Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1035 (2004) 227–236
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Oxidative effects induced by dediazoniation of the *p*-hydroxybenzenediazonium ion in a neutral aqueous medium

B. Quintero*, M.I. Martínez Puentedura[1], M.T. Megías, M.C. Cabeza, M.P. Gutiérrez, P.J. Martínez de las Parras

Department of Physical Chemistry, Faculty of Pharmacy, University of Granada, Campus Universitario de Cartuja, 18071 Granada, Spain

Received 3 November 2003; received in revised form 13 February 2004; accepted 17 February 2004

Abstract

The toxicity of arenediazonium ions is believed to result from the appearance of very reactive compounds during the dediazoniation process. In the case of the *p*-hydroxybenzenediazonium ion (PDQ), radical species generated during dediazoniation could potentially initiate lipid peroxidation. The data obtained in spectrophotometric experiments suggest that an interaction between PDQ and linoleic acid (LA) gives rise to the characteristic absorption of oxidized products deriving from LA, both in the presence and absence of a mixed micellar medium containing the surfactant Tween 20 (Tw20). Spectroscopic evidence also clearly points to the interference of these processes in the dediazoniation of PDQ. Analysis by reverse-phase, high-pressure liquid chromatography (HPLC) confirms that the decomposition of PDQ in a mixed micellar medium induces the peroxidation of both LA and methyl linoleate (MEL), thus causing the appearance of peaks characteristic of dienic conjugated hydroperoxides. The same products are observed after interaction between LA and the water-soluble 2,2′-azobis (2-amidinopropane), a frequently used initiator of lipid peroxidation. The proportion of isomers produced during the peroxidation process agrees well with that reported for reactions mediated by free radicals. A further chromatographic analysis of the decomposition of PDQ in the presence of 2-methylcyclohexa-2,5-diene-1-carboxylic acid (CHD) shows that phenol and quinone are the main products of the reaction. These results are discussed on the understanding that aryl and peroxyl radicals abstract a hydrogen atom from CHD, in accordance with our general scheme for PDQ dediazoniation described in a previous publication.

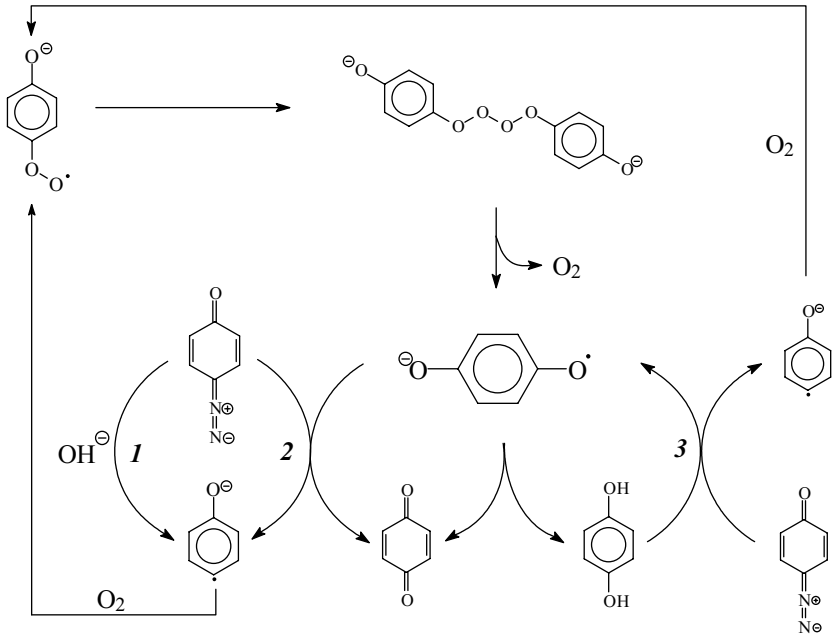
Keywords: Dediazoniation; Peroxidation; *p*-Hydroxybenzenediazonium ion; Linoleic acid; 2-Methylcyclohexa-2,5-diene-1-carboxylic acid; Aryl radical; Peroxyl radical

1. Introduction

Arenediazonium ions have mutagenic and carcinogenic capacity [1–10]. Nevertheless, the mechanisms involved in their genotoxicity are not fully understood. Thus in early studies it was suggested that the arenediazonium ions themselves exerted direct genotoxic activity [11,12]. Along similar lines indirect evidence has been found to suggest the formation of triazene adducts of adenine in DNA [13]. The formation of a triazene adduct has also been considered to be an intermediate step in the arylation of adenine residues in DNA [14]. The idea has also been put forward that the observed damage caused to DNA might be the result of the simultaneous action of arenediazonium ions and aryl radicals deriving from them [5]. A direct attack on DNA by aryl radicals would also explain the genotoxic activity attributed to arenediazonium ions in the light of the easy in vitro arylation caused by aryl radicals to both natural and synthetic nucleobases, nucleosides and polynucleotides [15] and their ability to produce unspecific DNA strand breakage [16–19]. It must also be born in mind that the presence in a biological medium of a number of reductants will increase the chance of aryl radical formation. Apart from this, other pathways have been suggested, such as the capacity of carbon-centered

* Corresponding author: Tel.: +34-958-249071; fax: +34-958-244090.
E-mail addresses: bqosso@ugr.es (B. Quintero), martinez@ugr.es (M.I. Martínez Puentedura).
[1] Co-corresponding author. Tel.: +34-958-243824; fax: +34-958-244090.

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.02.046



Scheme 1.

radicals deriving from the *p*-methylbenzenediazonium ion to activate the AP-1 system and thus stimulate the phosphorylation of ERK1, ERK2 and p38 proteins in a similar way to that observed for superoxide anion and hydroxyl radicals [20]. Whatever the final answer, it seems fairly evident that the toxicity of arenediazonium ions derives from the appearance during the dediazoniation processes of very reactive compounds, the aryl radical and aryl cation, which are the intermediates in the homolytic or heterolytic dediazoniation mechanisms, respectively [21]. It has been suggested by several authors [22] that both mechanisms may operate simultaneous and competitively, depending upon the experimental conditions under which dediazoniation occurs [23].

p-Hydroxybenzenediazonium (PDQ) is an arenediazonium ion present in the inedible mushroom *Agaricus xanthodermus* [24]. It cleaves DNA [17], is responsible for the formation of the C8-guanine adduct [25] and causes tumors when repeatedly administered as a sulphate salt by subcutaneous injection [26].

In a previous publication we reported on the dediazoniation of PDQ in a neutral aqueous medium [27]. We proposed that in darkness and in the absence of reductants dediazoniation occurs via a homolytic mechanism (see Scheme 1 further) started by the hydroxide anion (pathway 1 in Scheme 1) but accelerated by other reductants (pathways 2 and 3 in Scheme 1), namely hydroquinone and the semiquinone radical, which formed during the initial reaction.

In Scheme 1 peroxyl radicals are taken to be formed during PDQ dediazoniation. The commonly accepted mechanism for the initiation of lipid peroxidation involves a rapid reaction between molecular oxygen and carbon-centered radicals and thus the resulting peroxyl radical (ROO) is usually considered to be the initiating species responsible for abstracting hydrogen atoms from substrates susceptible to peroxidation. The process of lipid peroxidation, though still not completely understood, is associated with a number of pathological states, including diabetes, atherosclerosis, macular degeneration, ischemia-reperfusion lesions, cancer, and several neurological disorders [28–33]. This paper deals with possible oxidative effects induced by the appearance of radicals during the dediazoniation of PDQ in a neutral aqueous medium (Scheme 1).

2. Experimental

2.1. Materials

p-Hydroxybenzenediazonium tetrafluorborate (PDQ) was obtained according to the procedure described elsewhere [34] and stored at −18 °C in darkness. Linoleic acid [*cis*,*cis*-9,12-octadecadienoic acid (LA)], methyl linoleate (MEL), surfactant [polyoxyethylensorbitan sodium monolaurate (Tw20)], water-soluble azo initiator ABAP, 2,2′-azobis (2-amidinopropane) hydrochloride, (97% pure) and 2-methyl-2,5-cyclohexadiene-1-carboxylic acid (CHD) came from Sigma (Madrid, Spain). LA and MEL were stored at −4 °C under argon. Redistilled phenol (Ph) (99% pure) and 1,4 benzoquinone (Q) were from Aldrich (Madrid, Spain) and hydroquinone (HQ) from Panreac (Madrid, Spain). All reagents were used as received without further purification. Phosphate buffers of ionic strengths 0.1 and 0.05 M (pH 7.2) were used after filtering through Chelex 100 resin (50–100 dry mesh, sodium salt) from Aldrich (Madrid, Spain). Glacial acetic acid came from Panreac (Madrid, Spain), and acetonitrile and methanol (both high-pressure

liquid chromatography (HPLC) gradient grade) from Merck (Madrid, Spain) were used for chromatographic analyses. Doubly distilled water from a Millipore system was always used.

2.2. Instrumental

Spectrophotometric measurements were made in a Cintra 10 Spectrophotometer (GBC Scientific Equipment, Hucoa Erlöss, Madrid, Spain) equipped with a Frigomix external thermostatic water bath (B. Braun-Biotech S.A., Barcelona, Spain). HPLC was done in a Merck-Hitachi Liquid Chromatograph (Merck KgaA, Merck Farma y Química S.A.,VWR International S.L., Barcelona, Spain) equipped with a LaChrom L-7100 pump, an L-4500 diode array detector and a D-7000 interface. All computations were performed using the Merck D-7000 HSM data system. Aqueous phases were filtered through a Millipore system with 0.45 μm pore size. A Kromasil C18 analytical column (Technokroma, Spain), 250 mm × 4.6 mm, with 5 μm particle size, and a LiChrospher C18 100 column guard (Merck KgaA, Merck Farma y Química, S.A.VWR International S.L. Barcelona, Spain).

pH measurements were made in a calibrated Crison GLP 22 pH/potentiometer (Crison Instruments S.A., Barcelona, Spain). Calibrations were made with Crison buffer references (pH 4 and 7).

2.3. Methods

In experiments involving the use of Tw20, the surfactant concentrations were always above the critical micellar concentration, 0.006 g/100 ml, in water, at 20 °C [35] to ensure the formation of mixed micelles.

All samples containing PDQ were handled in darkness to avoid photodediazoniation [27]. Spectrophometric and chromatographic measurements involving PDQ were routinely carried out with aliquots of solutions kept at 37 °C in darkness.

2.3.1. Preparation of samples for spectrophotometric analysis of LA decomposition in an aqueous buffered medium

Spectrophotometric experiments made in the absence of mixed micelles consisted of mechanically stirring the appropriate quantities of LA (0.475 ml, 0.212 M) for 10 s in up to 50 ml Chelex-filtered phosphate buffer (0.1 M, pH 7.2) at 37 °C. These solutions were used to follow the decomposition of LA. Phosphate buffer was used as a blank.

2.3.2. Preparation of samples for spectrophotometric analysis of the influence of PDQ on LA decomposition in an aqueous buffered medium

The influence of PDQ was studied by preparing an LA solution in phosphate buffer as described above. Simultaneously a solution of PDQ (100 ml, 2.5×10^{-5} M) was prepared in the same buffer and divided into two aliquots (50 ml), into one of which the LA solution (0.475 ml, 0.212 M) was added up to the required concentration. The other aliquot received an equal volume of buffer to be used as a blank.

2.3.3. Preparation of samples for spectrophotometric analysis of the influence of PDQ on LA and MEL decomposition in an aqueous buffered medium in the presence of the surfactant Tw20

For experiments with mixed micelles a solution of the surfactant was prepared by stirring 0.008–0.020 g of Tw20 into 100 ml of a solution of PDQ (2.5×10^{-5} M) in phosphate buffer (0.1 M, pH 7.2) at 37 °C. From this solution equal volumes (50 ml) were separated and appropriate quantities of LA (0.475 ml, 0.21 M) or MEL (0.400 ml, 0.19 M) added to one of the aliquots. The other received the same volume of buffer. Appropriate volumes of a recently prepared PDQ solution were then added to both reaction mixtures. The solution containing only Tw20 + PDQ was used as a blank.

2.3.4. Preparation of samples for spectrophotometric analysis of LA and MEL decomposition in an aqueous buffered medium with the surfactant Tw20

A solution of Tw20 was prepared (0.008–0.020 g/100 ml phosphate buffer) and 50 ml aliquots were taken to be mixed with the appropriate quantities of either LA (0.475 ml, 0.21 M) or MEL (0.800 ml, 0.19 M). A blank aliquot received the same volume of buffer.

2.3.5. Preparation of samples for chromatographic analysis

A Tw20 solution (960–970 mg) was prepared in phosphate buffer (0.05 M, pH 7.2) and the required volume of a newly prepared solution of PDQ or ABAP was added. A suitable quantity of LA (28 μl) was then added to the reaction mixture. The samples were kept in darkness at 37 °C and shaken slowly for up to 72 h, during which time they were analysed by chromatography. A solution of CHD in phosphate buffer (0.05 M, pH 7.2) was used when required.

Prior to use, all solvents were degassed ultrasonically to exclude as much dissolved oxygen as possible. The headspaces of all flasks were purged with nitrogen to exclude oxygen. The injection volume was 20 μl. The mobile phases used were acetonitrile:water (30:70) and (70:30) with 1 ml min^{-1} isocratic flow in both cases. The spectral range of the analysis was 220–500 nm and the wavelength was set at 233 nm.

3. Results and discussion

3.1. Spectrophotometric analysis

The absorption spectra registered with aliquots of buffered solutions of PDQ (2.5×10^{-5} M in phosphate buffer 0.1 M, pH = 7.2) in the presence of LA 2.0×10^{-3} M, kept in darkness at 37 °C, are set out in Fig. 1. For every measurement

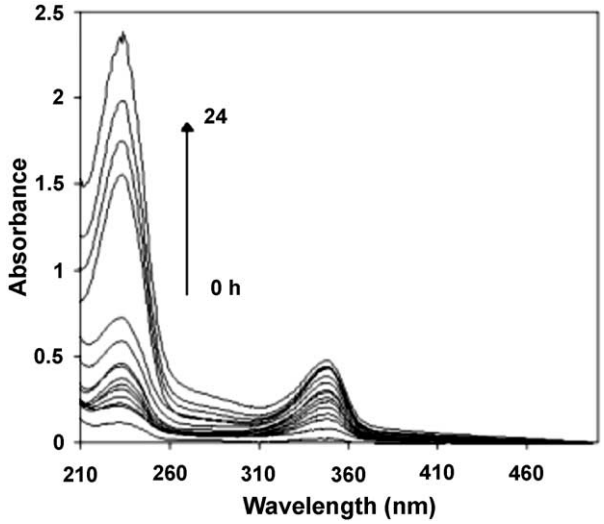


Fig. 1. Absorption spectra registered with aliquots of a buffered solution (phosphate buffer 0.1 M, pH 7.2) of PDQ (2.5×10^{-5} M) with LA (2.0×10^{-3} M), kept in darkness at 37 °C. Aliquots of a PDQ solution without LA were used as blanks in each measurement.

an aliquot of the PDQ solution without LA was used as a blank. It can be seen that absorbance ca. 233 and 350 nm increased significantly with time. The observed variation ca. 233 nm is much more pronounced than that obtained with a buffered solution of LA 2.0×10^{-3} M, as can be seen from the data plotted in Fig. 2. It is clear that LA is oxidised more quickly during PDQ dediazoniation, thus producing more absorption ca. 233 nm than during the normal process of auto-oxidation.

Likewise, the significant increase in absorbance measured ca. 350 nm (Fig. 1) is explicable bearing in mind that

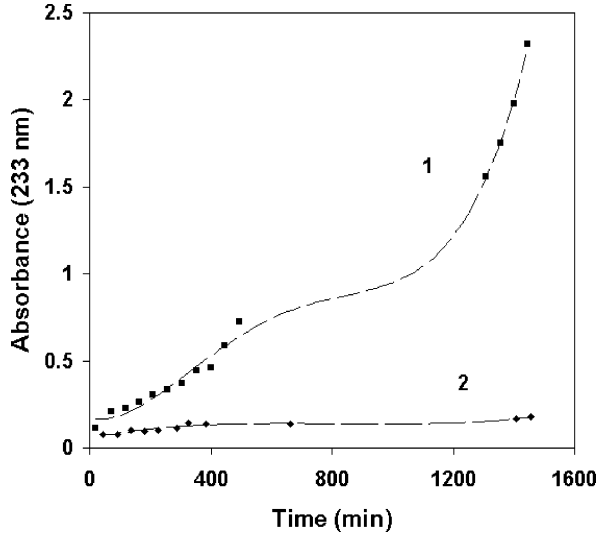


Fig. 2. Plots of the absorbance values measured at 233 nm vs. time. Spectroscopic data obtained with aliquots of buffered solutions (phosphate buffer 0.1 M, pH 7.2) of LA (2.2×10^{-3} M) kept in darkness at 37 °C (1) with PDQ (2.5×10^{-5} M); (2) without PDQ. Aliquots of the PDQ solution without LA were used as blanks for each measurement in (1) and the buffer was used as a blank in (2).

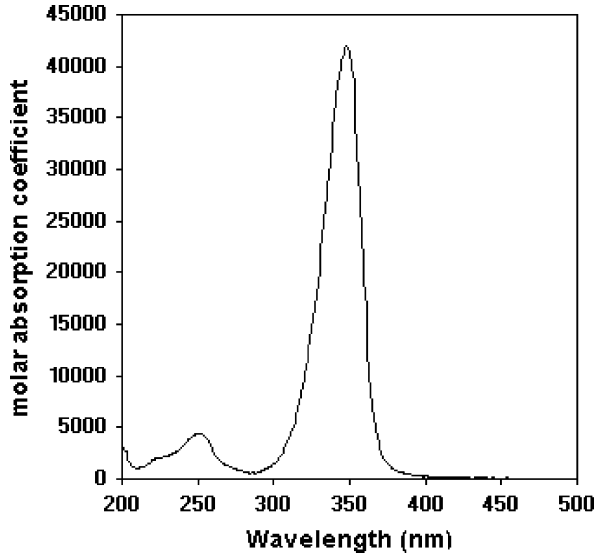


Fig. 3. Change in the molar-absorption coeffiecient with the wavelength for solutions of PDQ in phosphate buffer pH 7.2.

the absorption spectrum of PDQ in a phosphate-buffered aqueous medium (Fig. 3) presents a band with its maximum at 350 nm (ε: 41990 l mol^{-1} cm^{-1}) [34] and, as mentioned in Section 2.3.2., the spectra were recorded using the PDQ solution without LA as a blank. Therefore the observed increase in absorption ca. 350 nm is a consequence of a decrease in the dediazoniation rate of PDQ in the presence of LA, which concentrates the PDQ in the sample compared to the blank. We checked this by comparing the difference in absorbance at 350 nm between a sample containing LA and PDQ and one containing only PDQ. In both cases the blank was an aliquot of the PDQ solution. As expected the absorbance measurements made using both cuvettes with the same PDQ solution always gave absorbance readings close to zero whilst the absorbance measured using the sample containing PDQ and LA once more increased with time (Fig. 4). An increase in the absorption at 233 nm, the region where hydroperoxides absorb, might also be expected simultaneously with the changes in absorption observed at 350 nm, as a consequence of the decrease in the dediazoniation rate of PDQ. Nevertheless, no interference from PDQ should be found since the PDQ molar-absorption coefficient at 233 nm is about 2.3×10^{3} l mol^{-1} cm^{-1} (Fig. 3) whilst the hydroperoxides present a molar-absorption coefficient of about 2.5×10^{5} l mol^{-1} cm^{-1} [36]. Thus, taking the maximum value of absorbance observed at 350 nm (approximately 0.45, as shown in Fig. 1), it is possible to calculate the difference in PDQ concentration between the sample and the blank, which turns out to be 1.1×10^{-5} M and would thus give an absorbance value of 0.023 at 233 nm, which is very small compared to the observed value of absorbance of >2 (Fig. 1).

The spectrophotometric data strongly suggest that LA interferes with PDQ dediazoniation by diverting radicals derived from PDQ to LA peroxidation in such a way that

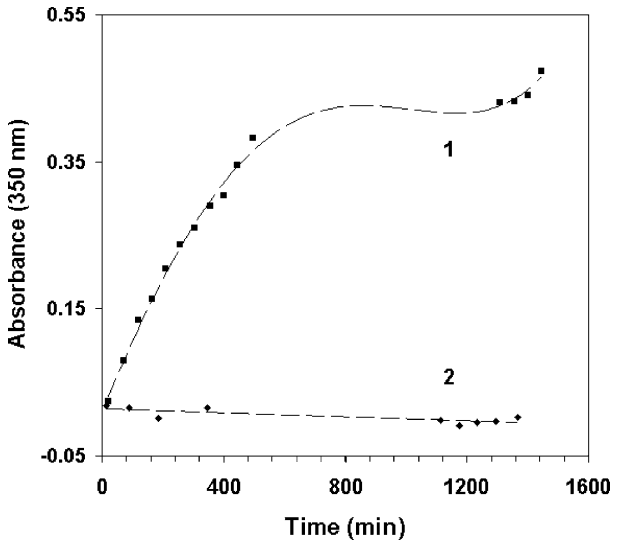


Fig. 4. Plots of the absorbance values measured at 350 nm vs. time. Spectroscopic data obtained with aliquots of buffered solutions (phosphate buffer 0.1 M, pH 7.2) of PDQ (2.5×10^{-5} M) kept in darkness at 37 °C (1) with LA (2.2×10^{-3} M); (2) without LA. In both cases aliquots of PDQ solutions without LA were used as blanks.

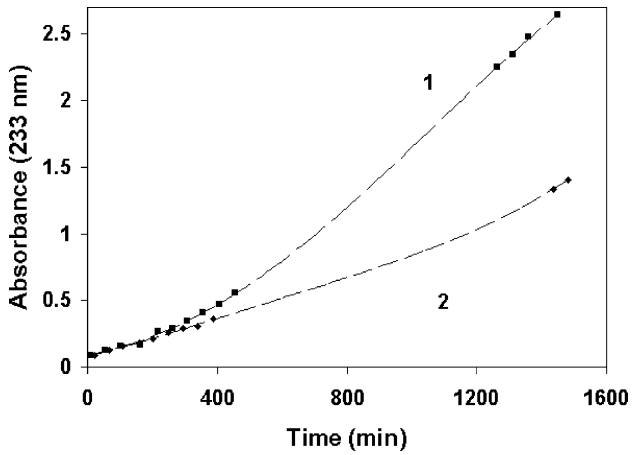


Fig. 5. Plots of the absorbance values measured at 233 nm vs. time. Spectroscopic data obtained with aliquots of LA (2.2×10^{-5} M) buffered solutions (phosphate buffer 0.1 M, pH 7.2) plus Tw20 (1.4×10^{-4} M), kept in darkness at 37 °C (1) with PDQ (2.5×10^{-5} M) and (2) without PDQ. In the first case aliquots of (PDQ + Tw20) solution without LA were used as blanks. In the second case the buffered solution of Tw20 was used as a blank.

they are prevented from forming active reductants such as semiquinone, which increase the PDQ dediazoniation rate, as depicted in Scheme 1.

A complementary spectrophotometric analysis of the interaction between PDQ and LA was also carried out in a mixed micellar medium made with the non-ionic surfactant Tw20, since this system is used in the subsequent chromatographic analysis. At the concentrations used in these experiments some small absorption caused by Tw20 was observed in the range of 233–236 nm (<0.01), which did not affect the spectrophotometric analyses because the blank employed in the measurements was a buffered solution containing Tw20 at the same concentration as that in the samples. It was also observed that 1.5×10^{-4} M Tw20 increased the auto-oxidation rates of LA (1.8×10^{-3} M), which in principle might be put down to hydroperoxide contaminants of Tw20 [37–39]. A quantitative analysis of hydroperoxides by reaction with KI [40] confirmed the presence of such compounds within a concentration range of 11–23 μmol/g Tw20.

Although the surfactant induces an increase in LA and MEL peroxidation, supported by evidence of low oxidative stability for both lipid substrates in Tw20 emulsion [41], the changes in absorbance at 233 nm registered in the same system but in the presence of PDQ were much more marked, especially after 24 h. Fig. 5 shows the variation in absorbance at 233 nm recorded with a buffered PDQ solution 2.5×10^{-5} M (phosphate buffer 0.1 M, pH 7.2) in the presence of Tw20 (1.4×10^{-4} M) and LA (2.0×10^{-3} M) compared to an identical solution without PDQ.

Similar results to those depicted in Fig. 5 were found when MEL 1.5×10^{-3} M was used as a peroxidation substrate (data not shown) but in this case auto-oxidation was practically negligible after 10 h and only rose to an absorbance value of 0.1 after 24 h. In the presence of PDQ, as with LA, the increase in absorbance was more noticeable, rising to 0.4 after 24 h.

Taking the spectrophotometric results as a whole, they agree in that the absorption spectra recorded with LA and MEL in the presence of PDQ register an increase in absorbance at 233 nm, which is characteristic of the appearance of peroxidation products, probably hydroperoxides, deriving from LA and MEL. The method used for this analysis also allowed us to establish that the interaction of PDQ with peroxidable substrates is concomitant with a decrease in the PDQ dediazoniation rate.

3.2. Chromatographic analysis

The spectroscopic monitoring of LA and MEL peroxidation processes has well-known limitations [42] since other compounds produced during peroxidation apart from hydroperoxides may absorb in this UV region, for which reason other methods of simultaneous analysis at different wavelengths have been proposed [43]. This prompted us to confirm the results obtained in our spectrophotometric analyses and so we made a direct chromatographic study of the decomposition of PDQ in a mixed micellar medium made with the non-ionic surfactant Tw20. Mixed micelles have been used in the past with satisfactory results in the separation of hydroperoxides derived from LA by micellar electrokinetic chromatography [44,45]. HPLC analysis indicates that PDQ (8.34×10^{-5} M) produces an increase in the peroxidation of LA (0.015 M) in a buffered medium (phosphate buffer 0.05 M, pH 7.2) plus Tw20 (0.13 M), as can be inferred from the appearance of four chromatographic signals with retention times of between 14 and 18 min (Fig. 6). The

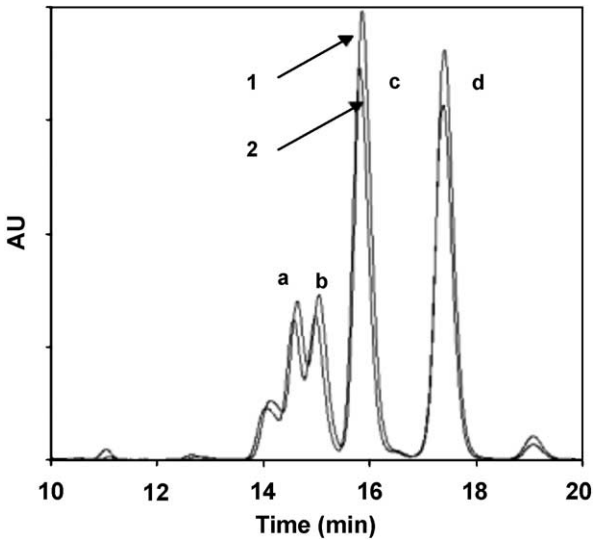


Fig. 6. Chromatograms registered with LA (0.015 M) buffered solutions (phosphate buffer 0.05 M, pH 7.2) plus Tw20 (0.13 M) with (1) PDQ (8.34×10^{-5} M) and (2) ABAP (4.26×10^{-3} M). Samples kept for 20 h in darkness at 37 °C in a thermostatically regulated bath with slow agitation. Wavelength: 233 nm.

signals agree excellently with those produced by the thermolabile azocompound ABAP (4.26×10^{-3} M), a frequently used initiator of lipid peroxidation [46].

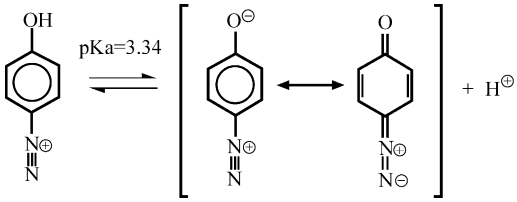
A similar four-signal response has been attributed to dienic conjugated hydroperoxides by other authors [36,37,47–51] and thus we made no further attempts at characterization. These chromatographic signals are the result of a hydrogen atom being abstracted from LA and the subsequent reaction of the LA radical with molecular oxygen to form a first group consisting of an unsolved couple of peaks (Fig. 6, peaks a and b), corresponding to isomers 9 and 13-*cis*-*trans* (*c*,*t*), dienic hydroperoxides characterised by an absorption maximum located at 235 nm, and a second group (Fig. 6, peaks c and d) formed by two well-separated peaks, corresponding to isomers 9 and 13-*trans*-*trans* (*t*,*t*), the absorption spectra of which display a maximum at 232 nm [51,52]. We calculated the values for the quotient of the areas corresponding to the chromatographic signals of isomers, *c*,*t*/*t*,*t*, from the chromatograms registered at different times for a sample containing LA, Tw20 and PDQ, kept in darkness at 37 °C, which were 0.15, 0.29 and 0.52 respectively. Although the surfactant can affect the stability of the hydroperoxides [53] and the proportion of isomers may undergo variations for various reasons, such as lipid temperature, concentration or isomerization reactions [54], the experimental values are close to the range of 0.18–0.24 considered to be characteristic of processes mediated by radicals and clearly different from that which might be expected of a peroxidation process involving an oxygen singlet [48].

In summary, the appearance in our chromatographic analysis of characteristic signals corresponding to dienic conjugated hydroperoxides indicates that LA peroxidation takes place in the presence of PDQ. Such products were also observed when peroxidation was induced by the thermolabile azocompound ABAP. Furthermore, the proportion of isomers generated is in accordance with a process mediated by radical species.

Additional experiments using MEL as a substrate gave similar results to those already described for LA.

Nevertheless, the peroxidation of LA and MEL is a chain reaction. Thus any possible interaction with the aryl and peroxyl radicals derived from the dediazoniation of PDQ is limited to the initial hydrogen-abstraction step, and the subsequent formation of hydroperoxides can occur together with (i) the formation of hydroperoxide as a result of LA and MEL auto-oxidation; (ii) the reduction of the hydroperoxydes, which would contribute to the propagation of the peroxidative effects; (iii) other reactions that provoke the transformation of hydroperoxides. As a consequence, the number of products resulting from the peroxidation of LA and MEL makes it difficult to distinguish which radical deriving from PDQ triggers the peroxidation process. To find out more about the oxidation induced by PDQ we made complementary experiments using CHD as a substrate, which also has one allylic position but permits a simpler mechanistic approach. This compound has given excellent results in chromatographic studies of photochemical processes affecting drugs with benzophenone-like chromophores [55]. A chromatographic analysis of the interaction between PDQ and CHD was made after changing the experimental conditions used in the study of LA and MEL peroxidation. Preliminary experiments consisted of choosing suitable mobile phase and reagent concentrations. Both reagents (PDQ and CHD) are ionic compounds which present an electrical charge under the experimental conditions used for our analysis. In fact CHD is present at pH 7 as a carboxylate anion because its pK_a value is 4.00 [56]. Likewise PDQ has a pK_a value of 3.33, as calculated from spectrophotometric measurements at 348 nm with the PDQ solutions in the pH range 1.03–11.20 (Fig. 7) according to the Henderson–Hasselbach equation.

According to the pK_a value for PDQ, the species present in solution at pH 7 is the deprotonated molecule, which can be formulated as a hybrid containing at least two canonical forms with a major contribution of the quinoid form [27].



Thus under the chromatographic conditions used, both compounds should elute with the solvent front together with all the other electrolytes. To satisfy our interest in finding out the number and distribution of the reaction product, whilst

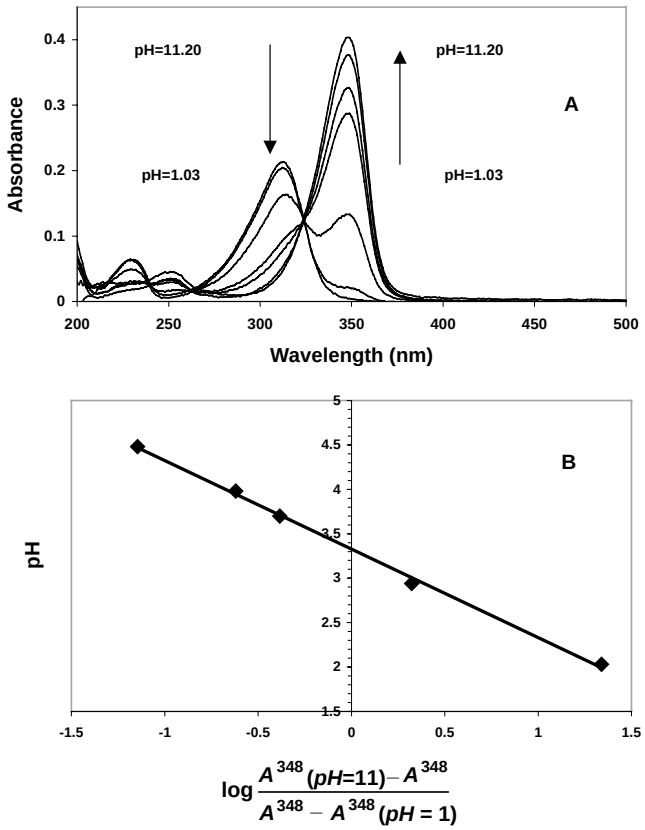


Fig. 7. (A) Absorption spectra registered with PDQ solutions (1.0×10^{-5} M) in a aqueous medium at different pH values (a) 1.03; (b) 2.03; (c) 2.94; (d) 3.70; (e) 3.98; (f) 4.48; (g) 11.20. (B) Plot of the pH values vs. the values of the logarithm of absorbances according to the Henderson–Hasselbach equation. A^{348}(pH = 11) is the absorbance measured at 348 nm with the PDQ solution at pH 11.20 and A^{348}(pH = 1) corresponds to the absorbance measured at 345 nm with the PDQ solution at pH 1.03. *A* represents the absorbance measured at an intermediate pH.

bearing in mind that some difficulties might be found in the quantification of CHD and PDQ, we made several different chromatograms with PDQ concentrations ranging from 7.3×10^{-5} M to 3.7×10^{-3} M and CHD concentrations from 7.5×10^{-4} M to 3.7×10^{-2} M. Too high a concentration of PDQ unfailingly produced saturation and widening of the chromatographic signal because of its high molar-absorption coefficient. Moreover, although a high PDQ concentration leads to more rapid decomposition, it also increases the incidence of side reactions. We finally chose concentrations of 4.9×10^{-4} M for PDQ and 4.0×10^{-3} M for CHD. When using acetonitrile:water as the mobile phase, the separation of some polar by-products could not be achieved due to the proximity of the reagents' chromatographic signals as well as the strong absorption of PDQ. Nevertheless, this mobile phase should allow a good separation of the somewhat more apolar main reaction products, i.e. hydroquinone (HQ), 1,4-benzoquinone (Q) and phenol (Ph). Experiments made with the reagents and these three main products allowed us to verify that their retention times when using acetonitrile:water as the mobile phase appear in the following

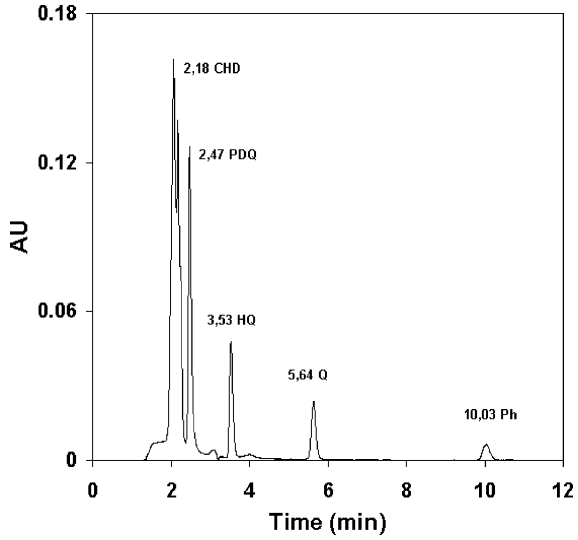


Fig. 8. Chromatogram registered with buffered solutions (phosphate buffer 0.05 M, pH 7.2) of PDQ (4.9×10^{-4} M) and CHD (4.0×10^{-3} M) plus Ph (1.0×10^{-3} M) and a solution of HQ partially oxidized to Q. Wavelength: 233 nm.

order: CHD < PDQ < HQ < Q < Ph. Fig. 8 shows the chromatogram obtained with these compounds using acetonitrile:water (30:70), where it can be seen that a satisfactory separation of the reaction products was achieved. Under experimental conditions similar to ours Q has been reported by other authors to elute before Ph [57–59]. In principle this phenomenon might be considered surprising because, although phenol ($pK_a = 9.98$) is practically non-dissociated at pH 7, its dipole moment is not equal to zero, as expected for Q. Nevertheless, the experimentally observed elution order could be determined not just by polarity but also by concomitant processes, such as adsorption for example, which would facilitate the retention of phenol.

Buffered solutions (phosphate buffer 0.05 M, pH 7.2) of PDQ (4.9×10^{-4} M) and CHD (4.0×10^{-3} M) kept in darkness at 37 °C were used for the analysis of the interaction of PDQ with CHD. Separate samples of PDQ and CHD at the same concentrations were also kept under identical experimental conditions. The CHD solution proved to be very stable during the time involved in our experiments. The data for the areas of the peaks (Table 1) indicate that the substrate remained practically untouched after 24 h. PDQ, on the other hand, clearly decomposed, apparently via a first-order reaction with $k_{obs} = 3.5 \times 10^{-3}\,min^{-1}$, calculated taking the area values (Table 1), in close accordance with $k_{obs} = 3.3 \times 10^{-3}\,min^{-1}$, obtained from spectroscopic measurements (data not shown), leading to the appearance of HQ and Q as described elsewhere [27]. In Table 1 it can be seen that HQ and Q appeared almost simultaneously. More HQ was generated than Q since the chromatographic signal corresponding to HQ is greater both in height and area than that for Q, even taking into account that the molar-absorption coefficient of HQ at 236 nm is approximately 10% of that displayed by Q. It is also important to indicate that in these

Table 1
Values of the areas of the peaks corresponding to the chromatographic signals obtained in the analysis at different times

	Time (min)							
	0	70	150	218	315	528	1420	
(A) CHD solution (4.0×10^{-3} M)								
Area CHD	581160	568681	642902	544503	601124	14577	739936	
Time (min)	Area*			Time (min)	Area**			
	PDQ	HQ	Q		PDQ	HQ	Q	Ph
(B) PDQ solution (4.9×10^{-4} M)								
0	751396	39552	25117	0	743180	0	2850	0
80	586667	74521	97845	80	751216	0	8840	0
150	492471	54645	81900	145	827228	0	20260	0
224	362937	122753	139307	225	839844	0	50350	6740
320	240110	129321	103801	410	836499	0	59855	11570
520	129989	162750	148337	615	731204	5605	61110	25770
1410	0	66555	99610	1500	32290	22447	4357	43740

Values of the same parameter measured for the dediazoniation products in the absence (*) and presence (**) of CHD (4.0×10^{-3} M). Wavelength: 233 nm.

chromatograms the signal corresponding to phenol could not be detected even when the detection capacity of the chromatograph was raised to its maximum (rank of absorbance units from 0 to 0.005) or the wavelength set at the maximum for phenol absorption (271 nm).

The chromatograms corresponding to mixtures of PDQ and CHD show the disappearance of the signal corresponding to PDQ followed by the appearance of Q, HQ and Ph. It can be seen in Table 1 that on this occasion HQ did not appear simultaneously with Q, although the height of the peak for Q is similar to that obtained in the experiment made with PDQ alone. Since it is possible to detect HQ, the process of interaction with CHD under our experimental conditions did not completely interrupt the pathway of PDQ dediazoniation via semiquinone (Scheme 1). Furthermore, it is important to note that the heterolytic formation of HQ can be ruled out because the appearance of its chromatographic signal was delayed until approximately 7 h.

To illustrate the experimental behaviour observed for the decomposition of PDQ the plots of the areas of the peaks measured at 233 nm during its decomposition both in the presence and absence of CHD are set out in Fig. 9, where it can clearly be seen that the presence of CHD delays the process.

As a whole, the results obtained in these experiments go to support the conclusions reached in the previous spectrophotometric and chromatographic studies using LA and MEL as peroxidation substrates. As far as the results obtained with CHD are concerned, it is very possible that the radicals formed during the dediazoniation of PDQ abstracted an allylic hydrogen from CHD, thus leading to the formation of phenol if the radical deriving from PDQ is the aryl radical, or phenylhydroperoxide if it is the peroxyl radical which abstracts the hydrogen atom (Scheme 2, A). An additional reaction (Scheme 2, B) explains how the

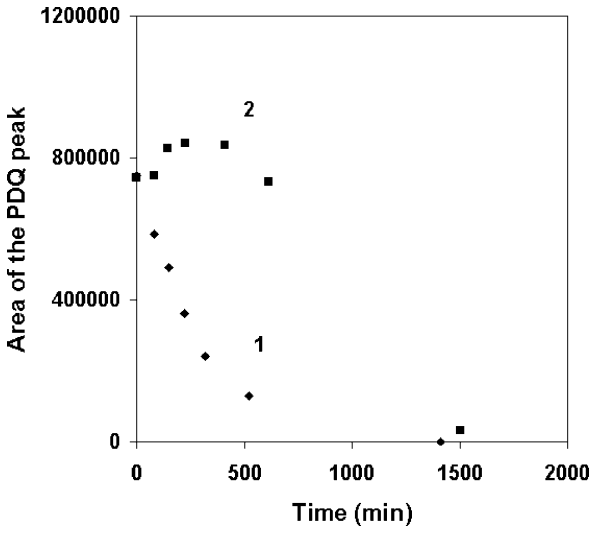
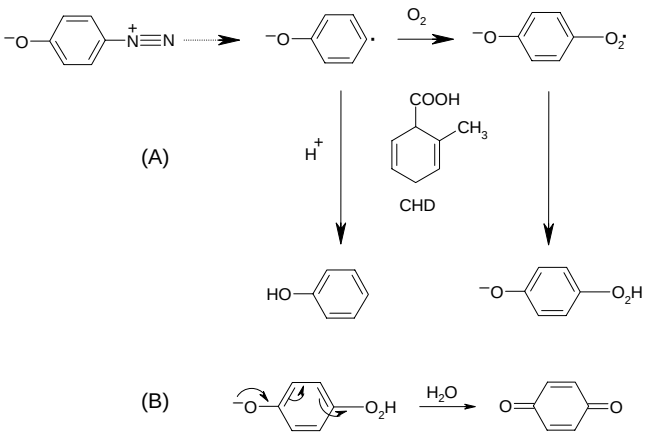


Fig. 9. Plots of the areas of the peaks corresponding to the chromatographic signal of PDQ measured at 233 nm at different times during the decomposition of PDQ (4.9×10^{-4} M) kept in darkness at 37 °C without (1) and with (2) CHD (4.0×10^{-3} M).



Scheme 2.

decomposition of phenylhydroperoxide would take place to form quinone.

The appearance of phenol can be put down to competition between CHD and molecular oxygen to react with the aryl radical. Since the concentrations of CHD and O_2 are similar ($1.2–1.3 \times 10^{-3}$ M for molecular oxygen at 760 Torr of O_2 and 25 °C [60]) and the reaction of the aryl radical with oxygen has a second-order rate constant of around $2.0 \times 10^9\ M^{-1}\ s^{-1}$ [61], the delay in the appearance of the chromatographic signal for phenol (Table 1) suggests that the reaction of the aryl radical with CHD is only viable when the oxygen concentration diminishes.

It is noteworthy that later chromatographic results can be put down to a reaction between the peroxyl radical and CHD (Scheme 2). It is well-known that peroxyl radicals play an important role in chemistry and biology [62]. They are involved in many radical chain reactions but their decomposition to form alkoxyl radicals [63–66] often renders them difficult to detect directly. The data presented in this paper indicate that the peroxidation of LA and MEL can be put down to a radical attack deriving from the dediazoniation of PDQ but they do not distinguish between effects caused by aryl radicals and those of peroxyl radicals. Nevertheless, the results of our simple chromatographic analysis using CHD as an oxidizable substrate allow us to make the reasonable assumption that peroxyl radicals abstract a hydrogen atom from CHD to form quinone and this may be taken as indirect evidence of the involvement of this radical in PDQ dediazoniation, as proposed in Scheme 1.

Acknowledgements

This work was supported by the Andalucían autonomous government and the University of Granada. M.T.M. thanks the University of Granada for a grant. The authors thank their colleague Dr. J. Trout for revising the English text.

References

[1] B.N. Ames, R. Magaw, L.S. Gold, Science 236 (1987) 271.
[2] K. Kikugawa, T. Kato, Y. Takeda, Mutat. Res. 172 (1987) 35.
[3] K. Kikugawa, T. Kato, Food Chem. Toxicol. 26 (1988) 209.
[4] H. Ohshima, C. Furihata, T. Matsushima, H. Bartsch, Food Chem. Toxicol. 27 (1989) 193.
[5] T. Lawson, P.M. Gannett, W.M. Yau, N.S. Dalal, B. Toth, J. Agric. Food Chem. 43 (1995) 2627.
[6] M. Stiborova, H. Hansikova, H.H. Schmeiser, E. Frei, Gen. Physiol. Biophys. 16 (1997) 285.
[7] B. Toth, P. Gannett, Mycopathologia 124 (1993) 73.
[8] B. Toth, K. Patil, J. Erickson, P. Gannett, In Vivo 12 (1998) 379.
[9] B. Toth, K. Patil, J. Erickson, P. Gannett, In Vivo 13 (1999) 125.
[10] B. Toth, In Vivo 14 (2000) 299.
[11] A. Chin, M.H. Hung, L.M. Stock, J. Org. Chem. 46 (1981) 2203.
[12] M.H. Hung, L.M. Stock, J. Org. Chem. 47 (1982) 448.
[13] S.R. Koepke, M.B. Kroeger-Koepke, C.J. Michejda, Chem. Res. Toxicol. 3 (1990) 17.
[14] P.M. Gannett, J.H. Powell, R. Rao, X. Shi, T. Lawson, C. Kolar, B. Toth, Chem. Res. Toxicol. 12 (1999) 297.
[15] J.P. Berh, J. Chem. Soc., Chem. Commun. (1989) 101.
[16] J. Griffiths, J.A. Murphy, J. Chem. Soc., Chem. Commun. (1992) 24.
[17] T. Kato, K. Kojima, K. Hiramoto, K. Kikugawa, Mutat. Res. 268 (1992) 105.
[18] C. Hazlewood, M.J. Davies, B.C. Gilbert, J.E. Packer, J. Chem. Soc., Perkin Trans. 2 (1995) 2167.
[19] K. Hiramoto, M. Kaku, T. Kato, K. Kikugawa, Chem. Biol. Interact. 94 (1995) 21.
[20] P.M. Gannett, J. Ye, M. Ding, J. Powell, Y. Zhang, E. Darian, J. Daft, Chem. Res. Toxicol. 10 (2000) 1020.
[21] H. Zollinger, Diazo Chemistry: Aromatic and Heteroaromatic Compounds, vol. I, Wiley, New York, 1994.
[22] C. Bravo Diaz, E. González Romero, Curr. Topics Colloid Interface Sci. 4 (2001) 58, and references cited therein.
[23] B. Quintero, M.C. Cabeza, M.I. Martínez Puentedura, P. Gutiérrez, P.J. Martínez de las Parras, J. Llopis, A. Zarzuelo, Ars Pharm. 44 (2003) 239.
[24] K. Dornberger, W. Ihn, W. Schade, D. Tresselt, A. Zureck, I. Radics, Tetrahedron Lett. 27 (1986) 559.
[25] K. Kikugawa, T. Kato, K. Kojima, Mutat. Res. 268 (1992) 65.
[26] B. Toth, K. Patil, J. Taylor, C. Stessman, P. Gannett, In Vivo 3 (1989) 301.
[27] B. Quintero, J.J. Morales, M. Quirós, M.C. Cabeza, M.I. Martínez-Puentedura, Free Radic. Biol. Med. 29 (2000) 464.
[28] P.A. Southorn, G. Powis, Mayo Clin. Proc. 6 (1988) 390.
[29] D.T. Dexter, C. Carter, F.R. Wells, F. Javoy-Agid, Y. Agid, A.J. Lees, P. Jenner, C.D. Marsden, J. Neurochem. 52 (1989) 381.
[30] H. Esterbauer, J. Gebicki, G. Puhl, G. Jurgens, Free Radic. Biol. Med. 3 (1992) 341.
[31] K.H. Cheeseman, Lipid Peroxidation and Cancer, In: B. Halliwell, O.I. Aruoma (Eds.), DNA and Free Radicals, Ellis Horwood Ltd., West Sussex, UK, 1993.
[32] T. Ito, M. Nakano, Y. Yamamoto, T. Hiramitsu, Y. Mizuno, Arch. Biochem. Biophys. 316 (1995) 864.
[33] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, third ed., Oxford University Press, Oxford, 1999.
[34] B. Quintero, M.C. Cabeza, M.I. Martínez, P. Gutiérrez, P.J. Martínez, Can. J. Chem. 81 (2003) 832.
[35] M. Carafa, E. Santucci, G. Lucania, Intern. J. Pharm. 231 (2002) 21.
[36] J. Aiken, T.A. Dix, Biochem. Biophys. 305 (1993) 516.
[37] H.W-S. Chan, G. Levett, Lipids 12 (1977) 99.
[38] J. Jaeger, K. Sorensen, S.P. Wolf, J. Biochem. Biophys. Methods 29 (1994) 77.
[39] J.R. Mancuso, D.J. McClements, E.A. Decker, J. Agric. Food Chem. 47 (1999) 4146.
[40] E.A. Lovaas, J. Am. Oil Chem. Soc. 69 (1992) 777.
[41] S.-W. Huang, A. Hopia, K. Schwarz, E.N. Frankel, J.B. German, J. Agric. Food Chem. 44 (1996) 444.
[42] P.M. Abuja, R. Albertini, Clin. Chim. Acta 306 (2001) 1.
[43] E. Schnitzer, I. Pinchuk, M. Fainaru, Z. Schafer, Biochem. Biophys. Res. Commun. 216 (1995) 854.
[44] O. Schmitz, S. Gäb, J. Chromatogr. A 767 (1997) 249.
[45] O. Schmitz, S. Gäb, J. Chromatogr. A 781 (1997) 215.
[46] Y. Yamamoto, S. Haga, E. Niki, Y. Kamiya, Bull. Chem. Soc. Jpn. 57 (1984) 1260.
[47] A. Hopia, S.-W. Huang, E.N. Frankel, Lipids 31 (1996) 357.
[48] R.L.C. Barclay, J.K. Grandy, H.D. MacKinnon, H.C. Nichol, M.R. Vinqvist, Can. J. Chem. 76 (1998) 1805.
[49] S.S. Pekkarinen, H. Stöckmann, K. Schwarz, M. Heinonen, A.I. Hopia, J. Agric. Food Chem 47 (1999) 3036.
[50] F. Boscá, M.A. Miranda, I.M. Morera, A. Samadi, J. Photochem. Photobiol. B Biol. 58 (2000) 1.
[51] A. Samadi, L.A. Martínez, M.A. Miranda, M.I. Morera, Photochem. Photobiol. 73 (2001) 359.

[52] E.N. Frankel, W.E. Neff, D. Weisleder, Determination of methyl 13 linoleate hydroperoxides by C nuclear magnetic resonance spectroscopy, in: L. Packer, A.N. Glazer (Eds.), Methods in Enzymology, vol. 186, Part B, Academic Press, San Diego, CA, 1990, pp. 380–387.
[53] O.T. Kasaikina, V.D. Kortenska, Z.S. Kartasheva, G.M. Kuznetsova, T.V. Maximova, T.V. Sirota, N.V. Yanishlieva, Colloids Surf. A Physicochem. Eng. Aspects 149 (1999) 29.
[54] N.A. Porter, B.A. Weber, H. Weenen, J.A. Khan, J. Am. Chem. Soc. 102 (1980) 5597.
[55] M.A. Miranda, L.A. Martínez, A. Samadi, F. Boscá, I.M. Morera Chem. Commun. (2002) 280.
[56] Calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67 (© 1994-2003 ACD).
[57] A. Alejandre, F. Medina, P. Salagre, A. Fabregat, J.E. Sueiras, Appl. Catal. B Environ. 18 (1998) 307.
[58] J. Gao, Y. Liu, W. Yang, L. Pu, J. Yu, Q. Lu, Plasma Sources Sci. Technol. 12 (2003) 533.
[59] Macherey-Nagel GmbH & Co., KG (http://www.mn-net.com/web/MN-WEB-Applikationen.nsf/0/9b56d2e842a8b47dc1256a530040dd3d!OpenDocument&TableRow= 1.1#1).
[60] R. Battino, IUPAC Solubility Data Series: Oxygen and Ozone, vol. 7, Pergamon Press, New York, 1981.
[61] Z.B. Alfassi, S. Marguet, P. Neta, J. Phys. Chem. 98 (1994) 8019.
[62] B. Frei, R. Stocker, B.N. Ames, Proc. Natl. Acad. Sci. USA 85 (1988) 9748.
[63] S.I. Dikalov, R.P. Mason, Free Radic. Biol. Med. 27 (1999) 864.
[64] S.I. Dikalov, R.P. Mason, Free Radic. Biol. Med. 30 (2001) 187.
[65] J.A. Howard, J.C. Tait, Can. J. Chem. 56 (1978) 176.
[66] E.G. Janzen, P.H. Krygsman, D.A. Lindsay, D.L. Haire, J. Am. Chem. Soc. 112 (1990) 8279.