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

Short title: Determination of plasma hydroperoxides by DPPP

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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-pyrenylphospine.*

Introduction

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 cycloxygenase [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). Dipheny-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 4ºC 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 2040 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 -80ºC 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 80ºC for 1h under continuous magnetic stirring (500 rpm). ORP samples were 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 60ºC 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 40ºC 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 40ºC. Plasma HP was determined as described earlier. The final concentrations of EDTA,

157 BHT and DPPP were 0.05%, 2 mM and 100 μM 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 40ºC, 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 50ºC and 60ºC was higher than that at 40ºC, 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 60ºC 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 40ºC 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 40ºC 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 DPPP 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 µL 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 µL; 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 µM 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

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

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 ± 0.8 μM 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 ± 0.37 μM 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 ± 0.4 μM 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 ±
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 ± 2.2 μM of CHP eq., but after consumption
324 of hypercholesterolemic diets plasma oxidation levels increased up to 31.0 ± 3.9 μM 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

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.

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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

[1] T.B. Kryston, A.B. Georgiev, P. Pissis, A.G. Georgakilas, Role of oxidative stress and DNA damage in human carcinogenesis, Mutat. Res. Fundam. Mol. Mech. Mutagen. 711 (2011) 193-201

[2] L. Vera-Ramirez, P. Sanchez-Rovira, M.C. Ramirez-Tortosa, C.L. Ramirez-Tortosa, S. Granados-Principal, J.A. Lorente, J.L. Quiles, Free radicals in breast carcinogenesis, breast cancer progression and cancer stem cells. Biological bases to develop oxidative-based therapies, Crit. Rev. Oncol. Hematol. 80 (2011) 347-368

[3] K. Sugamura, J.F. Keaney Jr., Reactive oxygen species in cardiovascular disease, Free Radic. Biol. Med. 51 (2011) 978-992

[4] R. Stocker, J.F. Keaney Jr., Role of oxidative modifications in atherosclerosis, Physiol. Rev. 84 (2004) 1381-1478

[5] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing, Nature 408 (2000) 239-247

[6] K.B. Pandey, S.I. Rizvi, Markers of oxidative stress in erythrocytes and plasma during aging in humans, Oxidative Medicine and Cellular Longevity 3 (2010) 2-12

[7] C.V. Smith, R.E. Anderson, Methods for determination of lipid peroxidation in biological samples, Free Radic. Biol. Med. 3 (1987) 341-344

[8] V.I. Lushchak, Free radical oxidation of proteins and its relationship with functional state of organisms, Biochemistry (Moscow) 72 (2007) 809-827

[9] W. Jessup, R.T. Dean, J.M. Gebicki, Iodometric determination of hydroperoxides in lipids and proteins, Methods Enzymol. 233 (1994) 289-303

[10] M.C. Dobarganes, J. Velasco, Analysis of lipid hydroperoxides, Eur. J. Lipid Sci. Technol. 104 (2002) 420-428

[11] H.H. Draper, E.J. Squires, H. Mahmoodi, J. Wu, S. Agarwal, M. Hadley, A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials, Free Radic.Biol.Med. 15 (1993) 353-363

379 [12] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.-. Lenz, B.-. Ahn, S.
380 Shaltiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins,
381 Methods Enzymol. 186 (1990) 464-478
382 [13] T. Miyazawa, K. Fujimoto, T. Suzuki, K. Yasuda, Determination of phospholipid
383 hydroperoxides using luminol chemiluminescence-high-performance liquid chromatography,
384 Methods Enzymol, 233 (1994) 324-332
385 [14] P.J. Marshall, M.A. Warso, W.E.M. Lands, Selective microdetermination of lipid
386 hydroperoxides, Anal. Biochem. 145 (1985) 192-199
387 [15] C.A. Gay, J.M. Gebicki, Measurement of protein and lipid hydroperoxides in biological
388 systems by the ferric-xylenol orange method, Anal. Biochem. 315 (2003) 29-35
389 [16] J. Nourooz-Zadeh, J. Tajaddini-Sarmadi, S.P. Wolff, Measurement of plasma
390 hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction
391 with triphenylphosphine, Anal. Biochem. 220 (1994) 403-409
392 [17] R. Bou, R. Codony, A. Tres, E.A. Decker, F. Guardiola, Determination of
393 hydroperoxides in foods and biological samples by the ferrous oxidation-xylenol orange
394 method: A review of the factors that influence the method's performance, Anal. Biochem. 377
395 (2008) 1-15
396 [18] K. Akasaka, H. Ohrui, Development of phosphine reagents for the high-performance
397 liquid chromatographic-fluorometric determination of lipid hydroperoxides, J. Chromatogr. A
398 881 (2000) 159-170
399 [19] K. Akasaka, A. Ohata, H. Ohrui, H. Meguro, Automatic-determination of
400 hydroperoxides of phosphatidylcholine and phosphatidylethanolamine in human plasma, J.
401 Chromatogr. B 665 (1995) 37-43

402 [20] R. Bou, B. Chen, F. Guardiola, R. Codony, E.A. Decker, Determination of lipid and
403 protein hydroperoxides using the fluorescent probe diphenyl-1-pyrenylphosphine, Food
404 Chem. 123 (2010) 892-900
405 [21] H.A. Sober, G.J. Mannering, M.D. Cannon, C.A. Elvehjem, E.B. Hart, Nutrition of the
406 Guinea Pig, The J. Nut. 24 (1942) 503-514
407 [22] K. Akasaka, H. Ohrui, H. Meguro, Simultaneous determination of hydroperoxides of
408 phosphatidylcholine, cholesterol esters and triacylglycerols by column-switching high-
409 performance liquid-chromatography with a postcolumn detection system, J. Chromatogr. B
410 622 (1993) 153-159
411 [23] J.H. Sohn, Y.S. Taki, H. Ushio, T. Ohshima, Quantitative determination of total lipid
412 hydroperoxides by a flow injection analysis system, Lipids 40 (2005) 203-209
413 [24] K. Akasaka, I. Sasaki, H. Ohrui, H. Meguro, A simple fluorometry of hydroperoxides in
414 oils and foods, Biosci. Biotechnol. Biochem. 56 (1992) 605-607
415 [25] H.-. Belitz, W. Grosch, P. Schieberle, Food Chemistry, Springer, Berlin, 2008.
416 [26] AOACS, AOAC Official Method 2003.05 Crude Fat Feeds, Cereal Grans, and Forages
417 [27] E. Södergren, J. Nourooz-Zadeh, L. Berglund, B. Vessby, Re-evaluation of the ferrous
418 oxidation in xylenol orange assay for the measurement of plasma lipid hydroperoxides, J.
419 Biochem. Biophys. Methods 37 (1998) 137-146
420 [28] J. Nourooz-Zadeh, Ferrous ion oxidation in presence of xylenol orange for detection of
421 lipid hydroperoxides in plasma, Methods Enzymol. 300 (1998) 58-62
422 [29] G.L. Long, J.D. Winefordner, Limit of detection: A closer look at the IUPAC definition,
423 Anal. Chem. 55 (1983) 712-724
424 [30] H. Meguro, K. Akasaka, H. Ohrui, Determination of hydroperoxides with fluorometric
425 reagent diphenyl-1-pyrenylphosphine, Meth.Enzymol. 186 (1990) 157-161

426 [31] K. Arab, J.-. Steghens, Plasma lipid hydroperoxides measurement by an automated
427 xylenol orange method, Anal. Biochem. 325 (2004) 158-163
428 [32] D. Banerjee, U.K. Madhusoodanan, M. Sharanabasappa, S. Ghosh, J. Jacob,
429 Measurement of plasma hydroperoxide concentration by FOX-1 assay in conjunction with
430 triphenylphosphine, Clinica Chimica Acta 337 (2003) 147-152
431 [33] Y. Dotan, D. Lichtenberg, I. Pinchuk, Lipid peroxidation cannot be used as a universal
432 criterion of oxidative stress, Prog. Lipid Res. 43 (2004) 200-227
433 [34] V. Chajès, W. Sattler, M. Stultschnig, G.M. Kostner, Photometric evaluation of lipid
434 peroxidation products in human plasma and copper oxidized low density lipoproteins:
435 Correlation of different oxidation parameters, Atherosclerosis 121 (1996) 193-203
436 [35] M.L. Fernandez, J.S. Volek, Guinea pigs: a suitable animal model to study lipoprotein
437 metabolism, atherosclerosis and inflammation., Nutr. Metab. 3 (2006)
438 [36] H.K. Vincent, K.E. Innes, K.R. Vincent, Oxidative stress and potential interventions to
439 reduce oxidative stress in overweight and obesity, Diabestes Obes. Metab. 9 (2007) 813-839
440
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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 40ºC. 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).

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

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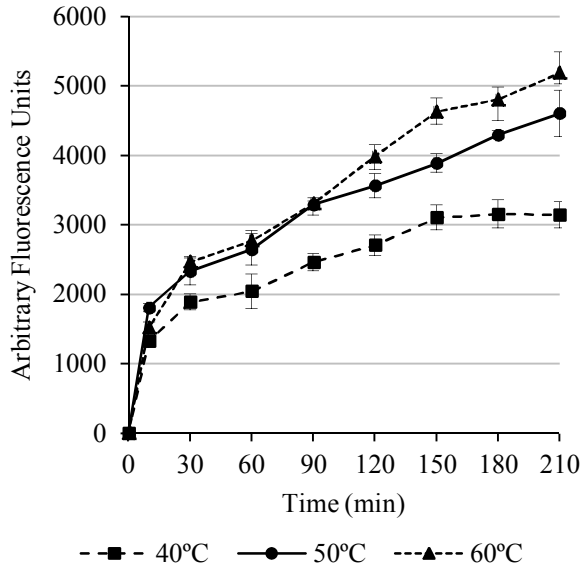

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Figure 2: Effect of sample amount

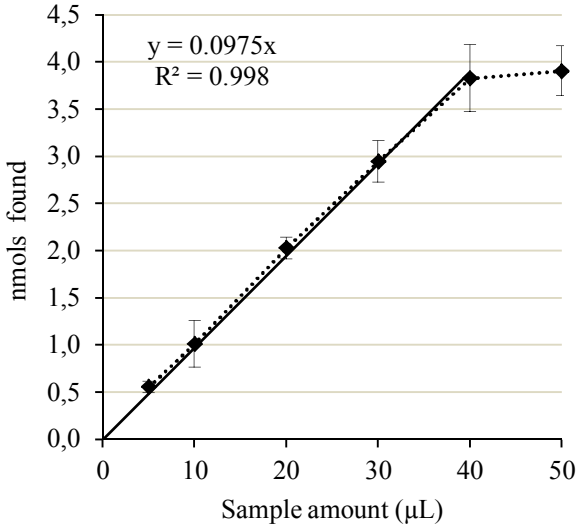
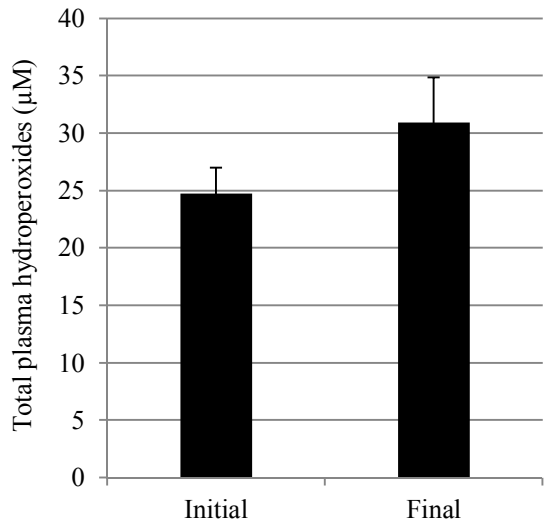


Figure 3: Plasma hydroperoxides in guinea pigs



467 **Table 1: Effect of antioxidant addition**[1]

		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 40ºC 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

Table 2. Relative standard deviation and recovery of hydroperoxides in fresh unoxidized rat plasma at two levels of cumene hydroperoxide (CHP) standard addition[1]

	Levels of CHP standard addition		
	0 μM	26 μM	52 μM
Concentration (μM CHP eq.)	14.50	37.97	62.4
RSD (%)	*5.2*	*4.5*	*4.4*
Recovery		90.5	92.3

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

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-xylenol orange	FOX
Guanidine hydrochloride	GdnHCl
Hydroperoxide	HP
Oxidized rat plasma	ORP
Phosphate-buffer saline	PBS
Triphenylphosphine	TPP
Unoxidized rat plasma	URP