

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

3 *Short title: Determination of plasma hydroperoxides by DPPP*

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19 **Abstract**

20 Plasma hydroperoxides (HP) are widely accepted to be good indicators of oxidative stress. By
21 means of the method proposed herein, which uses diphenyl-1-pyrenylphosphine (DPPP) as a
22 fluorescent probe, all types of plasma HP were determined. The detection and quantification
23 limits of the method were 0.08 and 0.25 nmols of cumene hydroperoxide (CHP) equivalents
24 in 40 μ L of plasma, respectively. The method is satisfactory in terms of precision (5.3% for
25 14.5 μ M CHP eq.; n=8) and the recoveries were 91% and 92% after standard additions of 26
26 and 52 μ M of CHP, respectively. The selectivity of the proposed method is higher than 96%.
27 Moreover, optimization of the reaction conditions and the addition of
28 ethylenediaminetetraacetic acid (EDTA) disodium salt and 2,6-di-tert-butyl-4-methylphenol
29 (BHT) prevented the formation of HP artifacts during the analysis. Therefore, the proposed
30 method is useful for simple and quantitative determination of total plasma HP.

31

32 *Keywords: oxidative stress, hydroperoxides, plasma, diphenyl-1-pyrenylphosphine.*

33

34 **Introduction**

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

46 Routine analysis techniques to estimate oxidative stress in biological samples include
47 iodometric assays [9], spectrophotometric determination of conjugated dienes [7, 10],
48 determination of thiobarbituric acid reactive substances values [11] and measurement of
49 carbonyl content [12]. However, these methods are usually criticized for their lack of
50 sensitivity and/or specificity. More sensitive methods, such as determination of
51 hydroperoxides HP using luminal chemiluminescence [13] or HP activation of cyclooxygenase
52 [14] have also been proposed. However, the application of these methods is sometimes
53 limited because they are often complex and require sophisticated instrumentation [13].
54 Alternatively, plasma lipid and protein HP can be determined by the formation of colored
55 metal complexes with thiocyanate or xylenol orange. These methods are satisfactory in terms
56 of sensitivity and simplicity [15, 16], but the procedures are subject to interference caused by
57 chelators, ferric iron, some redox compounds or the presence of other chromophores. The
58 ferrous oxidation-xylenol orange (FOX) method is commonly used for all kinds of biological

59 sample, but plasma samples contain many compounds that can react with the dye and thus
60 interfere with determination. Hence, it is common to use specific reducing agents for HP to
61 discriminate the background signal from authentic HP [16, 17].

62 However, DPPP is a non-fluorescent molecule that specifically reacts with HP, forming DPPP
63 oxide which then emits fluorescence at 380 nm (excitation wavelength 353 nm). In fact, the
64 use of DPPP has been proven to allow selective and very sensitive determination of lipid HP
65 in biological samples using flow injection and HPLC post-column methods [18, 19]. In
66 addition, it has recently been reported that lipid and protein HP can be determined by this
67 fluorescent probe using simple batch methods [20].

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

71

72 **Material and methods**

73 *Materials*

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

81

82 *Final procedure for determination of total plasma hydroperoxides*

83 The entire procedure was conducted under subdued light conditions and 1.5-mL microtubes
84 with safe-lock were used to avoid evaporation. First, 40 μ L of plasma was mixed with 160
85 μ L of a solution which contained 0.125% EDTA and 6M GdnHCl in PBS. Then, 100 μ L of 4
86 mM BHT in methanol was immediately added. After vortexing for 1 min, 100 μ L of 400 μ M
87 DPPP and 4 mM BHT in butanol were added and samples were vortexed again for 1 min.
88 These solutions were extemporaneously prepared. The final concentrations of EDTA, BHT
89 and DPPP were 0.05%, 2 mM and 100 μ M, respectively. The head-space of the microtubes
90 was flushed with nitrogen, immediately closed and then samples were incubated at 40°C for
91 3h under constant agitation. The reaction was stopped by placing the samples in an ice bath
92 for 20 min. Then, 1 mL of 6M GdnHCl in PBS was added and samples were vortexed for 1
93 min. One hundred μ L of the resulting solution was thoroughly mixed for 2 min with 1 mL of
94 butanol and samples were then centrifuged at 1500 x g for 10 min at 4°C. One hundred μ L of
95 the supernatant was immediately transferred to 96-microwell plates and fluorescence was
96 determined in a Fluostar Optima fluorimeter (BMG Labtech, Ortenberg, Germany) at 30°C
97 using the 360 ± 10 nm and 380 ± 10 nm fluorescence filters for excitation and emission,
98 respectively. The signal was consecutively measured at intervals of 2 min for 10 min. Since
99 the signal was observed to be stable, the average of the measurements was used for
100 calculations.

101

102 *Sample collection*

103 Blood samples were extracted by heart puncture from healthy Sprague-Dawley rats (6-8
104 months old) fed with 2014 Teklad Global 14% Protein Rodent Maintenance Diet (Teklan
105 Harlan, Madison, WI, USA). Samples were collected in heparin tubes as anticoagulant and
106 immediately centrifuged at 1500 x g for 15 min at 4°C for plasma separation. Plasma samples
107 used for method development were pooled and stored in aliquots at -80°C until analysis to

108 avoid oxidation (unoxidized rat plasma, URP). Plasma samples used to determine the
109 precision and recovery of the method were stored in the dark at 4°C and analyzed within 24h
110 after extraction (fresh URP).

111 Hypercholesterolemic blood samples were obtained from eight Dunkin Harley guinea pigs (4
112 weeks old) from Harlan Interfauna Ibérica (Barcelona, Spain). Animals were fed with 2040
113 Teklad Global Diet for guinea pigs (Teklan Harlan, Madison, WI, USA) for one week of
114 acclimation. Subsequently, blood samples from fasting animals were obtained from the
115 saphenous vein and collected in heparin tubes. Animals were switched to a
116 hypercholesterolemic experimental diet, the composition of which was as follows: protein
117 18.8%, fat 17.1%, carbohydrates (non-fiber) 45.6%, cellulose 12.0%, cholesterol 0.25%,
118 mineral mix 5.5% and vitamin mix 1.0%. The fat mix content of the diet was olive oil/palm-
119 kernel oil/safflower oil (1:2:1.8) and the carbohydrates were added as starch/sucrose (1:1.34).
120 The mineral and vitamin mix was designed to meet all the nutritional requirements of guinea
121 pigs [21]. After 4 weeks, blood samples from fasting animals were obtained from the
122 saphenous vein and collected in heparin tubes. Plasma from initial and final blood samples
123 was separated by centrifugation as described earlier and stored at -80°C until analysis. In
124 order to assess the hypercholesterolemic effect of the diet, plasma LDL cholesterol was
125 determined fluorimetrically using enzymatic kits from BioVision, Inc. (Millpitas, CA). All the
126 procedures were approved by the University of Barcelona's Animal Care and Use Committee.

127

128 *Preparation of oxidized plasma samples*

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

133

134 *Reaction kinetics*

135 Reaction time was studied by mixing 40 μL of diluted ORP samples (equivalent to 20 μL of
136 plasma) with 160 μL of 6M GdnHCl in PBS containing 0.063% of EDTA (final concentration
137 in the media of 0.025%), 100 μL of 2 mM BHT in methanol and 100 μL of 400 μM DPPP in
138 2 mM BHT in butanol. Then, samples were incubated at 40, 50 and 60°C and plasma HP
139 determined as described earlier. All sample kinetics studies were conducted in triplicate.

140

141 *Effect of antioxidant addition*

142 The influence of EDTA and BHT addition on the reaction was assessed by using a two-factor
143 four-level (2x4) experimental design. First, 40 μL of ORP samples (equivalent to 20 μL of
144 plasma) was diluted with 160 μL of 6M GdnHCl in PBS containing 0%, 0.0625%, 0.125% or
145 0.25% of EDTA (final concentration in the media of 0%, 0.025%, 0.05%, 0.1% respectively).
146 Then, 100 μL of 0, 2, 4 or 8 mM of BHT in methanol was added (final concentration in the
147 media of 0, 0.5, 1, and 2 mM respectively). Samples were incubated at 40°C for 3h and
148 plasma HP determined as described earlier. The experiment was replicated 4 times.

149

150 *Effect of sample amount*

151 The optimal amount of sample volume was studied by determining the HP of different
152 amounts of ORP. Equivalent plasma volumes of 5, 10, 20, 30, 40 and 50 μL were made up to
153 100 μL with 6M GdnHCl in PBS. Then, 100 μL 6M GdnHCl in PBS containing 0.2% of
154 EDTA and 100 μL of 4 mM BHT in methanol were added. Finally, 100 μL of 400 μM DPPP
155 in butanol containing 4 mM BHT were added and samples were then incubated for 3h at
156 40°C. Plasma HP was determined as described earlier. The final concentrations of EDTA,

157 BHT and DPPP were 0.05%, 2 mM and 100 μ M respectively. Studies were conducted in
158 triplicate.

159

160 *Selectivity of the method*

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

165

166 *Statistical analysis*

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

172

173 **Results and discussion**

174 *Reaction kinetics*

175 The fluorescence emitted by the DPPP probe over time at different incubation temperatures is
176 shown in **Figure 1**. At 40°C, fluorescence intensity reached a maximum at 2.5 h and then
177 remained stable for at least 3.5 h of incubation. Conversely, the recorded fluorescence
178 intensity at 50°C and 60°C was higher than that at 40°C, and did not stabilize even after 210
179 min. This different behavior was attributed to oxidation of the sample as a result of the
180 incubation step. Determination of HP by the DPPP method is commonly conducted at
181 reaction temperatures of 60°C for 1 h or more [20], although higher temperatures can also be

182 used for shorter times when using HPLC and flow injection analysis systems [22, 23].
183 However, it remains questionable whether high temperatures could lead to the formation of
184 HP artifacts during the incubation step, especially when a long incubation period is
185 considered. Therefore, a reaction temperature of 40°C was considered optimal for the
186 determination of plasma HP as this temperature allows the HP to react with the DPPP and
187 minimizes induced oxidation of the sample during the incubation step. Lower temperatures
188 also minimize a potential underestimation that could be due to decomposition
189 and/or volatilization of HP. Moreover, higher temperatures can lead to plasma protein
190 denaturalization and further aggregation and precipitation, which could interfere with the
191 analysis. In consequence, incubation of the sample for 3h at 40°C was considered to be the
192 optimum condition for determination of total plasma HP. In addition, flushing the head-space
193 of the vial with nitrogen efficiently minimized oxidation during the incubation step, thus
194 yielding a lower coefficient of variation within a run. It also resulted in slightly lower HP
195 values, especially at high incubation temperatures (data not shown).

196

197 *Effect of the antioxidant addition*

198 In order to further minimize the formation of HP artifacts, different doses of EDTA (0-0.1%)
199 and BHT (0-2 mM) were added. It has previously been reported that neither the addition of
200 BHT nor EDTA interferes in the development of fluorescence caused by oxidation of the
201 DPPP [20, 24]. As shown in **Table 1**, results reveal that the amount of HP found varied
202 depending on the final amount of EDTA and BHT in the reaction medium ($P<0.01$). Among
203 the different concentrations of EDTA, the highest HP values were obtained when BHT was
204 added at concentrations of 1 or 2 mM. This effect was explained by the protective effect of
205 BHT on the oxidation of the DPPP probe in the blanks, thus causing a reduction in the
206 background signal which in turn resulted in higher levels of relative fluorescence intensity. In

207 contrast to BHT, EDTA did not significantly change blank signals, but it increased the
208 fluorescence intensity of the samples. Since EDTA does not interfere, it is reasonable to
209 assume that it exerted a protective effect on endogenous plasma HP by chelating transition
210 metals, as these metals decompose HP into radicals [25]. Thus, their inactivation would lead
211 to higher recoveries of endogenous plasma HP. The possibility of adding EDTA to avoid HP
212 breakdown during analysis is a clear advantage over other methods such as the thiocyanate
213 and FOX methods where EDTA interferes with the analysis. Among the different tested
214 concentrations of EDTA, a final concentration of 0.05% was shown to be optimal, whereas a
215 lower efficiency was observed at higher amounts (**Table 1**). It is probable that when 0.1% of
216 EDTA was added, the high amount of solutes in the reaction media interfered with the
217 reaction. Thus, a final concentration in the reaction media of 0.05% EDTA and 2 mM BHT
218 was considered optimal to avoid oxidation of both the sample and the DPPH probe during the
219 incubation step.

220

221 *Effect of sample amount*

222 The effect of the matrix was studied by adding different volumes of ORP, equivalent to a
223 range from 5 to 50 μL . The plot of sample volume versus response showed that the reaction
224 was linear from 5 to 40 μL with an R^2 of 0.998 (**Figure 2**). Within this range, the method
225 afforded the same HP amounts with a coefficient of variation between different sample
226 volumes of 5.8%. When the assay was repeated with URP, it was observed that volumes
227 lower than 20 μL were below the limit of quantification of the method. However, the reaction
228 was linear from 20 to 40 μL ($R^2=0.994$) with a coefficient of variation between different
229 sample volumes of 5.3 %. Hence, the maximum plasma volume is limited to 40 μL , probably
230 because of the precipitation/denaturation of plasma proteins at higher concentrations. In fact,
231 the precipitation of plasma proteins seems to be a critical aspect, and this was avoided with

232 the appropriate solvent mixture. The presence of butanol significantly improves the response
233 of HP after reaction with DPPP [20]. Hence, DPPP is dissolved in butanol, but since it is not
234 miscible with aqueous solutions, the addition of methanol is critical to allow the reaction of
235 DPPP with plasma HP. Due to the denaturation of plasma proteins by alcohols, whether
236 methanol or butanol, a minimum amount of 50% of aqueous buffer is necessary. Likewise,
237 the addition of solubilizing agents, such as GdnHCl or urea, proved to be useful to avoid
238 protein precipitation in the selected solvent system. However, GdnHCl was more suitable than
239 urea because the solubility of the latter is lower due to the presence of alcohols, thus limiting
240 its addition at high concentrations.

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

244

245 *Linearity range and between days calibration*

246 The reaction was linear from 0.25 to 8 nmols of CHP eq. present in the reaction media (400
247 μ L; $R^2=0.997$). The relative standard deviation within this concentration range was from
248 16.8% to 2.04%. Since the linear range of the method depends on the concentration of DPPP
249 in the medium, it could easily be increased by addition of higher amounts of DPPP (data not
250 shown). Nevertheless, the addition of DPPP at 400 μ M was considered optimal because
251 higher amounts of HP are unlikely to be present in fresh plasma samples and the cost of the
252 assay would increase with increased concentrations of the fluorescent probe.

253 The slope of the calibration curve was constant between different days (relative standard
254 deviation = 0.97 %, n=3) although the background signal was observed to vary slightly
255 between assays. This was mainly attributed to oxidation of the DPPP probe, which can be
256 partially minimized by improving its storage conditions. It is therefore recommended that

257 independent aliquots of the probe are prepared and stored at -80°C in the presence of nitrogen
258 atmosphere and/or BHT. Moreover, it is recommended that a calibration curve is run daily
259 with at least five standard concentrations in order to obtain reliable results.

260

261 *Precision and recovery*

262 The precision of the method was assessed in fresh URP samples by determining total plasma
263 HP amount in 8 aliquots of 40 µL (**Table 2**). The relative standard deviation for fresh URP
264 samples was 5.3% (14.50 µM CHP eq.). The recovery of the method was studied by adding
265 two standard amounts of CHP to plasma samples (final standard concentrations were 26 and
266 52 µM). The recoveries of the method at these levels of addition were 91% and 92%,
267 respectively. It is noteworthy to indicate that recoveries were high only when the amount of
268 added GdnHCl was sufficient to maintain a homogenous reaction mixture. The resulting
269 precision and recovery of the method were comparable to those previously reported for
270 determination of lipid or protein HP by using the DPPP probe [20], and based on AOAC
271 recommendations are satisfactory as well [26]. Therefore, the proposed method represents an
272 alternative to the FOX method which is commonly used to measure HP in plasma [17]. The
273 latter method is more simple but it has been reported to have a higher variability (relative
274 standard deviation between 7-14%) and lower recoveries (about 77%) [16, 27, 28].

275

276 *Sensitivity and selectivity of the method*

277 The limit of detection and quantification of the method was determined as described
278 elsewhere [29]. Accordingly, the method allows the detection and quantification of amounts
279 as low as 0.08 nmols and 0.25 nmols of CHP eq. in the reaction medium (400 µL containing
280 40 µL of plasma). Thus, the limit of detection and the limit of quantification in plasma can be
281 set at 2.0 and 6.3 µM of CHP eq., respectively. These limits are slightly higher than those

282 determined by means of the same fluorescent probe to determine HP in isolated lipids and
283 proteinaceous samples [20]. However, it should be noted that first, a much more complex
284 matrix (plasma) was used in order to validate the present method and, second, this method
285 allows a high-throughput sample analysis. In addition, the novelty of the method is that does
286 not require sample preparation steps or separation techniques such as HPLC [19, 30].
287 Provided that TPP and its oxide have no fluorescence activity [18, 23], it can be used as a
288 reductant of organic HP and, therefore, allows to evaluate the selectivity of the method [20].
289 The amount of HP found in fresh URP was $14.5 \pm 0.8 \mu\text{M}$ CHP eq. After addition of TPP, the
290 concentration found was below the limit of detection of the method, which implies that the
291 background signal was lower than 4% and the method highly selective for organic HP. The
292 same samples were analyzed by means of the commonly used version of the FOX method
293 [16], which led to an overall HP concentration of $10.7 \pm 0.37 \mu\text{M}$ CHP eq. However, after the
294 addition of TPP to discriminate the background signal, this was shown to be approximately
295 55%, meaning that the concentration of authentic HP determined by the FOX method was 4.9
296 $\pm 0.4 \mu\text{M}$ CHP eq. This concentration was in the range of results previously published for
297 plasma from healthy humans [16, 31] and lower than the HP concentration determined by the
298 present DPPP method. These reported concentrations of HP determined by means of the FOX
299 method are close or slightly below the quantification limit of the proposed method. However,
300 it is important to note that these concentrations are determined after the background signal
301 subtraction, which is much higher than in the present method. Therefore, these reported low
302 concentrations are not consequence of lower quantification limits, which are usually not
303 reported for the FOX method. The selectivity of the proposed method is, therefore, a clear
304 advantage.

305 The methods to measure plasma HP show differences in precision, recovery and selectivity
306 due, in part, to the diversity of plasma HP, their low stability and their easy formation of

307 artifacts. Therefore, there is no methodological consensus on their determination and it is
308 difficult to compare the results obtained in different studies [32, 33, 34]. The use of
309 antioxidants as protective agents, the lack of extraction steps and the good recovery and
310 selectivity of the method confirmed that the present method is accurate for measurement of
311 total authentic HP. Moreover, the present method allows the determination not only of lipid
312 HP but also HP of protein origin. Given these findings, it is not surprising to find higher
313 levels of plasma HP when using the proposed method.

314

315 *Performance of the method by using guinea pig plasma samples*

316 Guinea pig plasma samples were analyzed because this animal responds to dietary cholesterol
317 in a similar manner to that of humans and, in contrast to other rodents, accumulates
318 cholesterol in the arterial wall and develops atherosclerosis, thus rendering it a suitable model
319 [35]. The results obtained for plasma samples from healthy and hypercholesterolemic guinea
320 pigs confirmed this fact (**Figure 3**), as plasma LDL cholesterol levels were 16 ± 6.3 and $266 \pm$
321 108 mg dL^{-1} , respectively. Furthermore, oxidative stress is commonly associated with obesity
322 and hypercholesterolemia [3, 36]. Using the proposed method, results for total HP from
323 healthy guinea pigs fed a standard diet were $24.7 \pm 2.2 \text{ }\mu\text{M}$ of CHP eq., but after consumption
324 of hypercholesterolemic diets plasma oxidation levels increased up to $31.0 \pm 3.9 \text{ }\mu\text{M}$ of CHP
325 eq. ($P=0.041$). This represents an increase of about 25%, consistent with findings previously
326 reported by Nourooz *et al.* [16].

327

328 **Conclusion**

329 The method developed for quantitative determination of total plasma HP was satisfactory in
330 terms of simplicity, sensitivity, precision and selectivity. The method requires small amounts
331 of sample and is not dependent on expensive equipment. A distinct advantage is that it omits

332 previous extraction of plasma HP, which allows good recoveries and accurate determination
333 of total HP rather than individual classes (i.e. lipid HP). In conclusion, the method offers a
334 useful alternative for determination of plasma HP which is of particular interest for assessing
335 oxidative stress.

336

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340

341 **Figures**

342 Figure 1. Reaction kinetics at different temperatures.

343 Figure 2: Effect of sample amount.

344 Figure 3: Plasma hydroperoxides in guinea pigs.

345

346 **Tables**

347 Table 1: Effect of antioxidant addition.

348 Table 2. Relative standard deviation and recovery of hydroperoxides in fresh unoxidized rat
349 plasma at two levels of cumene hydroperoxide standard addition.

350 Table 3. Abbreviation list

351

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441

442 **FIGURE LEGENDS**

443

444

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

447

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

450

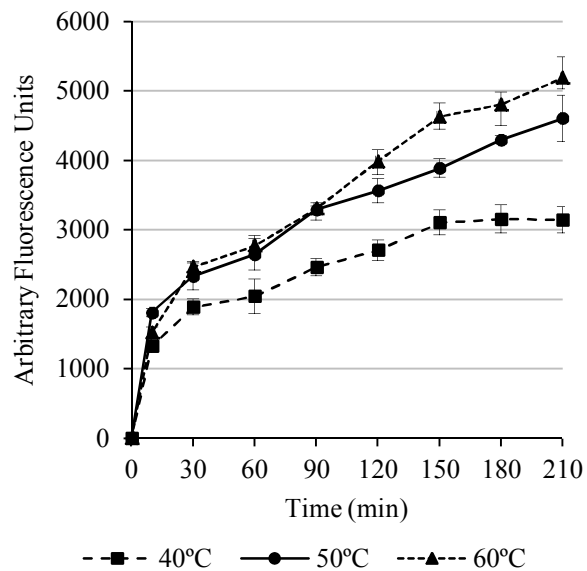
451 **Figure 3.** Initial and final plasma hydroperoxide content in guinea pigs fed with hypercholesterolemic diets for
452 28 days. Results are expressed in μM of cumene hydroperoxide equivalents. Error bars represent the SD of the
453 means (n=6). Results were statistically different based on paired samples *t*-Student's test ($P=0.041$).

454

455 **Figure 1. Reaction kinetics at different temperatures.**

456

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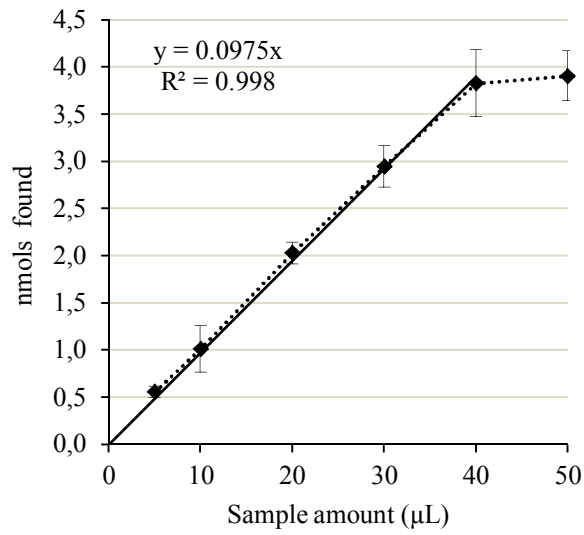


458

459

460 **Figure 2: Effect of sample amount**

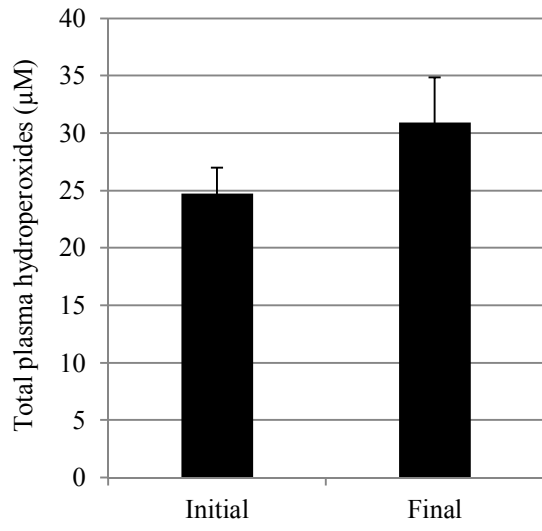
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464 **Figure 3: Plasma hydroperoxides in guinea pigs**



465

466

467 **Table 1: Effect of antioxidant addition¹**

		BHT			
		0 mM	0.5 mM	1 mM	2 mM
EDTA	0 %	1.06 ± 0.11 ^{a, x}	1.17 ± 0.10 ^{a, x}	1.40 ± 0.11 ^{a, y}	1.49 ± 0.02 ^{a, y}
	0.025%	1.42 ± 0.24 ^{a, x}	1.86 ± 0.17 ^{b, y}	2.05 ± 0.20 ^{b, yz}	2.35 ± 0.13 ^{b, z}
	0.05%	2.32 ± 0.10 ^{b, x}	2.48 ± 0.09 ^{c, x}	2.92 ± 0.09 ^{c, x}	3.06 ± 0.10 ^{c, y}
	0.1%	2.10 ± 0.04 ^{b, x}	2.24 ± 0.04 ^{c, xy}	2.29 ± 0.07 ^{b, xy}	2.34 ± 0.13 ^{b, y}

468

469 ¹ Results expressed as means ± SD in nmols of cumene hydroperoxide equivalents found in 20 µL aliquots of
 470 oxidized rat plasma after incubation at 40°C for 3 hours. Results in the same column that do not share the same
 471 superscript (^a, ^b or ^c) are significantly different based on Scheffé's *post hoc* test ($P < 0.05$) at different final
 472 concentrations of ethylenediaminetetraacetic acid (EDTA). Results in the same row that do not share the same
 473 superscript (^x, ^y or ^z) are significantly different based on Scheffé's *post hoc* test ($P < 0.05$) at different final
 474 concentrations of 2,6-di-tert-butyl-4-methylphenol (BHT).

475

476 **Table 2. Relative standard deviation and recovery of hydroperoxides in fresh unoxidized**
477 **rat plasma at two levels of cumene hydroperoxide (CHP) standard addition¹**

478

	Levels of CHP standard addition			
	0 μM	26 μM	52 μM	
479				
480	Concentration (μM CHP eq.)	14.50	37.97	62.4
	<i>RSD</i> (%)	5.2	4.5	4.4
481	Recovery		90.5	92.3

482

483 ¹ n=8 for non-spiked fresh unoxidized rat samples and n=6 for spiked fresh normal rat samples.

484

485

486 **Table 3. Abbreviation list**

Name	Abbreviation
2,6-di-tert-butyl-4-methylphenol	BHT
Cumene hydroperoxide	CHP
Diphenyl-1-pyrenylphosphine	DPPP
Ethylenediaminetetraacetic acid	EDTA
Ferrous oxidation-xylene orange	FOX
Guanidine hydrochloride	GdnHCl
Hydroperoxide	HP
Oxidized rat plasma	ORP
Phosphate-buffer saline	PBS
Triphenylphosphine	TPP
Unoxidized rat plasma	URP

487

488