effect of addition of EDTA and BHT and their interaction. The exact nature of the differences between levels of addition was determined by Scheffé’s multirange test. Differences between initial and final values of plasma HP from guinea pigs were determined by paired-sample Student’s t-test. Differences were considered significant at $P<0.05$.

Results and discussion

Reaction kinetics

The fluorescence emitted by the DPPP probe over time at different incubation temperatures is shown in Figure 1. At 40°C, fluorescence intensity reached a maximum at 2.5 h and then remained stable for at least 3.5 h of incubation. Conversely, the recorded fluorescence intensity at 50°C and 60°C was higher than that at 40°C, and did not stabilize even after 210 min. This different behavior was attributed to oxidation of the sample as a result of the incubation step. Determination of HP by the DPPP method is commonly conducted at reaction temperatures of 60°C for 1 h or more [20], although higher temperatures can also be
used for shorter times when using HPLC and flow injection analysis systems [22, 23].

However, it remains questionable whether high temperatures could lead to the formation of HP artifacts during the incubation step, especially when a long incubation period is considered. Therefore, a reaction temperature of 40ºC was considered optimal for the determination of plasma HP as this temperature allows the HP to react with the DPPP and minimizes induced oxidation of the sample during the incubation step. Lower temperatures also minimize a potential underestimation that could be due to decomposition and/or volatilization of HP. Moreover, higher temperatures can lead to plasma protein denaturation and further aggregation and precipitation, which could interfere with the analysis. In consequence, incubation of the sample for 3h at 40ºC was considered to be the optimum condition for determination of total plasma HP. In addition, flushing the head-space of the vial with nitrogen efficiently minimized oxidation during the incubation step, thus yielding a lower coefficient of variation within a run. It also resulted in slightly lower HP values, especially at high incubation temperatures (data not shown).

**Effect of the antioxidant addition**

In order to further minimize the formation of HP artifacts, different doses of EDTA (0-0.1%) and BHT (0-2 mM) were added. It has previously been reported that neither the addition of BHT nor EDTA interferes in the development of fluorescence caused by oxidation of the DPPP [20, 24]. As shown in Table 1, results reveal that the amount of HP found varied depending on the final amount of EDTA and BHT in the reaction medium ($P<0.01$). Among the different concentrations of EDTA, the highest HP values were obtained when BHT was added at concentrations of 1 or 2 mM. This effect was explained by the protective effect of BHT on the oxidation of the DPPP probe in the blanks, thus causing a reduction in the background signal which in turn resulted in higher levels of relative fluorescence intensity. In
contrast to BHT, EDTA did not significantly change blank signals, but it increased the fluorescence intensity of the samples. Since EDTA does not interfere, it is reasonable to assume that it exerted a protective effect on endogenous plasma HP by chelating transition metals, as these metals decompose HP into radicals [25]. Thus, their inactivation would lead to higher recoveries of endogenous plasma HP. The possibility of adding EDTA to avoid HP breakdown during analysis is a clear advantage over other methods such as the thiocyanate and FOX methods where EDTA interferes with the analysis. Among the different tested concentrations of EDTA, a final concentration of 0.05% was shown to be optimal, whereas a lower efficiency was observed at higher amounts (Table 1). It is probable that when 0.1% of EDTA was added, the high amount of solutes in the reaction media interfered with the reaction. Thus, a final concentration in the reaction media of 0.05% EDTA and 2 mM BHT was considered optimal to avoid oxidation of both the sample and the DPPP probe during the incubation step.

Effect of sample amount

The effect of the matrix was studied by adding different volumes of ORP, equivalent to a range from 5 to 50 µL. The plot of sample volume versus response showed that the reaction was linear from 5 to 40 µL with an $R^2$ of 0.998 (Figure 2). Within this range, the method afforded the same HP amounts with a coefficient of variation between different sample volumes of 5.8%. When the assay was repeated with URP, it was observed that volumes lower than 20 µL were below the limit of quantification of the method. However, the reaction was linear from 20 to 40 µL ($R^2=0.994$) with a coefficient of variation between different sample volumes of 5.3 %. Hence, the maximum plasma volume is limited to 40 µL, probably because of the precipitation/denaturation of plasma proteins at higher concentrations. In fact, the precipitation of plasma proteins seems to be a critical aspect, and this was avoided with
the appropriate solvent mixture. The presence of butanol significantly improves the response of HP after reaction with DPPP [20]. Hence, DPPP is dissolved in butanol, but since it is not miscible with aqueous solutions, the addition of methanol is critical to allow the reaction of DPPP with plasma HP. Due to the denaturation of plasma proteins by alcohols, whether methanol or butanol, a minimum amount of 50% of aqueous buffer is necessary. Likewise, the addition of solubilizing agents, such as GdnHCl or urea, proved to be useful to avoid protein precipitation in the selected solvent system. However, GdnHCl was more suitable than urea because the solubility of the latter is lower due to the presence of alcohols, thus limiting its addition at high concentrations.

Given these considerations, 40 µL of plasma sample is, therefore, recommended as the sample volume for both URP and ORP samples because this amount is large enough to provide sufficient HP and lies within the linearity range of the method.

**Linearity range and between days calibration**

The reaction was linear from 0.25 to 8 nmols of CHP eq. present in the reaction media (400 µL; \( R^2=0.997 \)). The relative standard deviation within this concentration range was from 16.8% to 2.04%. Since the linear range of the method depends on the concentration of DPPP in the medium, it could easily be increased by addition of higher amounts of DPPP (data not shown). Nevertheless, the addition of DPPP at 400 µM was considered optimal because higher amounts of HP are unlikely to be present in fresh plasma samples and the cost of the assay would increase with increased concentrations of the fluorescent probe.

The slope of the calibration curve was constant between different days (relative standard deviation = 0.97 %, \( n=3 \)) although the background signal was observed to vary slightly between assays. This was mainly attributed to oxidation of the DPPP probe, which can be partially minimized by improving its storage conditions. It is therefore recommended that
independent aliquots of the probe are prepared and stored at -80ºC in the presence of nitrogen atmosphere and/or BHT. Moreover, it is recommended that a calibration curve is run daily with at least five standard concentrations in order to obtain reliable results.

**Precision and recovery**

The precision of the method was assessed in fresh URP samples by determining total plasma HP amount in 8 aliquots of 40 µL (Table 2). The relative standard deviation for fresh URP samples was 5.3% (14.50 µM CHP eq.). The recovery of the method was studied by adding two standard amounts of CHP to plasma samples (final standard concentrations were 26 and 52 µM). The recoveries of the method at these levels of addition were 91% and 92%, respectively. It is noteworthy to indicate that recoveries were high only when the amount of added GdnHCl was sufficient to maintain a homogenous reaction mixture. The resulting precision and recovery of the method were comparable to those previously reported for determination of lipid or protein HP by using the DPPP probe [20], and based on AOAC recommendations are satisfactory as well [26]. Therefore, the proposed method represents an alternative to the FOX method which is commonly used to measure HP in plasma [17]. The latter method is more simple but it has been reported to have a higher variability (relative standard deviation between 7-14%) and lower recoveries (about 77%) [16, 27, 28].

**Sensitivity and selectivity of the method**

The limit of detection and quantification of the method was determined as described elsewhere [29]. Accordingly, the method allows the detection and quantification of amounts as low as 0.08 nmols and 0.25 nmols of CHP eq. in the reaction medium (400 µL containing 40 µL of plasma). Thus, the limit of detection and the limit of quantification in plasma can be set at 2.0 and 6.3 µM of CHP eq., respectively. These limits are slightly higher than those
determined by means of the same fluorescent probe to determine HP in isolated lipids and proteinaceous samples [20]. However, it should be noted that first, a much more complex matrix (plasma) was used in order to validate the present method and, second, this method allows a high-throughput sample analysis. In addition, the novelty of the method is that does not require sample preparation steps or separation techniques such as HPLC [19, 30]. Provided that TPP and its oxide have no fluorescence activity [18, 23], it can be used as a reductant of organic HP and, therefore, allows to evaluate the selectivity of the method [20]. The amount of HP found in fresh URP was 14.5 ± 0.8 µM CHP eq. After addition of TPP, the concentration found was below the limit of detection of the method, which implies that the background signal was lower than 4% and the method highly selective for organic HP. The same samples were analyzed by means of the commonly used version of the FOX method [16], which led to an overall HP concentration of 10.7 ± 0.37 µM CHP eq. However, after the addition of TPP to discriminate the background signal, this was shown to be approximately 55%, meaning that the concentration of authentic HP determined by the FOX method was 4.9 ± 0.4 µM CHP eq. This concentration was in the range of results previously published for plasma from healthy humans [16, 31] and lower than the HP concentration determined by the present DPPP method. These reported concentrations of HP determined by means of the FOX method are close or slightly below the quantification limit of the proposed method. However, it is important to note that these concentrations are determined after the background signal subtraction, which is much higher than in the present method. Therefore, these reported low concentrations are not consequence of lower quantification limits, which are usually not reported for the FOX method. The selectivity of the proposed method is, therefore, a clear advantage. The methods to measure plasma HP show differences in precision, recovery and selectivity due, in part, to the diversity of plasma HP, their low stability and their easy formation of
artifacts. Therefore, there is no methodological consensus on their determination and it is difficult to compare the results obtained in different studies [32, 33, 34]. The use of antioxidants as protective agents, the lack of extraction steps and the good recovery and selectivity of the method confirmed that the present method is accurate for measurement of total authentic HP. Moreover, the present method allows the determination not only of lipid HP but also HP of protein origin. Given these findings, it is not surprising to find higher levels of plasma HP when using the proposed method.

Performance of the method by using guinea pig plasma samples

Guinea pig plasma samples were analyzed because this animal responds to dietary cholesterol in a similar manner to that of humans and, in contrast to other rodents, accumulates cholesterol in the arterial wall and develops atherosclerosis, thus rendering it a suitable model [35]. The results obtained for plasma samples from healthy and hypercholesterolemic guinea pigs confirmed this fact (Figure 3), as plasma LDL cholesterol levels were 16 ± 6.3 and 266 ± 108 mg dL$^{-1}$, respectively. Furthermore, oxidative stress is commonly associated with obesity and hypercholesterolemia [3, 36]. Using the proposed method, results for total HP from healthy guinea pigs fed a standard diet were 24.7 ± 2.2 µM of CHP eq., but after consumption of hypercholesterolemic diets plasma oxidation levels increased up to 31.0 ± 3.9 µM of CHP eq. ($P=0.041$). This represents an increase of about 25%, consistent with findings previously reported by Nourooz et al. [16].

Conclusion

The method developed for quantitative determination of total plasma HP was satisfactory in terms of simplicity, sensitivity, precision and selectivity. The method requires small amounts of sample and is not dependent on expensive equipment. A distinct advantage is that it omits
previous extraction of plasma HP, which allows good recoveries and accurate determination of total HP rather than individual classes (i.e. lipid HP). In conclusion, the method offers a useful alternative for determination of plasma HP which is of particular interest for assessing oxidative stress.

Acknowledgements

J. Santas received a Torres Quevedo fellowship. The authors are grateful to X. Charles and G. Rodriguez for their technical support.

Figures

Figure 1. Reaction kinetics at different temperatures.
Figure 2: Effect of sample amount.
Figure 3: Plasma hydroperoxides in guinea pigs.

Tables

Table 1: Effect of antioxidant addition.
Table 2. Relative standard deviation and recovery of hydroperoxides in fresh unoxidized rat plasma at two levels of cumene hydroperoxide standard addition.
Table 3. Abbreviation list

References


[26] AOACS, AOAC Official Method 2003.05 Crude Fat Feeds, Cereal Grans, and Forages


Y. Dotan, D. Lichtenberg, I. Pinchuk, Lipid peroxidation cannot be used as a universal criterion of oxidative stress, Prog. Lipid Res. 43 (2004) 200-227


H.K. Vincent, K.E. Innes, K.R. Vincent, Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity, Diabestes Obes. Metab. 9 (2007) 813-839
**FIGURE LEGENDS**

**Figure 1.** Fluorescence intensity over time of oxidized rat plasma (ORP) samples incubated at different temperatures. Error bars represent the SD of the means (n=3).

**Figure 2.** Amount of hydroperoxides (nanomols of cumene hydroperoxide equivalents) found after incubation of different volumes of oxidized rat plasma for 3 h at 40°C. Error bars represent the SD of the means (n=3).

**Figure 3.** Initial and final plasma hydroperoxide content in guinea pigs fed with hypercholesterolemic diets for 28 days. Results are expressed in µM of cumene hydroperoxide equivalents. Error bars represent the SD of the means (n=6). Results were statistically different based on paired samples $t$-Student’s test ($P=0.041$).
Figure 1. Reaction kinetics at different temperatures.
Figure 2: Effect of sample amount

\[ y = 0.0975x \]

\[ R^2 = 0.998 \]
Figure 3: Plasma hydroperoxides in guinea pigs
## Table 1: Effect of antioxidant addition

<table>
<thead>
<tr>
<th>EDTA</th>
<th>0 mM</th>
<th>0.5 mM</th>
<th>1 mM</th>
<th>2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>1.06 ± 0.11</td>
<td>1.17 ± 0.10</td>
<td>1.40 ± 0.11</td>
<td>1.49 ± 0.02</td>
</tr>
<tr>
<td>0.025%</td>
<td>1.42 ± 0.24</td>
<td>1.86 ± 0.17</td>
<td>2.05 ± 0.20</td>
<td>2.35 ± 0.13</td>
</tr>
<tr>
<td>0.05%</td>
<td>2.32 ± 0.10</td>
<td>2.48 ± 0.09</td>
<td>2.92 ± 0.09</td>
<td>3.06 ± 0.10</td>
</tr>
<tr>
<td>0.1%</td>
<td>2.10 ± 0.04</td>
<td>2.24 ± 0.04</td>
<td>2.29 ± 0.07</td>
<td>2.34 ± 0.13</td>
</tr>
</tbody>
</table>

Results expressed as means ± SD in nmols of cumene hydroperoxide equivalents found in 20 µL aliquots of oxidized rat plasma after incubation at 40°C for 3 hours. Results in the same column that do not share the same superscript (a, b or c) are significantly different based on Scheffé’s post hoc test ($P<0.05$) at different final concentrations of ethylenediaminetetraacetic acid (EDTA). Results in the same row that do not share the same superscript (x, y or z) are significantly different based on Scheffé’s post hoc test ($P<0.05$) at different final concentrations of 2,6-di-tert-butyl-4-methylphenol (BHT).
Table 2. Relative standard deviation and recovery of hydroperoxides in fresh unoxidized rat plasma at two levels of cumene hydroperoxide (CHP) standard addition

<table>
<thead>
<tr>
<th>Levels of CHP standard addition</th>
<th>0 µM</th>
<th>26 µM</th>
<th>52 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (µM CHP eq.)</td>
<td>14.50</td>
<td>37.97</td>
<td>62.4</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>5.2</td>
<td>4.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Recovery</td>
<td>90.5</td>
<td>92.3</td>
<td></td>
</tr>
</tbody>
</table>

1 n=8 for non-spiked fresh unoxidized rat samples and n=6 for spiked fresh normal rat samples.
<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,6-di-tert-butyl-4-methylphenol</td>
<td>BHT</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>CHP</td>
</tr>
<tr>
<td>Diphenyl-1-pyrenylphosphine</td>
<td>DPPP</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid</td>
<td>EDTA</td>
</tr>
<tr>
<td>Ferrous oxidation-xylenol orange</td>
<td>FOX</td>
</tr>
<tr>
<td>Guanidine hydrochloride</td>
<td>GdnHCl</td>
</tr>
<tr>
<td>Hydroperoxide</td>
<td>HP</td>
</tr>
<tr>
<td>Oxidized rat plasma</td>
<td>ORP</td>
</tr>
<tr>
<td>Phosphate-buffer saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Triphenylphosphine</td>
<td>TPP</td>
</tr>
<tr>
<td>Unoxidized rat plasma</td>
<td>URP</td>
</tr>
</tbody>
</table>