



HAL
open science

Olive phenols efficiently inhibit the oxidation of serum albumin-bound linoleic acid and butyrylcholine esterase

Marjolaine Roche, Claire Dufour, Michele Loonis, Marianne Reist,
Pierre-Alain Carrupt, Olivier O. Dangles

► To cite this version:

Marjolaine Roche, Claire Dufour, Michele Loonis, Marianne Reist, Pierre-Alain Carrupt, et al.. Olive phenols efficiently inhibit the oxidation of serum albumin-bound linoleic acid and butyrylcholine esterase. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 2009, 1790 (4), pp.240-248. 10.1016/j.bbagen.2009.01.007 . hal-02662058

HAL Id: hal-02662058

<https://hal.inrae.fr/hal-02662058v1>

Submitted on 30 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

Olive phenols efficiently inhibit the oxidation of serum albumin-bound linoleic acid and butyrylcholine esterase

Marjolaine Roche,¹ Claire Dufour,^{1*} Michèle Loonis,¹ Marianne Reist,²
Pierre-Alain Carrupt,² and Olivier Dangles¹

¹ *UMR408 Safety and Quality of Plant Products, INRA, University of Avignon, Domaine St Paul, Site Agroparc, F-84914 Avignon*

² *University of Geneva - Section of pharmacy – LCT - Pharmacochimie, Quai Ernest-Ansermet 30, CH-1211 Geneva 4*

*Author to whom correspondence should be addressed

Claire Dufour, UMR408 Safety and Quality of Plant Products, INRA, University of Avignon, Site Agroparc, F-84914 Avignon, tel.: +33 432 72 25 15
fax: +33 432 72 24 92, E-mail address: Claire.Dufour@avignon.inra.fr

Keywords

Antioxidant, olive phenols, serum albumin, butyrylcholine esterase, lipid peroxidation, binding site.

1

2 **Abstract**

3 *Background* : Olive phenols are widely consumed in the Mediterranean diet and can be
4 detected in human plasma. Here, the capacity of olive phenols and plasma metabolites to
5 inhibit lipid and protein oxidations is investigated in two plasma models.

6 *Methods* : The accumulation of lipid oxidation products issued from the oxidation of
7 linoleic acid bound to human serum albumin (HSA) by AAPH-derived peroxy radicals is
8 evaluated in the presence and absence of phenolic antioxidants. Phenol binding to HSA is
9 addressed by quenching of the Trp214 fluorescence and displacement of probes (quercetin,
10 dansylsarcosine and dansylamide). Next, the esterase activity of HSA-bound
11 butyrylcholine esterase (BChE) is used as a marker of protein oxidative degradation.

12 *Results* : Hydroxytyrosol, oleuropein, caffeic and chlorogenic acids inhibit lipid
13 peroxidation as well as HSA-bound BChE as efficiently as the potent flavonol quercetin.
14 Hydroxycinnamic derivatives bind noncompetitively HSA subdomain IIA whereas no
15 clear site could be identified for hydroxytyrosol derivatives..

16 *Conclusion* : in both models, olive phenols and their metabolites are much more efficient
17 inhibitors of lipid and protein oxidations compared to vitamins C and E.

18 *General significance* : low postprandial concentrations of olive phenols may help to
19 preserve the integrity of functional proteins and delay the appearance of toxic lipid
20 oxidation products.

21

22

23

1 Introduction

2 Oxidative modification of proteins and polyunsaturated fatty acids (PUFA) play a
3 key role in the development of cardiovascular and neurodegenerative diseases [1-4]. These
4 deleterious oxidation processes involve reactive oxygen and nitrogen species (RONS) such
5 as superoxide, nitric oxide and peroxynitrite that can form from circulating blood
6 leukocytes in response to various inflammatory stimuli [5-6]. Fortunately, protection of
7 endogenous biomolecules is afforded by antioxidant enzymes and a broad range of
8 nonenzymatic antioxidants such as urate, glutathione, vitamins C and E and dietary
9 phenols [7]. Consistently, epidemiological data suggest that the so-called mediterranean
10 diet, typically rich in antioxidants from vegetables, fruits, cereals and olive oil, shows
11 significant protective health effects [8]. Olive oil and olives are rich in oleic acid, a
12 monounsaturated fatty acid, and a variety of phenolic antioxidants such as tocopherols,
13 caffeic acid as well as the more typical tyrosol, hydroxytyrosol and their elenolic esters
14 [9,10]. Interestingly, more than 50% of tyrosol and hydroxytyrosol were found to be
15 absorbed through the intestine after ingestion of olive oil by humans [11-13] and only
16 partially excreted in the urine under their native forms or as glucuronides of the native and
17 catechol-O-methylated forms [14]. On the other hand, caffeic acid is recovered in plasma
18 unchanged or metabolized by methylation, hydrogenation, glucuronidation and sulfation
19 [15,16]. Regarding possible health effects, ingestion by humans of extra virgin olive oil
20 significantly decreased inflammatory markers in plasma [17]. Furthermore,
21 hypocholesterolemic and antiatherosclerotic effects were observed after oral administration
22 of olive phenols to rats and rabbits [18,19]. Olive phenols also decrease levels of reactive
23 oxygen species in macrophages, melanoma and endothelial cells, thus preventing protein
24 damage and lipid peroxidation [20-22]. Part of these activities may involve their strong
25 capacity to scavenge free radicals and inhibit lipid peroxidation [23,24]. In particular,

1 plasma low-density lipoproteins were shown to be protected from oxidation after
2 absorption of a meal rich in olive phenols [25].

3 Human serum albumin (HSA) is the most abundant protein in serum and a potent
4 plasma antioxidant [26,27]. Albumin dimers have been proposed to be markers of
5 oxidative stress following plasma exposure to peroxides [28]. HSA plays an important role
6 in the transport of endogenous and exogenous ligands, especially fatty acids [29],
7 xenobiotics such as drugs [26] and dietary components including polyphenols [30-33].
8 Recently, we developed a physiologically-relevant model of antioxidant activity in the
9 plasma based on the ability of dietary antioxidants to inhibit the peroxidation of HSA-
10 bound linoleic acid [34,35].

11 The plasma protein butyrylcholine esterase (BChE) belongs to the ubiquitous
12 polymorphic family of choline esterases [36]. This enzyme, which is typically extracted in
13 association with HSA [37], catalyzes the hydrolysis of a variety of esters including
14 succinylcholine, a muscular relaxant, cocaine and heroin. BChE seems to be implicated in
15 lipoprotein metabolism since a strong choline esterase activity was detected in the serum of
16 hyperlipidaemic patients [38]. HSA-bound BChE was shown to be protected against
17 oxidation by several naturally occurring phenols [39].

18 The present study is aimed at determining the capacity of olive phenols to inhibit
19 the oxidation of HSA-bound linoleic acid and butyrylcholine esterase as potential lipid and
20 protein targets of RONS in plasma. The antioxidants selected are tyrosol, hydroxytyrosol,
21 oleuropein and caffeic acid as the major olive phenols, dihydrocaffeic acid and ferulic acid
22 as caffeic acid metabolites in the intestine and plasma, respectively. The antioxidant
23 potential of chlorogenic acid is addressed not only because it is the main dietary source of
24 caffeic acid but also in view of its demonstrated plasma availability [40]. The flavonol

1 quercetin, L-ascorbate, α -tocopherol and *n*-hexadecylcaffeate, a chemically synthesized
2 amphiphilic derivative of caffeic acid [24], are also investigated for comparison.

3

4 **Materials and Methods**

5 *Chemicals*

6 Hydroxytyrosol (HT) and *n*-hexadecylcaffeate were synthesized as previously described
7 [25]. Dansylsarcosine (DNSS), dansylamide (DNSA), caffeic acid, chlorogenic acid,
8 dihydrocaffeic acid, *p*-coumaric acid, (\pm) α -tocopherol, quercetin, L-ascorbic acid, 2,2'-
9 azo-bis(2-methylpropionamide dihydrochloride) (AAPH), moxisylyte, linoleic acid
10 (LH), 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid, 13(*S*)-hydroxy-(9*Z*,11*E*)-
11 octadecadienoic acid, human serum albumin (fatty acid-free, A-1887 and fraction V, A-
12 1653) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ferulic acid,
13 tyrosol and oleuropein were purchased from Extrasynthèse (Genay, France). BCECF
14 (2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein) was purchased from Calbiochem (La
15 Jolla, CA, USA). All reagents were of the highest purity available (95-99%) and were used
16 without purification. All solvents used were analytical grade.

17

18 *Inhibition of the peroxidation of HSA-bound linoleic acid*

19 HSA (fatty acid free) was dissolved in a 50 mM NaH₂PO₄ - 100 mM NaCl buffer at pH 7.4
20 (Millipore Q-Plus water) previously eluted on Chelex 100 (0.4 mequiv./mL - Bio-Rad) to
21 remove contaminating metal traces. To 3 mL of 0.5 mM HSA was added under stirring 30
22 μ L of a freshly prepared 200 mM solution of linoleic acid in MeOH. After 10 min of
23 homogenization, 30 μ L of a 5 mM solution of antioxidant in MeOH was added followed 1
24 min later by 42 mg of AAPH. The reaction mixture was stirred in an oven (225 rpm) at 37
25 °C for 10 h. Aliquots (200 μ L) were removed every hour for lipid analysis and every 10

1 min for antioxidant analysis then placed immediately on ice. Protein removal was achieved
2 by acidification with 10 μ L of 1 M HCl, addition of 400 μ L of a 500 μ M solution of L-
3 ascorbic acid in THF/MeCN (2:3), then centrifugation at 10000 g during 3 min. For HPLC
4 analyses, 30 μ L of the supernatant were immediately injected. All experiments were run in
5 triplicates.

6 *Analyses of lipid peroxidation products*

7 HPLC analyses were carried out on a Hewlett-Packard 1100 apparatus coupled to a UV-
8 Vis diode array detector. An Alltima C18 reverse phase column (150 mm x 4.6 mm, pre-
9 column of 4.6 x 7.5 mm, particle size 5 μ m, Alltech) was used for the chromatographic
10 separations at 35°C. The solvent system was a gradient of A (0.05% aqueous HCO₂H) and
11 B (MeCN) with 5% B at 0 min, 20% B at 4 min, 50% B at 13 min, 60% B at 23 min, 73%
12 B at 38 min and 100% B at 45 min with a flow rate of 1 mL/min. UV detection was set at
13 234 nm (HPODE and HODE), 280 nm (KODE) or 210 nm (linoleic acid LH). Calibration
14 curves provided the following conversion factors (defined as concentration (μ M) = peak
15 area (mAU/min) x Cf): Cf_{210nm} (LH) = 1.6, Cf_{234nm} (HPODE) = 10, Cf_{234nm} (HODE) = 10
16 and Cf_{280nm}(KODE) = 2 [41]. Cf values of HPODE and HODE were obtained with 13(S)-
17 hydroperoxy-(9Z,11E)-octadecadienoic acid and 13(S)-hydroxy-(9Z,11E)-octadecadienoic
18 acid, respectively. Cf of KODE was obtained using a mixture of 4 synthesized isomers.

19 *Antioxidant consumption*

20
21 The decay of the antioxidants was followed with the same HPLC conditions as above
22 except for the gradient which was 5% B at 0 min, 20% B at 4 min, 50% B at 13 min and
23 100% B at 15 min. The solvents were a solution of 50 mM KH₂PO₄, 5 mM
24

1 hexadecyltrimethylammonium bromide and 1 mM EDTA at pH 5 for L-ascorbic acid. and
2 isocratic MeOH for α -tocopherol.

3

4 *Fluorescence spectroscopy*

5 *Tryptophan fluorescence.* To 2 mL of 75 μ M HSA in a pH 7.4 buffer (50 mM phosphate –
6 100 mM NaCl) were added via syringe aliquots of 7.5 mM phenol solutions in MeOH. The
7 fluorescence spectra were monitored at 25 °C with a Xenius spectrofluorometer (SAFAS,
8 France) by excitation of the tryptophan residue at 295 nm. The excitation and emission slit
9 widths were set at 10 nm.

10 *Marker displacement.* To 2 mL of 75 μ M HSA in the above pH 7.4 buffer were added 20
11 μ L of 7.5 mM marker solutions in MeOH. After equilibration, aliquots of 30 mM phenol
12 solutions in MeOH were then added and the fluorescence spectra were monitored at 25 °C
13 by excitation of either HSA-bound quercetin at 450 nm or HSA-bound DNSS or DNSA at
14 360 nm [30].

15 Fluorescence intensities were corrected from the inner filter effect of the phenolic ligand
16 according to the following relation [42]:

$$17 F_{\text{corr.}} = F_{\text{meas.}} \cdot \varepsilon c (l_2 - l_1) \ln 10 / (10^{-\varepsilon l_1 c} - 10^{-\varepsilon l_2 c})$$

18 Where $F_{\text{corr.}}$ and $F_{\text{meas.}}$ are respectively the corrected and measured fluorescence intensities,
19 ε is the molar absorption coefficient of the added phenol at the excitation wavelength, l_1
20 and l_2 are the lower and upper distances from the bottom cell where light is transmitted to
21 the photomultiplier and c is the phenol concentration. In this system, l_1 and l_2 were
22 evaluated as 0.45 and 0.85 cm, respectively.

23 The respective molar absorption coefficients were evaluated in the buffer by UV-vis.
24 spectroscopy. They were calculated as the slope of the absorbance vs. concentration plot
25 for at least 5 incremental aliquots ($n = 2-3$). Couples of ε are given in $\text{M}^{-1}\text{cm}^{-1}$ at 295 and

1 360 nm, respectively: caffeic acid (14100, 900), chlorogenic acid (14000, 5750), ferulic
2 acid (14470, 600), dihydrocaffeic acid (495, NA), quercetin (9400, NA), oleuropein (690,
3 NA) and hydroxytyrosol (420, NA).

4

5 *Inhibition of butyrylcholine esterase oxidation* [39]

6 Fluorescence analyses were carried out on a SAFAS flx apparatus containing a ten-cell
7 holder (optical pathlength: 1 cm). The temperature was controlled by a water-thermostated
8 bath. To 3.4 mL of a pH 7.4 phosphate buffer (50 mM KH_2PO_4 - Na_2HPO_4 , ionic strength
9 set at the human blood value of 174 mM by KCl) were added 400 μL of a 250 μM solution
10 of HSA (fraction V) and 40 μL of an antioxidant solution in MeOH (except for n-
11 hexadecylcaffeate dissolved in MeOH/DMSO (1/1)) to a final concentration of 0.1-100
12 μM . The solution was incubated for 10 min at 40 °C under stirring (150 rpm). Then, 100
13 μL of a freshly prepared 80 mM solution of AAPH in phosphate buffer were added and the
14 solution was incubated for 120 min at 40 °C under stirring (150 rpm). Then, the incubated
15 solution was placed in a quartz cell and 15 μL of a 19.2 μM solution of BCECF in water
16 followed by 30 μL of a freshly prepared 0.1 M solution of moxisylyte in water were added.
17 After brief manual stirring, the fluorescence measurement was started at 37 °C over 15 min
18 (excitation wavelengths: 442 nm and 503 nm, emission wavelength: 530 nm). The
19 percentage of remaining activity P_{RA} was defined as $100 \times (p/p_{\text{control}})$, p_{control} being the slope
20 of the I_{BCECF} vs. time plot without antioxidant and AAPH, and p the slope of the I_{BCECF} vs.
21 time plot with the antioxidant and AAPH (slopes evaluated in the linear portion of the
22 curves between 4 and 12 min). P_{RA} was plotted as a function of logC (C: antioxidant
23 concentration, 8 values selected around the EC_{50}) to extract $\text{pEC}_{50} = -\log\text{EC}_{50}$.

24

25 *Data analysis*

1 The Prism V3.0 program (GraphPad Software, San Diego, USA) was used in the curve-
2 fitting procedures using a sigmoid equation for the calculation of EC50 values.

3

4 **Results**

5 In both antioxidant tests, a constant flow of hydrophilic peroxy radicals is
6 produced via the thermal decomposition of the diazo compound AAPH. The capacity of
7 olive phenols and other antioxidants (Fig. 1) to protect HSA-bound linoleic acid (LH) and
8 butyrylcholine esterase (BChE) against oxidation was evaluated.

9

10 **Inhibition of the peroxidation of HSA-bound linoleic acid by olive phenols**

11 The peroxidation of HSA-bound linoleic acid (C18:2) leads to a mixture of
12 hydroperoxyoctadecadienoic (HPODE), hydroxyoctadecadienoic (HODE) and
13 oxooctadecadienoic (KODE) acids. Within each family, a mixture of four (9/13) regio- and
14 (*Z/E*) diastereoisomers could be evidenced by mass spectrometry and NMR [42]. After 4
15 hours, additional products resulting from the cleavage of the hydrocarbon chain could be
16 detected (data not shown).

17 In the absence of antioxidant, a short lag phase of *ca.* 1 hour was observed in the
18 accumulation of HPODE, HODE and KODE (Fig. 2). Then, the rate of formation of the
19 oxidation products reached a maximal value and eventually decreased. The stronger trend
20 toward saturation was achieved for HPODE, which emerged as the main products of the
21 early phase of peroxidation and as likely precursors of HODE and KODE.

22 Lipid peroxidation was followed by HPLC for 10 hours with different
23 hydroxytyrosol concentrations (5 - 100 μ M). The lowest antioxidant concentration
24 showing significant inhibition was 10 μ M. In the presence of 50 μ M hydroxytyrosol, no
25 consumption of linoleic acid was detectable during the first 2 hours (Fig. 2). Peroxidation

1 then set in with a profile similar to that observed in the absence of antioxidants although
2 the rates of HPODE, HODE and KODE formation remained lower than the maximal rates
3 without antioxidant. In addition, the HODE and KODE concentrations at 10 h were found
4 to be decreased by, respectively, 17 % and 28 %.

5 For naturally occurring phenols having a 1,2-dihydroxybenzene (catechol) moiety,
6 the lag phase in HPODE formation (T_{lag}) is 2-3 times as long as the period required for
7 total antioxidant consumption (T_a) (Table 1), which shows that some of their oxidation
8 products still retain a capacity to inhibit the peroxidation of linoleic acid in agreement with
9 our previous observations [34,35]. This phenomenon reaches a maximum with quercetin,
10 which is most rapidly consumed (shortest T_a) and however emerges as the most efficient
11 inhibitor (longest T_{lag}). The small phenols hydroxytyrosol and caffeic acid display roughly
12 the same efficiency as their more hydrophilic conjugates oleuropein and chlorogenic acid
13 showing that the bulkiness of these hydrophilic substituents does not hinder the antioxidant
14 activity. It just seems that the oxidation products of caffeic acid are better inhibitors than
15 those derived from chlorogenic acid. Unlike catechols, tyrosol and α -tocopherol come up
16 as modest inhibitors, which moreover are not totally consumed after lipid peroxidation has
17 resumed ($T_{lag} < T_a$). Remarkably, the amphiphilic *n*-hexadecylcaffeate is both less efficient
18 and less rapidly consumed than caffeic acid. Its consumption coincides with the outburst of
19 peroxidation as if its oxidation products (formed during the lag phase) had no residual
20 antioxidant activity. Finally, the hydrophilic L-ascorbate is rapidly consumed and exerts
21 the weakest protection against lipid peroxidation.

22 Possible synergies between antioxidants were also checked. Combining L-ascorbate
23 (50 μ M) and hydroxytyrosol or caffeic acid (1 μ M) in concentrations close to the
24 circulating concentrations did not allow to detect the possible regeneration of the phenolic
25 antioxidants by L-ascorbate. Moreover, 25 μ M of hydroxytyrosol combined with 25 μ M of

1 caffeic acid gave an intermediate T_{lag} meaning additive antioxidant effects (data not
2 shown).

3 The antioxidants can be classified from the HPODE concentration accumulated in
4 the presence of a fixed antioxidant concentration and after a given peroxidation period
5 (Fig. 3A): quercetin > caffeic acid = oleuropein = chlorogenic acid = hydroxytyrosol =
6 dihydrocaffeic acid > *n*-hexadecylcaffeate = tyrosol = ferulic acid = α -tocopherol > L-
7 ascorbate. The same order was established from their capacity to delay KODE production
8 (Fig. 3B).

9

10 **Binding of olive phenols to HSA**

11 The binding of olive phenols to HSA was investigated by fluorescence spectroscopy. The
12 fluorescence of HSA single tryptophan (Trp214) was monitored at 340 nm (ex. 295 nm)
13 upon addition of increasing olive phenol concentrations. At a phenol/HSA ratio of 1,
14 chlorogenic, caffeic and ferulic acids promoted a strong quenching of the Trp214
15 fluorescence as observed for quercetin [30] (Fig. 4). By contrast, oleuropein,
16 hydroxytyrosol and dihydrocaffeic acid did not modify the hydrophobic environment of
17 Trp214, thus indicating no binding to the pocket accommodating the three
18 hydroxycinnamic acids. Because the various phenols absorb light at the excitation
19 wavelength, a correction for the inner filter effect was applied after evaluation of the
20 respective molar absorption coefficients. Trp214 is part of the so-called subdomain IIA
21 which is known to bind flavonoids [30-31] and drugs such as warfarin [26].

22 The binding site location for olive phenols was also investigated by displacement of probes
23 bound to HSA. DNSA and quercetin were selected as markers of subdomain IIA and
24 DNSS of subdomain IIIA [43-44]. In a first series of experiments, olive phenols were
25 added to albumin and quercetin held in equimolar concentrations (75 μ M). With a mean

1 binding constant of $125 \times 10^3 \text{ M}^{-1}$ [30-31], 72% of quercetin is bound to HSA. For an olive
2 phenol/albumin ratio of 20, the decrease of the fluorescence intensity of HSA-bound
3 quercetin at 530 nm (excitation at 450 nm) was less than 30% for ferulic, caffeic and
4 chlorogenic acids and around 10% for hydroxytyrosol and oleuropein. Although the three
5 hydroxycinnamic acids are more modest HSA ligands than quercetin [33], their inability to
6 displace quercetin from its binding site suggests that their interaction with HSA does not
7 take place in competition with quercetin and rather points to the formation of ternary
8 complexes and subsequent weak conformational changes of HSA. This may also be the
9 case for HT and its elenolic derivative oleuropein as evidenced by the fluorescence
10 patterns displayed at higher albumin-ligand molar ratios.

11 When olive phenols were added to DNSS/HSA complexes ($K_b = 1.3 \times 10^5 \text{ M}^{-1}$ [44], 73%
12 bound), the fluorescence was more strongly quenched than with the quercetin probe.
13 Chlorogenic acid efficiently displaces DNSS, ranked in the literature as a subdomain IIIA
14 probe, leading to a fluorescence quenching of 50% for a chlorogenic acid/HSA ratio of 3.
15 By contrast, hydroxytyrosol had no effect on the DNSS-HSA fluorescence. Unexpectedly,
16 similar fluorescence quenching patterns were observed for DNSA, which is considered a
17 subdomain IIA probe (data not shown). Thus, it can be proposed that either olive phenols
18 bind without selectivity to both subdomains IIA and IIIA or that DNSS and DNSA are in
19 fact markers for a same subdomain. Low-affinity binding sites for dansyl probes have also
20 been proposed [44] as observed for ANS (1-anilino-8-naphtalenesulfonamide), a
21 structurally related probe which binds both subdomain IIIA ($K_b = 8.7 \times 10^5 \text{ M}^{-1}$) and
22 subdomain IIA ($K_b = 7.9 \times 10^4 \text{ M}^{-1}$) [45].

23
24 **Inhibition of the oxidation of HSA-bound butyrylcholine esterase**

1 The experiments were conducted in the presence of moxisylyte, an ester substrate
2 of BChE, and the pH-sensitive fluorescent indicator BCECF [39]. The pH drop induced by
3 moxisylyte hydrolysis quenches the fluorescence of BCECF. In the presence of AAPH, the
4 oxidative degradation of BChE strongly attenuates the quenching of BCECF fluorescence.
5 Intermediate situations are observed in the presence of an antioxidant (Fig. 6). Over the
6 typical period of the enzymatic experiments, both moxisylyte and BCECF did not undergo
7 significant oxidative degradation (data not shown). Ferulic acid and the antioxidants
8 having a catechol moiety are the most efficient at inhibiting the oxidative degradation of
9 BChE ($EC_{50} < 10 \mu\text{M}$) (Table 2). The differences between them are not statistically
10 significant. Ascorbate and tyrosol are both much less active, a likely consequence of the
11 poor stability of the former and the weak reducing potential of the latter.

13 **Discussion**

14 After oral absorption, olive phenols are recovered (mostly as conjugates) into the
15 human blood stream [11-14] where they could develop interesting antioxidant activities (in
16 addition to more specific effects following distribution to other tissues). The blood plasma
17 contains important biomolecules that are sensitive to oxidative stress such as
18 polyunsaturated fatty acids and proteins. Moreover, the major plasma protein serum
19 albumin (0.53 - 0.68 mM in adult blood) is the transporter of free fatty acids and a wide
20 range of xenobiotics [26]. HSA displays three repetitive binding domains named I, II, III,
21 in which long-chain fatty acids are asymmetrically distributed over up to 7 binding sites
22 [29]. HSA-bound fatty acids circulate in blood plasma at a concentration near 1 mM.
23 Among them, PUFA are essential lipids that are sensitive to oxidation processes leading to
24 potentially toxic hydroperoxides and aldehydes *via* highly oxidizing intermediates such as
25 oxyl and peroxy radicals [46]. In plasma, such PUFA oxidation products could mediate

1 oxidative modifications of HSA (carbonylation, loss of Lys residues) [47,48]. In this work,
2 olive phenols and other common antioxidants are tested for their capacity to inhibit the
3 oxidative degradation of linoleic acid and butyrylcholine esterase in the presence of human
4 serum albumin.

5 Linoleic acid, the most abundant PUFA *in vivo*, was selected as the first potential
6 target of ROS in plasma. The linoleic acid/HSA molar ratio was set at 4:1 so as to mimick
7 metabolic conditions reached during strenuous exercise and in diabetes (normal
8 physiological ratio 2:1) [26]. The protection by antioxidants of HSA-bound linoleic acid
9 against hydrophilic peroxy radicals generated in the aqueous phase must be governed by
10 the two following factors:

11 - the intrinsic reactivity of the antioxidant at reducing peroxy radicals. H-atom or electron
12 transfer from the antioxidant to the peroxy radicals is largely governed by the stability of
13 the radical derived from the antioxidant. For phenolic antioxidants, it is well known that
14 semiquinone radicals derived from catechol (1,2-dihydroxybenzene) nuclei are stabilized
15 by a combination of electronic and intramolecular hydrogen bonding effects.
16 Consequently, antioxidants having a catechol group typically come up as more efficient
17 than analogs having a single phenolic OH group.

18 - the affinity of the antioxidant for HSA (measurement by the binding constant K_b) and its
19 availability to the peroxy radicals. Dietary flavonoids such as quercetin that are partially
20 absorbed from the gastro-intestinal tract are detected in plasma as HSA-bound conjugates
21 [49]. Up to now, binding constants to HSA were not available for the true circulating
22 polyphenol conjugates. For commercially available polyphenols (aglycones, glycosides)
23 and chemically synthesized models of conjugates (flavonoid sulfates), K_b values have been
24 evaluated in the range $10^4 - 10^5 \text{ M}^{-1}$ [30-33]. This moderate to low affinity is sufficient to
25 ensure that quercetin and the hydroxycinnamic acids investigated in this work are

1 predominantly bound to HSA given the high HSA/ligand molar ratio used, which is
2 expected to be even higher in the plasma conditions. Because data dealing with the affinity
3 of hydroxytyrosol derivatives towards HSA are lacking, an insight was brought into the
4 location and affinity of these small ligands. Ferulic acid, caffeic acid and its quinic ester
5 chlorogenic acid all quenched Trp214 fluorescence as efficiently as quercetin suggesting
6 binding in subdomain IIA or in proximity of the interdomain IIA/IIIA. By contrast,
7 dihydrocaffeic acid, HT and oleuropein showed no quenching and thus do not display a
8 high-affinity binding site in these regions. Displacement studies were next conducted to
9 refine the location of olive phenols. The three hydroxycinnamic acids are slightly weaker
10 HSA ligands than quercetin (K_b (ferulic acid/HSA) = $2.2 \times 10^4 \text{ M}^{-1}$, K_b (chlorogenic
11 acid/HSA) = $4.4 \times 10^4 \text{ M}^{-1}$) [33]. However, if they bind to HSA in competition with
12 quercetin, their affinity for HSA would be sufficient to totally quench the fluorescence of
13 HSA-bound quercetin given the high ligand/quercetin molar ratios used. This is by far not
14 the case and rather points to noncompetitive binding. Unexpected results were obtained in
15 the displacement of specific probes for subdomains IIA (DNSA) and IIIA (DNSS) in that
16 similar fluorescence patterns were observed. Again, the three hydroxycinnamic acids
17 deeply affected the binding of the dansyl ligands to HSA whereas the small-sized
18 hydroxytyrosol showed no effect. Multiple binding sites may be suggested for
19 hydroxycinnamic acids whereas no clear site could be identified for hydroxytyrosol and
20 oleuropein.

21 A detailed investigation has shown that quercetin bound to HSA ($K_b \approx 125 \times 10^3 \text{ M}^{-1}$) is
22 oxidized as rapidly as free quercetin under the flow of peroxy radicals produced by the
23 thermal decomposition of AAPH as if the HSA matrix exerted no screening between the
24 peroxy radicals generated in the aqueous phase and quercetin in its binding cavity within
25 subdomain IIA [35]. This observation suggests long-range electron transfer processes

1 operating between the periphery of the protein (the primary sites of radical attack) and the
2 quercetin binding site. The lag phase observed in the peroxidation of HSA-bound linoleic
3 acid has been interpreted as the period needed for AAPH-derived peroxy radicals to
4 oxidize some peripheral HSA residues thereby triggering a minimal conformational change
5 that leads to LH molecules being more exposed to the protein-borne radicals. After
6 quercetin, the most efficient peroxidation inhibitors are caffeic acid, chlorogenic acid and
7 the typical olive antioxidants hydroxytyrosol and oleuropein. Among these four
8 hydrophilic antioxidants, only the two first are known to be (weak) HSA ligands. Hence,
9 hydrophilic antioxidants with a catechol group clearly emerge as the best peroxidation
10 inhibitors as if their activity was governed by their intrinsic H-donating properties more
11 than by their binding to HSA. Moreover, the presence of a large hydrophilic sub-unit that
12 could be expected to alter the binding to HSA (caffeic acid vs chlorogenic acid,
13 hydroxytyrosol vs oleuropein) does not significantly affect the antioxidant efficiency.

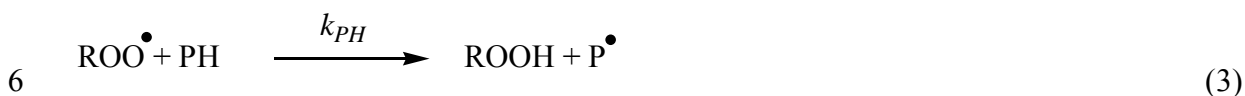
14 After the lag phase, the peroxidation rate quickly increases to reach 80 to 90% of
15 that of the uninhibited peroxidation ($R_p^{\max} \approx 1.08 \mu\text{M min}^{-1}$, Table 1). This value is much
16 lower than the initiation rate, i.e. the flow of AAPH-derived peroxy radicals in the
17 aqueous phase (based on the literature [35], $R_i \approx 14 \mu\text{M min}^{-1}$ in our conditions). Hence,
18 even when the peroxidation rate is maximal, linoleic acid is largely protected from the
19 attacking peroxy radicals by the HSA matrix. From the quasi-linear decay of the
20 antioxidant concentration, the rate of antioxidant consumption (R_a) can also be estimated
21 (Table 1). Potent antioxidants are rapidly consumed with R_a values in the range 0.8-1.3 μM
22 min^{-1} (period required for complete consumption of the antioxidant T_a in the range 50-60
23 min). By contrast, α -tocopherol and tyrosol, which are poor peroxidation inhibitors, are
24 both more stable ($R_a < 0.4 \mu\text{M min}^{-1}$, $T_a > 180$ min). Once more, the rates of antioxidant
25 consumption are much lower than the initiation rate as a consequence of the competition

1 between the antioxidant and other H-donating species during the lag phase. These species
2 include not only some exposed reducing residues of HSA but also some oxidation products
3 of the antioxidants in which the catechol nuclei are regenerated as a consequence of radical
4 recombination (dimerization, disproportionation) or water addition on quinone or
5 quinonemethide intermediates [24]. It is also noteworthy that the distribution of caffeic
6 acid oxidation products (mainly dimers with biaryl or biarylether linkages [24]) is
7 unaffected by the HSA matrix (data not shown). Those observations could be more
8 consistent with an inhibition mechanism involving the unbound fraction in the aqueous
9 phase. Remarkably, α -tocopherol, normally a potent inhibitor of lipid peroxidation, comes
10 up as a poor antioxidant in this model. Its good solubility in the medium (despite its
11 hydrophobic character) may be taken as a clue of its binding to HSA, which could
12 substantially mask its activity. It can also be added that α -tocopherol is especially potent at
13 inhibiting lipid peroxidation in systems where propagation can take place (chain-breaking
14 inhibition by scavenging of lipid peroxy radicals). However, the encounter between lipid
15 peroxy radicals (LOO^\bullet) and linoleic acid molecules (LH) is an unlikely event in this model
16 where each LH molecule is tightly sequestered within its binding pocket. Finally, L-
17 ascorbate is unique in that it is rapidly consumed while providing only a marginal
18 protection of linoleic acid. These observations are consistent with the high instability of L-
19 ascorbate in the medium reflecting its fast autoxidation rather than its efficient scavenging
20 of peroxy radicals.

21 A simple kinetic model can be developed to obtain a semi-quantitative picture of
22 the sequence of oxidative events taking place within the HSA-linoleic acid complex in the
23 absence or presence of an antioxidant. In our system, a large concentration of diazo
24 initiator AAPH ensures a constant flow of hydrophilic peroxy radicals ROO^\bullet (rate R_i) that

1 abstract H atoms from the antioxidant (AH), lipid (LH), or oxidizable protein residues
 2 (PH) [Eqs. (1)-(3)]:

3



7

8 The steady-state hypothesis for ROO^\bullet is expressed as [Eq. (4)]:

9

$$10 \quad R_i = [\text{ROO}^\bullet](k_{\text{AH}}[\text{AH}] + k_{\text{LH}}[\text{LH}] + k_{\text{PH}}[\text{PH}]) \quad (4)$$

11

12 From these relationships, the chemical rates for the consumption of AH and LH are readily
 13 deduced [Eqs. (5)-(6)]:

14

$$15 \quad R_{\text{AH}} = -\frac{d}{dt}[\text{AH}] = k_{\text{AH}}[\text{AH}][\text{ROO}^\bullet] = \frac{R_i}{1 + r_{\text{LH}}[\text{LH}]/[\text{AH}] + C_{\text{PH}}/[\text{AH}]} \quad (5)$$

16

$$17 \quad \text{With } r_{\text{LH}} = k_{\text{LH}}/k_{\text{AH}}, C_{\text{PH}} = k_{\text{PH}}[\text{PH}]/k_{\text{AH}}$$

18

$$19 \quad R_{\text{LH}} = -\frac{d}{dt}[\text{LH}] = k_{\text{LH}}[\text{LH}][\text{ROO}^\bullet] = R_{\text{AH}} r_{\text{LH}} \frac{[\text{LH}]}{[\text{AH}]} \quad (6)$$

20

21 In fact, an antioxidant of stoichiometry n can be modelled as n identical AH sub-units that
 22 all transfer a single H-atom with rate constant k_{AH} . We may also write: $[\text{AH}] = nC_a$, $R_{\text{AH}} =$

1 nR_a , C_a being the true antioxidant concentration (typically, 50 μ M) and R_a the true rate of
2 antioxidant consumption (estimated by HPLC titrations). Hence, we have:

$$3 \quad R_a = \frac{R_i}{n + r_{LH}[LH]/C_a + C_{PH}/C_a} \quad (7)$$

5
6 In fact, the antioxidant is consumed during the lag phase, i.e. when LH consumption is
7 negligible. Hence, Eq. (7) can be simplified as follows:

$$8 \quad R_a = \frac{R_i}{n + C_{PH}/C_a} \quad (8)$$

10
11 After the lag phase, LH consumption is maximized as the consequence of the consumption
12 of the antioxidant and some specific HSA residues (responsible for the lag phase observed
13 in uninhibited peroxidation). Hence, only the bulk of oxidizable HSA residues are left to
14 compete with linoleic acid for the attacking peroxy radicals:

$$15 \quad R_{LH}^{\max} = \frac{R_i}{1 + C_{PH}^{bulk}/r_{LH}[LH]} = \frac{R_i}{1 + AE_{PH}^{bulk}[PH^{bulk}]/[LH]} \quad (9)$$

17
18 With $AE_{PH}^{bulk} = k_{PH}^{bulk}/k_{LH}$

19
20 If only the oxidation of the antioxidant and specific HSA residues are considered during
21 the lag phase, we may write:

22

$$R_i = \frac{[PH^{SP}] + nC_a}{T_{lag}} = \frac{[PH^{SP}]}{T_{lag}^0} \quad (10)$$

2

3 Taking $R_i = 14 \mu\text{M min}^{-1}$, $T_{lag}^0 = 60 \text{ min}$ (no antioxidant), we obtains: $[PH^{SP}] = 0.84 \text{ mM}$,
 4 i.e. 1.7 residues per HSA molecule. For potent antioxidants ($50 \mu\text{M}$), $T_{lag} \approx 150 \text{ min}$. Thus,
 5 we have: $n \approx 25$, which is unrealistic. Consequently, we must assume that PH residues
 6 from the bulk already contribute. Taking a more reasonable n value of 4 [24], we obtain
 7 from Eq. (10): $[PH] = 1.9 \text{ mM}$, i.e. almost 4 residues per HSA molecule.

8 For potent antioxidants during the lag phase, we also have: $R_a \approx 1 \mu\text{M min}^{-1}$.

9 From Eq. (8), we thus deduce: $C_{PH} = k_{PH}[PH]/k_{AH} \approx 10C_a = 0.5 \text{ mM}$. Hence, $k_{AH}/k_{PH} \approx 4$.

10 Finally, we have: $R_p^{\max} \approx 1 \mu\text{M min}^{-1}$. Taking $[LH] = 1.8 \text{ mM}$ at R_p^{\max} , we deduce from Eq.
 11 (9): $AE_{PH}^{\text{bulk}} [PH^{\text{bulk}}] = 23 \text{ mM}$. Taking 1.9 mM as an approximate value for $[PH^{\text{bulk}}]$, we
 12 may write: $AE_{PH} = k_{PH}/k_{LH} \approx 12$.

13 Although very crude, these calculations provide a deeper insight in the mechanisms at
 14 work. In summary:

- 15 - Uninhibited peroxidation starts after a minimal oxidation of HSA at 1-2 critical residues
- 16 during the lag phase.
- 17 - In the presence of potent antioxidants, the lag phase is much longer and likely involves
- 18 additional HSA residues. However, the oxidation of the antioxidant is faster than that
- 19 taking place on the protein.
- 20 - After the lag phase, peroxidation of linoleic acid is significant but remains slow because
- 21 of its competition with oxidation at several HSA residues (*ca.* 4), which as a whole is one
- 22 order of magnitude faster. Potential targets of radical attack on HSA are Trp-214, Cys-34,
- 23 Tyr (18 residues), His (16 residues) and Met (6 residues) [27,50,51].

1 Finally, olive phenols are able to inhibit the oxidation of butyrylcholine esterase co-
2 extracted with HSA (Table 2) [39]. EC₅₀ values of all phenolic antioxidants except tyrosol
3 lie in the range 2.5 - 6.9 μ M. This poor discrimination between the antioxidants despite
4 their structural diversity and variable affinity for HSA once more suggests that a strong H-
5 donating capacity is the main factor for a potent inhibition, which in most cases is likely to
6 operate via direct scavenging of the AAPH-derived peroxy radicals by the unbound
7 antioxidant fraction in the aqueous phase.

9 **Conclusion**

10 The biological significance of the antioxidant activity of dietary phenols has been strongly
11 questioned based on their relatively low bioavailability and extensive conjugation after
12 intestinal absorption (a process that may lower the reducing activity of phenolic nuclei).
13 Anyway, the inhibition of lipid and protein oxidation remains an important challenge in
14 terms of preserving important nutrients and avoiding the formation of potentially toxic
15 hydroperoxides and carbonyl compounds. A deeper insight in the chemical mechanisms
16 sustaining the antioxidant activity of dietary phenols in biologically-relevant systems
17 involving lipids and proteins brought some unexpected results. Catechol-bearing dietary
18 phenols, whether strongly or weakly bound to serum albumin (*e.g.*, quercetin and
19 hydroxytyrosol), are much more efficient at inhibiting lipid and protein peroxidation than
20 recognized antioxidants such as vitamins C and E. Finally, although the postprandial
21 plasma concentration of dietary phenols is much lower than the circulating concentrations
22 of α -tocopherol and ascorbate, dietary phenols could help protect HSA itself against
23 oxidative modifications, thereby maintaining its function as a transport protein and a
24 plasma antioxidant.

25

1 **Acknowledgments**

2 M. Roche is grateful to the General Council of the PACA region and to INRA for financial
3 support and AFIDOL for their interest in this study.

5 **List of Abbreviations**

6 HSA, human serum albumin; BChE, butyrylcholine esterase; PUFA, polyunsaturated fatty
7 acid; HODE, hydroxyoctadecadienoic acid; HPODE, hydroperoxyoctadecadienoic acid;
8 KODE, oxooctadecadienoic acid; AAPH, 2,2'-azo-bis(2-methylpropionamide
9 dihydrochloride).

11 **References**

- 12 [1] Z. Serdar, K. Aslan, M. Dirican, E. Sarandöl, D. Yesilburca, A. Serdar, Lipid and
13 protein oxidation and antioxidant status in patients with angiographically proven coronary
14 artery disease, *Clinical Biochem.* 39 (2006) 794-803..
- 15 [2] G. Spiteller, Peroxyls radicals: inductors of neurodegenerative and other inflammatory
16 diseases. Their origin and how they transform cholesterol, phospholipids, plasmalogens,
17 polyunsaturated fatty acids, sugars, and proteins into deleterious products, *Free Radic.*
18 *Biol. Med.* 41 (2006) 362-387.
- 19 [3] D.A. Butterfield, C.M. Lauderback, Lipid peroxidation and protein oxidation in
20 Alzheimer's disease brain: potential causes and consequences involving amyloid β -peptide-
21 associated free radical oxidative stress, *Free Radic. Biol. Med.* 32 (2002) 1050-1060.
- 22 [4] A. Tappel, A. Tappel, Oxidant free radical initiated chain polymerization of protein and
23 other biomolecules and its relationship to diseases, *Med. Hypothesis* 63 (2004) 98-99.
- 24 [5] D.L. Gilbert, C.A. Colton, *Reactive oxygen species in biological systems: an*
25 *interdisciplinary approach*, Kluwer Academic, New-York, 1999.

- 1 [6] M.M. Elahi, B.M. Matata, Free radicals in blood: evolving concepts in the mechanism
2 of ischemic heart disease, *Arch. Biochem. Biophys.* 450 (2006) 78-88.
- 3 [7] B. Frei, R. Stocker, B.N. Ames, Antioxidant defenses and lipid peroxidation in human
4 blood plasma, *Proc. Natl. Acad. Sci.* 85 (1988) 9748-9752.
- 5 [8] R.W. Owen, A. Giacosa, W.S.E. Hull et al., Olive-oil consumption and health: the
6 possible role of antioxidants, *Lancet Oncology* 1 (2000) 107-112.
- 7 [9] R. Limioli, R. Consonni, A. Ranalli, G. Bianchi, L. Zetta, ¹H-NMR study of phenolics
8 in the vegetation water of three cultivars of *olea europaea*: similarities and differences, *J.*
9 *Agric. Food Chem.* 44 (1996) 2040-2048.
- 10 [10] A. García, M. Brenes, P. García, C. Romero, A. Garrido, Phenolic content of
11 commercial olive oils, *Eur. Food Res. Technol.* 216 (2003) 520-525.
- 12 [11] M.N. Vissers, P.L Zock, A.J. Roodenburg, R. Leenen, M.B. Katan, Olive oil phenols
13 are absorbed in humans, *J. Nutr.* 132 (2002) 409-417.
- 14 [12] F. Visioli, C. Galli, F. Bornet et al., Olive oil phenolics are dose-dependently absorbed
15 in humans, *FEBS Lett.* 468 (2000) 159-160.
- 16 [13] E. Miró-Casas, M.I. Covas, M. Fitó, M. Farré-Albadalejo, J. Marrugat, R. de la Torre,
17 Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin
18 olive oil in humans, *Eur. J. Clin. Nutr.* 57 (2003) 186-190.
- 19 [14] E. Miró-Casas, R. de la Torre, Capillary GC-MS quantitative determination of
20 hydroxytyrosol and tyrosol in human urine after olive oil intake, *Anal. Biochem.* 294
21 (2001) 63-72.
- 22 [15] P. Cremin, S. Kasim-Karakas, A. Waterhouse, A. L. LC/ES-MS detection of
23 hydroxycinnamates in human plasma and urine, *J. Agric. Food Chem.* 49 (2001) 1747-
24 1750.

- 1 [16] A.R. Rechner, J.P.E. Spencer, G. Kuhnle, U. Hahn, C. Rice-Evans, Novel biomarkers
2 of the metabolism of caffeic acid derivatives in vivo, *Free Radic. Biol. Med.* 30 (2001)
3 1213-1222.
- 4 [17] P. Bogani, C. Galli, M. Villa, F. Visioli, Postprandial, anti-inflammatory and
5 antioxidant effects of extra virgin olive oil, *Atherosclerosis* 190 (2007) 181-186.
- 6 [18] I. Fki, M. Bouaziz, Z. Sahnoun, S. Sayadi, Hypocholesterolemic effects of phenolic-
7 rich extracts of Chemlali olive cultivar in rats fed a cholesterol-rich diet, *Bioorg. Med.*
8 *Chem.* 13 (2005) 5362-5370.
- 9 [19] M. Gonzalez-Santiago, E. Martin-Bautista, J.J. Carrero et al., One-month
10 administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to
11 hyperlipidemic rabbits improves blood lipid profile, antioxidant status and reduces
12 atherosclerosis development, *Atherosclerosis* 188 (2006) 35-42.
- 13 [20] C.L. Léger, N. Kadiri-Hassani, B. Descomps, Decreased superoxide anion production
14 in cultured human promonocyte cells (THP-1) due to polyphenol mixtures from olive oil
15 processing wastewaters, *J. Agric. Food Chem.* 48 (2000) 5061-5067.
- 16 [21] S. D'Angelo, D. Ingrosso, V. Migliardi et al., Hydroxytyrosol, a natural antioxidant
17 from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in
18 melanoma cells, *Free Radic. Biol. Med.* 38 (2005) 908-919.
- 19 [22] J.S. Perona, R. Cabello-Moruno, V. Ruiz-Gutierrez, The role of olive oil components
20 in the modulation of endothelial function, *J. Nutr. Biochem.* 17 (2006) 429-445.
- 21 [23] O. Bouzid, D. Navarro, M. Roche et al., Fungal enzymes as a powerful tool to release
22 simple phenolic compounds from olive oil by-product, *Process Biochem.* 40 (2005) 1855-
23 1862.

- 1 [24] M. Roche, C. Dufour, N. Mora, O. Dangles, Antioxidant activity of olive phenols:
2 mechanistic investigation and characterisation of oxidation products by mass spectrometry.
3 *Org. Biomol. Chem.* 3 (2005) 423-430.
- 4 [25] M.I. Covas, K. de la Torre, M. Farre-Albaladejo et al., Postprandial LDL phenolic
5 content and LDL oxidation are modulated by olive oil phenolic compounds in humans,
6 *Free Radic. Biol. Med.* 40 (2006) 608-616.
- 7 [26] T. Peters, All about albumin: biochemistry, genetics and biomedical applications,
8 Academic Press, San Diego, 1996.
- 9 [27] E. Bourdon, N. Loreau, L. Lagrost, D. Blache, Differential effects of cysteine and
10 methionine residues in the antioxidant activity of human serum albumin, *Free Radic. Biol.*
11 *Med.* 39 (2005) 15-20.
- 12 [28] Y. Ogasawara, T. Namai, T. Togawa, K. Ishii, Formation of albumin dimers induced
13 by exposure to peroxides in human plasma: a possible biomarker for oxidative stress,
14 *Biochim. Biophys. Acta* 340 (2006) 353-358.
- 15 [29] I. Petitpas, T. Grüne, A.A. Bhattacharya, S. Curry, Crystal structures of human serum
16 albumin complexed with monounsaturated and polyunsaturated fatty acids, *J. Mol. Biol.* 314
17 (2001) 955-960.
- 18 [30] C. Dufour, O. Dangles, Flavonoid-serum albumin complexation: determination of
19 binding constants and binding site by fluorescence spectroscopy, *Biochim. Biophys. Acta*
20 172 (2005) 164-173.
- 21 [31] F. Zsila, Z. Bikadi, M. Simonyi, Probing the binding of the flavonoid, quercetin to
22 human serum albumin by circular dichroism, electronic absorption spectroscopy and
23 molecular modelling methods, *Biochem. Pharmacol.* 65 (2003) 447-456.

- 1 [32] M. Jiang, M.-X. Xie, D. Zheng, Y. Liu, X.-Y. Li, X. Chen, Spectroscopic studies on
2 the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin, *J.*
3 *Mol. Struct.* 692 (2004) 71-80.
- 4 [33] J. Kang, Y. Liu, M.-X. Xie, S. Li, M. Jiang, Y.-D. Wang, Interactions of human serum
5 albumin with chlorogenic acid and ferulic acid, *Biochim. Biophys. Acta* 1674 (2004) 205-
6 214.
- 7 [34] C. Dufour, M. Loonis, Flavonoids and their oxidation products efficiently protect
8 albumin-bound linoleic acid in a model of plasma oxidation, *Biochim. Biophys. Acta*, 1770
9 (2007) 958–965.
- 10 [35] C. Dufour, M. Loonis, O. Dangles, O. Inhibition of the peroxidation of linoleic acid
11 by the flavonoid quercetin within their complex with human serum albumin, *Free Radic.*
12 *Biol. Med.* 43 (2007) 241-252.
- 13 [36] H. Soreq, S. Seidman, P.A. Dreyfus, D. Zevin-Sonkin, H. Zakut, Expression and
14 tissue-specific assembly of human butyrylcholine esterase in microinjected *Xenopus laevis*
15 oocytes, *J. Biol. Chem.* 264 (1989) 10608-10613.
- 16 [37] N. Chapuis, C. Brühlmann, M. Reist, P.-A. Carrupt, J.M. Mayer, B. Testa, The
17 esterase-like activity of serum albumin may be due to cholinesterase contamination,
18 *Pharmaceut. Res.* 18 (2001) 1435-1439.
- 19 [38] J. Kálmán, A. Juhász, Z. Rakonczay et al., Increased serum butyrylcholinesterase
20 activity in type IIb hyperlipidaemic patients, *Life Sci.* 75 (2004) 1195-1204.
- 21 [39] A. Salvi, C. Brühlmann, E. Migliavacca, P.-A. Carrupt, K. Hostettmann, B. Testa,
22 Protein protection by antioxidants: development of a convenient assay and structure-
23 activity relationships of natural polyphenols, *Helv. Chim. Acta* 85 (2002) 867-881.

- 1 [40] M. Monteiro, A. Farah, D. Perrone, L.C. Trugo, C. Donangelo, Chlorogenic acid
2 compounds from coffee are differentially absorbed and metabolized in humans, *J. Nutr.*
3 137 (2007) 2196-2201.
- 4 [41] C. Dufour, M. Loonis, Regio- and stereoselective oxidation of linoleic acid bound to
5 serum albumin: identification by ESI-mass spectrometry and NMR of the oxidation
6 products, *Chem. Phys. Lipids*. 138 (2005) 60-68.
- 7 [42] D.E. Epps, T.J. Raub, V. Caiolfa, Determination of the affinity of drugs toward serum
8 albumin by measurement of the quenching of the intrinsic tryptophan fluorescence of the
9 protein, *J. Pharm. Pharmacol.* 51 (1999) 41-48.
- 10 [43] G. Sudlow, D.J. Birkett, D.N. Wade, Further characterization of specific drug binding
11 sites on human serum albumin, *Mol. Pharmacol.* 12 (1976) 1052-1061.
- 12 [44] D.E. Epps, T.J. Raub, F.J. Kezdy, A general, wide-range spectrofluorometric method
13 for measuring the site-specific affinities of drugs toward human serum albumin, *Anal*
14 *Biochem.* 227 (1995) 342-350.
- 15 [45] L.A. Bagatolli, S.C. Kivatinitz, G.D. Fidelio, Interaction of small ligands with human
16 serum albumin IIIA subdomain. How to determine the affinity constant using easy steady
17 state fluorescent method, *J. Pharm. Sciences* 85 (1996) 1131-1132.
- 18 [46] E. Niki, Y. Yoshida, Y. Saito, N. Noguchi, Lipid peroxidation: mechanisms,
19 inhibition, and biological effects, *Biochem. Biophys. Res. Comm.* 338 (2005) 668-676.
- 20 [47] W. Liu, J.-Y. Wang, Modifications of protein by polyunsaturated fatty acid ester
21 peroxidation products, *Biochim. Biophys. Acta* 1752 (2005) 93-98.
- 22 [48] H.H.F. Refsgaard, L. Tsai, R. Stadtman, Modifications of proteins by polyunsaturated
23 fatty acid peroxidation products, *Proc. Natl. Acad. Sci.* 97 (2000) 611-616.

1 [49] C. Manach, C. Morand, V. Crespy et al., Quercetin is recovered in human plasma as
2 conjugated derivatives which retain antioxidant properties, *FEBS Lett.* 426 (1998) 331-
3 336.

4 [50] P. Filipe, P. Morliere, L.K. Patterson et al., Mechanisms of flavonoid repair reactions
5 with amino acid radicals in models of biological systems: a pulse radiolysis study in
6 micelles and human serum albumin, *Biochim. Biophys. Acta* 1572 (2002) 150-162.

7 [51] J.A. Silvester, G.S. Timmins, M.J. Davis, Photodynamically generated bovine serum
8 albumin radicals: evidence for damage transfer and oxidation at cysteine and tryptophan
9 residues, *Free Radic. Biol. Med.* 24 (1998) 754-766.

10

11

12

13

1 **Figure legends**

2 Fig. 1. Chemical structures of the selected antioxidants.

3

4 Fig. 2. Oxidation of HSA-bound linoleic acid at 37 °C. Open symbols: inhibition by
5 hydroxytyrosol (HT), close symbols: control without antioxidant. **A:** Time dependence of
6 total lipids (\blacktriangle, Δ) and linoleic acid (\blacksquare, \square). **B:** HPODE accumulation (\blacksquare, \square) and HT
7 consumption (\circ). **C:** HODE accumulation. **D:** KODE accumulation. Initial concentrations:
8 0.5 mM HSA, 2 mM LH, 50 mM AAPH, 50 μ M HT.

9

10 Fig. 3. Inhibition of the oxidation of HSA-bound linoleic acid by the selected antioxidants.
11 **A:** concentrations of HPODE accumulated after 3 h. **B:** concentrations of KODE
12 accumulated after 4 h (antioxidant concentration = 50 μ M).

13

14 Fig. 4. Quenching of HSA Trp fluorescence by the selected phenols. Dihydrocaffeic acid
15 (open circle), hydroxytyrosol (close square), oleuropein (close diamond), caffeic acid
16 (cross), ferulic acid (close circle), quercetin (open square), chlorogenic acid (close triangle)
17 and MeOH (dashed line). HSA concentration = 75 μ M. $n = 2$ (mean dev.<10%)

18

19 Fig. 5. Influence of selected phenols on the fluorescence of HSA-bound quercetin (A) and
20 dansylsarcosine (B). Hydroxytyrosol (close square), oleuropein (close diamond), caffeic
21 acid (cross), ferulic acid (close circle), chlorogenic acid (close triangle) and MeOH (dashed
22 line). HSA and marker concentrations = 75 μ M. $n = 2$ (mean dev.<10%)

23

24 Fig. 6. Inhibition of the oxidative degradation of HSA-bound BChE by hydroxytyrosol
25 (HT). A: Time dependence of the BCECF fluorescence intensity at 530 nm in the presence

Postprint

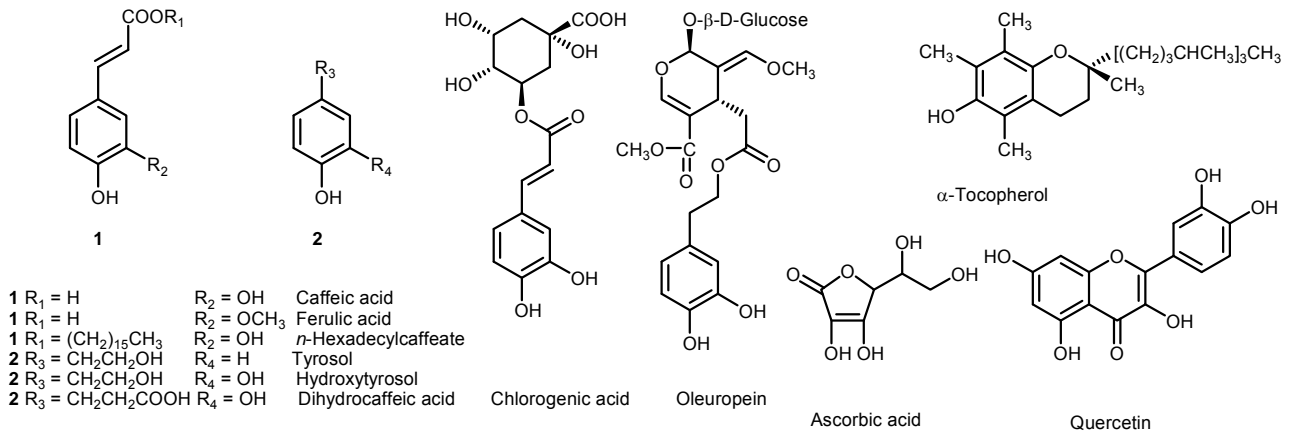
Olive phenols as antioxidants

Version définitive du manuscrit publié dans / Final version of the manuscript published in : *Biochimica et Biophysica Acta General Subjects*, 2009, vol.1790, no.4, 240-248 , DOI : 10.1016/j.bbagen.2009.01.007

- 1 of different HT concentrations (C) at 40°C. B: Plot of the percentage of remaining BChE
- 2 activity as a function of logC.
- 3

1

2



3

4

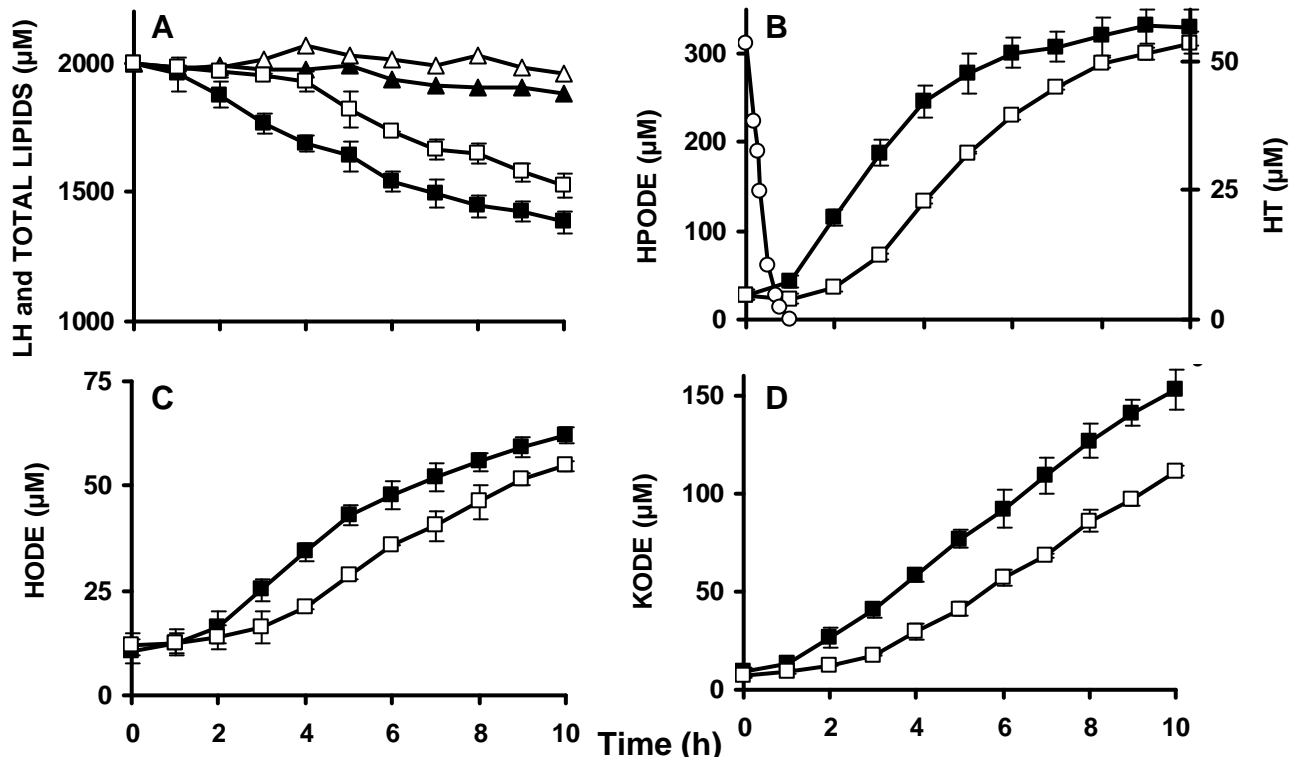
5 **Fig. 1**

6

1

2

3



4

5

6

7 Fig. 2

8

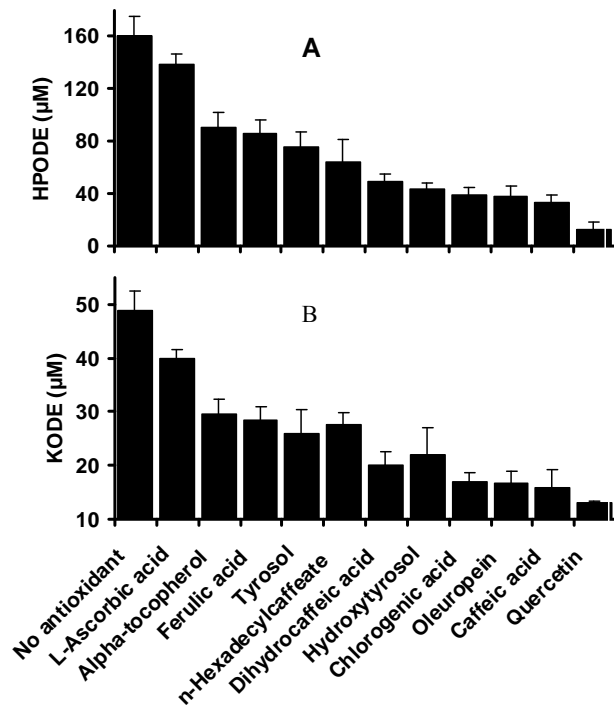
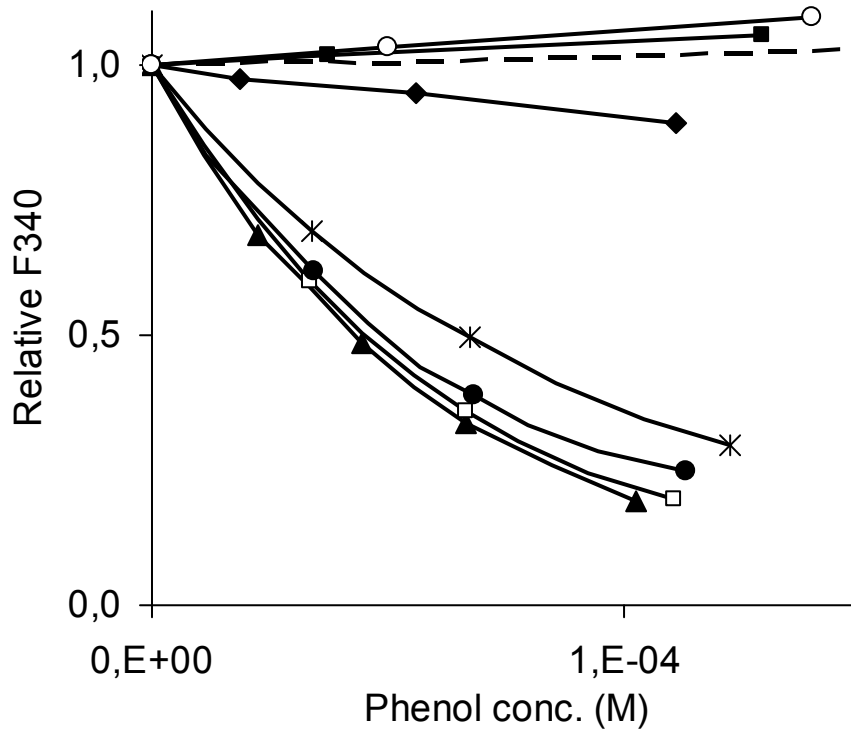


Fig. 3

1



2

3

Fig. 4

4

5

6

7

8

9

10

11

12

13

14

15

16

17

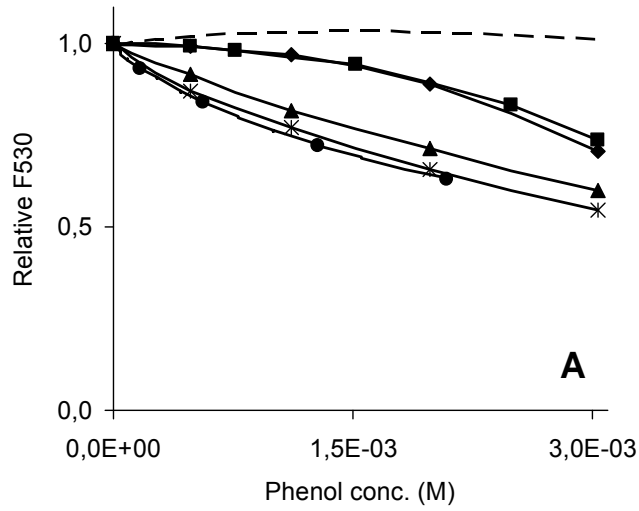


Fig. 5A

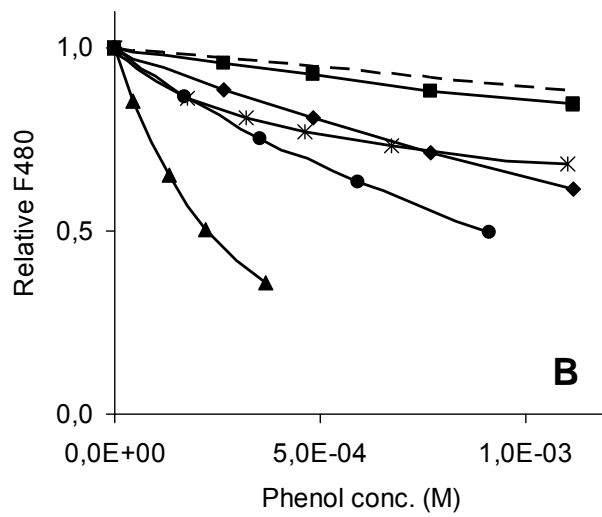


Fig. 5 B

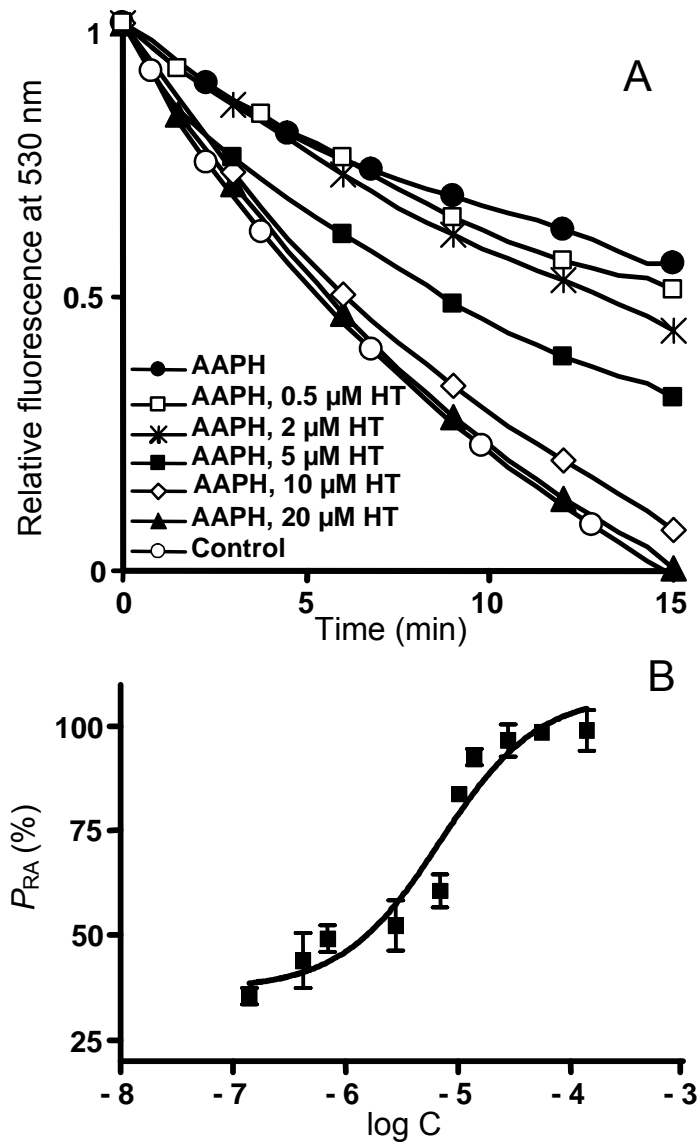


Fig. 6

1

2

Table 1. Inhibition of the peroxidation of HSA-bound linoleic acid

3

 (initial concentrations: 0.5 mM HSA, 2 mM LH, 50 mM AAPH, 50 μ M antioxidant).

Antioxidant	T_{lag} (min) ^a	T_a (min) ^b	R_p^{max} (μ M/min) ^c	R_a (μ M/min) ^d
Quercetin	163 (\pm 3)	50	0.92 (\pm 0.06)	1.11(\pm 0.02)
Caffeic acid	153 (\pm 11)	60	0.95 (\pm 0.06)	0.81 (\pm 0.03)
Chlorogenic acid	141 (\pm 4)	60	0.91 (\pm 0.14)	0.89 (\pm 0.02)
Oleuropein	142 (\pm 9)	60	0.83 (\pm 0.01)	0.80 (\pm 0.01)
Hydroxytyrosol	141 (\pm 2)	50	0.92 (\pm 0.02)	1.33 (\pm 0.11)
Dihydrocaffeic acid	131 (\pm 1)	60	0.86 (0.10)	0.91 (0.01)
n-Hexadecylcaffeate	118 (\pm 10)	120	1.01 (\pm 0.05)	0.41 (\pm 0.01)
Ferulic acid	110 (\pm 8)	80	1.19 (\pm 0.08)	0.61 (\pm 0.04)
Tyrosol	86 (\pm 1)	240	0.78 (\pm 0.08)	0.26 (\pm 0.01)
α -Tocopherol	88 (\pm 2)	180	0.92 (\pm 0.07)	0.39 (\pm 0.02)
L-Ascorbate	68 (\pm 7)	40	1.12 (\pm 0.01)	1.03 (\pm 0.03)
Control	56 (\pm 2)	-	1.08 (\pm 0.09)	-

4

^a Lag phase of the [HPODE] vs. time curve; $n = 2 - 4$. ^b Period required for complete consumption of the

5

 antioxidant (estimated error from duplicates = \pm 10 min). ^c Maximal peroxidation rate, calculated from $t = 3$ to

6

 5 h (from $t = 1$ to 3 h for the control) of the [HPODE] vs. time curve. Values are means (SD), $n = 2 - 4$. ^d Rate

7

of antioxidant consumption.

8

1 Table 2. Inhibition of the oxidation of HSA-bound BChE (initial concentrations: 25 μ M
2 HSA, 2 mM AAPH, 72 nM BCECF, 750 μ M moxisylyte).

3

Compound	pEC ₅₀ ^a	EC ₅₀ (μ M) ^b
Quercetin	5.61 (\pm 0.12)	2.5 [1.4 - 4.3]
Dihydrocaffeic acid	5.48 (\pm 0.09)	3.3 [2.1- 5.1]
Chlorogenic acid	5.46 (\pm 0.13)	3.5 [1.9 - 6.6]
Caffeic acid	5.32 (\pm 0.10)	4.8 [3.0 - 7.5]
Oleuropein	5.31 (\pm 0.15)	4.9 [2.4 - 10.0]
<i>n</i> -Hexadecylcaffeate	5.31 (\pm 0.27)	4.9 [1.3 - 19.1]
Ferulic acid	5.27 (\pm 0.10)	5.4 [3.5 - 8.5]
Hydroxytyrosol	5.16 (\pm 0.11)	6.9 [4.1 - 11.7]
L-Ascorbate	4.46 (\pm 0.10)	35 [21.5 - 56.9]
Tyrosol	< 3.84	> 140 [nd]

4 ^a pEC₅₀= -logEC₅₀, values are means (SD) n = 4; ^b confidence interval of EC₅₀ is valid at 95 %.