

## Characterization by NMR of Ozonized Methyl Linoleate

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No presente estudo o linoleato de metila ozonizado com índice de peróxidos de 1.800 mmol-equiv. kg<sup>-1</sup> foi caracterizado quimicamente. Os efeitos do ozônio sobre o linoleato de metila produz hidroperóxidos, ozonídeos e aldeídos, os quais foram identificados por técnicas de ressonância magnética nuclear de <sup>1</sup>H e <sup>13</sup>C, mono- e bidimensional. O linoleato de metila padrão e o linoleato de metila ozonizado mostram espectros muito similares, excetuando os valores de ressonância ( $\delta$  9,7 e  $\delta$  9,6) que correspondem aos hidrogênios aldeídicos ( $\delta$  5,7 e  $\delta$  5,5) e olefínicos de hidroperóxidos ( $\delta$  5,2). Outras atribuições estão baseadas nas conectividades fornecidas pelas constantes de acoplamento. Estes resultados indicam que a espectrometria de RMN pode oferecer informação valiosa a respeito da quantidade de compostos oxigenados formados à partir do linoleato de metila ozonizado para o uso em ozonoterapia e na química de óleos vegetais ozonizados.

In the present study ozonized methyl linoleate with peroxide index of 1,800 mmol-equiv kg<sup>-1</sup> was chemically characterized. Ozonation of methyl linoleate produced hydroperoxides, ozonides and aldehydes which were identified by <sup>1</sup>H and <sup>13</sup>C NMR two-dimensional. The standard methyl linoleate and ozonized methyl linoleate shown very similar <sup>1</sup>H NMR spectra except for the signals at  $\delta$  9.7 and  $\delta$  9.6 that correspond to aldehydic hydrogen,  $\delta$  5.7 and  $\delta$  5.5 (olefinic signals from hydroperoxides) and  $\delta$  5.2 ppm (multiplet from ozonides methynic hydrogen). Other resonance assignments are based on the connectivities provided by the hydrogen scalar coupling constants. These results indicate that NMR spectroscopy can provide valuable information about the amount of formed oxygenated compounds in the ozonized methyl linoleate in order to use it to follow up ozone therapy and chemistry of ozonized vegetable oil.

**Keywords:** methyl linoleate, ozone, ozonides, aldehydes, NMR

### Introduction

Linoleic acid is one of the most important components of vegetable oils and lipids.<sup>1</sup> The determination of molecular structure of ozonized fatty acids is one of aim of scientists which work with ozonized vegetable oils and ozone therapy.<sup>2</sup>

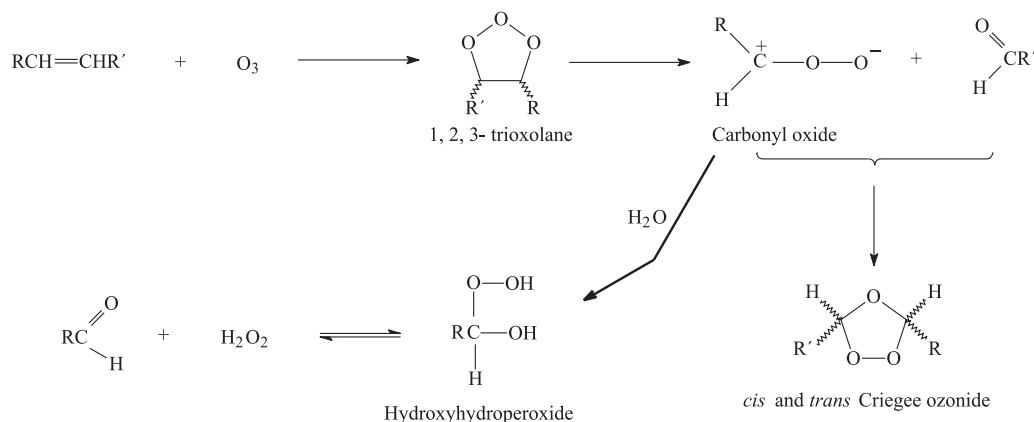
Many studies have been carried out to develop new analytical techniques that, with very little or any manipulation of the sample, can afford the identification of the vegetable oil and lipids.<sup>3,4</sup> Specifically, one spectroscopic technique with a high potential in this field is High-Resolution Nuclear Magnetic Resonance Spectroscopy. Both <sup>1</sup>H and <sup>13</sup>C NMR have already been

employed in the analysis of vegetable oil and biological samples such as lipids.<sup>5,6</sup> However, data on the spectra of ozonized fatty acids is scarce in the literature.

The reaction of ozone with vegetable oil and lipids occurs almost exclusively with the carbon-carbon double bonds present in unsaturated fatty acids. Different kinds of oxygenated products are formed (hydroperoxides, ozonides and aldehydes) that probably are responsible for the biological activity of ozonized fatty acids.<sup>7-9</sup> The mechanism of this reaction is well known (Criegee mechanism, Figure 1), as well as the conditions necessary to enhance the preferential formation of any of these oxygenated compounds.<sup>10,11</sup>

Of all the natural fatty acids, linoleic acid is one of the most widely distributed and is present in practically all lipids. For this reason, methyl linoleate was chosen as a model compound to be ozonized and chemically

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**Figure 1.** Mechanism of reaction of ozone with unsaturated fatty acids.

characterized. In this study the products of ozonized methyl linoleate were analyzed applying  $^1\text{H}$ ,  $^{13}\text{C}$  and 2D COSY NMR spectroscopy.

## Experimental

### General ozonization procedure

A mixture of 1.6 mL ( $0.0048 \text{ mol L}^{-1}$ ) of methyl linoleate (99%) and 0.16 mL of water were introduced into a bubbling reactor where ozone reaction took place at room temperature.<sup>7,8</sup> The reaction was continued during 7.25 minutes and one sample was taken at applied ozone doses  $245 \text{ mg g}^{-1}$ . The samples were stored at  $-80^\circ\text{C}$  until NMR analysis. Methyl linoleate were purchased from Sigma Chemical Co. (St. Louis, MO).

### Ozone generation

Ozone was generated by passing oxygen through a 12-02 model ozone generator of Trailigaz Company (France) at a fixed voltage (170 V) and constant flow rate of  $42 \text{ L h}^{-1}$ . The initial ozone concentration ( $69 \text{ mg L}^{-1}$ ) was determined by an Ozomat model equipment of Anseros Company (Germany).

### Measurement of NMR spectra

$^1\text{H}$ ,  $^{13}\text{C}$ , DEPT 135 and 2D COSY NMR spectra were obtained in a BRUKER 9.4 Tesla AVANCE Spectrometer with  $\text{CDCl}_3$  as solvent and tetramethylsilane (TMS) as internal reference. The  $^1\text{H}$  NMR spectra were obtained at 5 kHz spectral width, 60 degree pulse width (5  $\mu\text{s}$ ), 8 scans, and 64 kbytes of memory were used to obtain the spectra.  $^{13}\text{C}$  NMR spectra were recorded operating at 100 MHz and were obtained using the following

acquisition parameter: 64 k of acquisition point; spectral width 220 ppm; relaxation delay, 2 s; a total of 800 scans was collected for sample with a  $45^\circ$  excitation pulse. The experiment (distortionless enhancement by polarization transfer, DEPT) were obtained using variable pulse  $\theta = 135^\circ$ . The 2D  $^1\text{H}$ - $^1\text{H}$  correlation spectroscopy (COSY) and Heteronuclear Simple Quantum Correlation (HSQC) spectra were obtained with a digital resolution of 5.425 Hz after zero filling. Zero filling (one) were done in the F1 dimension of a  $512 \times 512$  matrix, the data were 2D transformed and the magnitude spectra multiplied by a sine window in each dimension and symmetrized along the diagonal.<sup>12</sup>

### Peroxide index determination

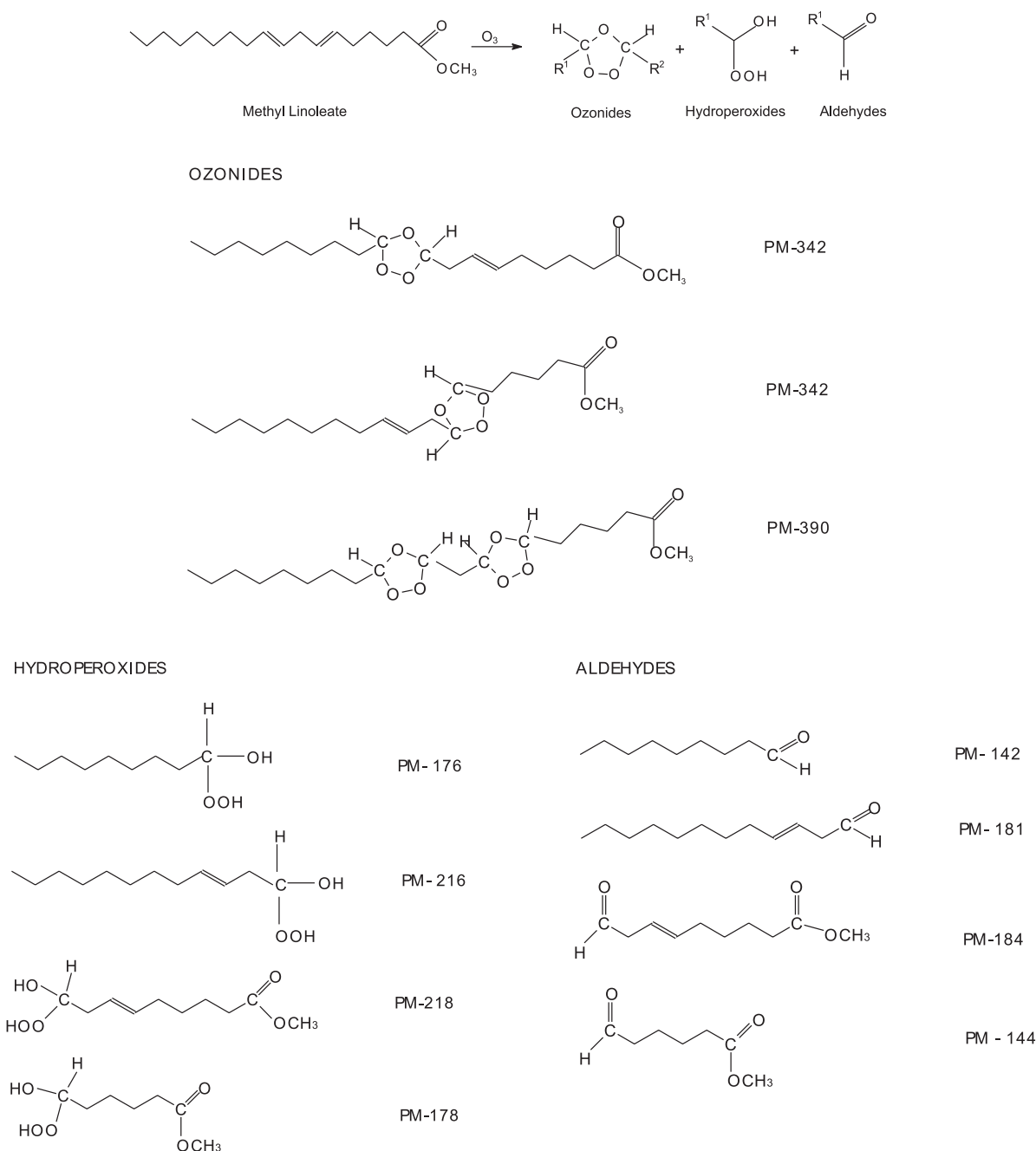
The peroxide index represents the number of mmol-equivalents of active oxygen that expresses the amount of peroxide contained in 1.000 g of the methyl linoleate. Briefly, a sample of 5 g was mixed with 30 volumes of glacial acetic, 20 volumes of chloroform and 0.5 mL of saturated potassium iodide solution. The mixture was shaken for exactly 1 minute, mixed with 30 mL of water and slowly titrated; shaking continuously, with  $0.01 \text{ mol L}^{-1}$  sodium thiosulphate until the yellow color almost disappears. The peroxide index values were obtained from the expression  $10 v/m$  where  $v$  is the volume of sodium thiosulphate in mL consumed in the titration, and  $m$  is the weight in g, of substance taken.<sup>13</sup> The peroxide index (PI) was expressed in mmol-equiv.  $\text{kg}^{-1}$ .

## Results and Discussion

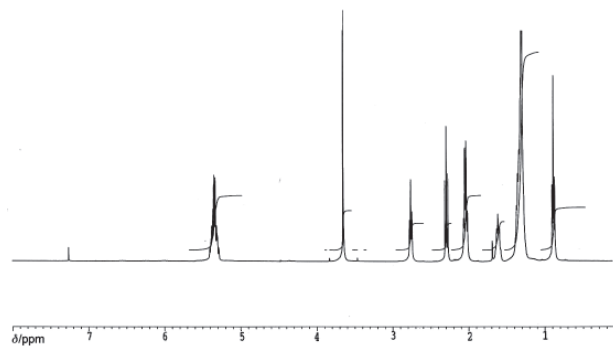
The aim of this study was to characterize oxygenated products in ozonized methyl linoleate. Since vegetable oils and lipid consist almost entirely of triglycerides

molecules, no appreciable difference was anticipated between the chemical shift values of these vegetable oils and their component esterified fatty acids.<sup>14</sup> In our experiment for preparation of ozonized methyl linoleate, about 245 mg g<sup>-1</sup> of ozone was absorbed *per* 1.6 mL of methyl linoleate which seemed to be enough to obtain ozonized methyl linoleate with 1,800 mmol-equiv. kg<sup>-1</sup> of peroxide index. Scheme 1 shows possible ozonides, hydroperoxides and aldehydes can be obtained in ozonized methyl linoleate.

Figure 2 shows <sup>1</sup>H NMR spectrum from methyl linoleate, presenting a single peak at  $\delta$  7.3 which belongs to the chloroform-d, multiplet peaks at  $\delta$  5.3 which belong to olefinic signals from the fatty acid,  $\delta$  3,6 (single peak from methylic hydrogen of ester),  $\delta$  2.7 (triplet from methylenic group between olefinic hydrogen);  $\delta$  2.3 (triplet from methylenic groups in  $\alpha$  position with respect to carbonylic group);  $\delta$  2.0 (multiplet from methylenic group in both sides of olefinic hydrogen);  $\delta$  1.6 (multiplet from methylenic group in  $\beta$  position with respect to carbonylic



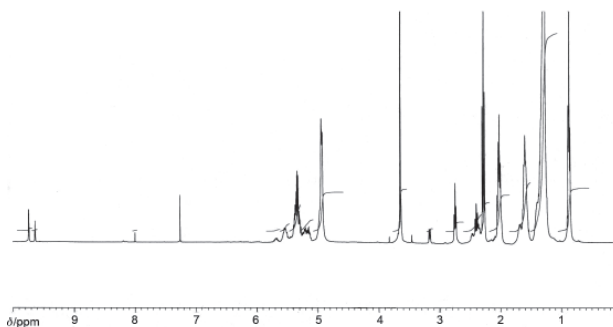
**Scheme 1.** Oxygenated compounds obtained of the reaction of ozone with methyl linoleate.



**Figure 2.**  $^1\text{H}$  NMR spectrum of methyl linoleate in  $\text{CDCl}_3$  in 9.4 Tesla equipment.

group);  $\delta$  1.3 (signal from methylenic groups in fatty acid chain); and  $\delta$  0.9 (triplet from terminal methyl group).

$^1\text{H}$  NMR spectrum from ozonized methyl linoleate is displayed in Figure 3. This spectrum has the same observed signals in methyl linoleate (Figure 2) and additionally other eight signals at  $\delta$  9.7 and  $\delta$  9.6 from aldehydic hydrogen,  $\delta$  5.7 and  $\delta$  5.5 (olefinic hydrogen signal from hydroperoxides),  $\delta$  5.2 (multiplet from ozonides),  $\delta$  3.2 (doublets from methylenic hydrogen allylic of olefinic hydrogen),  $\delta$  2.4 and  $\delta$  1.62 (multiplet from formed ozonides hydrogen). These formed additionally signals are oxygen compounds which seem to be responsible for the germicide effect of ozonized vegetable oil and metabolites generated by application of systemic ozone therapy.<sup>15-17</sup>

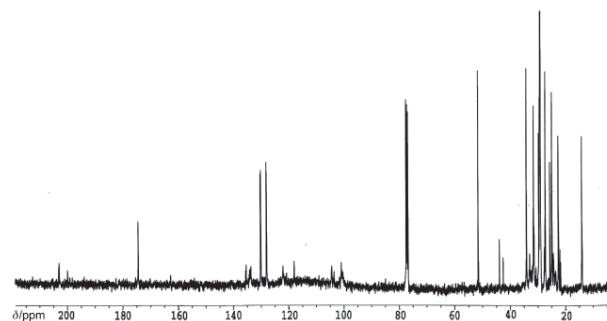


**Figure 3.**  $^1\text{H}$  NMR spectrum of ozonized methyl linoleate in  $\text{CDCl}_3$  in 9.4 Tesla equipment.

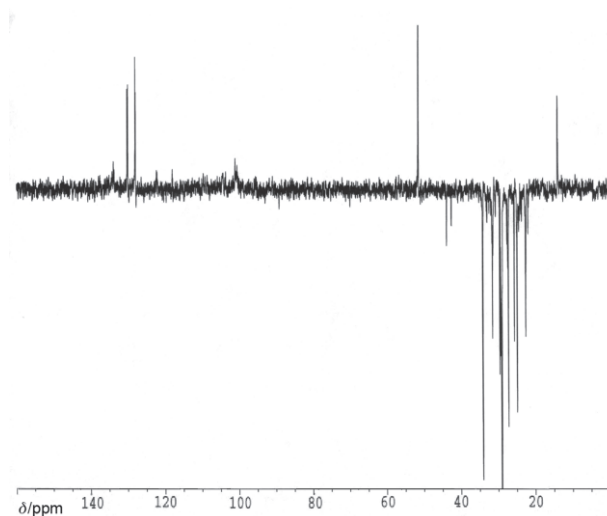
The  $^{13}\text{C}$  NMR spectrum of methyl linoleate contains resonance similar of carbons from the triglyceride fraction of vegetable oil, *i.e.*, the fatty acid signals.<sup>18,19</sup> However a new group of signals were found in the spectrum from ozonized methyl linoleate (Figure 4). The  $^{13}\text{C}$  NMR spectrum are grouped in four sets of signals, aldehyde carbons resonating from  $\delta$  199.0 to  $\delta$  203.0, carbonyl carbon resonance from  $\delta$  174.4, unsaturated carbons in the range from  $\delta$  127.0 to  $\delta$  136.0 ppm, methylenic carbons corresponding to ozonides at  $\delta$  100.0 to  $\delta$  104.0, and aliphatic carbons from  $\delta$  14.0 to  $\delta$  44.0.

### Spectral region $\delta$ 14.0-44.0

Figure 4 and 5 shows  $^{13}\text{C}$  and DEPT NMR spectra from ozonized methyl linoleate. The aliphatic carbons of methyl and methylenic groups resonate in the range of  $\delta$  14.0-44.0. The different signals are resolved on the basis of chain double bond numbers: linoleic chains (C18:2 9,12c) resonate from high to low frequency. The chemical shift can be predicted by the additive relationship for the normal alkanes based upon the number of  $\alpha$ ,  $\beta$  and  $\gamma$  carbon atoms in the molecule.<sup>20</sup> The terminal methyl carbon shift C-18 is found at  $\delta$  14 ppm. Four methylene groups are readily identified: C-17 at  $\delta$  22.5 methylenic acyclic chains; C-3 at  $\delta$  24.9,  $\delta$  24.3 and  $\delta$  24.2 methylenic group in  $\beta$  position with respect to carbonylic group; C-11 at  $\delta$  25.6 methylenic group between olefinic hydrogen of linoleic chains; C-11, C-14, and C-8, at  $\delta$  27.1,  $\delta$  27.5 allylic carbons of linoleic chains;  $\delta$  29.0-29.5 methylenic groups in fatty acid central chain; C-16 at  $\delta$  31.5 methylenic acyclic chains  $\omega$ ; C-2 at  $\delta$  34.0 linoleic as a single signal but well resolved from saturated chains. New signals were observed at  $\delta$  24.9,  $\delta$  24.3, and  $\delta$  24.2 belong



**Figure 4.**  $^{13}\text{C}$  NMR spectrum of ozonized methyl linoleate in  $\text{CDCl}_3$  in a 9.4 Tesla equipment.



**Figure 5.** DEPT135 NMR spectrum of ozonized methyl linoleate in  $\text{CDCl}_3$  in a 9.4 Tesla equipment.

to methylenic carbons of ozonides and methylenic carbons of ozonides and hydroperoxides at  $\delta$  42.5 and  $\delta$  43.8.

#### Spectral region $\delta$ 127.0-136.0

The resonance of unsaturated carbons of long chain of ozonized methyl linoleate spread over the chemical shift range from  $\delta$  127.0-136.0. The carbons C-9 at  $\delta$  127.9, C-10 at  $\delta$  130.0, C-12 at  $\delta$  128.0 and C-13  $\delta$  130.1 corresponding to linoleic unsaturated carbons. The additional signals at  $\delta$  133.6,  $\delta$  135.3 and  $\delta$  135.5 methylenic carbons corresponding to ozonides and hydroperoxides.

#### Spectral region signals $\delta$ 102.0-105.0

In this spectral region signals corresponding to methylenic carbons of ozonides appear at  $\delta$  103.5 and  $\delta$  104.3.

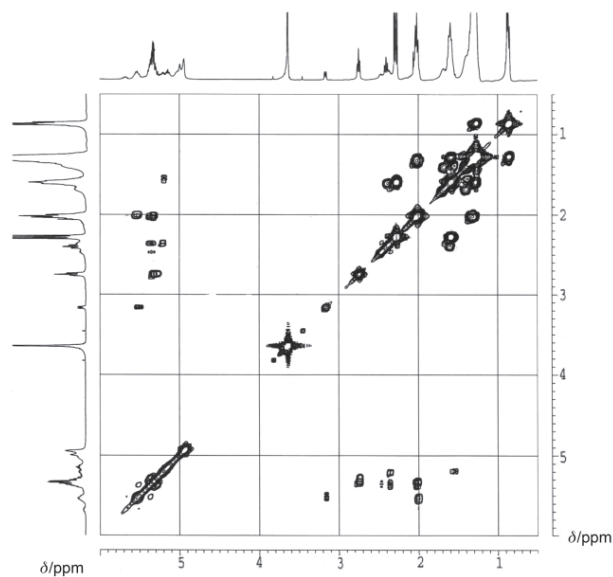
#### Spectral region $\delta$ 174.0-203.0

In this spectral zone the carbonyl carbons at  $\delta$  174.4 belong to ester function can be observed and also the aldehydes carbons resonating at  $\delta$  199.8,  $\delta$  202.9 and  $\delta$  203.1.

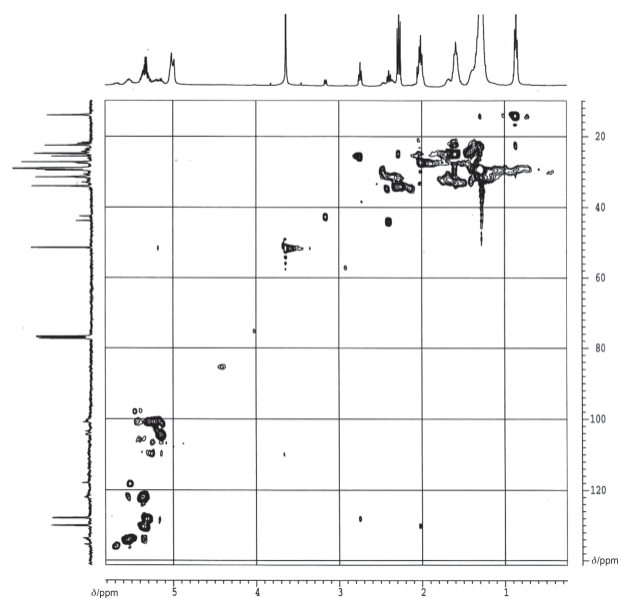
The DEPT experiment was applied to obtain  $^{13}\text{C}$  NMR spectra over the whole carbon-13 frequency range with the purpose of producing  $^{13}\text{C}$  NMR resonance for better structural elucidation. The only drawback was the loss of carbonyl carbons resonance, which is not detected by the DEPT sequence (Freeman<sup>21</sup>).

The assignments of the various signals were accomplished by using a combination of 2D COSY techniques  $^1\text{H}$ - $^1\text{H}$  and HSQC (Figure 6 and 7). To explain the detailed interpretation of the  $^1\text{H}$ - $^1\text{H}$  NMR COSY spectra, the  $^1\text{H}$  NMR spectrum showed six new signals:  $\delta$  5.7,  $\delta$  5.5,  $\delta$  5.2,  $\delta$  3.2,  $\delta$  2.4 and  $\delta$  1.6. From the  $^1\text{H}$ - $^1\text{H}$  NMR COSY correlation spectrum (Figure 6), it was clear that the multiplet at  $\delta$  5.5 (olefinic hydrogen signal could belong to hydroperoxides) was correlated to the hydrogen at  $\delta$  3.2 (doublets from methylenic hydrogen allylic of olefinic hydrogen); and  $\delta$  5.2 (multiplet from ozonides) was correlated to the  $\delta$  2.4 and  $\delta$  1.6 (multiplet from formed ozonides hydrogen).

The  $^{13}\text{C}$  spectrum showed various new signals:  $\delta$  135.5-133.6;  $\delta$  122.0-118.0;  $\delta$  104.4-100.9;  $\delta$  43.8;  $\delta$  42.5;  $\delta$  23.5-23.9. From the HSQC correlation spectrum (Figure 7), we observed that the multiplet at  $\delta_{\text{H}}$  5.7 was correlated to the carbon atoms  $\delta_{\text{C}}$  135.5-135.3 and the multiplet  $\delta_{\text{H}}$  5.5 was correlated to the carbon atom  $\delta_{\text{C}}$  133.6 spectral region of unsaturated carbons. The multiplet  $\delta_{\text{H}}$  5.2 was correlated to the carbon atoms  $\delta_{\text{C}}$  104.4,  $\delta_{\text{C}}$  103.5 and  $\delta_{\text{C}}$  100.9, they belong to methylenic carbons



**Figure 6.**  $^1\text{H}$ - $^1\text{H}$  NMR correlation spectroscopy 2D NMR spectrum of ozonized methyl linoleate in 9.4 Tesla equipment.

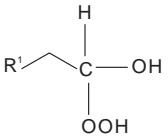
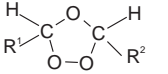
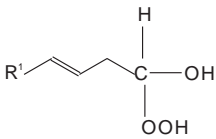
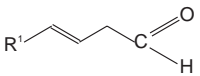
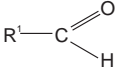


**Figure 7.** HSQC correlation spectrum of ozonized methyl linoleate in 9.4 Tesla equipment.

from ozonides and oligomers. These assignments are similar to those reported by Miura working with ozonated olive oil.<sup>22</sup> Signal of methylenic carbon at  $\delta_{\text{C}}$  43.8 was correlated to the  $\delta_{\text{H}}$  2.4 of ozonides. Other signal of methylenic carbon at  $\delta_{\text{C}}$  42.5 was correlated to the  $\delta_{\text{H}}$  3.2 (doublets from methylenic hydrogen allylic of olefinic hydrogen).

For a better explanation, Table 1 shows the main chemical shift and structures of the oxygenated compound of ozonized methyl linoleate. In the structural elucidation of the ozonized methyl linoleate, the assignments of various signals (ozonation products) were accomplished by using a combination of 2D COSY

**Table 1.** Ozonized methyl linoleate functional group chemical shift (ppm)

functional group	$\delta$ $^1\text{H}$	$\delta$ $^{13}\text{C}$
$-\text{CH}_3$	0.9	14
$-(\text{CH}_2)_n$	1.30	29-29.5
$-\text{CH}_2-\text{CH}_2-\text{CO}-$	1.6	24.9
$-\text{CH}_2-\text{CH}=\text{}$	2.0	27.1-27.5
$-\text{CH}_2-\text{CO}-$	2.3	22.5
	2.4	43.8
$-\text{CH}=\text{CH}-\text{CH}_2-$	2.7	25.6
$\text{R}'\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}(\text{OH})\text{OOH}$	3.2	42.5
$-\text{COOCH}_3$	3.6	51.4
	5.2	100-104
$-\text{CH}=\text{CH}-$	5.3	127-130
	5.5	133.6-135.5
	9.6	199.8
	9.7	202.9-203.1

techniques  $^1\text{H}$ - $^1\text{H}$  and HSQC with  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. In this study all functional groups of the ozonation products were well characterized as ozonides, hydroperoxides and aldehydes present in ozonized methyl linoleate. These reaction products were identified according to Criegee mechanism.<sup>10</sup> The ozonides of methyl oleate have been considered compounds with biological activity, which has been demonstrated by Díaz *et al.*<sup>15</sup> The elucidation and chemical characterization of reaction products from ozonized methyl linoleate are important for ozone therapy follow up and new ozonation strategies with vegetable oils.

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