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Quantitation of oxidized triglyceride monomers and dimers as an useful measurement for early and advanced stages of oxidation

By **G. Márquez-Ruiz, M. Martín-Polvillo and M.C. Dobarganes**

Instituto de la Grasa (CSIC). Avda. Padre García Tejero, 4. 41012 Sevilla, Spain

SUMMARY

Quantitation of oxidized triglyceride monomers and dimers as an useful measurement for early and advanced stages of oxidation

Quantitation of oxidized triglyceride monomers and dimers is reported as a good measurement for early and advanced stages of oxidation. Applicability of this approach to follow-up oxidation was tested in samples of trilinolein and methyl linoleate stored at either room temperature or 60°C for different periods of time. Oxidized monomers, dimers and polymers were determined in 50 mg-samples by adding monostearin as internal standard and applying a combination of adsorption chromatography, using silica cartridges, followed by high-performance size-exclusion chromatography. Additionally, peroxide values and tocopherol contents were measured. Results showed that a significant rise of dimeric compounds denoted the end of the induction period while oxidized monomers were the only group of compounds showing a progressive increase during the early stages of oxidation.

KEY-WORDS: High performance size exclusion chromatography – Oxidation(stage) – Oxidized triglyceride dimers – Oxidized triglyceride monomers.

1. INTRODUCTION

Oxidative deterioration of edible oils and fatty foods is of great concern since it affects the wholesomeness of foods due to the development of rancidity (Frankel, 1985a; Addis, 1986; Grosch 1987) as well as to the decay of nutritional value and food safety (Eriksson, 1987; Kubow, 1992; Esterbauer, 1993).

The mechanism of autoxidation of unsaturated lipids is a catalytic process which proceeds via free radicals and involves the reaction of oxygen with the unsaturated fatty acid moieties of triglycerides or any other lipid molecule. Following formation of hydroperoxides and further oxidation, decomposition and polymerization reactions, a wide range of oxidation intermediates and end compounds are produced (Foote, 1985; Frankel, 1985b; Frankel, 1991), hence the great difficulties encountered to evaluate oxidative alteration and the present demand for new analytical procedures of general application.

Among the classical methods most commonly used to evaluate early oxidation, peroxide value, UV absorption and assay of thiobarbituric acid-reactive substances probably stand out, all focused on partial and distinct aspects of the oxidation process, formation of hydroperoxides in the first case and secondary products in the others. On the other hand, through application of chromatographic techniques, analyses of specific oxidation compounds can be attained (Frankel, 1979; Rossell, 1989; Marini, 1992). Given the complexity of the reactions involved, the chromatographic methods most commonly applied are headspace analysis of volatiles obtained after hydroperoxide scission, hexanal and pentane standing out (Frankel, 1982, 1984). With respect to the sensory methods, although they are of enormous interest in relation to consumer acceptance, they are mainly of utility once rancidity has appeared.

Each of the methods cited above is, however, applicable to particular stages of the process and the information obtained depends on the type of method chosen (Frankel, 1993). The methods currently used have been reviewed by several authors, and it is generally concluded that, in spite of the multitude of assays available, there is no universal method which allows evaluation of the extent of oxidation throughout the entire process. Therefore, the need arises for improving methodologies to reevaluate aspects of particular concern, such as the effectiveness of antioxidants and, in general, the influence of the different variables which modify the rate of oxidative reactions (Gray, 1978; Löliger, 1989; Holmer, 1991; Kanner and Rosenthal, 1992; Halliwell and Chirico, 1993; Frankel, 1993).

During the last years, we have developed and widely applied a methodology based on combination of adsorption and size exclusion chromatographies to study alteration of used frying oils, which enables quantitation of oxidized and polymeric compounds, as well as hydrolytic products, i.e. diglycerides and fatty acids (Dobarganes et al., 1988). Application of this procedure is not limited to fats and oils heated at high temperature and hence it was later proved to be of great utility for quality evaluation of refined oils (Dobarganes et al., 1989; Hopia, 1993a) and

quantitation of oxidation compounds in fats and fatty foods at low temperature (Pérez-Camino et al., 1990, 1991, Hopia, 1993b; Hopia et al., 1993). Recently, a modification of this methodology has been introduced directed to the utilization of small quantities of samples and solvents, by using an internal standard for quantitative purposes (Márquez-Ruiz et al., in press). The high reproducibility achieved for samples of low alteration levels adds further possibilities of application to initial stages of oxidation.

In this paper, quantitation of oxidized triglyceride monomers and dimers is reported as a good measurement for early and advanced stages of oxidation since it provides information on the oxidation extent by evaluating concomitantly primary and secondary oxidation products. Samples tested included trilinolein and methyl linoleate stored at ambient conditions and subjected to oxidation at 60°C for accelerated tests. It is concluded that a significant rise of dimeric compounds denotes the end of the induction period while oxidized monomers are the only group of compounds showing a progressive increase during the early stage of oxidation.

2. EXPERIMENTAL

Samples and treatments

Trilinolein (LLL) and methyl linoleate (ML) were purchased from Nu-Chek-Prep (Elysian, MN, USA) and used as model triglyceride and fatty acid methyl ester, respectively. Samples of both were placed in open beakers (surface-to-volume ratio of 10 cm^{-1}) and either heated in an oven at 60°C or stored at room temperature for different periods of time.

Analytical determinations

Separation of polar fractions by adsorption chromatography

LLL samples were fractionated using silica cartridges for solid-phase extraction (Sep-pak columns supplied by Waters Associates, Milford, MA, USA). The methodology was described in detail, including precision, accuracy and recovery data in a recent publication (Márquez-Ruiz et al., in press). Briefly, 2 mL of the sample solution, containing 50 mg of sample and 1 mg of monostearin, used as internal standard, were placed on the column and the solvent was passed through while the sample was retained on the column. Next, the non-polar fraction was eluted with 15 mL of petroleum ether:diethyl ether 90:10. A second fraction containing polar compounds and the internal standard was eluted with 15 mL of diethyl ether. Non-polar and polar fractions were evaporated under reduced pressure and redissolved in 1 mL of tetrahydrofuran for further analyses, i.e., TLC to check the efficiency of the separation, and high-performance size-exclusion chromatography (HPSEC).

High-performance size-exclusion chromatography

Fractions of polar compounds from LLL samples, obtained as outlined above, as well as total ML samples, were analyzed by HPSEC in a Konik 500A chromatograph (Konik SA, Barcelona, Spain) with a 10 µL sample loop. A refractive index detector (Hewlett Packard, Pittsburgh, PA, USA) and two 100 and 500 Å Ultrastyragel columns (Waters Associates, Milford, MA, USA) connected in series operated at 35°C. The columns were 25 cm x 0.77 cm inner diameter, packed with a porous, highly cross-linked styrenedivinylbenzene copolymer (< 10 µm). High-performance liquid chromatography grade tetrahydrofuran served as the mobile phase with a flow of 1 mL/min.

Peroxide value

Measurements of peroxide values were carried out by means of the iodometric assay (AENOR, 1991), starting from 100 mg sample.

α-Tocopherol content

α-tocopherol levels were quantitated by HPLC (IUPAC, 1992).

3. RESULTS AND DISCUSSION

Figure 1 shows representative HPSEC chromatograms of polar fractions of LLL stored at moderate temperature (60°C), at the initial (A) and three different points of the oxidation process (B,C and D). Changes in the groups of compounds resolved are well illustrated given that polar fractions were dissolved in equal volumes of solvent. Thus, it can be observed that the peak corresponding to monostearin, used as internal standard, is of similar magnitude in all chromatograms. The first chromatogram corresponds to the polar fraction of the starting, unoxidized LLL, composed exclusively of the internal standard added (1 mg) since LLL was otherwise recovered in the non-polar fraction. The polar fraction B was separated from a sample taken out during the early oxidation period (peroxide value = 52.6 meq/Kg), and showed the presence only of oxidized triglyceride monomers, in this case LLL oxidized monomers, accounting for 2.3 % on total sample. The peak of oxidized triglyceride monomers is comprised of those monomeric triglycerides containing at least one oxidized fatty acyl, either a peroxide group or any other oxygenated function, such as epoxy, keto, hydroxy, etc. Hence it provides a global measurement of both primary and secondary oxidation products. Sample C (peroxide value = 92.0 meq/Kg) was withdrawn around the end of the induction period, as shown by the appearance of dimers (0.2% on total sample), thus indicating that the sample was entering the period of advanced oxidation. However, rancidity was not still detected at this point, under these conditions. In turn, LLL oxidized monomers had reached a level of 5.4% on total

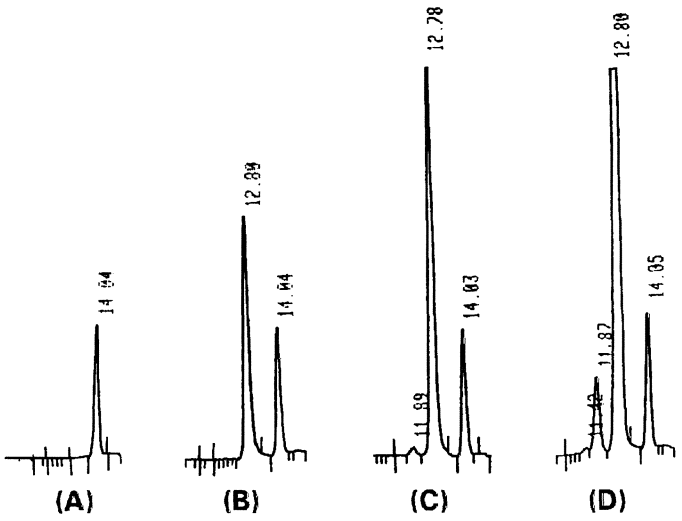

Figure 1
High-performance size exclusion chromatograms of polar fractions isolated from starting trilinolein (A) and from trilinolein samples withdrawn at different points during oxidation at 60°C (B, C and D). Retention times (min): 11.4, LLL polymers; 11.8, LLL dimers; 12.8, LLL oxidized monomers and 14.0, monostearin (internal standard)

sample. Finally, polar fraction D, separated from a sample taken out further within the advanced oxidation stage (peroxide value = 516.3 meq/Kg), presented a significant increase in oxidized monomers (17.7% on total sample) and dimers (1.4% on total sample). Also, polymers could be already detected (Rt: 11.4 min). It is worthy to remark the great utility offered by this methodology, which enables the concominant evaluation of initial and decomposition compounds through quantitation of oxidized triglyceride monomers and dimers. Thus, it is possible to determine the degree of oxidation at any time during the course of oxidation.

Figure 2 presents a representative profile of the evolution of oxidation at 60°C, for LLL samples, without (A) and with 250 mg/Kg α-tocopherol added (B). LLL was used as a model unsaturated triglyceride in order to test the possibilities of the analytical procedure to evaluate modifications throughout oxidation, without the interference of minor compounds of potential prooxidant or antioxidant effects which could be present in oils. Accelerated oxidative tests were carried out at 60°C in an oven as it has been reported that, at this temperature, results correlate well with evaluations of actual shelf lifes while a number of side reactions occurring at higher temperature are minimized (Ragnarsson and Labuza, 1977). Under these conditions, testing stability is not only much more rapid but also easier than it is under ambient conditions since there are many variables difficult to control over prolonged storage (Frankel 1993).

As can be observed, LLL oxidized monomers showed a progressive increase during the earlier stages of oxidation, which was parallel to peroxide value evolution. Thus, an excellent correlation between both determinations was found (Y = -15.5387 + 2.8911X, r = 0.9941 for LLL (A) and Y = -23.2369 + 3.0560X, r = 0.9910 for LLL (B)). Hydroperoxides are the primary oxidation products formed and hence peroxide value is the most common method for early oxidation. During the early stages of oxidation, LLL oxidized monomers would be primarily comprised of hydroperoxides and hence their increase was closely parallel to that of peroxide value. However, peroxides are intermediate and labile products, readily converted into a multitude of secondary oxidation products. Among them, radical recombination gives rise to oxygenated side products of the same chain lenght than the parent hydroperoxide, here quantitated globally as LLL oxidized monomers, because of their similar polarity and molecular weight. Quantitation of this group of compounds can be therefore of great utility not only to detect initially formed oxidized products, even before rancidity, but also to follow-up oxidation during further stages.

In the course of oxidation it is clearly observed that, at a certain point, oxidation is accelerated, as shown by the sharp increase of LLL oxidized monomers and initiation of polymerization. This occurred at around 9 h in LLL devoid of α-tocopherol versus approximately 95 h in the case of LLL with α-tocopherol. At the same time, α-tocopherol was practically consumed in the sample containing it initially, what indicates it exerted a protective effect till it was exhausted. Expressing the induction period as the time interval before oxidation proceeds rapidly, presenting a sudden change in the rate of oxidation, it

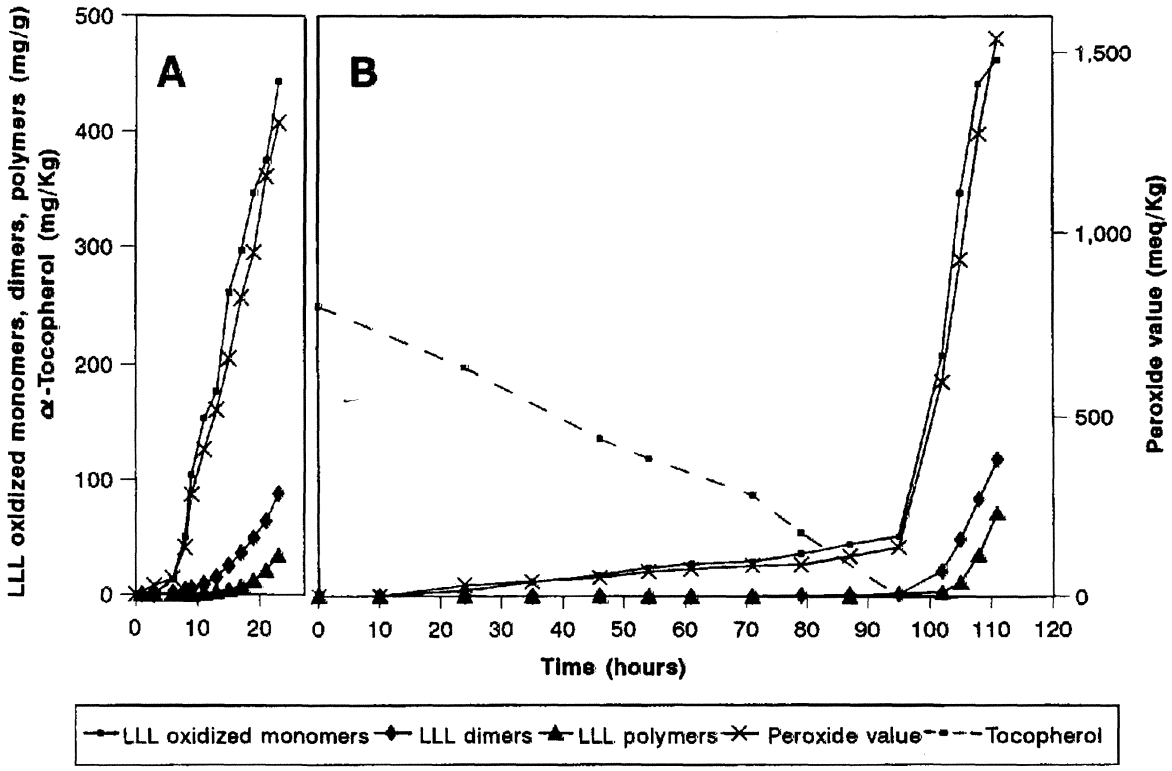

Figure 2
Evolution of oxidized monomers, dimers, polymers, peroxide value and α-tocopherol content in samples of trilinolein (LLL) without (A) and with (B) α-tocopherol initially added, throughout oxidation at 60°C

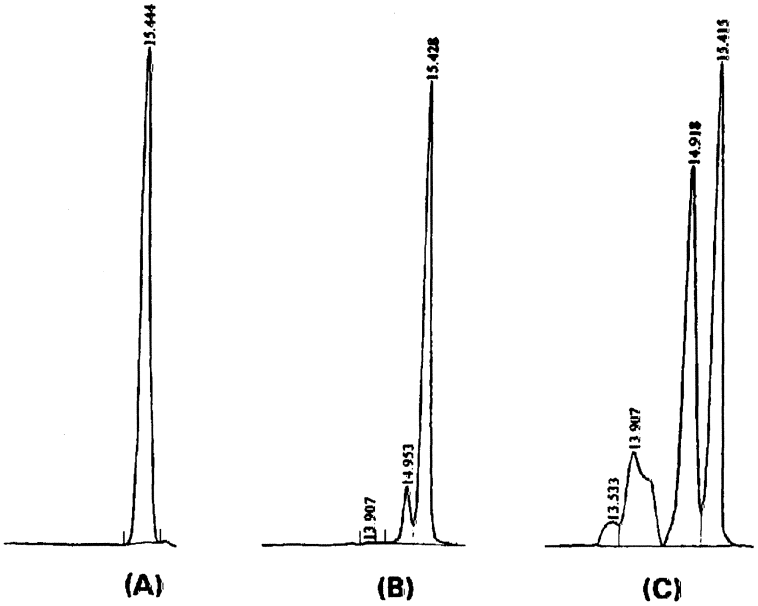

Figure 3
High-performance size exclusion chromatograms of polar fractions isolated from starting methyl linoleate (A) and from methyl linoleate samples withdrawn at different points during oxidation at room temperature (B and C). Retention times (min): 13.5, ML polymers; 13.9, ML dimers; 14.9, ML oxidized monomers and 15.4, unoxidized ML

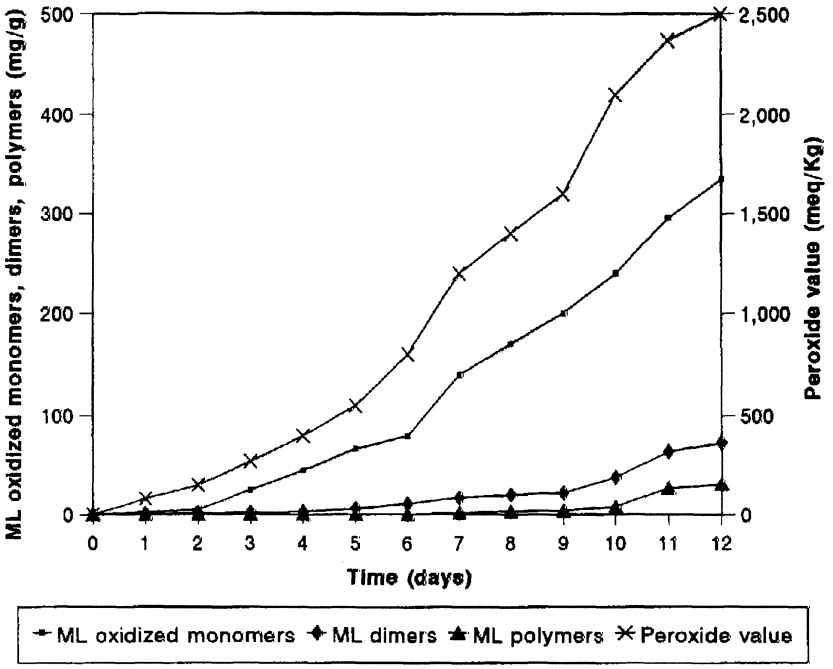

Figure 4
Evolution of oxidized monomers, dimers, polymers and peroxide value in samples of methyl linoleate (ML) throughout oxidation at room temperature

is observed that, under these conditions, around 50 mg/g of oxidized monomers denotes the end of the induction period and the onset of advanced oxidation. A significant rise in dimers indicates additionally that the course of oxidation has entered a second and accelerated phase.

The development of oxidation was considerably delayed in LLL samples with α-tocopherol added but, interestingly, once tocopherol was used up, these samples and their counterparts without α-tocopherol showed a similar evolution pattern.

A different and simpler approach was used to assess model methyl esters because we found it was not necessary to carry out a preliminary separation of the nonpolar fraction by adsorption chromatography but only a direct HPSEC injection of the entire sample. Figure 3 shows HPSEC chromatograms of the starting sample of ML (A) and samples withdrawn after oxidation at room temperature, of low (B) and high (C) oxidation level. As can be observed, unoxidized and oxidized monomers eluted at distinct retention times by virtue of the clear differentiation in molecular weight between the intact compounds and the oxidized monomeric molecules, mostly hydroperoxides under these conditions. Therefore, through a simple analysis by HPSEC run in just 15 min, a complete picture of the oxidative state can be attained.

This approach can be applied as a complementary analysis to triglyceride or oil samples following transesterification, with the aim of evaluating specifically the altered fatty acyl groups (Márquez-Ruiz et al. 1990). Determined as methyl esters, smaller amounts of oxidized and dimeric compounds would be found as compared to those of oxidized and dimeric triglycerides, given that from the three fatty acyls included in the oxidized triglyceride molecule, roughly two of them would be expected to remain unchanged (Márquez-Ruiz et al., 1995).

Figure 4 shows the results obtained for ML at room temperature, achieved through direct analysis by HPSEC. As observed for LLL at 60°C, there was a continuous increase of oxidized monomers from the initial stages of oxidation, which supports the great utility of this measurement for early oxidation under ambient conditions also. A parallel rise of peroxide value was monitored. Under these conditions, dimers did not appear until high amounts of oxidized monomers were accumulated, i.e. 70 mg/g specifically of oxidized methyl esters, and high peroxide values (close to 500 meq/Kg) had been reached. This suggests that temperature may have an important influence on the oxidation kinetics.

In conclusion, quantitation of oxidized monomers provides an useful measurement for early oxidation stages and parallel quantitation of dimers gives a good indication of the onset of advanced oxidation. A large number of experiments along this line are currently underway in our laboratory, using model systems and oils, wherein this methodology is being

applied to study the useful storage life and determine the efficacy of antioxidants.

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