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A Novel Approach to Study Linoleic Acid Autoxidation: Importance of Simultaneous Detection of the Substrate and its Derivative Oxidation Products

SEBASTIANO BANNI, MARIA S. CONTINI, ELISABETTA ANGIONI, MONICA DEIANA, MARIA A. DESSÌ, MARIA P. MELIS, GIANFRANCA CARTA and FRANCESCO P. CORONGIU*

Dipartimento di Biologia Sperimentale, Sez. Patologia Sperimentale, Universita' di Cagliari, Cagliari, Italy

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In this paper we have proposed a novel approach for studying the reaction of lipid oxidation by using the simplest chemical system available. Neat linoleic acid was incubated for 24 hours at 37°C in the air. The course of lipid oxidation was followed by measuring simultaneously by HPLC with a diode array detector 1) linoleic acid decrease, 2) the products formed by radical attack, namely four hydroperoxy-octadeca-dienoic acid (HPODE) isomers, two c,t (c,t) and two trans, trans (t,t). 3) the byproducts formed by HPODE degradations, the four oxo-octadeca-dienoic acid (oxo-ODE) isomers. In HPODEs the presence of conjugated diene chromophore was confirmed by second derivative spectrophotometry. c,t HPODEs were also identified for their positional isomerism, while for t,t molecules the lack of suitable reference compound makes unfeasible the identification of their positional isomerism. As in the case of the latter two c,t and two t,t oxo-ODE isomers were characterized. This simple system appears to be useful for studying the activity exherted by lipophilic molecules that, like α-tocopherol, may act as antioxidants and/or as hydrogen atom donating molecules. The presence of α-tocopherol in different concentration for 24 hours in the reaction environment, shifts the reaction of linoleic acid autoxidation towards different byproduct formations. From the results obtained it is evident that α-tocopherol acts as hydrogen atom donor at all concentration tested, shifting the reaction toward a prevalent formation of c,t isomer of both HPODEs and oxo-ODEs. At concentration lower than 40 nmoles, when the ratio between α-tocopherol and linoleic acid was 1:100, the reaction of autoxidation is strongly inhibited, while at higher concentration α-tocopherol acted as a prooxidant. In these experimental conditions, α-tocopherylquinone was spectrophotometrically identified as the predominant oxidation product of α-tocopherol.

Key words: linoleic acid, linolelaidic acid, conjugated dienes, linoleic hydroperoxide isomers, oxo linoleic isomers, HPLC, diode array detector, second derivative UV spectrophotometry.

Abbreviations: c,t, cis, trans; t,t, trans, trans; HPODE, hydroperoxy-octadeca-dienoic acid; oxo-ODE, oxo-octadeca-dienoic acid; PUFA, polyunsaturated fatty acid

Mailing Address: Dipartimento di Biologia Sperimentale, Sez. Patologia Sperimentale, Universita' di Cagliari, Viale Regina Margherita, 45, Cagliari 09124, Italy, Telephone #: 011/39/70/665993, Fax #: 011/39/70/662875, E-Mail Corongiu @ Vaxca 1. Unica.it

*To whom correspondence should be addressed. Prof. Francesco P. Corongiu, Dipartimento di Biologia Sperimentale, Sez. Patologia Sperimentale, Universita' di Cagliari, Viale Regina Margherita, 45, Cagliari 09124, Italy.

INTRODUCTION

Oxidation of polyunsaturated fatty acids (PUFA) is a well studied radical chain reaction. This process is responsible for fat deterioration and can lead, in living systems, to cell damage.[1] Monitoring lipid oxidation reaction is quite simple in chemical systems and becomes more and more difficult as the complexity of the system increases. A number of techniques have been developed but in the case of living systems, as stated by Halliwell and Gutteridge 'no one method by itself can be said to be an accurate measure of lipid oxidation'.[2]

PUFA autoxidation is a rather complex process that proceeds readily with the conversion of a non conjugated diene fatty acid to a conjugated fatty acid hydroperoxide.[3–5] The process involves the formation of PUFA peroxyl radicals with different geometrical and positional isomerisms (Figure 1) according to the chemico-physical conditions which the reaction takes place, such as temperature and, particularly the presence of hydrogen atom donors in the reaction environment.[5] However, PUFA oxidation is often considered a reaction that produces hydroperoxides and their oxidation products without taking in account their molecular isomerism. Since it has been demonstrated that only cis, trans form of PUFA hydroperoxide isomers possess biological activities[6] more attention should be paid on the formation of different hydroperoxide isomers. Furthermore, must be point out that α-tocopherol, the biological active form of vitamin E, widely present in biological membranes is one of the most powerful hydrogen atom donor in the lipophilic environment.

Due to this property, when α-tocopherol is present during PUFA autoxidation, the reaction shifts towards c,t conjugated diene hydroperoxides.[4,5] It has been hypothesized that the same shift may occur in vivo.[7]

Fatty acid hydroperoxides are quite unstable, and produce radicals that can either initiate the autoxidation, as is the case in our experimental conditions, or propagate it,[8] giving rise to a host

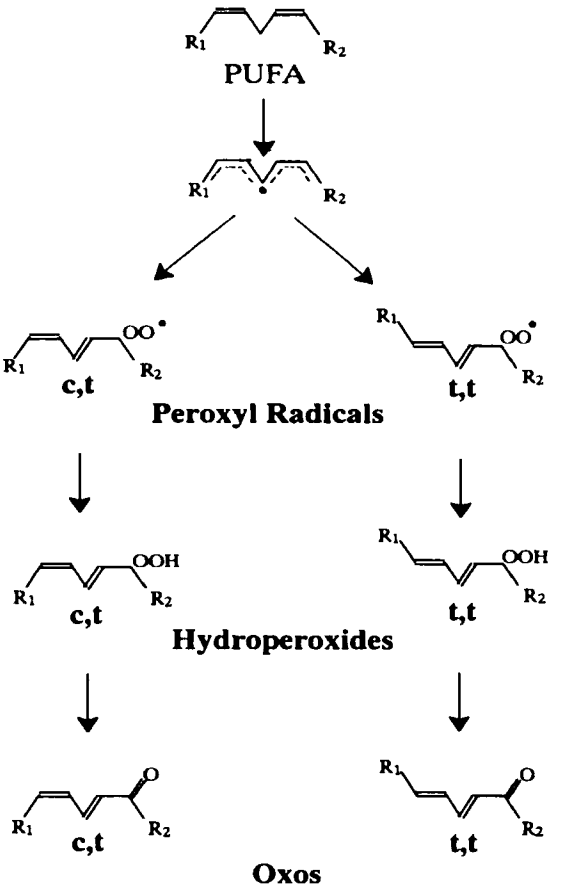

FIGURE 1 Formation of hydroperoxides and oxos from PUFA autoxidation.

of degradation products which are responsible for the rancidity of fats.[9] One of the primary products of hydroperoxide degradations are the oxo-fatty acids[8,9] (Figure 1).

The number of possible hydroperoxide isomers increase upon the number of double bonds present in the PUFA. Linoleic acid which contains two double bonds, can give rise to four hydroperoxy-octadecadienoic acid (HPODE) isomers.[10,11]

Characterization of HPODE isomers during autoxidation of linoleic acid has been already described,[10,11] however to our knowledge there is

no report on simultaneous detection of linoleic acid, HPODE and oxo-octadecadienoic acid (oxo-ODE) isomers during this process. In this paper we have monitored autoxidation of neat linoleic acid at 37°C with or without α-tocopherol by measuring the formation of HPODE and oxo-ODE isomers and the consumption of linoleic acid. The compounds were simultaneously analyzed by HPLC with diode array detector. This technique allows the identification of each eluting compound by taking the simple and second derivative spectra. In fact the conjugated diene structure present in HPODEs is easily detectable by its characteristic second derivative UV spectrum with minima at around 233 and 242 nm,[12] while oxo-ODEs, due to the double bond C=0 adjacent to the conjugated diene gives rise to a conjugated triene structure with a maximum UV absorption at around 280 nm.

The same technique was used for measuring α-tocopherol disappearance and α-tocopheryl-quinone formation when linoleic acid was autoxidized in presence of α-tocopherol.

MATERIALS AND METHODS

All solvents were HPLC grade and purchased from Carlo Erba, Milano, Italy.

Linoleic acid and linolelaidic acid (the trans, trans isomer of linoleic acid) were purchased from SIGMA, St. Louis, MO, USA.

cis, trans-13-Hydroperoxy-octadeca-dienoic acid (c,t-13-HPODE); cis, trans-9-Hydroperoxy-octadeca-dienoic acid (c, t-9-HPODE); cis, trans-13-oxo-octadeca-dienoic acid (c,t-13-oxo-ODE); cis, trans-9-oxo-octadeca-dienoic acid (c,t,-9-oxo-ODE) were purchased from Cascade Biochem. LTD, London α-tocopherol was purchased from Fluka (Buchs, Switzerland) and α-tocopheryl-quinone was purchased from Eastman Kodak (Rochester NY, USA).

Autoxidation of linoleic or linolelaidic acid

1 ml of linoleic or linolelaidic acid solutions (1 mg/ml CH_3CN) was dried down in a round bottom test tube (I.D. 16 mm and length 180 mm) under vacuum. The samples were incubated at 37°C for 4,8,12,16,20 and 24 h, in a Dubnoff water bath where samples were immersed 4 cm in the water; controls were kept at 0°C. Artificial light exposure was kept throughout the experiment. However no significant differences were noted between samples kept in the dark or exposed to artificial light.

Autoxidation of linoleic acid in presence of α-tocopherol

a-tocopherol was dissolved in solution along with linoleic acid and processed as described above. Different concentrations 20, 40, 80 and 160 nmoles per mg of fatty acids were incubated at 37°C or 0°C for 24 h.

Second derivative spectrophotometric analyses

Fatty acids were redissolved in cyclohexane and their simple and second derivative U.V. absorption spectra between 220 and 300 nm were taken using a Perkin Elmer (Norwalk, Connecticut, USA) model Lambda 15. The height of the two signals with a minimum at around 233 and 242 nm were measured and added together. The concentration of conjugated dienes in the samples was determined with reference to a standard curve.[12]

HPLC diode array detector analyses

Fatty acid separations were carried out with a Hewlett-Packard 1050 liquid chromatography equipped with a diode array detector 1040M (Hewlett Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column (Alltech Europe, Eke, Belgium) 5 μm particle size, 250×4.6 mm, was used for all separations, at a flow rate of 1.5 ml/min, and a mobile phase of $CH_3CN/H_20/CH_3COOH$ (70/30/0.12 V/V/V).

HPODEs were detected at 234 nm, PUFAs at 200 nm, and oxo-ODEs were detected at 280 nm (Figure 2).

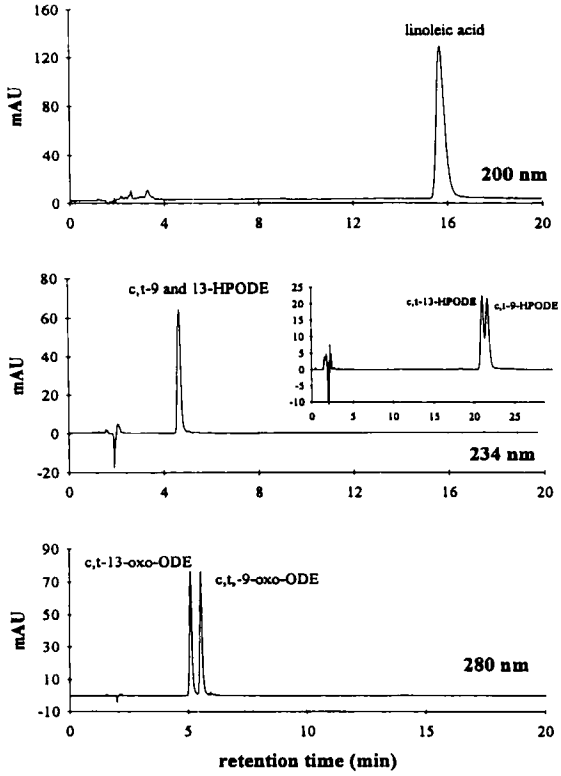

FIGURE 2 HPLC chromatograms of reference linoleic acid and its cis, trans hydroperoxides and oxos, recorded at 200 nm (upper panel), 234 nm (middle panel) and 280 nm (lower panel). insert in the middle panel shows HPLC chromatogram of c,t-HPODEs ran with a different mobile phase ($CH_3CN/H_2O/CH_3COOH$-(50/50/0.12, V/V/V).

α-tocopherol and α-tocopherylquinone were eluted with 100% of methanol at a flow rate of 1.5 ml/min and were detected with the same instruments and the same column but different settings. α-tocopherol was detected at 293 and α-tocopherylquinone at 268 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 sec and were electronically stored. Second derivative spectra were generated using the Phoenix 3D HP Chemstation software.

Second derivative as well as conventional UV spectra were taken in order to confirm the identification of the peaks.

A further identification of peaks was performed by adding pure reference compounds, when available, to the samples (data not shown).

Statistical analyses

INSTAT software (GraphPad software, San Diego, CA, USA) was used to calculate the mean and standard deviation of samples from three independent experiments, involving triplicate analyses for each sample/condition. One-way ANOVA was used to test whether the group means differed significantly. This program uses the Bonferroni method: the threshold for 'significance' is the traditional value ($p < 0.05$) divided by the number of comparisons. Thus we set a more strict (lower) threshold of significance for each comparison, so that there would be an overall probability of 5% that random chance could make any one (or more) of the differences be 'significant'.

RESULTS

Analyses of standard reference linoleic acid, its hydroperoxides and oxos

Figure 2 shows HPLC chromatograms recorded at 200, 234 and 280 nm of a mixture of linoleic acid, c,t-13-HPODE, c,t-9-HPODE, c,t-13-oxo-ODE and c,t-9-oxo-ODE. At 200 nm only linoleic acid could be detected, at 234 nm one peak is evident corresponding to the mixture of c,t-13-HPODE and c,t-9-HPODE. Under this chromatographic conditions we were not able to separate the two isomers. A better separation was achieved with a different mobile phase ($CH_3CN/H_2O/CH_3COOH$ (50/50/0.12, V/V/V), as shown in Figure 2, where the 13 and 9 isomers eluted at 21 min and 22 min respectively, but unfortunately with these chromatographic conditions linoleic acid eluted with too long a retention time. Therefore we have used the latter mobile phase only to determine the distribution of c,t-13-HPODE, c,t-9-HPODE. However, no

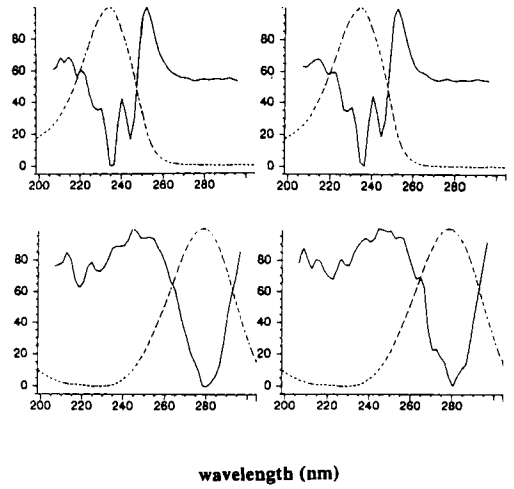

FIGURE 3 Upper panels: simple (- - -) and second derivative (——) spectra of peaks shown in the middle panel of Figure 2 corresponding to c,t-13-HPODE (left) and c,t-9-HPODE (right). Lower panel: simple (——) and second derivative (- - -) spectra of peaks shown in the lower panel of figure 2 corresponding to c,t-13-oxo-ODE (left) and c,t-9-oxo-ODE (right).

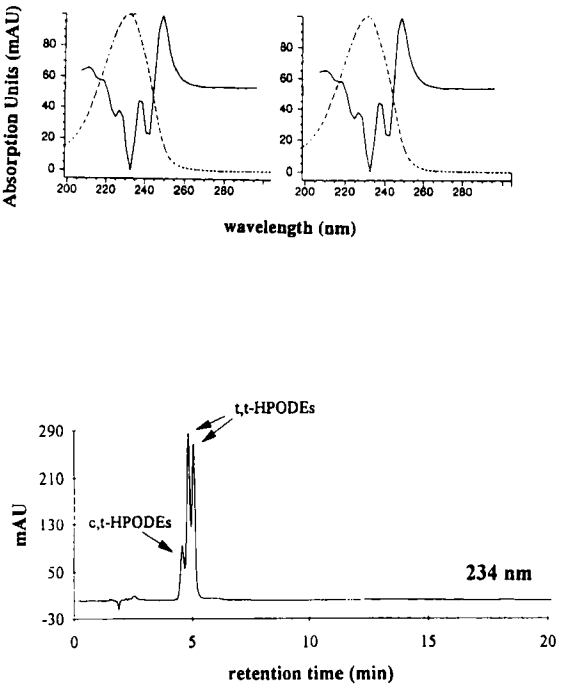

FIGURE 4 Lower panel: HPLC chromatogram of autoxidized linolelaidic acid recorded at 234 nm. Upper panel: simple (- - -) and second derivative (——) spectra of t,t HPODE peaks eluting at 4.9 min (right) and 5.4 min(left).

significant differences were seen in quantitative results between samples ran with different mobile phases (data not shown). At 280 nm the two peaks eluting at 5.1 min and 5.9 min (Figure 2) correspond to c,t-13-oxo-ODE and c,t-9-oxo-ODE. c,t-13-HPODE and c,t-9-HPODE show a typical second derivative spectrum of conjugated dienes, with two minima at 236 nm and 245 nm, while c,t-13-oxo-ODE and c,t-9-oxo-ODE show a minimum at 280 nm (Figure 3).

Autoxidation of linoleic and linolelaidic acids

A chromatogram of autoxidized linolelaidic acid recorded at 234 nm (Figure 4) shows 2 major peaks eluting at 4.9 min and 5.4 min. Both peaks display the same minima in second derivative spectra at 233 nm and 242 nm. These two fatty acids show different retention times and different spectra with respect to c,t-HPODEs. They are most likely the t,t-HPODE isomers. The minor peak eluting at 4.5 min has same retention time and same spectrum of c,t-HPODEs. In fact it has been demonstrated that linolelaidic autoxidation produces t,t-HPODEs and small amounts of c,t-HPODEs.[4] At 280 nm (Figure 5), there are two peaks at 5.5 min and 6.2 min, with different retention times and different minima (275 nm) in second derivative spectra of c,t 9 and 13 oxo-ODEs (compare Figure 2 and 3 with Figure 5). Differences in retention times and second derivative spectra with respect to c,t isomers, as well as the predominant appearance of t,t-HPODEs in the chromatogram recorded at 234 nm, supports the hypothesis that the two peaks in the chromatogram recorded at 280 nm are the t,t-oxo-ODEs derived from the corrispective HPODE isomers.

HPLC analyses of autoxidized linoleic acid (Figure 6) showed at 234 nm 3 major peaks. The first peak eluting at 4.5 min shows the same retention time and second derivative spectrum of reference compounds, c,t-HPODEs, (compare Figure

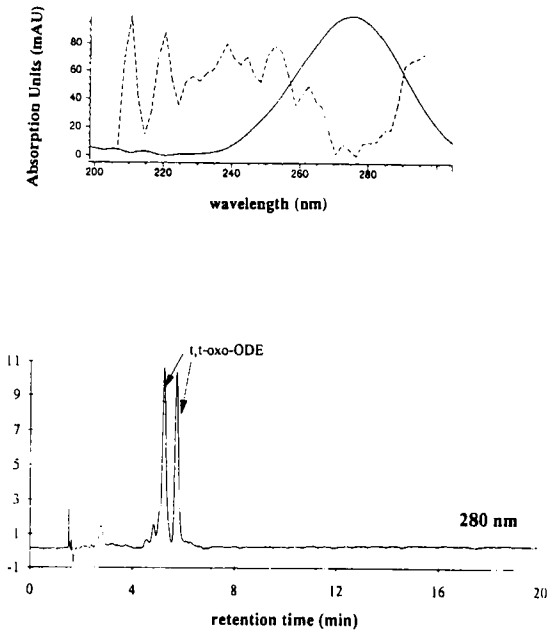

FIGURE 5 Lower panel: HPLC chromatogram of autoxidized linolelaidic acid (the t,t isomer of linoleic acid) recorded at 280 nm. Upper panel: simple (——) and second derivative (- - -) spectra of t,t-oxo-ODEs peaks.

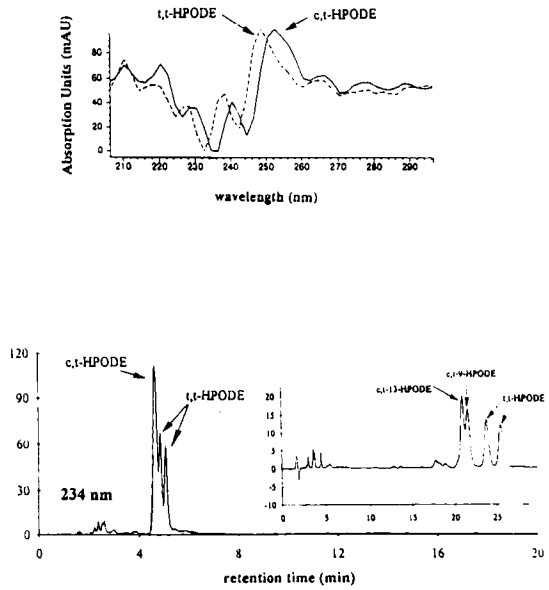

FIGURE 6 Lower panel: HPLC chromatogram of autoxidized linoleic acid recorded at 234 nm. In the insert is shown the HPLC chromatogram of autoxidized linoleic acid recorded at 234 nm ran with a different mobile phase ($CH_3CN/H_2O/CH_3COOH$) (50/50/0.12, V/V/V), Upper panel: second derivative spectra of peaks corresponding to c, t (——) and to t,t (- - -) HPODEs.

2 and 3 with Figure 6), whereas the other two peaks eluting at 4.9 min and 5.4 min have the same retention time and the same second derivative spectra of those present in the chromatogram at 234 nm of autoxidized linolelaidic acid attributed to t,t-HPODEs (compare Figure 4 with Figure 6). Again using the more polar mobile phase the first peak was partially separated in two peaks with same retention time of reference c,t-13 HPODE and c,t,-9 HPODE (Figure 6).

In the chromatogram recorded at 280 nm (Figure 7), four peaks are evident. Two peaks, those eluting at 5.1 min and 5.9 min have same retention time and same second derivative spectra of 9 and 13 c,t oxo-ODEs (compare Figure 2 and 3 with Figure 7), while the peaks eluting at 5.5 min and 6.2 min show the same retention time of the peaks present in the chromatogram recorded at 280 nm of autoxidized linolelaidic acid (compare Figure 5 with Figure 7).

Figure 8 and Table 1 show the distribution of HPODE and oxo-ODE geometrical isomers during linoleic acid autoxidation. The distribution of all isomers is nearly the same for both products as the time of incubation is longer than 8 hours.

The time course of HPODE and oxo-ODE formations during linoleic acid autoxidation (Figure 8) shows an almost linear HPODEs increase up to 12–16 hours followed by a decrease at 20 and 24 h, while oxo-ODEs keep increasing up to 24 h. Linoleic acid displays a linear decrease throughout the autoxidation process (Figure 8).

Quantitative analyses of HPODE were confirmed by measuring conjugated dienes by second derivative UV spectrophotometry (data not shown).

Autoxidation of linoleic acid in presence of α-tocopherol

In the presence of α-tocopherol the reaction of autoxidation of linoleic acid shifts toward the

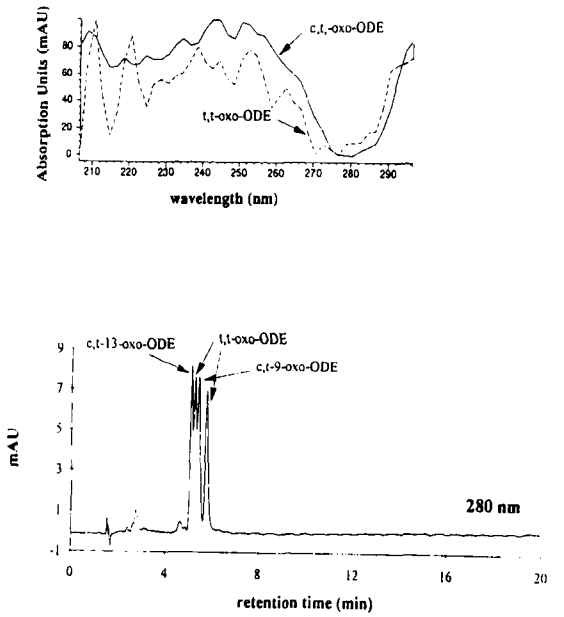

FIGURE 7 Lower panel: HPLC chromatogram of autoxidized linoleic acid recorded at 280 nm. Upper panel: second derivative spectra of peaks corresponding to c,t (——) and to t,t (- - -) oxo-ODEs.

formation of c,t isomers of both HPODEs and oxo-ODEs (Figure 9 and Table 1).

As you can see in the Figure 9 all the products formed during the reaction of autoxidation of linoleic acid in the presence of α-tocopherol at 24 hours, follow a biphasic pattern except for both t,t isomers of HPODE and oxo-ODE, the formation of which are almost completely blocked because of the specific activity of hydrogen atom donor exherted by α-tocopherol. The inversion of pattern seems to be dependent by the molar ratio α-tocopherol: linoleic acid = 1:100, that correspond, in our experimental condition, at the presence of 40 nmols of α-tocopherol in the reaction environment. At lower concentration the production of c,t HPODEs, t,t HPODEs, c,t, oxo-ODEs and t,t oxo-ODEs significantly decrease while at higher concentrations significantly increases only c,t products.

The action exherted by α-tocopherol during the autoxidation of linoleic acid can be clearly seen if both the residual linoleic acid present after 24 hours of oxidation and the production of α-

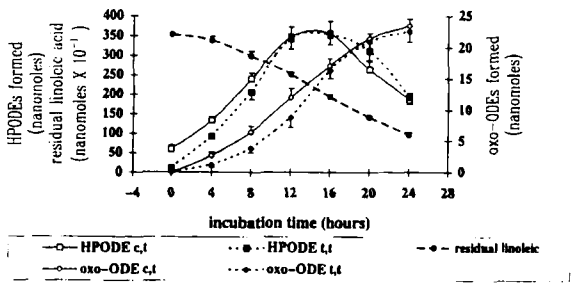

FIGURE 8 Time course of HPODEs, oxo-ODEs formation and linoleic acid consumption during 24 hour autoxidation at 37°C.

tocopherylquinone is considered (Table 1). In fact the amount of linoleic acid measured after 24 hours of oxidation increases from ≈22% (no α-tocopherol) to reach ≈88% (40–160 nmoles of α-tocopherol). Furthermore, the sum of residual linoleic acid, HPODEs and oxo-ODEs after linoleic autoxidation, accounts for ≈30% of the initial linoleic nanomoles. This suggests that ≈70% of other oxidation products other than HPODEs and oxo-ODEs are formed, while the presence of α-tocopherol lowers the formation of these compounds down to ≈9%. The production of the molecule derived from α-tocopherol oxidation, α-tocopherylquinone, increases significantly and linearly, while residual α-tocopherol decrease to detectable amount only when the initial concentration range from 80–160 nmoles (Table 1).

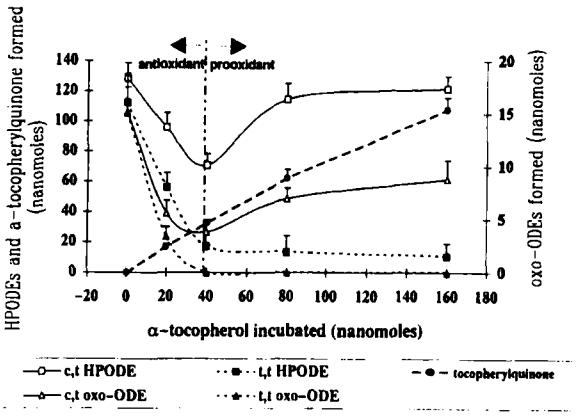

FIGURE 9 HPODEs, oxo-ODEs and α-tocopherylquinone formation when α-tocopherol is incubated with linoleic acid at 37°C for 24 hours.

TABLE 1

α-Tocopherol[§]	residual linoleic[§§]	HPODE c,t[§§§]	HPODE t,t[§§§]	oxo-ODE c,t[§§§]	oxo-ODE t,t[§§§]	residual α-Tocopherol[@]	α-Tocopherylquinone[@@]
0	782.03 ± 74.64^{b,c,d,e}	128.40 ± 10.21^{b,c}	112.30 ± 12.05^{b,c,d,e}	15.16 ± 3.65^{b,c,d,e}	15.06 ± 2.96^{b,c,d,e}	n.d.	n.d.
20	2859.29 ± 159.78^{a,c,d}	95.86 ± 9.71^{a,c,d,e,*}	56.07 ± 7.45^{a,c,d,e}	5.57 ± 1.22^{a,c,e,*}	3.40 ± 0.89^{a,c,d,e}	n.d.	16.77 ± 2.44^{a,c,d,e}
40	3161.48 ± 147.27^{a,b}	70.71 ± 7.53^{a,b,d,e,*}	17.25 ± 1.44^{a,b}	3.81 ± 0.76^{a,b,d,e,*}	n.d.[$]	n.d.	32.32 ± 2.49^{a,b,d,e}
80	3086.02 ± 81.22^{a,b}	114.20 ± 10.81^{b,c,*}	13.39 ± 1.89^{a,b}	6.94 ± 1.02^{a,b,c,e,*}	n.d.	3.13 ± 0.64^{a,b,c,e}	62.15 ± 5.84^{a,b,c,e}
160	3001.01 ± 43.93^{a}	121.59 ± 8.58^{b,c,*}	10.55 ± 1.61^{a,b}	8.79 ± 1.82^{a,b,c,*}	n.d.	8.33 ± 2.14^{a,b,c,d}	107.54 ± 8.20^{a,b,c,d}

Superscript letters indicate significant difference from: [a]0 nanomoles, [b]20 nanomoles, [c]40 nanomoles, [d]80 nanomoles, [e]160 nanomoles of α-tocopherol in the incubation mixture

[$] not detectable

*significant difference from t,t isomer

[§] nanomoles of α-tocopherol incubated with 1 mg of linoleic acid at 37°C for 24 h

[§§] nanomoles of linoleic acid left from the initial 1 mg (equals to 3566 nanomoles) after incubation with α-tocopherol at 37°C for 24 h

[§§§] All data are expressed in nanomoles; mean ± SD of three independent experiments, involving triplicate analyses for each sample/condition.

[@] nanomoles of α-tocopherol left from the initial concentrations shown in the first column after incubation with 1 mg of linoleic acid at 37°C for 24 h

[@@] nanomoles of α-tocopherylquinone formed from different concentration of α-tocopherol after incubation with 1 mg of linoleic acid at 37°C for 24 h

DISCUSSION

PUFA autoxidation is a rather complex process that proceeds readily with the conversion of a non conjugated diene fatty acid to conjugated fatty acid hydroperoxide isomers.[3,4] In the absence of metal ions, as occurs in our experimental conditions, it is expected a slower formation of compounds derived from the degradation of lipid peroxides. Thus detection of conjugated diene structure in PUFA molecules would represent an excellent index for measuring lipid autoxidation.

Nevertheless we decided to study the reaction of autoxidation of linoleic acid by examining contemporarily more than one parameter in order to have a clear picture of how the reaction proceeds. For this reason we studied the time course of the reaction by measuring the disappearance of linoleic acid, the formation of both the isomeric hydroperoxides and their isomeric oxo-derived compounds. With the same aim the reaction of autoxidation has also been studied in the presence of different amounts of α-tocopherol. The qualitative characterization and the quantitative determination of the different compounds has been performed by HPLC coupled with a diode array detector. This technique permits to detect simultaneously the eluting compounds at more than one wavelength. In our case we were able to detect simultaneously different classes of compounds having different characteristic UV absorption, namely linoleic acid, c,t hydroperoxides, t,t hydroperoxides, and their oxo-derivatives. In addition the contemporary disappearance of α-tocopherol and the contemporary appearance of their oxidative compound α-tocopherylquinone were also monitored. In addition to these analytical advantages the utilization of this method enable us to greatly improve our knowledge about the correct proceeding of the reaction of autoxidation of PUFA, particularly in chemical model systems, although, in our opinion, the same course of the reaction may occur when PUFA are oxidized in living cells.

Our results demonstrate that hydroperoxide formations during linoleic acid oxidation shows a biphasic time-course (Figure 8). The first part, up to 12–16 hours, in which HPODEs increase constantly, and the second one, up to 24 hours, in which they decrease. A similar curve has been shown to occur also during any PUFA oxidation, and is accounted for by hydroperoxide susceptibility to undergo degradation.[8] Therefore, when the oxidation is monitored after 16 hours, in the descending part of the curve, although HPODEs decrease, oxidation proceeds, since linoleic acid concentration decreases linearly and oxo-ODEs, a degradation product of HPODEs, increase throughout the 24 hours of oxidation.

These results suggest that simultaneous detection of linoleic acid together with its autoxidation byproducts allows to have a correct picture of how the reaction of autoxidation proceeds.

The use of this technique, coupled with the second derivative spectrophotometry, enables us to discriminate geometrical HPODE isomers, namely c,t from t,t conjugated dienes. In fact c,t isomers show minima at 236 and 245 nm while t,t isomers at 233 and 242 nm. A similar shift has also been shown by other authors.[10,13] In contrast to these results in a previous report[7] we attributed the two minima at 233 nm and 242 nm to t,t an c,t conjugated diene isomers respectively. This hypothesis was supported by data obtained from *in vivo* experiments, even though in very different experimental conditions. While confirming that the two signals with minima at 233 and 242 nm are due to a conjugated diene chromophore, it is not clear why in particular experimental conditions *in vivo*, only one peak with minima at 242 nm appears. Monitoring the formation of different isomers allows to study in detail how the reaction proceeds and how other components present in the reaction environment can affect this process. Our results show that while the distribution of different HPODE positional isomers are nearly the same throughout the period of oxidation, geometrical isomers show a different pattern. It seems that the distribution of geometrical isomers is directed toward c,t hydroperoxide formations by

the hydrogen aton donor activity exherted by linoleic acid. In fact, during the first 8 hours of autoxidation, when the molar ratio between residual linoleic acid and HPODEs is about 7:1, c,t hydroperoxides are the predominant isomers formed, whereas thereafter, when the ratio decreases to 3:1, the difference between the two geometrical isomers are not anymore significant (Figure 8). The activity is even more evident when a strong hydrogen atom donor such as α-tocopherol is present in the reaction environment (Figure 9). The hydrogen atom donating activity of α-tocopherol is exherted at all the concentrations tested. In fact the concentration of c,t hydroperoxide isomers is always significantly higher than t,t isomers. At initial 40 nanomoles/mg of linoleic acid or lower concentrations, α-tocopherol acts as a strong antioxidant as verified by the drastic decrease of all hydroperoxide and oxo isomers formed and the increase of residual linoleic acid. At higher concentration, besides the property of α-tocopherol as hydrogen atom donor, is also quite evident a prooxidant activity. In fact only c,t hydroperoxides and c,t oxos significantly increase along with the production of α-tocopherylquinone that in our experimental conditions is the main oxidation product formed from α-tocopherol via α-tocopheryl radical.[14] The prooxidation effect of α-tocopherol has been already observed,[15–17] and it has been attributed to the abstraction of hydrogen atom from fatty acid hydroperoxide and/or fatty acid itself by the tocopheryl radical.[17] The activity of α-tocopherol as hydrogen atom donor in this condition might be explained by the presence of residual α-tocopherol in the reaction environment.

The formation of c,t isomers of both HPODE and oxo-ODE during linoleic acid autoxidation in presence of α-tocopherol as well as the detection of c,t oxo-ODE during autoxidation of c,t HPODE standard confirms that oxo-ODE is one of the primary byproducts of HPODEs and that oxo-ODEs maintain the same isomerism of the HPODEs they come from.

Interesting α-tocopherol seems very selective in lowering the formation of oxidation products. While it is very effective in lowering the t,t isomers of both HPODEs and oxo-ODEs and other oxidation products, it is less effective with c,t isomers. This result raises indeed the question of whether c,t and t,t isomers have or not the same pathological significance when formed *in vivo*. Most likely *in vivo* during a lipoperoxidative process, the major isomers produced are in c,t form due to the presence of α-tocopherol and the high ratio between PUFA and its hydroperoxides. Moreover during cyclooxygenase reaction where, a residue of Tyr, an atom hydrogen donor, is involved in the formation of PUFA peroxyl radicals, only c,t conjugated dienes are formed.[6] It has been demonstrated that c,t is the biological active form of hydroperoxide and oxo fatty acids.[6,18] Thus, only in extreme conditions, most likely incompatible with cell viability, the t,t form might prevail.

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