Behavior of Phenolic Antioxidants in a Partitioned Medium: Focus on Linoleic Acid Peroxidation Induced by Iron/Ascorbic Acid System

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ABSTRACT: The peroxidation of linoleic acid was induced by the Fe^{2+}/ascorbic acid system in a micellar medium, and the kinetics of the produced conjugated dienes were followed. The ascorbic acid concentration was adjusted to a weak level (0.7 μM) in order to initiate only the sequential oxidative reactions without superimposed antioxidant activity. Other conditions were established to obtain a simple kinetic profile. With a linoleic acid/Fe^{2+} ratio of 10 and an Fe^{2+}/ascorbic acid ratio of 23 in the medium emulsified by Tween and saturated in oxygen, no limiting effect of substrates was observed for several hours. Fe^{2+} disappeared in less than 5 min, while a linear propagation rate was reached after 1 h for up to 15 h. Two parameters were chosen to quantify the level of the oxidation: the slope of the linear phase, and the extrapolated absorbance of this step at zero time. These two parameters were used in order to compare the antioxidant power of different phenolic compounds in this partitioned medium.

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KEY WORDS: Conjugated dienes, iron/ascorbic acid system, linoleic acid, lipid peroxidation, partitioned medium.

The effectiveness of antioxidants on lipid peroxidation greatly depends on the physical state of the substrate, i.e., continuous or dispersed phases. Many foods or biological media are polyphasic systems with low-density lipoproteins of blood, liposomal membranes, micelles, fat droplets, or emulsions. In such systems, segregation phenomena and migrations of substrates and products may occur during oxidation and antioxidation of lipids.

The interfacial phenomena are at the center of the oxidation reactions in dispersed media. It has been demonstrated that more unsaturated fatty acids oxidize more slowly in oil-in-water emulsions than the less unsaturated ones (1,2), while the opposite was found in bulk oils (3,4).

In a partitioned medium, the location of the antioxidants and their concentrations in the different regions depend on their polarity and solubility properties. Many authors found that the efficiency order of several antioxidants tested in bulk oils was reversed in an emulsified medium (5–8). These results support the general rule, "polar paradox" (5,9).

To screen the efficacy of potential antioxidants or to determine the oxidative stability of fats and oils, it is customary to accelerate the peroxidation. Most often, samples are heated to 100–120°C to reach a significant level of oxidized products in less than one day. These methods are subject to criticism because they are not representative of normal storage. Accelerated tests may modify the nature of the final products. Generators of radicals are also often used to induce chemical oxidations. Pryor *et al.* (10) reported that in sodium dodecyl sulfate (SDS) micelles, the partition of the 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) into the micellar phase is not very different from the linoleic acid one. Transition metal ions are also remarkable promoters of free-radical reactions because their oxidative state changes through a single electron transfer. Either iron or copper is used to enhance the rate of oxidation, principally by homolytic cleavage of the weak O–O bond in hydroperoxides and subsequent formation of radicals. The iron/ascorbate system is currently used (11–16). In such a system, Fe(II) reacts with traces of hydroperoxides by means of a Fenton-like reaction to give primary radicals:

$$ROOH + Fe^{2+} \longrightarrow RO^{\bullet} + Fe^{3+} + OH^{-} \quad [1]$$

If Reaction 1 is fast, the next is very slow:

$$ROOH + Fe^{3+} \longrightarrow ROO^{\bullet} + Fe^{2+} + H^{+} \quad [2]$$

The alkoxyl radicals $RO^{\bullet}$ trigger the initiation reaction while Fe^{2+} is regenerated by ascorbic acid (AsA):

$$RO^{\bullet} + RH \longrightarrow ROH + R^{\bullet} \quad [3]$$

$$\text{AsA-}Fe^{3+} \longrightarrow \text{AsA-}Fe^{2+} \quad [4]$$

AsA AsA•

Many researchers have used this initiator system in dispersed

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lipids emulsified in microdroplets with different surfactants. Fukuzawa *et al.* (15) studied the iron/ascorbate induced lipid peroxidation in phospholipid vesicles and proposed a reaction mechanism. Ponginebbi *et al.* (16) used $FeSO_4$/AsA as an oxidation catalyst of linoleic acid (LA) micelles and studied the effect of relative concentrations of substrate and emulsifier. As generally much more AsA than iron was added in the medium, the AsA had multiple roles: reducing Fe(III) to Fe(II), chelating iron in the aqueous phase, transferring it to the interface, or scavenging the peroxide radicals**.**

To suppress the AsA antioxidant activities, we searched for the lowest concentration of AsA allowing a rapid production of conjugated dienes. Our goal was first, to define the role of the different reagents during the reaction, under these new conditions, and second, to determine some suitable physical parameters to quantify the supplied antioxidant activity. In fact, many published papers present a simple comparison of the kinetic oxidation curves that only rank the antioxidants according to their respective efficacy. The sequential steps of this system will be described in this paper, while behavior and structure-activity relationships of many phenolic compounds will be related in a second paper.

EXPERIMENTAL PROCEDURES

Reagents. LA came from one single production lot (Sigma, St. Quentin Fallavier, France, L-1268, 95% purity) and was stored in 5-g portions under nitrogen at −30°C. Ferrous chloride tetrahydrate (F-2130, 99% purity), polyoxyethylenesorbitan monolaurate (Tween 20, P-7949), piperazine-*N,N′*-bis (2-ethane sulfonic acid) (PIPES, P-8658), triphenylphosphine (T-1136), and 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4 triazine monosodium salt (Ferrozine, P-9762) were obtained from Sigma. AsA (AO 228, 99.7% purity) and ethyl acetate (99.8% purity) were obtained from OSI (Oulchy le Chateau, France); methanol (high-performance liquid chromatography grade) was obtained from Carlo Erba (Val de Reuil, France); and sodium chloride (99.5% purity, 27810.295) was obtained from Prolabo (Fontenay-sous-Bois, France). Conjugated octadecadienoic acid**s** were a mixture of *cis*- and *trans*-9,11 and 10,12 linoleic acids (O-5507, Sigma). Nitrogen (HP 45, 99.995% purity) and oxygen (HP 48, 99.998% purity) were purchased from Carboxique (Trappes, France).

Preparation of the emulsified medium. In distilled water, 1.5 g of PIPES, 900 mg of NaCl, 7.5 mL of 1 N NaOH, and 850 mg of Tween 20 were dissolved up to a volume of 100 mL. The pH was adjusted to 6.4 and this buffer was stored at 4°C. Then 200 µL of a methanolic solution of 0.05 M LA was evaporated under nitrogen and mixed with 2 mL of PIPES solution. The emulsification was performed by means of a vortex agitator (VTX 400) from Bioblock (Illkirch, France) at 40 Hz for 2 min. This emulsion had to be used within a 30-min interval.

Measurement of the conjugated dienes. Spectrophotometric data were obtained from an Uvikon 810 double beam spectrophotometer (Kontron, Switzerland). Two quartz cuvettes, equipped with Teflon stoppers, were carefully washed with detergent (1% MUCAPUR 2000), 0.15% EDTA, then 1 N nitric acid, with many rinses between each operation. Next, 100-µL aliquots of the emulsion were pipetted into the cuvettes, diluted with 2.9 mL of distilled water previously filtered on a Millipore membrane (HVLP 0.45 µm) (Molsheim, France), and oxygenated for 40 min by bubbling 7 $mL \cdot min^{-1}$ of pure oxygen. The two cuvettes were stoppered and placed in a water bath at 30°C ± 0.02 for 15 min, then put inside the cuvette holder of the spectrophotometer thermostated at 30°C. Then, 50 mg of $FeCl_2$ and 97 mg of AsA were dissolved separately in 10 mL of distillated water. Next, 1 mL of $FeCl_2$ and 20 µL of AsA solutions were pipetted into a vessel and adjusted to 10 mL, then 20 µL of this mixture was added to the reaction cuvette just before running the acquisition data. Table 1 gives the final concentrations of the reagents in the cuvette.

The change in absorbance at 233 nm was read after 90 s and then each 3 or 6 min for 2 h. Each value was the mean of the absorbance measured along 25 s. The reference cuvette (blank) containing 170 µM emulsified LA, without iron/ascorbate initiator, corrected the scattering effect of light in the sample cuvette, plus the absorbance of the dienes formed during the emulsification. Thus, the measured absorbance was only due to the conjugated dienes formed from the reactions induced by Fe^{2+}/ascorbate.

To determine the initial concentration of conjugated dienes present in the emulsion before induction by Fe^{2+}/ascorbate, two calibration curves were performed from a commercial mixture of conjugated octadecadienoic acids**.** Concentrations between 0 and 25 µM of 9,11- and 10,12-dienoic acids were added to the PIPES buffer alone or to the PIPES buffer containing 170 µM LA. Then, the emulsions were prepared as usual and the absorbances were measured at 233 nm. The distance between the two parallel spectrophotometric curves gives the absorbancy value due to the already existing hydroperoxides in the substrate, plus those formed during the emulsification. The ε coefficient of the emulsified conjugated octadecadienoic acids was found to be 23,000, near the 24,000–28,000 values reported in the literature for the linoleic dienes in solution.

Measurement of Fe^{2+} concentration in the reaction medium. The disappearance of ferrous ions during the oxidation steps was followed by complexing the remaining Fe^{2+} with ferrozine. During the oxidation of emulsified LA induced by iron/ascorbate, 2-mL aliquots were pipetted at 30-s intervals and put into disposable cuvettes containing 20 µL of

TABLE 1
Final Composition of the Emulsified Medium

Compound	Concentration
Linoleic acid	170 µM
Iron (II) chloride (Fe^{2+})	17 µM
Ascorbic acid (AsA)	0.7 µM
NaCl	5.15 mM
Tween 20	244 µM

10 mM ferrozine. The absorbance at 562 nm was read after 15 min at ambient temperature against a blank without Fe^{2+}/ascorbate.

RESULTS AND DISCUSSION

Conditions of the test. The composition of the medium permitted measurement of the conjugated dienes directly in the emulsion. The micelles were so finely dispersed that any visual turbidity was seen for several hours. No droplets were visible under the microscope, at 1000× magnification, supporting the idea that the size of the micelles was less than 1 µm. Ponginebbi *et al.* (16), using 80 to 800 µM Tween 20 and similar conditions of emulsification, found that the mean diameter of the oil droplets decreased with increasing amounts of emulsifier (from 0.30 ± 0.04 to 0.15 ± 0.03 µm), without any effect on the oxidation rate. Coupland *et al.* (17), homogenizing emulsions of ethyl linoleate in buffer with 2% of Tween 20 by means of ultrasound, found a size distribution centered around 0.6 µm.

LA/iron ratio, (*LA/Fe^{2+}*). Performing the measurements directly in the cuvette of the spectrophotometer is possible if the diene absorbance does not exceed the apparatus capability at the end of the assay. Higher concentrations of dienes would require extraction and dilution of the sample prior to measurement, or following the kinetics for only a short time like Pryor *et al.* (10) and Foti *et al.* (18). On the other hand, quantifying the antioxidant activity during the initiation and propagation phases requires responses of sufficient intensity. The amplitude of the absorbance at the end of the assay depends mainly on the initial concentration of LA. Seven LA/Fe^{2+} molar ratios have been tested: 0.5, 1, 1.6, 2, 3.2, 4.3, and 10. For each ratio, several concentrations of LA and subsequent iron were analyzed. In each case, for a given molar ratio, the higher the level of LA, the faster the reaction and the greater the amplitude of absorbance in the first steps (data not shown).

For LA/Fe(II) ratios less than 2, the diene production was very fast and the kinetic curve reached a plateau after a few minutes because, in such conditions, all the LA was rapidly oxidized and became limiting (Fig. 1, line a). Moreover, the absorbance at the plateau was directly proportional to the concentration of LA. For a ratio of 10 (Fig. 1, line c), the whole reaction was much longer, showing a hyperbolic profile over the first 20 to 30 min, and then a linear phase running for more than 2 h without reaching a plateau. The intermediate ratios (Fig. 1, line b) gave a complex curve that looked like the sum of two different kinetic plots and was more difficult to interpret. We chose the conditions of a two-phase kinetic. With 170 µM LA and 17 µM Fe^{2+}, the rate was variable during the first 30 min and then remained constant. The absorbance was around 0.4 after 2 h. Miller and Aust (19), Fukuzawa *et al.* (15), and Miyashita *et al.* (2) also used a lipid/Fe^{2+} molar ratio around 10.

Figure 2 shows the evolution of the conjugated dienes in the sample cuvette (with initiator) and in the reference cuvette (without initiator) over a long period of time. As mentioned above, the sample curve results from an automatic correction performed by the double beam spectrophotometer, and it is only due to the Fe^{2+}/ascorbate-catalyzed reactions. The LA autoxidation, represented by the curve without iron/ascorbate, increased strongly between 12 and 40 h, while the maximum of the catalyzed conjugated dienes was reached at 25 h. This means that during the first 25 h, the production rate of hydroperoxides was higher than the decomposition rate. Thus, for the first 2 h taken into account in our test, only weak decomposition can occur.

AsA. Generally, researchers used 5- to 20-fold more AsA than Fe^{2+} to reduce Fe^{3+} produced in Reaction 1 and to maintain a high level of oxidation. However, in order to evaluate the antioxidant efficiency of a compound, we thought it necessary to eliminate the antioxidant activity of AsA. Thus, the minimal quantity required to transform Fe^{3+} present in the commercial $FeCl_2$ was measured. Ferrozine is a complexing agent of the Fe^{2+} species that forms a colored product with an absorbance maximum at 562 nm and an extinction coefficient of 27,900 $M^{-1}\cdot cm^{-1}$ (19,20). For 27 µmol of $FeCl_2$, 0.3 µmol of AsA would be sufficient to completely reduce Fe^{3+} (Fig. 3). That means that only a few Fe^{3+} species were pre-

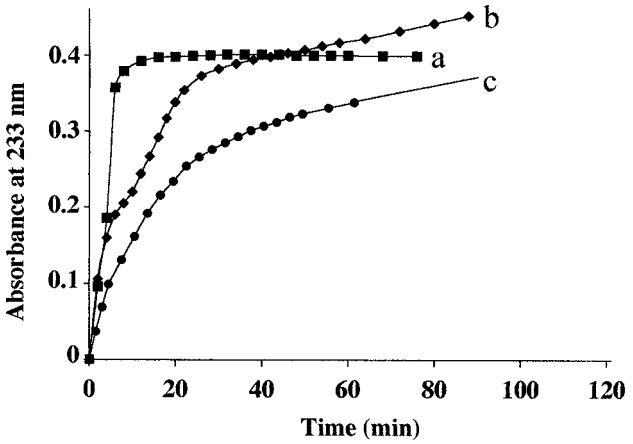

FIG. 1. Kinetics of conjugated diene appearance in a micellar medium, at three linoleic acid (LA)/$FeCl_2$ molar ratios. LA/Fe = 0.5 (a), = 3.2 (b), = 10 (c). Fe(II)/ascorbic acid (AsA) = 23. pH = 6.4, 30°C.

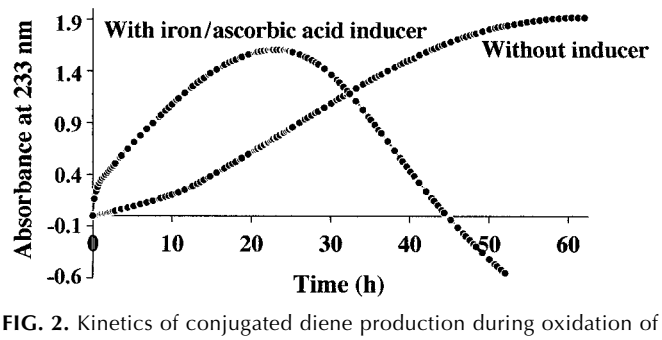

FIG. 2. Kinetics of conjugated diene production during oxidation of dispersed LA, in presence or in absence of iron/ascorbate inducer. Conditions: 170 µM LA, inducer = 17 µM $FeCl_2$ + 0.7 µM AsA, pH = 6.4, 30°C. For abbreviations see Figure 1.

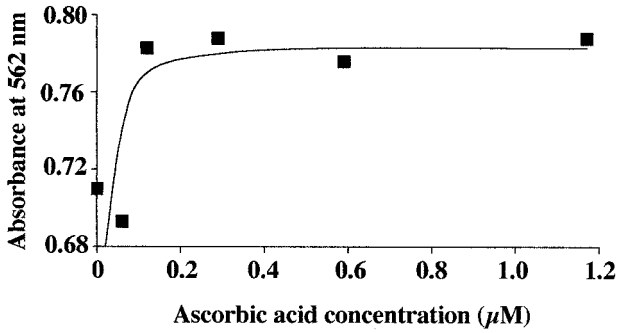

FIG. 3. Determination of the smallest AsA concentration required to reduce Fe^{3+} species in a 27 μM $FeCl_2$ solution, using ferrozine Fe^{2+} complexation. For abbreviation see Figure 1.

sent in the fresh solution of $FeCl_2$. At the same time, such a molar ratio leads to a quantity of conjugated dienes produced for the first hours that is too small (data not shown). A molar ratio Fe^{2+}/AsA of 23, as retained, constitutes a good compromise, since less than 5% Fe^{3+} species newly produced during Reaction 1 could be regenerated through Reaction 4. Antioxidant reactions of AsA were greatly reduced and the amplitude of the absorbance was satisfying.

Mechanisms. (i) Role of Fe(II). Figure 4 shows a standard kinetic curve of conjugated diene production. No lag period was observed. Fukuzawa *et al.* (15) reported that, in their liposome system, some hydroperoxides were necessary to initiate lipid peroxidation by AsA/Fe^{2+}. In our assay, the level of the already existing linoleate hydroperoxides in the reaction medium was around 7 μM. These initial hydroperoxides may be quickly decomposed in $RO^{\bullet}$ by Fe(II) as they are preferentially located at the interface. The produced $RO^{\bullet}$ induce $R^{\bullet}$ and form the first conjugated dienes that increase the absorbance and allow the propagation phase to occur. Residual Fe(II) from the initial 17 μM may also decompose some newly formed hydroperoxides in the first minutes, increasing the rate of propagation. According to Buettner (21), the reaction of ROOH with Fe(III) is thermodynamically unfavorable with a redox potential of −890 mV. Fukuzawa *et al.* (15) reported that 8 μmol of triphenylphosphine could completely block the formation of new hydroperoxides by reducing the initial hydroperoxides into their alcoholic form (15). Conversely, in our study, an addition of 30 μM triphenylphosphine in the dispersed medium did not prevent the starting reaction, but only stopped the chain propagation (Fig. 4). Triphenylphosphine does not seem to prevent the primary action of Fe^{2+} on ROOH. In order to better specify the iron action, the level of Fe^{2+} was followed in the medium by complexation with ferrozine (Fig. 5). Fe(II) quickly decreased and fell to zero after 5 min. In parallel, the diene absorbance increased up to 0.1 absorbance unis (AU). Therefore, Fe(II) triggers the initiation phase, and perhaps, the beginning of the propagation. Then, the autocatalyzed reaction continues without help of any initiator. A new addition of 17 μM $FeCl_2$ and 0.7 μM AsA during the second phase restored the initiation period, reproducing exactly the profile of the first phase (Fig. 6). This result also demonstrated that LA was not limiting for several hours.

(ii) Oxygen. As the propagation phase consumed oxygen, the emulsion was initially prepared with distilled water saturated in oxygen before starting up the peroxidation.

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \quad [5]$$

$$ROO^{\bullet} + R'H \longrightarrow ROOH + R'^{\bullet} \quad [6]$$

In order to verify that oxygen did not constitute a limiting factor, an additional supply of bubbling oxygen was added to the reaction cuvette after 4 h, i.e., during the linear part of the propagation. No increase of the slope was observed, showing that oxygen was still in excess (Fig. 7). The rate of the reaction in the linear phase is related to the $R^{\bullet}$ appearance and disappearance. So, as long as the partial oxygen pressure is high, the $R^{\bullet}$ concentration is low because the rate constant of Reaction 5 is much higher than Reaction 6, and the termination reactions are weak.

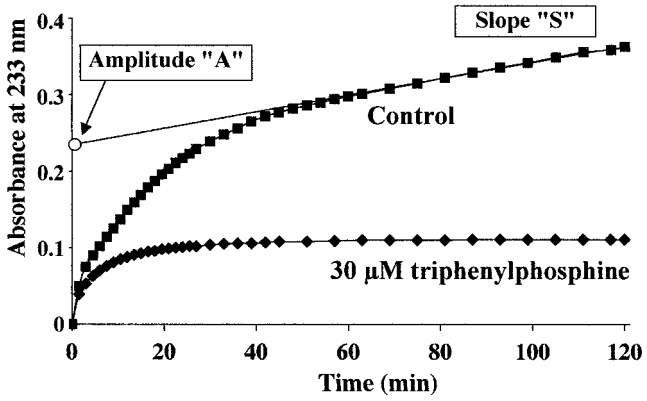

FIG. 4. Kinetics of conjugated diene production during iron/ascorbate-induced oxidation of dispersed LA, in the absence (control) or presence of triphenylphosphine. Conditions: 170 μM LA, 17 μM $FeCl_2$, 0.7 μM AsA, pH = 6.4, 30°C. For abbreviations see Figure 1.

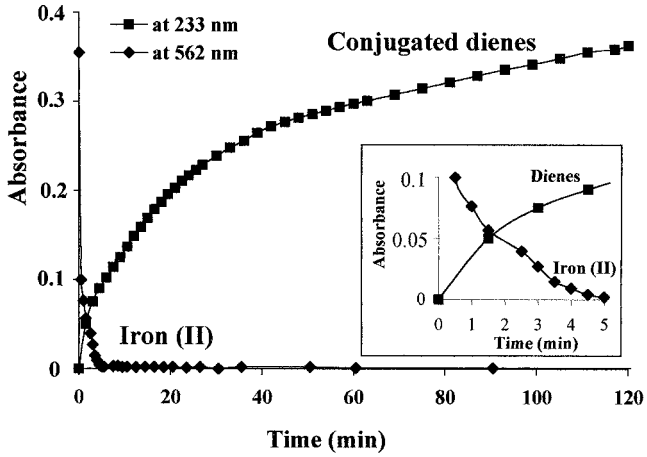

FIG. 5. Evolution of the Fe^{2+} species during the iron/ascorbate-induced oxidation of LA. Conditions: 170 μM LA, 17 μM $FeCl_2$, 0.7 μM AsA, pH = 6.4, 30°C. For abbreviations see Figure 1.

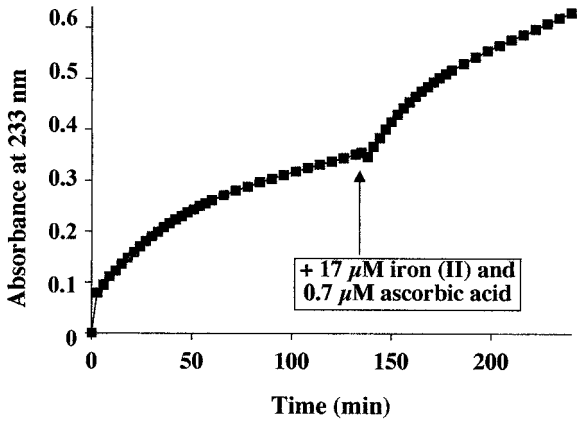

FIG. 6. Influence of an additional supply of iron/ascorbate inducer during the propagation phase of LA oxidation. Conditions: 170 μM LA, 17 μM $FeCl_2$, 0.7 μM AsA, pH = 6.4, 30°C. For abbreviations see Figure 1.

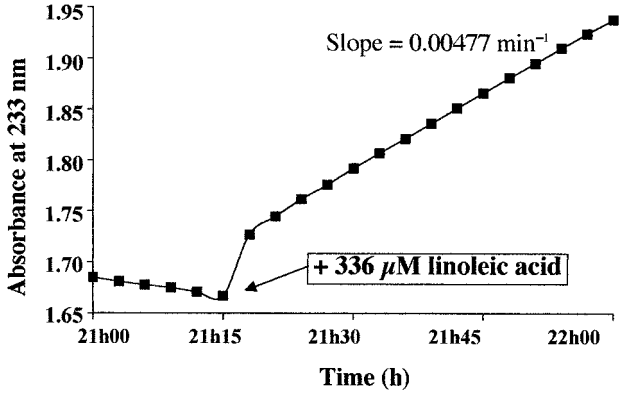

FIG. 8. Influence of an additional supply of emulsified substrate at the end of the LA peroxidation. Conditions: 170 μM LA, 17 μM $FeCl_2$, 0.7 μM AsA, pH = 6.4, 30°C. For abbreviations see Figure 1.

The decrease of the propagation phase occurred after 25 h, when the decomposition of the dienes exceeded their production, and all the LA was oxidized. A new addition of fresh emulsion in the cuvette restarted the linear propagation phase with a new supply of substrate (Fig. 8). No novel initiation phase was detectable in the absence of an initiator. As the added amount of LA was two times more than its initial concentration, the slope of the curve also doubled.

(iii) *Parameters of the kinetic phases.* As illustrated in Figure 4, the curve of the LA oxidation results from different steps. First, the induction of Fe^{2+} occurs during the first 5 min and leads to a moderate production of dienes (up to approximatively 0.1 AU). Initiation and propagation reactions run simultaneously at a decreasing speed, and finally, a steady-state of autocatalyzed propagation reactions set up and run for several hours. Two parameters were retained to quantify the antioxidant efficiency: the slope (*S*) of the linear part of the curve, representative of the steady-state propagation rate, and the absorbance (*A*) of this step, extrapolated at zero time. These two values were preferred over those for initial speed and amplitude of the crossing point between starting and secondary slopes because they were more discriminant for antioxidants (22) and presented the lowest standard deviations. The analysis of variance on 50 standard curves gave variations of 7% for *A* value and 6% for *S*.

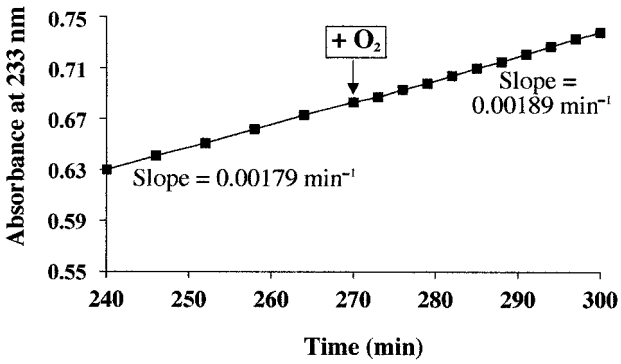

FIG. 7. Influence of an additional supply of oxygen during the propagation phase of LA oxidation. Conditions: 170 μM LA, 17 μM $FeCl_2$, 0.7 μM AsA, pH = 6.4, 30°C. For abbreviations see Figure 1.

This system is now well characterized, stable, and suitable to be used for measuring antioxidant activity. It could be expected that antioxidants would act at different levels of the reaction sequence by chelating iron, and thus preventing the initiation, or by stabilizing the peroxyl radicals preferentially. The partition of the antioxidants inside the different locations of the medium could also play a major role in their activity. Therefore, this test could provide precious information about their behavior. Nevertheless, we must keep in mind that this micellar model is very diluted, allowing all the reactants to have a high degree of mobility, which is not the case in most food emulsions, such as mayonnaises or sauces.

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