2017-11-15

Characterisation of Cold Plasma Treated Beef and Dairy Lipids Using Spectroscopic and Chromatographic Methods

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Abstract: The efficacy of cold plasma for inactivation of food-borne pathogens in foods is established. However, insights on cold plasma-food interactions in terms of quality effects, particularly for oils and fats are sparse. This study evaluated plasma induced lipid oxidation of model matrices namely dairy and meat fats. Product characterization was performed using FTIR, 1H NMR and chromatographic techniques. The oxidation of lipids by cold plasma followed the Criegee mechanism and typical oxidation products identified included ozonides, aldehydes (hexanal or pentenal, nonanal, nonenal) and carboxylic acids (9-oxononanoic acid, octanoic acid, nonanoic acid along with hydroperoxides (9-and13-hydroperoxy- octadecadienoylglycerol species). However, these oxidation products were only identified following extended treatment times of 30min and were also a function of applied voltage level. Understanding cold plasma interactions with food lipids and the critical parameters governing lipid oxidation is required to design appropriate industrial adoption of the technology for food products with high fat contents.
Characterization of cold plasma treated beef and dairy lipids using spectroscopic and chromatographic methods

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Running Title: Characterization of cold plasma effects on food lipids
Highlights

- This study evaluated cold plasma induced lipid oxidation of model matrices namely dairy and meat fats.
- The oxidation of lipids by cold plasma followed the Criegee mechanism.
- Oxidation products and pathways were determined using FTIR, NMR and GC-MS analysis.
- A detailed mechanism of formation of oxidation products has been proposed.
Characterization of cold plasma treated beef and dairy lipids using spectroscopic and chromatographic methods

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Running Title: Characterization of cold plasma effects on food lipids
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DBD</td>
<td>Dielectric barrier discharge plasma</td>
</tr>
<tr>
<td>ACP</td>
<td>Atmospheric cold plasma</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform Infrared radiation spectroscopy</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
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<td>GC</td>
<td>Gas chromatography</td>
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<td>GC-FID</td>
<td>Gas chromatography-flame ionization detector</td>
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<td>Hierarchal cluster-Principal Component</td>
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<td>SFA</td>
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Abstract
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However, insights on cold plasma-food interactions in terms of quality effects, particularly for
oils and fats are sparse. This study evaluated plasma induced lipid oxidation of model matrices
namely dairy and meat fats. Product characterization was performed using FTIR, $^1$H NMR and
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acid along with hydroperoxides (9-and13-hydroperoxy- octadecadienoylglycerol species).
However, these oxidation products were only identified following extended treatment times of
30min and were also a function of applied voltage level. Understanding cold plasma interactions
with food lipids and the critical parameters governing lipid oxidation is required to design
appropriate industrial adoption of the technology for food products with high fat contents.

Key words: Cold plasma, dairy and beef fat, FTIR, $^1$H NMR, chromatography, lipid oxidation
1. Introduction

Consumer demands for high quality food and the detrimental effects associated with existing thermal technologies drive the development of alternative non thermal process technologies. Cold plasma technology has been widely used in etching and deposition of electronics, bonding of plastics, dying in textiles (Korner, Beck, Dommann, Onda, & Ramm, 1995; Naebe, Cookson, Rippon, Brady, Wang, Brack, et al., 2010). It has also demonstrated efficacy in bio-decontamination (Ziuzina, Patil, Cullen, Keener, & Bourke, 2014), treatment of food packaging materials (Pankaj, Bueno-Ferrer, Misra, Milosavljević, O'Donnell, Bourke, et al., 2014) and processing of foods (Sarangapani, Devi, Thirundas, Annapure, & Deshmukh, 2015). Plasma is a partially or wholly ionized state which consists of positively and negatively charged ions, free electrons, free radicals and intermediate highly reactive species, atoms, molecules and UV photons (Thirumdas, Sarangapani, & Annapure, 2015). Cold plasma can be generated under both atmospheric and low pressure conditions. However, for food processing, given the need for economical and continuous processing, atmospheric conditions are likely to be more suitable. The effects of the plasma reactive species in any biological or chemical system are likely to continue and diversify over a longer time frame than the initial plasma discharge resulting from formation of more stable secondary reactive species and subsequent chemical pathways. Cold plasma has been used for treatment of complex dyes, wastewater (Jiang, Zheng, Qiu, Wu, Zhang, Yan, et al., 2014), degradation of mycotoxins (Park, Takatori, Sugita-Konishi, Kim, Lee, Han, et al., 2007).

Oils and fats form an important component of the human diet containing essential fatty acids. Polyunsaturated fatty acids cannot be made by our bodies, however, these are nutritionally important (Wang, Zhu, Lyu, Panigrahy, Ferrara, Hammock, et al., 2014). Unsaturated fatty acids
start decomposing upon isolation from their natural environment, resulting in rancidity (Wisowicz, Gramza, Hes, Jeleń, Korczak, & Malecka, 2004). This lipid oxidation occurs in pure fats and oils, but also in fat dense foods including peanuts, pork scratching’s, oatmeal, muesli, milk and meat products (Jensen, Danielsen, Bertelsen, Skibsted, & Andersen, 2005). Therefore, oxidation is a concern for dairy and meat products owing to changes in the structure of proteins, fatty acid composition, reduced nutrient value, and degradation of sensory quality. These foods undergo changes in chemical composition as a function of time, process related conditions (heat, UV treatment, photolysis) or other interactions with environmental conditions. The consequent thermal degradation and autoxidation of fats leads to the formation of primary, secondary and tertiary oxidation products such as aldehydes, ketones, carboxylic acids. Ozone processing can lead to the formation of ozonides and carbonyl oxides. Nevertheless, these technologies are used in processing of oils and fats (Soriano Jr, Migo, & Matsumura, 2003; Soriano, Migo, & Matsumura, 2003). Several authors have reported on the efficacy of cold plasma for biocontrol of hams and cheeses (Song, Kim, Choe, Jung, Moon, Choe, et al., 2009), and raw meat (Han et al., 2016). However, an understanding of the impact on chemical quality parameters is also required. A limited number of studies have described how cold plasmas have controlled and accelerated oxidation using complex matrices such as fish oil (Vandamme, Nikiforov, De Roose, Leys, De Cooman, & Van Durme, 2016). However, the mechanisms of lipid oxidation due to plasma processing have not been fully established. The aim of this work is to investigate atmospheric air plasma induced lipid oxidation in relation to dairy and meat products by assessing fatty acid composition and to identify any primary and secondary products formed. Insights into cold plasma induced reaction pathways are provided by identifying changes in the functional groups
using FTIR spectroscopy along with identification of lipid oxidation products using $^1$H NMR spectroscopy and chromatographic techniques.

2. Materials and Methods

2.1. Materials

Dairy fat (butter oil) and beef fat (99% pure) were purchased from a local supermarket (Dublin, Ireland) and samples were kept under refrigerated conditions (-20 °C) until used for analysis. Hexane, methanol (≥99.9% capillary GC-grade), sodium hydroxide, 50% boron trifluoride in methanol, Chloroform-d (CDCl$_3$) with 0.03% (v/v) TMS (Tetramethylsilane) were purchased from Sigma-Aldrich, Ireland.

2.2. Atmospheric air cold plasma treatment

The high voltage in package atmospheric cold plasma-dielectric barrier discharge (ACP-DBD) system employed for this work is described in Sarangapani et al., (2016). Fat samples (15 ± 1g) were placed in petri dishes and the samples were subjected to different doses of direct plasma treatment. The atmospheric air condition at the time of treatment was 40 ± 1% relative humidity (RH) and 16 ± 2°C, measured using a humidity-temperature probe connected to a data logger (Testo 176T2, Testo Ltd., UK). Atmospheric air was used as the working gas. Plasma treatment was performed at variable voltage (60-80 kV) and treatment duration (3-30 min). After processing, containers were stored at room temperature of 16-18 °C for 24 h in line with our previous findings that a sealed retention time is useful for biocontrol. This allows contact time of the generated and contained chemical reactive species with the samples. Control samples were not plasma treated.
2.3. FTIR spectroscopy
The IR spectra were recorded in absorbance mode at 4 cm\(^{-1}\) resolutions, using a Spectrum GX FT-IR (Perkin Elmer, Dublin, Ireland) equipped with an attenuated total reflectance (ATR) over the frequency range 4000–400 cm\(^{-1}\). The sample measurements were replicated for all the individual samples of each treatment class. Analyses were carried out at room temperature of 25°C. The background was collected before every sample was measured.

2.4. Fatty acid composition
Samples were evaluated for fatty acid composition using the GC-FID. Individual fatty acid methyl esters (FAME) were identified using FAME standards (Sigma Chemicals, Ireland) were used to calculate the percentage of fatty acids based on its peak area. A BRUKER SCION 456 GC equipped with a flame ionization detector and Zebron ZB-5MS capillary column with dimension 30 m x 0.25 mm I.D and 0.25 µm thickness is employed. Helium was used as the carrier gas and the flow rate was set at 1mL min\(^{-1}\). Samples were injected with a split ratio (1:10). The GC oven temperature was programmed as: 50°C held for 1 min, 50°C ramp to 200°C and held for 1min, ramp to 230°C at a rate of 8°C min\(^{-1}\) held for 20 min. The injector and detector temperatures were set at 250 and 280°C, respectively. Data was collected and integrated with a personal computer using MS workstation GC Software. The GC–MS analysis of the target compounds was performed using a Varian 3800 GC (JVA analytical Ltd. Ireland) with a 2200 Varian ion trap MS was used to analyze the samples. Chromatography was conducted on the same chromatographic column and under the chromatographic conditions described above. The mass detector was operated in the electron impact (EI) mode at 70 eV and electron multiplier voltage of 1.25 kV. The mass fragments of the derivatives were obtained in the full scan mode in the scan range from \(m/z\) 35 to 350. Data were collected using Varian software. The compounds present were tentatively identified based on computer matching.
against commercial National Institute of Standards and Technology (NIST) libraries and spectral library (Dalton, Dragoset, & Wiersma) of pure substances and literature available.

2.5. NMR analysis
For $^1$H and $^{13}$C NMR spectroscopy, about 50 mg of the sample was accurately weighed and dissolved in CDCl$_3$ containing TMS as standard. Both spectra were obtained using a Bruker Avance 400MHz Spectrometer. Trace amount of CHCl$_3$ in the solvent used exhibits signals at 7.26 and 77.0 ppm in $^1$H and $^{13}$C NMR, respectively.

2.6. Determination of peroxide value
The peroxide value is determined by the procedure of Kirk & Sawyer (1991).

2.7. Statistical analysis
The results were analyzed by one-way ANOVA using SPSS (IBM statistical analysis Version 19), and the significance amongst the samples was compared at p<0.05 by the least significant difference post-hoc comparison, SPSS 19 version. Results represent the means of at least two separate experiments.

3. Results and discussion
3.1. FTIR analysis
The changes in the functional groups for both dairy and beef fat were revealed using FT-IR analysis. The representative spectra of the control and plasma treated dairy and beef fats are presented in Fig.1. The analytical evaluation of the dairy and beef fat spectra were previously reported (M. a. D. Guillén & Ruiz, 2001). The IR spectra demonstrate that triglycerides were dominant. The major triglyceride peaks observed were around 2937 cm$^{-1}$ corresponding to C–H stretching (asymmetry), 2856 cm$^{-1}$ assigned to C–H stretching (symmetry), and a carbonyl peak 1746 cm$^{-1}$ (C=O stretching) (Adeyemi, Mohiuddin, Mirghani, & Jameel, 2012; Yang, Irudayaraj, & Paradkar, 2005). Moreover, a weak signal was observed at 3000 cm$^{-1}$ associated
with –C=CH stretching groups of cis-unsaturation (Ahmad Fadzlillah, Che Man, Rohman, Ismail, Mustafa, & Khatib, 2013). A weak peak around 1650 cm\(^{-1}\) was observed and corresponds to C=C stretching (cis) and a major peak at 1454 cm\(^{-1}\) assigned to C–H bending (scissoring). The stretching vibrations of the C–O bond of esters and bending vibrations of the methylene group were present around 1300–1000 cm\(^{-1}\). Other major peaks observed were at 1166 cm\(^{-1}\) which corresponds to C–O (stretching) and C–H (bending), and 723 cm\(^{-1}\) which corresponds to C–H bending (rocking) (M. D. Guillén & Cabo, 1997). The peak at 1117 cm\(^{-1}\) corresponds to C–H (deformation) and 1097 cm\(^{-1}\) (C–H bending) vibration of fatty acids, respectively. The peak at 966 cm\(^{-1}\) has been previously reported as a marker band of trans fatty acids in fats and oils and the peak is associated with –HC=CH out-of-plane deformation vibrations (Ahmad Fadzlillah, Che Man, Rohman, Ismail, Mustafa, & Khatib, 2013). These frequencies observed in the FTIR spectra were used as the basis for investigating the changes in the functional groups of plasma treated fat samples.

Several changes were observed in the IR spectra of the plasma treated samples. ACP-DBD plasmas are a source of a wide range of active species and reactive species such as O\(_3\), H\(_2\)O\(_2\), OH. These plasma species can cleave double bonds of unsaturated fatty acids. The IR spectra of the intensities corresponding to C=C decreased with increases in applied voltages and plasma treatment times. These include the relatively weak band at 1650 cm\(^{-1}\) see Fig.1. (I and II) and the =C–H stretch and bend at 3008 cm\(^{-1}\) and broadened peak at 723 cm\(^{-1}\), respectively. The dairy samples were more susceptible to plasma treatment than the beef samples, with changes evident within shorter treatment times of 3 and 6 min for all applied voltages. This is attributed to the higher concentration of unsaturated fatty acids (45%) in beef fat than dairy (31%). Similar trends were reported for ozonation of sunflower methyl esters by Soriano et al., (2003). Several
authors have reported the formation of carbonylic compounds as by-products of plasma treatment. Dairy and meat triglycerides have a strong absorption band at 1743 cm\(^{-1}\) attributed to stretching vibration of the carbonyl group (Moreno, Olivares, López, Martínez, & Reig, 1999). As observed in Fig.1 (c and d), increasing plasma treatment time and applied voltage widens the carbonyl band, which suggests the formation of new carbonyl compounds (Gao, Sun, Wan, Yu, & Li, 2013). This is attributed to the production of saturated aldehyde, carboxylic acids or other secondary oxidation products. The new aldehyde or ketone compounds formed come from the oxidation of C=C bonds in existing unsaturated triglycerides. The emergent band around 1725 cm\(^{-1}\) suggests the formation of aldehyde in the plasma treated dairy fat samples, where a shoulder was observed at 1700 cm\(^{-1}\) in plasma treated beef fat samples. However, the weak band at 1725 cm\(^{-1}\) in the beef samples, overlaps with the stretching vibration at 1746 cm\(^{-1}\) of the ester carbonyl functional group. Moreover, the presence of an aldehydic C-H stretch around 2900-2700 cm\(^{-1}\) confirms the formation of aldehyde and other oxidation products. The presence of new oxidation products in both the dairy and beef samples especially the carbonyl group (C=O) can be evidenced by broadened bands around 725 cm\(^{-1}\). This peak could be ascribed to complicate oligomerization of olefins (Soriano Jr, Migo, & Matsumura, 2003). Interestingly, a new band around 1105 cm\(^{-1}\) (Fig.1. a and b) was observed across all the plasma treated dairy and beef samples, which was not observed for control or the blank. The intensity of this new band increased with plasma treatment time and applied voltage. This peak likely corresponds to C-O stretching of ozonide (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003). This suggests that the majority of oxidation of fats using ACP-DBD occurs by direct attack of ozone to produce ozonide, as described by the Criegee mechanism (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003; Ledea, Díaz, Molerio, Jardines, Rosado, & Correa, 2003; Sega, Zanardi,
Chiasserini, Gabbrielli, Bocci, & Travagli, 2010). The ozonide formed during plasma treatment could be the 1,2,4-trioxolane ring which was reported by several authors (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003; Soriano Jr, Migo, & Matsumura, 2003; Soriano, Migo, & Matsumura, 2003) during ozonolysis of fatty acids methyl esters. Two other sharp bands appear at 1175 cm⁻¹ and 1195 cm⁻¹ which also correspond to C-O stretching and O-C stretching of ozonides (Soriano, Migo, & Matsumura, 2003). The other changes observed in the IR spectrum associated with plasma treatment were peaks at 1379 cm⁻¹ and 975 cm⁻¹, most likely associated with C-O-C and stretching vibration of O-O, respectively (Georgiev, Anachkov, Batakliev, & Rakovsky, 2013). The new bands formed at 969 and 829 cm⁻¹ and small changes around 3470 cm⁻¹ may be due to the formation of peroxide groups (Bellamy, 2013). The plasma treatment also induced major changes in the region 1350-1475 cm⁻¹. These changes correspond to the aldehydic group (C-H bend) at 1381 cm⁻¹ and C-O-H bending at 1440 cm⁻¹ to 1395 cm⁻¹, which is adjacent to the carbonyl group (Bailey, 2012). The scission of either initial or final ozonide leads to the formation of aldehyde.

While trends can be observed in these IR spectra, chemometric analysis, specifically hierarchical clustering, of the spectral data using principal component analysis (PCA) was adopted to classify these plasma treated fats based on their FT-IR spectra. For this purpose, the region between 700-1800 cm⁻¹ was selected as representing the majority of the changes induced in the functional groups such as C–H bending, C=O stretching, and C=C stretching and also the unsaturated C=C bond. Principal component analysis of spectra within this region revealed two principal components, explaining 87.3% of the data variance for the dairy sample. The cluster analysis algorithm divided the dairy sample sets into six clusters (see Fig. 2.(a)), where a clear separation between the control and the plasma treated samples was found. The replicates of the
dairy control sample are located at positive scores of PC1 and negative scores of PC2 and were
252 grouped into cluster 1 and majority of the samples treated for 9 min at all the applied voltages
253 (60, 70 and 80 kV) grouped into cluster 2 with 70 kV for 9 min (2nd replicates as center).
Moreover, the majority of samples treated for 3 min and 6 min were grouped into cluster 3.
However, it may be noted that some of the replicates of treated samples are separately grouped
257 into cluster 4, which might be due to overall variability in the plasma process (Misra, Pankaj,
Frias, Keener, & Cullen, 2015). It can be observed that all replicates of samples treated for 60 kV
for 30 min are cluster 5 similarly 70 kV for 30 min samples are cluster 6. Therefore, treatment
time and applied voltage played an interactive role in the formation of oxidation products in
dairy fat at higher treatment times.
262 It can be observed in Fig.2.(b) that two PCA scores for the beef samples explained 70.9% data
263 variance and replicates of control sample are located in positive score of PC1 and negative score
264 of PC2. There was a similar separation for the beef samples which are grouped into cluster 1.
However, there was no clear separation obtained among the plasma treated beef samples. The
266 majority of replicates of samples treated for 9 min or 30 min at all applied voltages were grouped
267 into cluster 2 with the exception of the 70 kV 9 min samples. However, 70 kV 9 min samples
268 were grouped into cluster 4 with two replicates of 60 kV 30 min, 80 kV 9 min and 80 kV 6 min.
269 The majority of 3 min replicates of 60 kV and 70 kV were grouped into cluster 3 whereas 3 min
270 replicates of 80 kV were grouped together with 6 min samples of 60 kV and 70 kV into cluster 5.
271 The variability in the samples can be attributed to plasma induced changes in the functional
groups such as formation of aldehydes around 1725 cm\(^{-1}\), ozonide at 1105 cm\(^{-1}\), peroxides at
272 969 cm\(^{-1}\) and 829 cm\(^{-1}\) in the beef fat samples. In addition, the natural variability in the
273 composition of fatty acids among dairy and beef fats should be considered.
3.2. Fatty acid analysis
The fatty acid composition of control (untreated) and plasma treated dairy and beef fat are summarized in Table 1. The dairy and beef fats are mainly composed of oleic, palmitic and stearic acids. It can be observed that plasma treatment decreased the relative amount of unsaturated fatty acids from 34.06% to 28.47% for the dairy samples and from 44.05% to 38.60% for the meat samples. Ozone and hydroxyl radicals are considered the principal active species which cleave the double bonds of unsaturated fatty acids primarily oleic, palmitoleic and linoleic acid leading to oxidation. There was a decrease in the ratio of unsaturated to saturated fatty acids (UFA/SFA) with increasing applied voltage level, which may be attributed to the increased dissociation reactions to form free radicals, reactive species and unstable compounds at higher voltages. A similar observation with a reduction in the unsaturated fatty acids was also observed in ozonated vegetable oil and pork lard (Jurado-Alameda, García-Román, Altmajar-Vaz, & Jiménez-Pérez, 2012; Soriano, Migo, & Matsumura, 2003). This result supports the changes seen in the 1H NMR and IR spectra of the plasma treated samples which is suggested to follow the Criegee mechanism (Soriano, Migo, & Matsumura, 2003). Several authors have reported that plasma treatment of organic chemicals results in the formation of new by-products (Gao, Sun, Wan, Yu, & Li, 2013; Sarangapani, Misra, Milosavljevic, Bourke, O’Regan, & Cullen, 2016). The GC-MS analysis of plasma treated fats was performed to identify the plasma degraded products. The unsaturated fatty acids were identified at retention times of; 16.49 min for palmitoleic, 19.19 for oleic, 20.09 for linoleic respectively. Interestingly, the analysis of chromatogram showed the formation of new peaks caused by the attack of reactive oxygen and nitrogen on the double bond of unsaturated fatty acids. Oleic acid was the primary fatty acid susceptible to oxidation by plasma species. The reaction of ozone to oleic acid forms primary ozonide (molozonide) which further decomposes into two Criegee intermediate radicals. These
results were supported by the $^1$H NMR at the chemical shift $\delta$ 5.15 ppm and the IR spectra at 1105 cm$^{-1}$, 1195 cm$^{-1}$. It is known that decomposition of the primary ozonides leads to the formation of aldehydes (or ketones) and carbonyl oxides (or Criegee intermediates) (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003). The formation of nonanal and 9-oxononanoic acid in plasma treated samples support this hypothesis. Nonanal (aldehyde) compound (c) was identified at a retention time of 9.69 min and exhibited [M]$^+$ ion at 142 and fragments ions at m/z 124 [M-18(loss of water)], m/z 114[M-28(loss of ethylene)], m/z 98[M-44(loss of CH$_2$=CH-OH)] with other prominent peaks at m/z 95, 81, 71, 69, 43 and 41. Compound (b) was identified as 9-oxononanoic acid (Carbonyl oxide) with mass fragments at [M]$^+$ 172, and fragments ions at m/z 154[M-18(loss of water)], m/z 144 [M-28 (loss of ethylene)], m/z 129 [M-44 (loss of CH$_2$=CH-OH)] (Supplementary matrial Fig.S7.) and other prominent peaks at m/z 111, 98, 87, 73, 59, 45, 41. Katrib et al., (2004) reported that the Criegee intermediates undergo reactions with oleic acid to form C27 molecules. Moreover, other products were also identified in the MS spectra of plasma treated samples. The compound (a) nonanoic acid (more than 70% match) was identified at 9.88 min in all the plasma treated samples and exhibited [M]$^+$ ion at 172, and other major fragments at m/z 129, 5, 101, 87, 74, 55, 43, 41. Compound (d) was identified as azelaic acid (96.6% match) exhibited [M]$^+$ ion at 185, and other fragments at m/z 152, 143, 124, 111, 97, 74, 55, 41. Interestingly, another compound identified at 9.206 min corresponds to octanoic acid (more than 75% match) which exhibited [M]$^+$ ion at 158, 127[M-31(OCH$_3$)], m/z 101, 87, 74, 41. These compounds could be formed by the isomerization of Criegee intermediates (Hung, Katrib, & Martin, 2005). A possible oxidation pathway see Fig.2 (c) for oleic is proposed based on the intermediates formed. Further possible recombination of the carbonyl oxide and the aldehyde (or ketone) yield secondary ozonides. The formation of 9-oxononanoic acid and
nonanal were also observed in the reaction of oleic acid droplets with nitrate radicals by Hung et al., (2005). However, no nitrate radical reactions forming long chain carbon molecules and formation of peroxides including hydroperoxides were seen in the MS spectra. Extensive information on the formation of hydroperoxides can be found elsewhere (Bailey, 2012).

3.3. NMR analysis

Fig. 3 (a and b) shows the typical $^1$H NMR spectra of the control and plasma treated samples of the dairy and beef fat respectively. $^1$H NMR assignments of the main components of pure fats are $\delta$(ppm): 0.90, $-\text{CH}_3$ (fatty acid terminal groups); 1.40-1.15, $-(\text{CH}_2)_n$− (saturated fatty acid acyl groups); 1.71-1.50, $-\text{O}(=\text{O})-\text{CH}_2-\text{CH}_2-$ (acyl groups); 2.10-1.90, $-\text{CH}_2-\text{CH}=\text{CH}$− (acyl groups); 2.35-2.20, $-\text{OCO}-\text{CH}_2-$ (acyl groups); 2.80-2.70, $=\text{CH}-\text{CH}_2-\text{CH}(=\text{O})$ (acyl groups); 4.32-4.10, $-\text{CH}_2\text{OC}(=\text{O})\text{R}$ (C1 and C3 glyceryl CH$_2$ groups); 5.26-5.20, $-\text{CH}=\text{CH}$− (acyl groups) (M. a. D. Guillén & Ruiz, 2001). Thus, the chemical shifts are assigned to the saturated fatty acid component of triglycerides and free fatty acids between 0-4 ppm while 4-6 ppm are assigned to proton signals of glycerol backbone as well as olefin components of unsaturated fatty acids and triglycerides. It was evident from the NMR analysis that fats underwent structural changes upon plasma treatment. New signals at 9.76 ppm found in the $^1$H NMR spectra of plasma treated dairy and beef correspond to aldehydic protons (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003). The formation of aldehydes were also observed at 1725 cm$^{-1}$ and C-H stretch around 2900-2700 cm$^{-1}$ in IR spectra. It was also observed that the area of these peaks increased with increases in plasma treatment time and applied voltage. The 9.3-10.5 ppm region of $^1$H NMR spectra of plasma treated beef and dairy samples contained some doublet resonances at 9.49, 9.52, 9.67 ppm (see supplementary material Fig.S19 and Fig.S31). Claxson et al., (1994) demonstrated that these signals arise from
unsaturated aldehydes and correspond to trans-2-alkenals, alka-2,4-dienals and 4-hydroxy-trans-2-alkenals, respectively. The signal at 9.76 ppm corresponds to saturated aldehydes and it is attributed to hexanal or pentanal whereas, 9.49, 9.52 ppm signals are a mixture of trans-2-heptenal, -octenal or -nonenal (Haywood, Claxson, Hawkes, Richardson, Naughton, Coumbarides, et al., 1995). A relatively weak signal at 9.67 ppm was observed in the 30 min treated samples and showed coupling patterns at 6.15 ppm that could possibly be assigned to 4-hydroxy-trans-2-nonenal. The possible generation of these compounds is due to the oxidation of unsaturated fatty acids (particularly linoleic acid) by reactive oxygen and nitrogen species. Similar results were observed in ozonolysis of sunflower and sesame oil (Sega, Zanardi, Chiasserini, Gаббrielli, Bocci, & Travagli, 2010; Soriano Jr, Migo, & Matsumura, 2003; Soriano, Migo, & Matsumura, 2003). The distinct singlet resonances at 8.9, 8.8, 8.2, 8.0 and 6.2 ppm in the dairy samples and 9.20, 8.2, 8.0, 6.45 and 6.2 ppm detected in beef samples were assigned to hydroperoxide group (-OOH) protons. Similar hydroperoxide groups were detected in thermally stressed linoleate samples by Claxson et al., (1994). There was an increase in the peak intensity with increases in plasma treatment time and applied voltage. The resonances at 8.2 and 8.0 ppm could be attributed to the reaction between oxidizing radicals (eg. •H, •O, •OH) and oleic acid in beef fat to form hydroperoxides. The reaction proceeds (see Fig.4(a)) with a free radical attack at C-8 and C-11 of oleic acid and hydrogen abstraction which leads to allylic radicals which upon reaction with reactive oxygen species would result in formation of 9-hydroperoxy-trans-10-,11-hydroperoxy-cis-9,10-hydroperoxy-trans-8-, 8-hydroperoxy-cis-9-octadecenoates (Frankel, 1984). These results are in agreement with reports by Neff et al., (1990) for autoxidation of olive oil and triolein. The chemical shift in values of 8.9 and 8.81 ppm observed in the dairy samples may correspond to hydroperoxide group protons of linoleic acid.
producing a mixture of 9-hydroxy-trans-10, cis-12- and 13-hydroxy-cis-9, trans-1-octadecadienoate (Frankel, 1984). The evidence of these hydroperoxy species were confirmed by resonances centered at 4.05, 5.56, 6.35, 5.90 and 5.30 ppm which correspond to 13, 12, 11, 10, 9 position protons respectively, of conjugated diene cis, trans-13-hydroxydiene isomer (Haywood, et al., 1995). The steps involved (see Fig.4(b)) include selective hydrogen abstraction to form a pentadienyl hybrid radical which upon reaction with plasma reactive species would result in 9- and 13-hydroperoxy- octadecadienoylglycerol species (Frankel, 1984). Similar hydroperoxy species were detected and a mechanism of formation explained by Chan et al., (1980). Similar resonances were also detected in the oxidation of trilinolenin in the presence of oxygen with the formation of hydroperoxy epidioxy adducts identified as the main products (Chan, Matthew, & Coxon, 1980; Neff, Frankel, & Miyashita, 1990). It is reported that the resonances at 8.9 and 8.8 corresponds to 12- and 9-position methine proton multiplets of 13-hydroperoxy-9,12-epidioxy-10-octadecenoates and the 10- and 13-position methine proton multiplets of 9-hydroperoxy-10,13- epidioxy-11-octadecenoate (Haywood, et al., 1995). These signals were also detected in thermally treated methyl linoleate, methyl oleate, soyabean oil and deteriorated oil (Claxson, et al., 1994; M. a. D. Guillén & Ruiz, 2001). The formation of these hydroperoxy epidioxide products were also reported in photosensitized methyl linoleate samples by Neff et al.,(1983). However, the absence of the signals at 4.66 ppm in both the dairy and beef $^1$H NMR spectra rules out the formation of hydroperoxy epidioxide as plasma oxidized products. Further investigation was also carried out to elucidate the possible formation of other dihydroperoxides. As stated earlier, the resonances at 8.25, 8.12 ppm in both plasma treated samples corresponds to hydroperoxy group. Moreover, the multiplets centered at 6.45, 6.25, 4.87, 4.30-4.40, 3.67, 2.31, 1.50-1.80 and 0.92 ppm correspond to 8-13-dihydroperoxy-trans-9,trans-11- and 9,14-
The formation of hydroperoxides and diperoxide was further confirmed by the peroxide value experiments. The plasma treatment increased the peroxide value of both dairy and beef fat. The peroxide value of control samples was 7.5 mEq O₂/ kg for dairy and 10 mEq O₂/ kg for beef fat. The 80 KV 30 min of plasma treatment increased the peroxide value to 23 mEq O₂/ kg for dairy and 17 mEq O₂/ kg for beef fat respectively. The formation of these peroxides is due to reaction of plasma species with unsaturated fatty acid following the Criegee mechanism. However, the mechanism of formation of these dihydroperoxides after plasma treatment should requires further investigation.

A new signal at 5.15 ppm was seen in the ¹H NMR spectra of plasma treated dairy and beef samples. This resonance was assigned to the ring proton of 1,2,4-trioxolane (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003; Sega, Zanardi, Chiasserini, Gabbielli, Bocci, & Travagl, 2010) and the formation of this ozonide was also supported by bands at 1105 cm⁻¹, 1170 cm⁻¹, 1195 cm⁻¹ in the IR spectra. From Fig.3 (a) and (b), this new signal resonance increased with plasma treatment time and voltage. Moreover, there was also an increase in resonances at 2.35–2.45 and 2.04–2.18 ppm upon plasma treatment. The signals at 2.35–2.45 ppm are attributed to methylene bridge protons connecting the 1,2,4-trioxolane ring to the double bond. However, the chemical shifts at 2.04–2.18 ppm correspond to methylene bridge protons now connecting two carbon rings (Sega, Zanardi, Chiasserini, Gabbielli, Bocci, & Travagl, 2010). The ozonolysis of methyl oleate (Ledea, Díaz, Molerio, Jardines, Rosado, & Correa, 2003), methyl linoleate (Díaz, Hernández, Ledea, Sazatornil, & Moleiro, 2003), sunflower oil (Soriano Jr, Migo, & Matsumura, 2003; Soriano, Migo, & Matsumura, 2003), sesame oil (Sega, Zanardi, Chiasserini, Gabbielli, Bocci, & Travagl, 2010) has been reported with the formation
of ozonides. The oxidation of fat by plasma treatment can be described by the Criegee mechanism. The electrophilic ozone molecule or other plasma active species attack the C=C double bonds of unsaturated fatty acid, leading to the formation of an unstable cyclic intermediate called an unstable initial ozonide (1-2-3-trioxolane). The unstable intermediate can be further decomposed to a cabonylic compound (aldehyde or ketone) to form stable 1-2-4 trioxolane, diperoxides and hydroperoxides (Ledea, Díaz, Molerio, Jardines, Rosado, & Correa, 2003). It was found that degradation of linoleic acid by ozone was found to be 1.5 times higher than that of oleic acid. The presence of greater amounts of unsaturated fatty acids (45%) in beef fat compared to dairy would result in relatively small increases in the reactivity of the C=C double bond due to a high steric effect. The reaction of plasma species such as ozone with fats to form products depends upon the composition of fatty acids and type of medium (solvent). The reaction of pure oleic acid with carbonyl oxide, by inter and intra molecular reactions, readily forms acyloxyalkyl hydroperoxides whereas, under dilute conditions favours ozonide formation and hydroperoxide. It was reported that ozonation of sunflower oil in presence of water yielded alkyl hydroperoxide and prevented the formation of ozonide (Soriano Jr, Migo, & Matsumura, 2003). Ledea et al., (2003) studied the ozonation of methyl oleate and reported the formation of ethoxy hydroperoxides in the presence of ethanol and ozonides and aldehydes in the presence of water. Considering the surrounding milieu of dairy and beef fats in food matrices, the interactions within aqueous environments require further investigation.

An extensive explanation into the formation of new products and their kinetics as a result of plasma treatment is proposed. The integral ratios of the proton signals of aldehydes, peroxides, ozonides and methylene groups with reference to the integral of TMS that remains constant during the process of some key protons resonances was evaluated. The formation of secondary
oxidation products with plasma treatment time and applied voltage was modelled using a first-order kinetic regression model (see supplementary material Fig.S33 and Fig.S34). It can be observed from Table 1 that the model is in agreement with the experimental data with high correlation coefficients explaining the increase in formation of new products in tandem and relation to plasma process parameters.

4. Conclusion

This work provides insights into ACP-DBD air plasma induced lipid oxidation with FTIR spectra of treated samples showing plasma induced changes in the functional properties of dairy and beef fat. In this present study the formation of secondary oxidation products were only seen at extended plasma treatment times of 30min. A linear trend explains the extent of the changes induced by the process conditions. The increase in ozonide band at 1105 cm\(^{-1}\), 1195 cm\(^{-1}\), formation of aldehydes 1725, 2950 cm\(^{-1}\) and 829, 969, 3470 cm\(^{-1}\) is attributable to the formation of hydroperoxides. These changes were dependent on treatment time and applied voltage.

\(^1\)H NMR analysis also identified the formation of several lipid oxidation products, of which aldehydes, such as hexanal or pentenal, mixtures of trans-2-heptenal, -octenal or -nonenal and 4-hydroxy-trans-2-nonenal are the most important. For the first time the formation of hydroperoxides of oleic acid was identified as 9-hydroperoxy-trans-10, 11-hydroperoxy-cis-9,10-hydroperoxy-trans-8-, 8-hydroperoxy-cis-9 octadecenoates and linoleic acid as 9- and 13-hydroperoxy- octadecadienoylglycerol species. Therefore, it is suggested that atmospheric air plasma treated fatty acids follow the Criegee mechanism. With the reaction of ozone and active species almost exclusively with carbon double bonds present in unsaturated fatty chains. Fatty acid composition analysis identified the reduction in oleic, palmitoleic and linoleic acid along with oxidation products as nonenal, azelaic acid, 9-oxononanoic acid, nonanoic acid and
octanoic acid. Moreover, these products can be used as oxidation markers for the chosen dairy and beef fats.

Acknowledgment

The funding for this research was provided by the Food Institutional Research Measure (FIRM) project entitled ‘Cold Plasma treatment of waste water’ (project number: 13/F/442) under the National Development Plan administered by the Department of Agriculture, Fisheries and Food, Ireland.
References


**Figure legends**

**Fig.1.** Overlaid raw FT-IR spectra of control and plasma treated of fat samples over region 600-3500 cm\(^{-1}\) (I) dairy (II) beef fat, where the spectra over region 950-1300 cm\(^{-1}\) was shown in (a) dairy and (b) beef and the spectra over region 1650-1800 cm\(^{-1}\) was shown in (c)dairy and (d) beef. Arrow indicates increase in plasma treatment

**Fig.2.** HC-PC of the raw data (700-1800cm\(^{-1}\)) of control and plasma treated (a) dairy (b) beef fat. In the data markers, the first digit indicates the voltage, the second time, and the last following underscore indicates the replicate Where (c) represents proposed oxidation mechanism of oleic acid from GC-MS.

**Fig.3.** 1H NMR spectra of control (untreated) a) plasma treated dairy fat and b) plasma treated beef fat samples. Note the bottom spectrum showing control and the expansion showing the oxidized products, the first digit indicates the applied voltage, the second digit indicates treatment

**Fig.4.** Proposed mechanism of hydroperoxides from a) oleic acid (Frankel, 1984) and b) linoleic acid (Frankel, 1984; Neff, Frankel, Selke, & Weisleder, 1983)
Table legends:

Table 1: Fatty acid profiles of the control (untreated) and plasma treated fats.

Table 2: Linear regression (y = ax + b, R² parameter) of integral of selected proton chemical shifts.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Table 1

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$^z$ Plasma treatment time of 30 min; Only fatty acids with a relative amount higher than 1% are shown where control is untreated sample. UFA: unsaturated fatty acid; SFA: saturated fatty acid. All the data are expressed as mean ± standard deviation. Means with the different superscript letters differ significantly (P < 0.05)
Table 2

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