Characterization of ozonated vegetable oils by spectroscopic and chromatographic methods

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Received 28 June 2007; received in revised form 18 October 2007; accepted 18 October 2007
Available online 25 October 2007

Abstract

In this work the effect of ozonation on olive oil, soybean oil, oleic-, linoleic- and linolenic acid was studied. The effects of ozonation time on the oils and acids were analyzed by \textsuperscript{1}H, \textsuperscript{13}C NMR. Further, the peroxide- and acid values, the viscosity and the molar mass were determined for pure and ozonated oils.

The fatty chains in both ozonated oils showed a gradual decrease of unsaturation with the gradual increase of ozonation time. Reaction products were identified according to Criegee mechanism. The major product in the early stage of the reaction was ozonide. The disappearance of unsaturation and formation of ozonide was almost equal. Ozonation increased the peroxide and acid values for both oils, the increase being higher for soybean oil. After long ozonation times higher molar mass species, as well as low molar mass species were observed. These are interpreted as oligomeric ozonides and cross-ozonides, respectively.

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Keywords: Ozonated soybean oil; Ozonated olive oil; Ozonated unsaturated fatty acids; NMR; GPC; Criegee mechanism

1. Introduction

The unsaturated triglycerides in vegetable oils give the oil many favorable properties. The double bonds are frequently also used for chemical modification of the oil, a typical example of which is hydrogenation. Ozonation of unsaturated triglycerides is another reaction that has gained much attention recently and ozonated vegetable oils have been proposed to be used in a variety of applications, e.g. as modifiers in biodiesel fuel (Soriano and Migo, 2005, 2006; Matsumura, 2002) and cutting fluid emulsions (John et al., 2004). Further, ozonated vegetable oils have been attributed antibacterial and fungicidal effects with applications in, e.g. food-, cosmetic- and pharmaceutical industry (Sechi et al., 2001; Geweely, 2006; Rodríguez et al., 2007; Díaz et al., 2006a,b; Guzel-Seydim et al., 2004; Ruiz et al., 2006; Valacchi et al., 2005; Bocci, 2006). The therapeutic use of ozonated vegetable oil is, however, still a rather controversial subject: although ozone and ozonated vegetable oils have been attributed many favorable therapeutic effects, their use has not been widely accepted in orthodox medicine (Bocci, 2006).

Several methods are available for characterization of ozonated vegetable oils, e.g. \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, FT-IR, determination of peroxide- and acidity values, viscosity measurements, GPC, GC, GC-MS, etc. The reaction of ozone with vegetable oils produces several oxygenated compounds such as hydroperoxides, ozonides, aldehydes, peroxides, di- and polyperoxides (John et al., 2004; Díaz et al., 2005, 2006a; Soriano et al., 2003a,b). The reaction of ozone with unsaturated fatty acid ester in triglycerides is well described by the Criegee mechanism (Criegee, 1975; Bailey, 1978).

In this work olive and soybean oil were ozonated for different periods of time and analyzed. Further, oleic, linoleic and linolenic acid, the esters of which are present in the triglycerides in said oils, were similar ozonated for different periods of time and analyzed. The effects of ozonation and ozone absorption on the chemical composition of the oils and fatty acids were studied.

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by $^1$H and $^{13}$C NMR. Further, the viscosities, molar masses, peroxide and acid values were determined for the pure and ozonated oils.

2. Experimental

2.1. Solvents and reagents

Oleic acid (98%), linoleic acid (99%), linolenic acid (70%), soybean oil were obtained from Sigma–Aldrich. Olive oil and acetic acid (99.5%) was obtained from Fluka, potassium iodide (99.5%) from FF-Chemicals Oy Ab and ethanol from Altia Corporation. Chloroform (99.5%), sodium thiosulfate, starch, phenolphthalein, sodium hydroxide and sulfuric acid (96%) were obtained from J.T. Baker. Air (dry and oil free) was obtained from Woikoski Oy Ab. The chemicals were used without further purification.

2.2. Ozonation procedure

Ozone was produced by flowing air at a constant flow-rate of 5 l/min through a Gebr. Hermann Köln ozone generator. The voltage was set to 150 V for all experiments. The generated ozone/air mixture was led to a reaction vessel where ozone was bubbled through the oils or the fatty acids, respectively. The off-gas containing non-reacted ozone was led to an absorption vessel. Ozone reacted with iodide to form free iodine:

$$\text{O}_3 + 2I^- + H_2O \rightarrow 2OH^- + O_2 + I_2$$

The formed iodine was then titrated with sodium thiosulfate

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}$$

with starch as indicator. The equivalence point was indicated as a color change from purple to colorless.

Thus, the ratio of the amount of ozone to the amount of thiosulfate for total reaction was

$$n(O_3) : n(S_2O_3^{2-}) = 1 : 2$$

The rate of ozone production was determined by absorbing ozone directly into acidic potassium iodide solution in the absorption vessel in a blank experiment. The amount of ozone produced, $n_{\text{PROD}}(O_3)$, was thus given by

$$n_{\text{PROD}}(O_3) = 0.5V_{\text{BLANK}}(S_2O_3^{2-})c(S_2O_3^{2-})$$

where $V_{\text{BLANK}}(S_2O_3^{2-})$ is the titrated volume thiosulfate solution in the blank experiment and $c(S_2O_3^{2-})$ is the concentration of the thiosulfate solution.

The amount of ozone consumed by the oils or fatty acids, $n_{\text{CONSUMED}}(O_3)$, is given by the difference of the amount of ozone produced during the respective ozonation procedure and the non-reacted ozone that was absorbed

$$n_{\text{CONSUMED}}(O_3) = 0.5[V_{\text{BLANK}}(S_2O_3^{2-})$$

$$- V_{\text{OZONATION}}(S_2O_3^{2-})]c(S_2O_3^{2-})$$

where $V_{\text{OZONATION}}(S_2O_3^{2-})$ is the volume thiosulfate solution used to titrate the non-reacted ozone from the ozonation of the oils/fatty acids (Eaton et al., 1998).

2.4. Peroxide value (PV)

Peroxide value is the number that expresses, in milliequivalents of active oxygen, the quantity of peroxide contained in 1000 g of the substance (British Pharmacopoeia, 2000a). The PV of each sample was determined using the American Oil Chemists’ Society (AOCS) official method. The chemical reactions of the AOCS official PV are presented as follows

$$2KI + 2CH_3COOH \rightarrow 2HI + 2CH_3COO^-K^+$$

$$R^*OO^*H + 2HI \rightarrow ROH + H_2O + I_2$$

Peroxides (R$^*$OO$^*$H) are reacted with potassium iodide in the presence of acetic acid, and the liberated iodine is titrated with sodium thiosulfate solution according to reaction (2). AOCS peroxide value is calculated by employing the following formula (AOCS, 1998):

$$PV = Vc \frac{1000}{w}$$

where PV is the peroxide value (meq/kg fat), $V$ the volume Na$_2$S$_2$O$_3$ used, $c$ the concentration of Na$_2$S$_2$O$_3$, $w$ is the mass of fat.

2.5. Acid value (AV)

Acid value is the number of mg of sodium hydroxide required to neutralize the free acids in 1.0 g of the substance (British Pharmacopoeia, 2000b).

Thus, the acid value indicates the degree to which the triglycerides in the oil have broken down to release free fatty acids.

$AV$ of the oil samples was determined by dissolving 2 g of each oil in 2 ml of neutral alcohol in a measuring flask to obtain a solution, which was titrated with sodium hydroxide in triplicate, using phenolphthalein as indicator. The acid value was calculated from

$$AV = Mc \frac{V}{w}$$
Fig. 1. Double bond peaks in the $^{13}$C spectrum for pure oleic acid, linoleic acid, and linolenic acid. Peaks for each characteristic double bond carbon are assigned the letters A for the peak found in all acids, B for the peak found in both linoleic and linolenic acid, and C and C' for the peaks found only in linolenic acid.

where AV is the acid value (mg NaOH/g fat), $M$ the molar mass of NaOH, $c$ the concentration NaOH, $V$ the volume of NaOH used, $w$ is the mass of oil sample.

2.6. NMR analysis

High resolution NMR spectroscopy was used to identify the oil compositions as a function of ozonation time. A Bruker Avance spectrometer operating at 600 MHz was used. The $^1$H NMR spectra were obtained at 9.6 kHz spectral width, 30° pulse width (3 μs), 8 scans and 32 kb of memory were used to obtain the spectra. $^{13}$C spectra were recorded at 238.3 ppm spectral width, relaxation delay 2 s, a total of 500 scans was collected for each sample with a 30° excitation pulse.

2.6.1. Calculation of composition

Fig. 1 reveals the alkene carbon peaks of the $^{13}$C spectra of the three reference fatty acids. Oleic acid possesses one double bond which gives two distinct peaks at 129 ppm (peaks A). Linoleic acid on the other hand has two separate double bonds and therefore has additionally two distinct peaks at 127.5 ppm (peaks B). Finally, linolenic acid has three separate double bonds giving additional peaks at 126.5 ppm (peaks C) and 131 ppm (peaks C’). These peaks were used to identify the alkene composition of the ester chains in the triglycerides. As a basis for such a calculation the integral ratios of the peaks for the pure acids has to be found. In linolenic acid the ratio of peak C to peak B is $R_{CB} = I_C/I_B = 0.277$. Compared to peak A the ratio of peak C is $R_{CA} = I_C/I_A = 0.562$. In linoleic acid the two pairs of distinct peaks have the same intensity. When the $^{13}$C spectra of the triglycerides are analyzed by integration of the peaks it is easy to find the double bond composition in the ester chains by first finding the fraction with three double bonds, then the fraction with two double bonds, and finally the fraction with one double bond.

2.7. GPC-measurements

Molar masses were determined with a Shimadzu GPC instrument with a Sedex 85 Sedere LT-ELSD detector.

The instrument had one guard column (Jordi RP-DVB SM-500, 7.5 mm × 4.6 mm) and two analytical columns, both 30 cm × 7.8 mm, with Jordi Gel DVB 500A and TSG-gel G300, respectively. HPLC-grade tetrahydrofuran was used as carrier solvent at a flowrate of 1.0 ml/min. The samples were dissolved in HPLC-grade tetrahydrofuran to a concentration of 0.5 mg/ml. C18 triglycerides, C17-cholesterol-ester and C18 fatty-acids were used for calibration. The results were analyzed with Shimadzu Class-VP (v. 6.12 SP5) software. The molar masses were calculated from the retention times and the relative amounts from the relative peak areas.

2.8. Density and viscosity measurements

The density measurements were carried out with an Anton-Paar DSA5000 vibrating tube densitometer and the viscosity was measured with Ostwald capillary viscometers at 25–40 °C.

3. Results and discussion

Fig. 2a and b shows the $^1$H and $^{13}$C NMR spectra of non-ozonated and ozonated linolenic acid. Tables 1a and 1b summarize the assignments of all pertinent peaks seen in the
Table 1a

<table>
<thead>
<tr>
<th>13 C Shift [ppm]</th>
<th>1H Shift [ppm]</th>
<th>Functional group</th>
<th>Assignments</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>179</td>
<td></td>
<td>Carboxylic acid</td>
<td>All unsaturated fatty acids</td>
<td>1</td>
</tr>
<tr>
<td>128–130</td>
<td>5.3</td>
<td>CH=CH</td>
<td>All unsaturated fatty acids</td>
<td>1</td>
</tr>
<tr>
<td>61.2; 68.33</td>
<td>4.22; 4.42</td>
<td>CH=OCOR</td>
<td>Triglycerides</td>
<td>1</td>
</tr>
<tr>
<td>24.9</td>
<td>2.8</td>
<td>CH=CHCH=CH</td>
<td>Linolenyl and linoleyl chains</td>
<td>1</td>
</tr>
<tr>
<td>33.19</td>
<td>2.32</td>
<td>CH2–COOH</td>
<td>All acyl chains</td>
<td>1</td>
</tr>
<tr>
<td>26.5</td>
<td>2.04</td>
<td>CH2–CH2COOH</td>
<td>All acyl chains</td>
<td>1</td>
</tr>
<tr>
<td>22.1</td>
<td>1.63</td>
<td>(CH3)3</td>
<td>All acyl chains</td>
<td>1</td>
</tr>
<tr>
<td>29–31</td>
<td>1.3–1.5</td>
<td>CH=CHCH3</td>
<td>Linolenyl chains</td>
<td>1</td>
</tr>
<tr>
<td>33.19</td>
<td>0.95</td>
<td>CH=CH2CH3</td>
<td>Linolenyl chains</td>
<td>1</td>
</tr>
<tr>
<td>13.35</td>
<td>0.89</td>
<td>CH3</td>
<td>All acyl chains except linolenyl</td>
<td>2</td>
</tr>
</tbody>
</table>

studied NMR spectra (Soriano et al., 2003b; Díaz et al., 2003; Ledea et al., 1997). The integrated intensity of the signals was normalized to the terminal methyl group of the acid moiety. This functionality was chosen because it was not affected by ozonation.

A new signal at 5.1 ppm was found in the 1H NMR spectra of ozonated samples. It was assigned to the ring proton of 1,2,4-trioxolane (Soriano et al., 2003a; Díaz et al., 2005) and at 104.5 ppm in 13C NMR spectra, the ring carbon in the same structure. The formation of 1,2,4-trioxolane with linoleic and linolenic acid with several double bonds in the chain shifted the signal at 5.3–5.5 ppm. Ozonated samples exhibited also peaks at 9.6 and 2.5 ppm in 1H NMR corresponding to the aldehydic proton. In 13C NMR, the carbonyl carbon resonated at 200 and 43 ppm, respectively.

The same peaks as for the pure acids are observed also for the oils and in addition peaks originating from the triglycerides. The effect of the ozonation time for the oils is shown in Fig. 3a and b.

The reaction with ozone is suggested to follow the Criegee mechanism. The decrease of the signal at 5.3 ppm in 1H NMR and at 128–130 ppm in 13C NMR is interpreted as the consumption of carbon–carbon bond. This is accompanied by the formation of 1,2,4-trioxolane. The disappearance of unsaturation and the formation of ozonide were almost equal. Further the ozonide to aldehyde ratio, regardless of ozonation time, was always above 90%. This together indicates that the major product in the early stage of the reaction is ozonide. After 2 h ozonation a new peak at 100 ppm in 13C NMR spectra occurs, which is interpreted as formation of small oligomers. This is in good agreement with GPC results (Fig. 4a and b), which show that new molar mass size classes occur, corresponding to dimers respectively trimers. After 20 h ozonation compounds with even higher molar masses were found. A small fraction of compounds with lower molar masses appears after 7 h and after 20 h of ozonation this fraction becomes a bit larger. This is again in good agreement with the Criegee mechanism: cross-ozonation would produce species of low molar mass as well as species of higher molar mass. This is in agreement with the relatively low peroxide and acid values, indicating that only a small part of the reaction results in acid and oligomeric peroxide fragments.

Fig. 5 shows the consumption of the carbon–carbon bond and in Table 2 the concentration of the non-reacted ozone absorbed given as a function of the ozonation time for the studied acids and oils. The ozonation time for complete consumption of carbon–carbon bonds varies from 2.26 h for oleic acid, 2.65 h for linoleic acid and 2.95 h for linolenic acid. The corresponding times for the oils were 20 h. The same trend was obtained by the titration method (Table 2). The difference between the pure acids and the oils could partly be explained by the difference in the volumes of the ozonated samples, but some kind of steric hindrance of the reaction in the oils can not be ruled out.

According to the method described in the experimental section, the composition of the oils is calculated from NMR spectra and is given as a function of the ozonation time in Table 3.

Table 1b

<table>
<thead>
<tr>
<th>13 C Shift [ppm]</th>
<th>1H Shift [ppm]</th>
<th>Functional group</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>104.5</td>
<td>5.1</td>
<td>1,2,4-Trioxolane</td>
<td>D</td>
</tr>
<tr>
<td>202</td>
<td>9.6</td>
<td>Aldehyde</td>
<td>E</td>
</tr>
<tr>
<td>43</td>
<td>2.50</td>
<td>α-Methylene group</td>
<td></td>
</tr>
</tbody>
</table>
The triglyceride fatty chain composition in both ozonated oils showed a gradual decrease in the amount of unsaturated fatty chain with the gradual increase of ozonation time.

Table 4 shows peroxide and acid values for the untreated and ozonated oils studied. The values for non-ozonated oils are in agreement with other published data for corresponding systems. The peroxide values for the ozonated oils are however not as high as reported by others (Diaz et al., 2006a). The lower peroxide values can partly be explained by the fact that no protic solvents were used in this work. An increase in peroxide and acid values was observed in both oils, but it was higher in ozonated soybean oil. These results can be explained by the higher proportion of unsaturated fatty chains in soybean oil than in olive oil.

The viscosity is usually used to characterize the fluid texture. As is seen in Fig. 6, in vegetable oils, viscosity decreases with increasing unsaturation of triglyceride fatty chains. The viscosity decreases with temperature increase. This is due to a higher thermal movement among molecules, reducing intermolecular forces, making flow among them easier. The difference in unsaturation is also reflected in the temperature dependency of the viscosity for untreated oils, but is almost the same for both ozonated oils. The increased viscosity for ozonated oils indicated that the double bonds in the oil molecules reacted with ozone form species of higher molar masses, as also seen in the molar mass measurements.

<table>
<thead>
<tr>
<th>Duration of ozonation</th>
<th>Concentration of non-reacted ozone absorbed [mmol O₃/ml]</th>
<th>Duration of ozonation</th>
<th>Concentration of non-reacted ozone absorbed [mmol O₃/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oleic acid</td>
<td>Linoleic acid</td>
<td>Linolenic acid</td>
</tr>
<tr>
<td>5 min</td>
<td>0.088</td>
<td>0.064</td>
<td>0.036</td>
</tr>
<tr>
<td>2 h</td>
<td>0.032</td>
<td>0.074</td>
<td>0.098</td>
</tr>
<tr>
<td>2 h</td>
<td>0.00085</td>
<td>0.00085</td>
<td></td>
</tr>
</tbody>
</table>

Table 3
Percentage composition of unsaturated fatty acids in both oils

<table>
<thead>
<tr>
<th>Unsaturated fatty acids</th>
<th>Olive oil</th>
<th>Ozonezd olive oil after 7 h</th>
<th>Soybean oil</th>
<th>Ozonezd soybean oil after 7 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:1 Oleic acid</td>
<td>78</td>
<td>60.6</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>C18:2 Linoleic acid</td>
<td>10</td>
<td>7.9</td>
<td>54</td>
<td>39.4</td>
</tr>
<tr>
<td>C18:3 Linolenic acid</td>
<td>7</td>
<td></td>
<td>7</td>
<td>4.48</td>
</tr>
<tr>
<td>1,2,4-Trioxolane</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>13.5</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4
Peroxide values and acid values for pure and oils ozonated for 7 h

<table>
<thead>
<tr>
<th></th>
<th>Peroxide value meq O₂²⁻/kg fat</th>
<th>Acid value mg NaOH/g fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated oil means ± S.D.</td>
<td>7 h ozonated oil means ± S.D.</td>
</tr>
<tr>
<td>Olive oil</td>
<td>8 ± 0.2</td>
<td>28 ± 0.1</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>3.2 ± 0.1</td>
<td>37 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 6. The dynamic viscosity of olive oil, soybean oil, 7 h ozonated olive oil and 7 h ozonated soybean oil at different temperatures.

4. Conclusions

Ozonolysis of fatty acids, olive oil and soybean oil was suggested to follow the Criegee mechanism. The reaction of ozone with vegetable oils occurs almost exclusively with carbon-carbon double bonds present in unsaturated fatty chains. The disappearance of unsaturation and the formation of ozonide were almost equal. The ozonide to aldehyde ratio was always above 90%, which indicates that the major product in the early stage of the reaction was ozonide. The ozonization time for complete consumption of the double bonds was ten times longer for the oils than for the pure fatty acids.

Pure, non-ozonated olive and soybean oil consists almost entirely of molecules with a molar mass 900–1000 g mol⁻¹, which is in agreement with the composition of triglycerides in the oils. Ozonating the oils for 2 h decreases the amount of these components and new molar mass size classes corresponding to ozonide dimers and trimers occur. After 20 h of ozonation compounds with even higher molar masses were found. The presence of species of low molecular weight species after long ozonation times indicated that cross-ozonides were formed.

An increase in peroxide and acid values was observed in both oils, but it was higher in ozonated soybean oil. The relatively low peroxide and acid values together with the small fraction of compounds with low molar masses observed in the chromatography experiments indicate that the scission of ozonide was small.

The viscosity measurements showed that viscosity is a function of molecules’ dimension and orientation. The decrease in the degree of unsaturation and the increase in molar mass both contribute to the increase in viscosity of the ozonated oils.

Acknowledgments

The Socrates-Erasmus program for European graduate student exchange and The Graduate School of Materials Research, Turku, Finland are acknowledged for financial support. Mrs. Gertrud Kiwilsza is thanked for laboratory assistance and Mrs. Päivi Pennanen is thanked for help with the NMR measurements.

References

British Pharmacopoeia, 2000b. Appendix XF, IA, IB. Acid value.