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DETERMINATION OF OXIDATION PRODUCTS OF N-PHENYLLINOLEAMIDE: SPANISH TOXIC OIL SYNDROME STUDIES

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SUMMARY

The early identification of fatty acid anilides in suspect oils directed attention to their possible role in the Spanish toxic oil syndrome. These anilides or their oxidized derivatives could have been spontaneously formed during the handling and/or storage of the oil. The exact cause of the intoxication is still unknown but free radical and peroxidative mechanisms have been implicated in its etiology. Epoxy-hydroxylated derivatives from linoleic acid anilide were obtained using a model of accelerated oxidation. Their mass spectral patterns agree with the trimethylsilyl ethers of N-phenyl-9,10-epoxy-11-hydroxy-12-octadecenamide and N-phenyl-12,13-epoxy-11-hydroxy-9-octadecenamide, respectively. These compounds were also identified is rapeseed oil samples supplemented with N-phenyllinoleamide and submitted to the reported accelerated oxidation method.

INTRODUCTION

The Spanish toxic oil syndrome (TOS) constitutes one of the most important massive food intoxications detected in a developed country. The early identification of fatty acid anilides in suspect oils directed attention to their possible role in the etiopathogenesis of TOS [1]. These anilides could have been formed spontaneously during the refining, transport and/or storage of the oil [1,2]. However,

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their implication in the pathogenesis of TOS is still not clear. In this regard, it has been suggested that an overproduction of free radicals, concomitant with an increased biosynthesis of metabolites of arachidonic acid, could explain part of the TOS symptomatology [3]. Current WHO guidelines for research on the causes of TOS are oriented towards the development of chemical models simulating the processes that rapeseed oil, denatured with aniline for industrial uses, could have undergone during fraudulent refining and storage for commercial distribution.

Along these lines we have studied the formation of oxidative products from N-phenyllinoleamide, the anilide of linoleic acid [(*Z*,*Z*)-9,12-octadecadienoic acid], using a model of accelerated oxidation. Some of the products formed have been characterized by gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Solvents and reagents

N-Phenyllinoleamide (NPLA) and N-[ring-G-^{3}H]phenyllinoleamide (^{3}H-NPLA) (154 mCi/mmol) were prepared as described previously [4,5]. Other products used were linoleic acid (LA) (Merck, Darmstadt, F.R.G.), scintillation liquid and acetonitrile (Koch-Light, Colnbrook, U.K.), 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich-Chemie, Steinheim, F.R.G.), bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (Sigma, St. Louis, MO, U.S.A.). Other reagents were obtained from Scharlau (Barcelona, Spain) or Merck. The rapeseed oil (Croix Jaune, Boulgne-Billancourt, France) was free from additives and antioxidants.

Gas chromatography

The gas chromatograph was a Hewlett-Packard 5790A equipped with a flame ionization detector and a Ross-type injection system [6]. Esterified fatty acids were separated on a Supelco SP-2330 glass capillary column (30 m$\times$0.2 mm I.D.). The initial and final temperatures were set at 150 and 220°C, respectively, with a 1.7°C min gradient. The injector was held at 220°C and the detector at 230°C. The products arising from the oxidation of LA acid and NPLA were analysed on a Hewlett-Packard OV-1 glass capillary column (20 m$\times$0.3 mm I.D.). The initial and final temperatures were 190°C for 1 min and 280°C with a gradient of 2.5°C/min. The injector was maintained at 285°C and the detector at 295°C.

High-performance liquid chromatography (HPLC)

The HPLC apparatus was a Kontron system equipped with two Model 414 pumps, a Series 200 programmer and a Uvikon 722 UV detector. Reversed-phase HPLC was carried out using a 30 cm$\times$0.46 cm I.D. column (10 μm particle size) packed with ODS C_{18} (Tracer Analitica, Barcelona, Spain). The eluent consisted of acetonitrile–acetic acid buffer (pH 3.4) at a flow-rate of 1.5 ml/min. Elution was performed with a stepwise gradient programme consisting of 35 to 65% acetonitrile in 7 min, 65 to 95% acetonitrile in 14 min and 95 to 100% acetonitrile in 1 min. Eluates were monitored by UV detection at 237 nm, which is the absor-

bance maximum for the anilide peroxides. The radioactivity of the labelled eluate fractions collected from the HPLC column was determined with an LKB 1217 RackBeta scintillation counter following the addition of 8 ml of Unisolve cocktail (Koch-Light) to each fraction prior to counting.

Thin-layer chromatography (TLC)

Pre-coated silica gel GF254 (0.25 mm layer) glass plates were used (Merck). Samples were spotted diluted in hexane–chloroform (2:1, v/v). The mobile phase was *n*-hexane–diethyl ether–methanol–acetic acid (90:20:3:2, v/v). Radioactive spots were measured with an IM 3000 radio TLC analyzer (Isommess, Stanbenhardt, F.R.G.).

Gas chromatography–mass spectrometry

A Hewlett-Packard Model 5995 gas chromatograph–mass spectrometer was used. The GC column was the same as that used for the GC determination of oxidation products from NPLA. The initial column temperature was 60°C, increased first to 150°C at 15°C/min, then to 265°C at 6°C/min and finally to 280°C at 1.5°C/min. The temperatures of the injector and transfer line were 285°C. The electron energy was 70 eV and the ion source was kept at 210°C. Samples were methylated and silylated with diazomethane [7] and BSTFA [8] for the determination of hydroxy fatty acids.

Accelerated oxidation system

Oil samples were oxidized in glass tubes (150×10 mm, or 60×4 mm for the oxidation of NPLA dissolved in LA) by bubbling a stream of pure synthetic air (SEO, Barcelona, Spain) at a rate of 0.5–1 ml/s and a constant temperature of 90°C. The tubes were fitted with reflux condensers to prevent losses of volatile materials. The following samples were subjected to oxidation: rapeseed oil, rapeseed oil supplemented with NPLA (500 ppm), rapeseed oil with ^{3}H-NPLA (500 ppm, 12 mCi/mmol) and LA with NPLA (6%, w/w).

Esterified fatty acids

The semi-quantitative assay of esterified fatty acids in control oils and in oils oxidized for various periods of time was carried out by GC. The procedure was based on the transesterification of fatty acids followed by silylation [8] in accordance with described procedures.

Fractionation of oil samples containing NPLA

After oxidation in air, rapeseed oil or LA samples containing ^{3}H-NPLA or NPLA were purified using Florisil silica cartridges (Sep-Pak; Millipore, Waters Assoc. Division, Milford, MA, U.S.A.). Briefly, 100 μl of the oxidized sample were loaded on the cartridges, which were then eluted consecutively with 2×10 ml of *n*-hexane (fractions I and II), 2.5 ml of *n*-hexane–chloroform (4:1, v/v) (fraction III), 16 ml of *n*-hexane–chloroform (1:3, v/v) (fraction IV), 10 ml of chloroform–ethanol (3:1, v/v) (fraction V), 10 ml of ethanol (fraction VI) and 10 ml of water adjusted to pH 3 with acetic acid (fraction VII).

The eluate fractions (I–VII) were monitored by liquid scintillation counting.

Those fractions with the highest content of radioactivity were rechromatographed on TLC plates or analysed by HPLC according to methods described previously. For the HPLC determinations fractions of 2 ml were evaporated to dryness and the residue resuspended in 250 μl of acetonitrile. The elution profile of the labelled material was established by measuring the radioactivity in consecutive 30-s fractions.

RESULTS AND DISCUSSION

Determination of esterified fatty acids

The degree of oxidation of a given oil sample could be efficiently monitored by measuring the changes in the ratios of unsaturated to saturated fatty acids. As shown in the GC profiles in Fig. 1, a decrease in the peaks corresponding to linoleic and linolenic acids and the appearance and time-dependent increase of new peaks (a, b and c in Fig. 1) at the far end of the chromatogram are evident with the oxidized samples. Also, as illustrated in Fig. 2, after an initial interval during which unsaturated fatty acids (UFAs) are mostly preserved, the rate of loss of these components on oxidation is in agreement with the degree of unsaturation. The decrease in UFAs is given as a percentage of the content in control oils (oxidation time 0 min). Palmitic acid was taken as a non-oxidizable internal reference substrate. The more unsaturated acids are less resistant to oxidation losses. The initial 24-h period of resistance to oxidation is probably due to the effect of the natural antioxidants present in the oil.

Semi-preparative isolation of fractions

The radiochemical assay of the Sep-Pak fractions collected from samples of oil supplemented with ^{3}H-NPLA after an oxidation period of 120 h shows that most of the counts recovered are concentrated in fraction 4 (*n*-hexane–chloroform, 1:3), with 16% of the total radioactivity, and fraction 5 (chloroform–methanol, 3:1), with 64% of the total counts. The amount recovered in the other fractions and the residual cartridge radioactivity were negligible and in all instances lower than 4%. Subsequent analysis of the various fractions by HPLC and TLC (Fig. 3) indicates that the radioactive component of fraction IV is ^{3}H-NPLA (Fig. 3A and C) whereas in fraction V we found the presumed oxidation products derived from NPLA (Fig. 3B and D).

The radioactivity profile in Fig. 3D shows a major peak eluting at 13 min (fraction numbers 25–30) arising from oxidation of the ^{3}H-NPLA and other components present in lower concentrations which elute on both sides of this peak (fraction numbers 15–20 and 30–40). The monitoring of the time course of oxidation of NPLA indicates that the last eluting peaks (the less polar compounds) are those formed first during oxidation.

GC–MS determination

The GC–MS study of the oxidation of NPLA dissolved in linoleic acid simplifies the interpretation of the results, minimizing the masking effect that could be expected from a more complex whole oil matrix. Also, this model prevents the

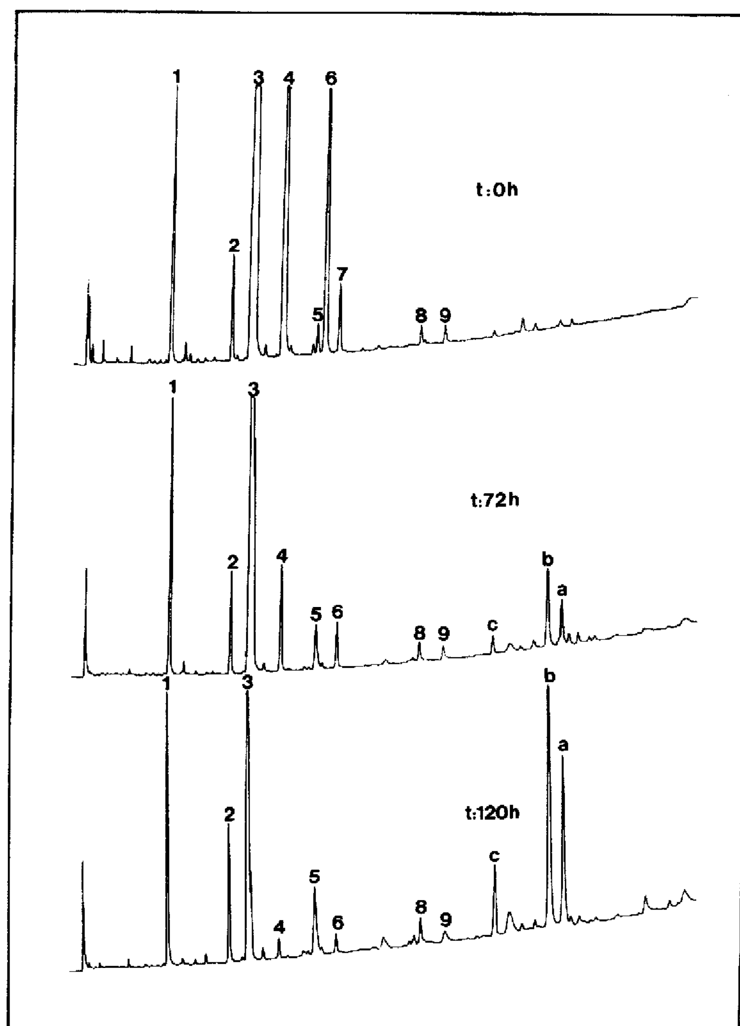

Fig. 1. GC profiles of rapeseed oil before ($t=0$ h) and after 72 and 120 h of oxidation in a stream of air. Fatty acids: 1 = 16:0; 2 = 18:0; 3 = 18:1; 4 = 18:2; 5 = 20:0; 6 = 18:3; 7 = 20:1; 8 = 22:0; 9 = 22:1.

formation of transesterification products with other fatty acids. The accelerated oxidation of LA by itself gave rise to monohydroxy and epoxymonohydroxy derivatives of LA, as would be expected from previous studies [9]. The identification of these compounds was based on the molecular ions and fragmentation patterns of the corresponding methylated and silylated derivatives. Compounds specifically identified were 9,10-epoxy-11-hydroxy-12-octadecenoic acid with a molecular ion at m/z 398 and characteristic fragment ions at m/z 383, 308, 241 and 199 (base peak) (Fig. 4) corresponding to the loss of CH_3, TMSOH, $(CH_2)_7COOCH_3$ and $C_2H_2O(CH_2)_7COOH_3$ groups, respectively. The positional 12,13-epoxylated isomer was also positively identified by its related fragmentation pattern with the base peak at m/z 285 corresponding to the loss of a $CH_3(CH_2)_4C_2H_2O$ group. These compounds have also been reported as metab-

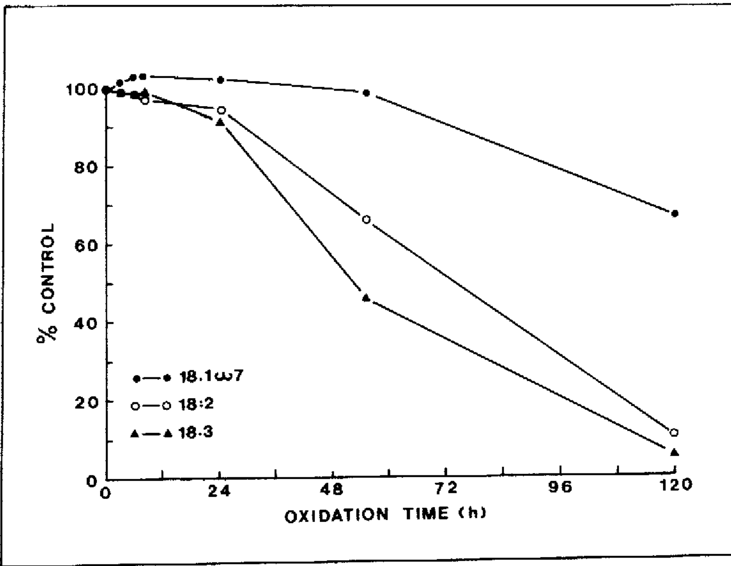

Fig. 2. Rate of loss of UFAs relative to control values on continuous oxidation in air.

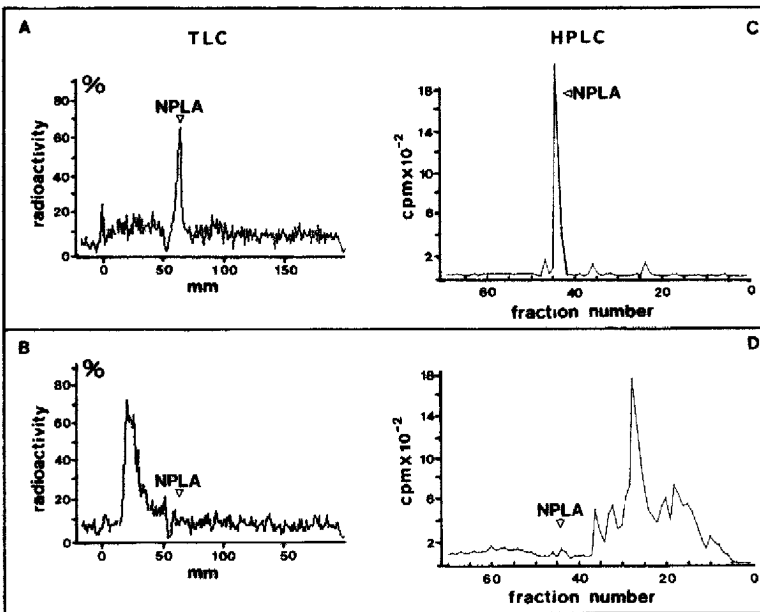

Fig. 3. (A) TLC radiochromatogram of fraction IV from rapeseed oil supplemented with ^{3}H-NPLA (500 ppm) and (B) the same sample after oxidation for 120 h; (C) HPLC radiochromatogram of rapeseed oil supplemented with ^{3}H-NPLA (500 ppm) and (D) same sample after oxidation for 120 h.

olites of LA with prostaglandin endoperoxide synthase [10]. The GC–MS study of the components of HPLC fractions 25–30 (see Fig. 3D) obtained by oxidation of NPLA in an LA matrix (see Fig. 3) showed the presence of the same family of

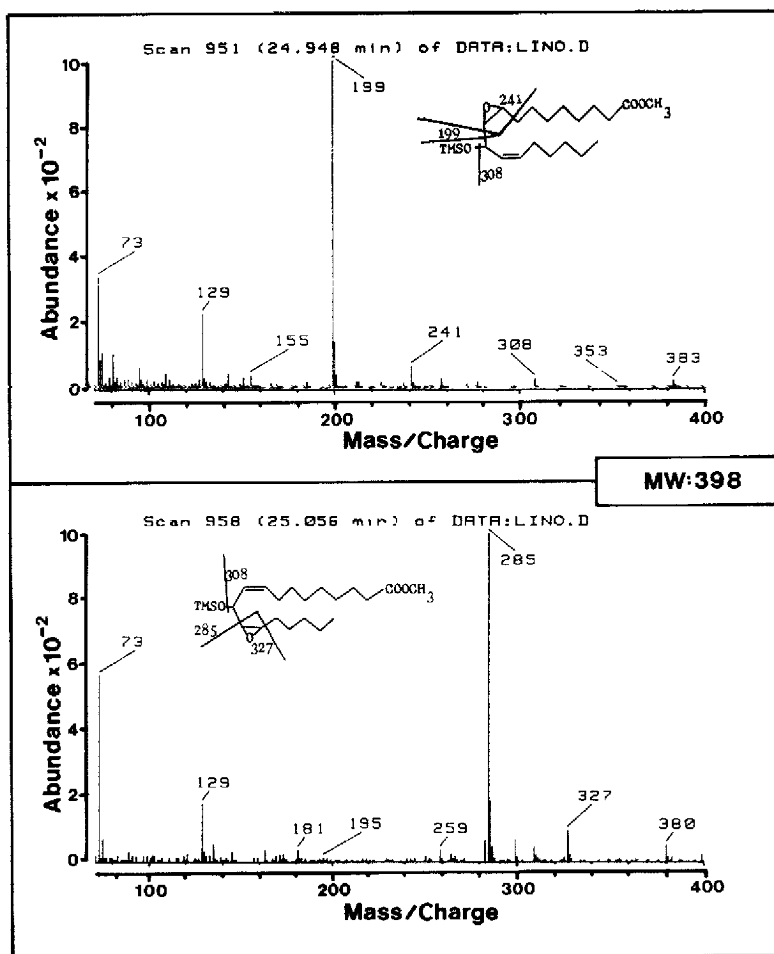

Fig. 4. Mass spectra from the GC peaks identified as 9,10-epoxy-11-hydroxy-12-octadecenoic acid (top) and its 12,13 positional isomer (bottom).

epoxy and hydroxy derivatives of NPLA as those obtained from the oxidation of LA. The compounds that have been identified from their mass spectral patterns are N-phenyl-9,10-epoxy-11-hydroxy-12-octadecenamide and N-phenyl-12,13-epoxy-11-hydroxy-9-octadecenamide (Fig. 5) together with 13-hydroxy-9,11-octadecaenoic acid. In the first instance the base peak is still at *m/z* 199, with additional ions arising from the anilide moiety such as the aniline fragment at *m/z* 93. With the 12,13-epoxylated octadecenamide isomer the base peak appears at *m/z* 346, corresponding the the *m/z* 285 fragment in the mass spectrum of the parent acid (see above).

These oxidation products were also indentified in samples of rapeseed oil with added NPLA and subjected to the same oxidation process as described for the mixture of LA–NPLA. The identification of these compounds proves that NPLA undergoes oxidative processes similar in nature to those affecting linoleic acid.

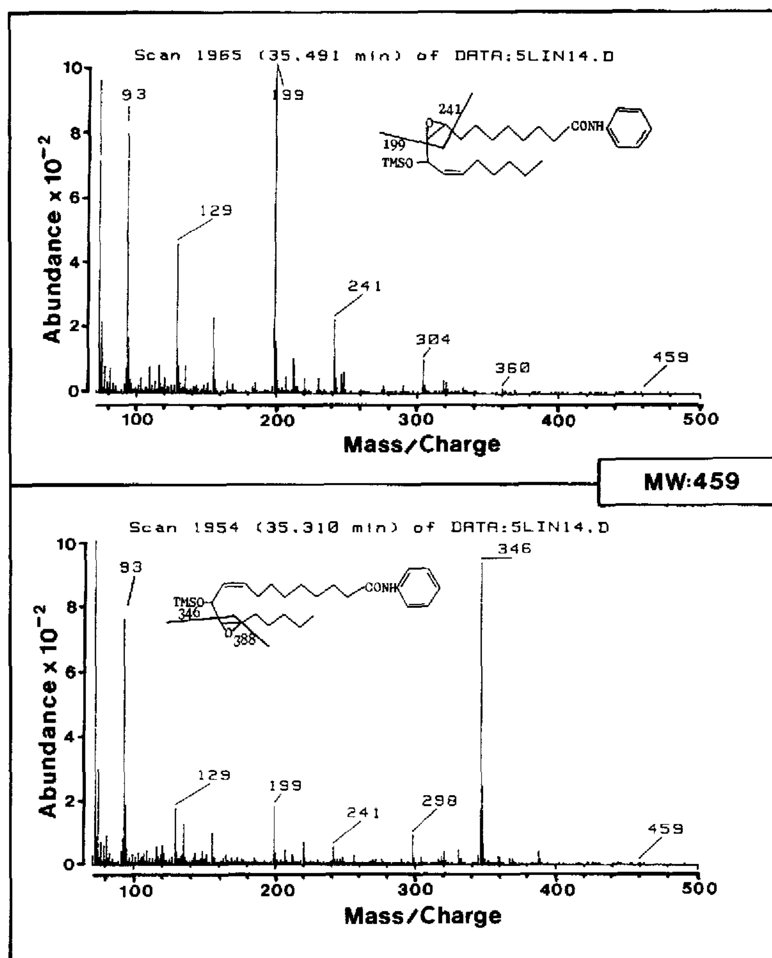

Fig. 5. Mass spectra from the GC peaks identified as N-phenyl-9,10-epoxy-11-hydroxy-12-octadecenamide (top) and its 12,13-epoxy isomer (bottom). These GC peaks were obtained after derivatization of HPLC fractions 25—30 (see text and Fig. 3).

In the process, intermediate hydroperoxides are generated and these could have a bearing on the etiopathogenesis of the toxic oil syndrome. In this regard, preliminary results from this laboratory indicate that NPLA can be metobolized by the lipoxygenase systems [11]. These oxidized metabolites might themselves exert a stimulating effect on arachidonic acid metabolism, which could be associated with the toxic activity of NPLA.

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