Gas Chromatographic Study on High-Temperature Thermal Degradation Products of Methyl Linoleate Hydroperoxides

Tullia Gallina Toschi*, Arianna Costa, and Giovanni Lercker

University of Bologna, Istituto di Industrie Agrarie, 40126 Bologna, Italy

ABSTRACT: Chromatographic techniques were used to separate secondary products generated by thermal degradation of methyl linoleate hydroperoxides (MLHP). The MLHP were obtained by oxidation, selected, and concentrated by solid-phase extraction (SPE) and thin-layer chromatography (TLC). The purified MLHP were then thermo-degraded in the gas-chromatographic glass liner and analyzed on-line by gas chromatography–mass spectrometry (GC–MS). The MLHP were also thermo-degraded and collected in a short silicic acid-packed column, eluted, separated by TLC, and then analyzed by GC. By considering the elution in TLC, the GC retention times and the GC–MS analyses, it was possible to characterize the mono- and the dioxygenated secondary products, particularly those having a boiling point higher than methyl linoleate. The peaks that corresponded to the mono-oxygenated products (epoxy, hydroxy, and keto) were identified, and, on the basis of their MS spectra, molecular structures were proposed. A specific elution order was suggested for keto derivatives: 9-keto,$\Delta^{10,12}$- and 13-keto,$\Delta^{9,11}$-octadecadienoate. The hydroxy derivatives, which show the typical fragmentations of 9-hydroxy,$\Delta^{10,12}$- and 13-hydroxy,$\Delta^{9,11}$-octadecadienoate, were also identified. On the other hand, identification of the di-oxygenated compounds was more difficult, and, therefore, it was not possible to indicate each positional isomer; however, their elution order could be epoxy-hydroxy and epoxy-keto derivatives.

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KEY WORDS: Autoxidation, capillary gas chromatography, fatty acids, hydroperoxides, linoleate hydroperoxides, methyl linoleate, oxidation.

The oxidative degradation of mono- and polyunsaturated lipids is an important problem because it produces substances with offensive odors that could also decrease the nutritional value and generate certain compounds with proven toxicity.

Linoleic acid, one of the most important fatty acids, nutritionally and biologically, is present at considerable levels in oils and fats of the human diet. For this reason, the thermal oxidation of methyl linoleate model systems has been studied by high-performance liquid chromatography (HPLC) and gas chromatography (GC) (1–3) and from a theoretical view (4–6). Most of the work on chromatographic separation has dealt with products of linoleic acid hydroperoxides (MLHP) (7,8) and their stable hydroxy derivatives (9,10). Other papers have been published on secondary products of methyl linoleate oxidation (11) and, more recently, on cyclic products (12).

The present work describes capillary GC separation of thermo-degradation products of MLHP, with the purpose to identify the most important oxidation products. The approach followed was similar to that of previous studies on methyl oleate (13–16). The products taken into consideration were those having boiling points and GC retention times higher than methyl linoleate. The knowledge of their GC position, together with their behavior during the oxidation, could provide useful information for quality control of fat-containing foods and for better understanding and monitoring of the oxidative process.

EXPERIMENTAL PROCEDURES

Materials and reagents. Linoleic acid standard (99% pure) and soybean lipoxygenase-1 were supplied by Fluka Chemie (Buchs, Switzerland); methyl laurate standard (99.5% pure) was supplied by Sigma Chemical Co. (St. Louis, MO); methyl linoleate standard (99% pure) was supplied by Nu-Chek-Prep Inc. (Elysian, MN). Analytical-grade reagents and solvents were supplied by Carlo Erba (Milan, Italy) and Prolabo (Paris, France). Silicic acid 100 mesh was supplied by Mallinckrodt (St. Louis, MO). Thin-layer chromatography (TLC) plates (20 × 20 cm, 0.25 mm film thickness Stratocrom SI, without fluorescence indicator) were supplied by Merck (Darmstadt, Germany). SPE columns, packed with 500 mg silica gel, were supplied by SPE-ED (Lehigh Valley, PA).

*Preparation of linoleic acid 13-hydroperoxide (9-*cis*,11-*trans*) by enzymatic synthesis.* This synthesis was performed according to Jacazio *et al.'s* method (17). Linoleic acid (280 mg) was introduced in a Schlenck tube (supplied by an artisan in Bologna, Italy), and 10 mL of a sodium tetraborate decahydrate 0.1 N solution was added. The tube was then submersed in an ice bath, and the mixture was vigorously stirred by a magnetic stirrer. After 10 min, 20 mg of soybean lipoxy-

*To whom correspondence should be addressed at University of Bologna, Istituto di Industrie Agrarie, Via San Giacomo 7, 40126 Bologna, Italy.

genase-1 was introduced, and oxygen pressure equal to 4 bars was applied. This procedure created optimal conditions of pH, temperature, and pressure for lipoxygenase activity. The reaction was controlled by using ultraviolet-visible (UV-vis) spectrophotometry. After 1 h, distilled water (250 mL) was added to the mixture, then the hydroperoxides, together with unreacted methyl linoleate, were extracted twice with diethyl ether (250 mL). The total ether extract was dried with anhydrous sodium sulfate and evaporated under nitrogen flow. The residue containing the four hydroperoxide isomers was dissolved in *n*-hexane. The isomeric yield of linoleic acid 13-hydroperoxide (9-*cis*,11-*trans*) was 95%.

*Preparation of linoleic acid 13-hydroperoxy-9-*cis*,11-*trans *methyl ester (13-MLHP).* The methyl ester was obtained by adding the mixture containing the 13-hydroperoxide, previously evaporated under a weak nitrogen flow, to a solution of diazomethane in ether.

Preparation and purification of MLHP obtained by thermooxidation. Methyl linoleate (500 mg) was oxidized (150°C for 1 h) in a polytetrafluoroethane-lined screw-cap tube. The sample was then dissolved in 1 mL *n*-hexane and loaded onto an SPE silica column that was previously conditioned with 5 mL *n*-hexane. The column was eluted with 5 mL *n*-hexane (fraction 1) and then with 10 mL of a mixture of *n*-hexane/diethyl ether 1:1 (vol/vol) (fraction 2). Fraction 1 contained most of the unoxidized methyl linoleate, whereas fraction 2 contained all oxidation polar products, including MLHP. Fraction 2 was evaporated under nitrogen flow, dissolved in 200 µL acetone, and then spotted on a TLC plate. The eluant was a mixture of *n*-hexane/diethyl ether 65:35 (vol/vol). The band of the MLHP was identified by using a saturated solution of KI and visualizing under UV light (254 nm) after being sprayed with a 0.2% ethanolic solution of 2,7-dichlorofluorescein sodium salt. The band was then scraped off and extracted with chloroform, and the solvent was evaporated at room temperature under nitrogen flow.

Preparation of hydroperoxide (MLHP) secondary degradation products. The MLHP were degraded in a GC glass liner at 310°C, and the secondary products were collected in a short column (16 cm × 4 mm i.d.) packed with silicic acid. The oven temperature was set at 70°C. The products were then extracted three times with chloroform and analyzed by TLC.

Synthesis of methyl linoleate and methyl linoelaidate mono-epoxides. Two mL of *m*-chloroperbenzoic acid in chloroform (8 mg/mL) were separately added to methyl linoleate and methyl linoelaidate (20 mg each) and kept for 4 h at room temperature. The epoxy derivatives were extracted twice with diethyl ether and purified by preparative TLC, according to Gunstone *et al.* (18).

Preparation of trimethylsilyl derivatives (TMS ethers). Silylation was performed according to Sweeley *et al.* (19). A mixture of dried pyridine, hexamethyldisilazane and trimethylchlorosilane (5:2:1, vol/vol/vol; *ca.* 0.1 mL) was added to the hydroxy and hydroxy-epoxy derivatives. Samples were reacted at room temperature for 15 min; they were then evaporated under nitrogen flow at 90°C for 10 min and dissolved in *n*-hexane.

Derivatization with BF_3-methanol. To investigate epoxy derivatives, the ring was opened with 10% BF_3 in methanol and held at 52°C for 6 min (20). The products were silylated and then analyzed by GC–MS.

GC and GC–MS. Hydroperoxy derivatives of methyl linoleate and the single TLC bands of the secondary degradation products were analyzed by GC and GC–MS. GC analyses were performed on a Carlo Erba model 5300 Mega Series and a model 4160, both equipped with a split-splitless injector and a flame-ionization detector (FID). A fused-silica capillary column (25 m × 0.25 mm i.d., film thickness 0.1 µm) coated with 5% diphenyl-/95% dimethyl-siloxane (Chrompack, Middelburg, The Netherlands) was used. The temperature was programmed from 70 to 300°C with a gradient of 8°C/min; the injector and detector temperatures were both 310°C, and helium was the carrier gas. For GC–MS analysis, a Varian (Walnut Creek, CA) gas chromatograph model 3300/3400, equipped with a split-splitless injector and coupled to a mass spectrometric detector model Finningan MAT ITS40 (San Jose, CA), was used. The temperature was programmed from 70 to 200°C with a gradient of 10°C/min and from 200 to 290°C with a gradient of 5°C/min and a final isotherm for 10 min. The injector and the transfer line temperatures were both 300°C, and helium was the carrier gas. The analyses were performed by electron impact (EI), and a low-bleed fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm) coated with poly-(5% diphenyl-/ 95% dimethyl-siloxane) (Supelco Inc., Bellefonte, PA) was used. To obtain and collect the thermoxidation products of MLHP, a DANI gas chromatograph (Milan, Italy) model Dani 3600, equipped with a 4-mm i.d. glass-liner injector, was used.

RESULTS AND DISCUSSION

The four MLHP isomers, prepared by thermoxidation and subsequently purified by SPE and TLC, were degraded in the GC glass liner at 310°C (Fig. 1). In the present work, the group of peaks with boiling points and gas-chromatographic retention times higher than those of methyl linoleate was considered (Fig. 1B); whereas the volatiles, that eluted before methyl linoleate (Fig. 1A) were not studied in depth. Analysis of the products of interest was carried out first by evaluating their chromatographic behavior, which was primarily linked to their polarity and to the number of oxygen atoms, and by considering the prevalent peaks or the group of peaks. The procedure for identifying the peaks included evaluation of the TLC and GC retention times, which were compared with well-known chromatographic behavior of the corresponding methyl oleate derivatives (15).

The products were also subjected to specific reactions to reveal the presence of hydroxy (TMS derivatization) and epoxy groups (BF_3–methanol reaction); GC–MS fragmentations were used for their final identification.

The first preparative step degraded the MLHP in a GC liner at 310°C, and the secondary products were collected in

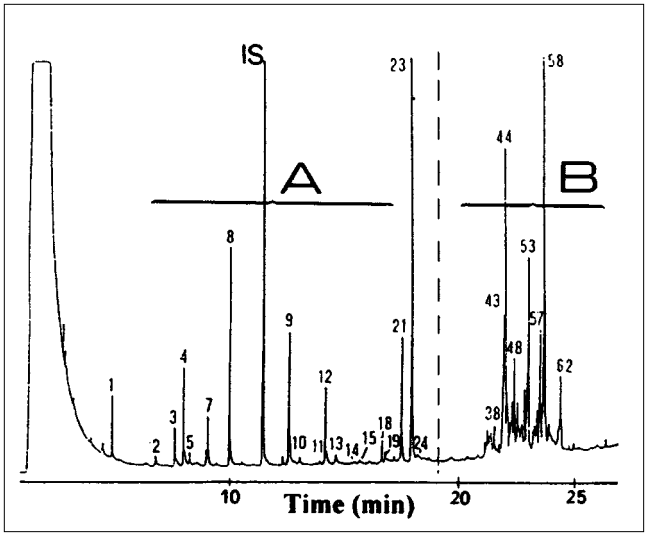

FIG. 1. Gas chromatographic trace of the total hydroperoxides mixture. A) volatiles; B) nonvolatiles. See text for peak identification.

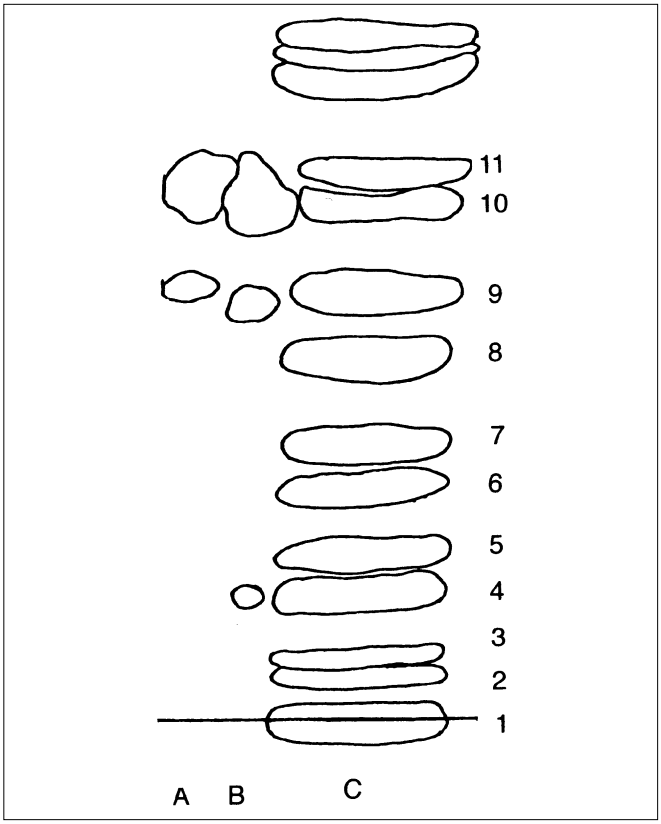

FIG. 2. Thin-layer chromatography of the glass-liner thermo-degraded products of methyl linoleate hydroperoxides: A) methyl linoleate; B) 13-MLHP; C) spots of thermo-degraded products of MLHP. Spot identifications: 10 and 11, methyl linoleate isomers; 8 and 9, epoxy derivatives; 6 and 7, keto and epoxy-keto derivatives; 4 and 5, hydroperoxide isomers; 2 and 3, hydroxy and epoxy-hydroxy derivatives.

a short packed silicic acid glass column. The products were then extracted and analyzed by TLC (Fig. 2), and the spots were compared to those of methyl oleate (15). The identification of the spots, in decreasing order of TLC retention factor was: 10 and 11, methyl linoleate isomers; 8 and 9, epoxy derivatives; 6 and 7, keto and epoxy-keto derivatives; 4 and 5, hydroperoxide isomers; 2 and 3, hydroxy and epoxy–hydroxy derivatives.

The components, resolved by TLC, were subsequently analyzed by GC to isolate and identify each peak.

Spot numbers 10 and 11 show the presence of peaks 26 and 27, recognized as methyl linoleate isomers, on the basis of their MS fragmentations.

GC–MS analyses of spot numbers 8 and 9, which could correspond to the epoxy-derivatives, did not give any significant information. However, GC isolation of the epoxy derivatives was difficult because of their low concentration in this mixture; in fact, they are supposed to be primarily generated by a bimolecular reaction between methyl linoleate and hydroperoxides (21). Based on this reaction mechanism, thermo-oxidized methyl linoleate (150°C for 3 h) was used for identification of the corresponding epoxy derivatives; Figure 3 shows a part of the GC trace of this total mixture. High amounts of peaks a, b, c, and d are observed in this trace; moreover, these peaks are located in the elution region of peaks 40–44 of the previous total hydroperoxides trace. Their identity was confirmed by the positive reaction with boron trifluoride in methanol (22), which results in the opening of the oxirane rings, and was supported by the negative response to the silylation procedure. On the basis of their quantitative rates, peaks a and b were supposed to be the threo (thermodynamically favored), and the c and d were the erythro geometrical isomers. Their geometric configuration was, in fact, confirmed by synthetizing mono-epoxides from pure methyl linoleate and methyl linoelaidate. Regarding the position of the epoxide bridge, it is known that, for methyl linoleate, the more favored positions are 9,10 and 12,13, but other isomers, such as the epoxy derivatives of conjugated linoleate, can be present in low amounts.

The single TLC spot numbers 6 and 7 were recognized as mono- and di-oxygenated compounds, such as keto- and epoxy-keto derivatives. The GC trace of those spots (Fig. 4) showed the presence of peak numbers 51, 52, and 58, which could not be silylated. Moreover, the corresponding MS spectra of peak numbers 51 and 52 showed typical fragmentations of diunsaturated keto esters isomers (7): molecular peak at *m/z* 308, and fragments at *m/z* 151, 277 ($M^+ - 31$), 237, 209 (237–28). Furthermore, considering the fragmentations at *m/z* 99, 177 of peak 52 and at *m/z* 166 (23), 185 of peak 51, it is reasonable to propose the following elution order: peak 51 = 9-keto,$\Delta^{10,12}$- and peak 52 = 13-keto,$\Delta^{9,11}$-octadecadienoate. The TLC spots that corresponded to the keto derivatives also contained peak number 53. In fact, this peak is more abundant in the total hydroperoxides and thermo-oxidated methyl linoleate GC traces than in that of the 13-hydroperoxide; this might be due to the coelution of more than one component, part of which undergo silylation. Furthermore, as observed in its mass spectrum, it displayed typical fragmentation of a keto derivative.

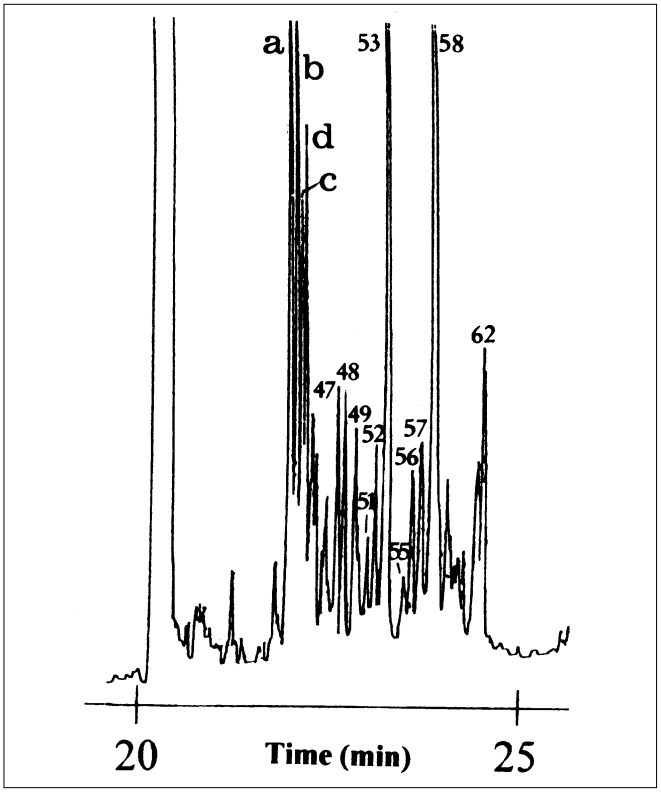

FIG. 3. Detail of the gas chromatographic trace of thermo-oxidized (150°C for 3 h) methyl linoleate.

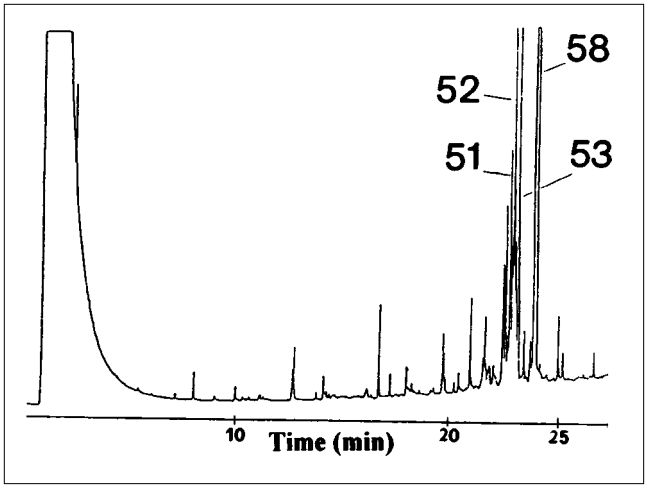

FIG. 4. Gas chromatographic trace of thin-layer chromatographic spot number 7 that shows the keto derivatives (peak numbers 51, 52, and 58).

With respect to peak number 58, it did not undergo silylation and showed a typical fragmentation of a keto derivative. Because of the presence of a fragment at *m/z* 253 in its mass spectrum, it could probably be a mixture of keto-epoxy isomers, the most important being 9,10-epoxy-13-keto,Δ^{11}-octadecenoate and 9-keto-12,13-epoxy,Δ^{10}-octadecenoate (4). To confirm the identification of the peak as an epoxy-keto derivative, spots 6 and 7 were treated with BF_3–methanol and then silylated. The fragments at *m/z* 173, 255 and 185, characteristic of 12-methoxy-9-oxo-13-trimethylsilyloxy-10-octadecenoate, and those at *m/z* 99, 259, 169, and 242 (169 + 73), typical of the isomer 10-methoxy-13-oxo-9-trimethylsilyloxy-11-octadecenoate (24), confirmed the coelution of the keto-epoxy positional isomers as peak 58.

TLC fraction numbers 5 and 4 corresponded to the MLHP; in fact, fraction number 4 shows the same Rf as methyl linoleate 13-hydroperoxide (Fig. 2B). The GC traces of these fractions, as well as the total hydroperoxides trace, contain all degradation and volatile products. The quantitative differences between the traces of these two spots could suggest a partial TLC separation of the 9- and 13-positional isomers.

The GC trace of the last TLC spots (numbers 3 and 2) (Fig. 5) revealed the presence of peak numbers 48, 55 and 56, which correspond to hydroxy derivatives. The mass spectrum of peak 48 shows a molecular fragment at *m/z* 310, as well as the following characteristic fragments: *m/z* 292 ($M^+ - 18$), 279 ($M^+ - 31$), 261 (292–31), 239 {$[M^+] - [CH_3(CH_2)_4\text{-}]$}, 221 (239–18), 153 {$[M^+] - [-(CH_2)_7COOCH_3]$}, 135 (153–18) (10). For these reasons, it could be identified as one of the most representative mono-hydroxy derivatives and, due to its mass fragmentation and to its high concentration in the 13-hydroperoxide GC trace, it could be proposed that peak 48 corresponds to the 13-hydroxy isomer. The identification of peak 48 was confirmed by the silylation procedure and the typical *m/z* 311 and 225 fragments of the corresponding mass spectra (3). According to its mass spectrum and the relative intensities of the fragments at *m/z* 225 and 311, peak 47 (Fig. 3) can be identified as 9-hydroxy,$\Delta^{10,12}$-octadecadienoate. On the other hand, peaks 55 and 56 have the typical fragmentations of di-oxygenated compounds, which can easily lose water: *m/z* 308 [326 (M^+) – 18], 277 (308–31), 255, 237 (255–18), 151 (169–18). On the basis of some other fragments, such as *m/z* 213 and 195 (213–18), it could be suggested that peak 55 corresponds to 9-hydroxy-12,13-epoxy,Δ^{10}-, whereas peak 56, showing a fragment

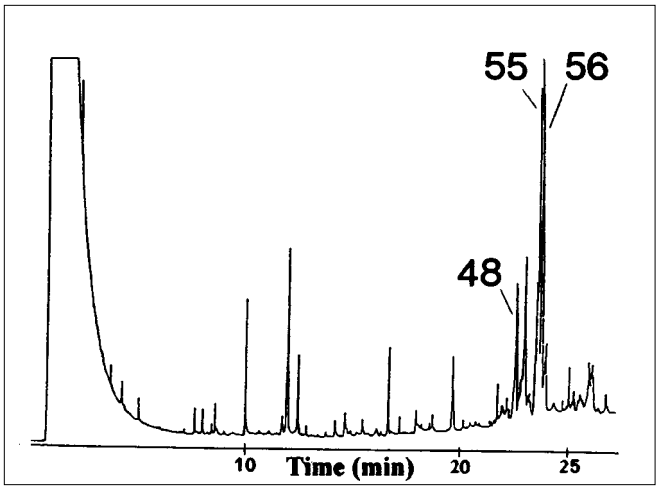

FIG. 5. Gas chromatographic trace of thin-layer chromatographic spot number 2 that shows the hydroxy derivatives (peak numbers 48, 55, and 56).

at *m/z* 109 (127–18), could be 9,10-epoxy-13-hydroxy,Δ^{11}-octadecenoate. GC–MS analysis of the silylated mixture confirms these identifications. In fact, peak 55 (as TMS ether) showed typical fragments at *m/z* 259, 241 and 99, whereas MS of peak 56 yielded fragments at *m/z* 185, 173, and 103 (24). Presence of the epoxide ring was underlined by positive reaction with BF_3–methanol.

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