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Chemiluminescent assay of lipid hydroperoxides quantification in emulsions of fatty acids and oils

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ABSTRACT

Lipid hydroperoxides (LOOH) are relatively stable intermediates which arise during oxidation of fats and lipids. The luminol-enhanced chemiluminescent (LCL) method has been applied to detect the LOOH formed during thermal oxidation. The method was optimized for best signal to noise ratio what gave reliable and sensitive data up to 50 pM of detected hydroperoxides (approximately 1 μmol hydroperoxides/kg lipid). The observed LCL signal was calibrated with 13-HPODE (13-(S)-hydroperoxy-9Z,11E-octadecadienoic acid) as an external hydroperoxide standard. This allowed quantitative determination of LOOH formed during thermal oxidation of linoleic acid and phosphatidylcholine (PC) in emulsion and during storage of linseed oil. The obtained LCL results were validated using HPLC, spectrophotometric assay for conjugated dienes at 234 nm and iodometric titration peroxide value (PV) methods.

The described LCL method is direct, sensitive, fast and simple, however, it is not specific to the lipids and may be used to determine the presence of the other types of hydroperoxides or the other oxidizing agents what finally gives overall total antioxidant estimation of the sample.

1. Introduction

Lipid oxidation is considered a free radical chain reaction, with the first step being hydrogen abstraction, followed usually by lipid hydroperoxide formation. In the absence of decomposing factors as redox-active metals, heat, ultraviolet light or antioxidants hydroperoxides are relatively stable products of lipid oxidation. In such case the formation of hydroperoxides is a good measure to detect early stages of lipid oxidation, however, the reaction condition and solvent should be taken into account. There are many analytical methods available to measure oxidation of lipids. The choice of the method depends on what parameters are being measured and which phase of oxidation we want to monitor. It seems that among different authors emerged the opinion that the total hydroperoxide content is a good measure of oxidation progress. This may be measured by a variety of methods including iodometric titration (Antolovich, Prenzler, Patsalides, Mcdonald, & Robards, 2002; Simic, Jovanovic, & Niki, 1992), colorimetric detection with the FOX method (Dobarganes & Velasco, 2002; Frankel, 1991), FTIR spectroscopy (Dauben, Lober, & Fullerton, 1969; Sedman, van de Voort, & Ismail, 1997), conjugated dienes and trienes (Antolovich et al., 2002; Wanasundara, Shahidi, & Jablonski, 1995; Waters, 1971). However, each of those methods has some drawbacks. Chemiluminescence (CL) offers some advantages to overcome shortcomings of the other methods as superior sensitivity and simplicity.

The use of CL method for analyzing lipid peroxidation was reported by Vladimirov and Petrenko (1976). From this time this method has been modified to avoid some drawbacks and was adopted to study the quality of food. Review of chemiluminescent methods used in food analysis were given (Jimenez & Navas, 2002; Navas & Jimenez, 1996) and some information was included in reviews regarding lipid oxidation (Dodeigne, Thunus, & Lejeune, 2000; Roginsky & Lissi, 2005; Wheatley, 2000).

CL originates from excited state product of two ground state molecules. The chemical energy gained is translated into electronically excited state which finally, trough photon emission, relaxes to ground state. One mechanism suggests the coupling of two peroxy radicals ($ROO^{\cdot}$) to form tetroxodie (ROOOOR), which further decomposes it results in photon emission, so called Russel mechanism. Another mechanism indicates that degradation of hydroperoxides (ROOH) will produce alkoxy radicals ($RO^{\cdot}$) or similar reactive species that in next step will lead to β-scission mechanism followed by photon emission. This ultraweak CL is related to only part of the oxidation process where decomposition reactions generate excited carbonyls, which eventually relax and emit photons. It has been shown that ultraweak CL is accompanied during oxidation of many compounds including hydrocarbons and lipids (Navas & Jimenez, 1996). Due to the very low quantum yield (less than 10^{-4}) the detection of CL intensity requires sophisticated methods like single photon counting method. At early stages of oxidation

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the reaction of addition to double bonds dominates since reactive conjugated dienes are present at this stage. However, these addition reactions do not cause any chemiluminescence. In order to increase the CL intensity and avoid specialized apparatus the luminol and lucygenin as light amplifiers have been introduced. The luminol-enhanced chemiluminescence (LCL) involves oxidation of luminol in basic solution generating a free radical intermediate which reacts with flux of oxidizing agents present in the system, e.g. H_2O_2, lipid hydroperoxides. This leads to formation luminol-derived product in excited state which eventually returns to ground state emitting strong blue light at 430 nm. In general, the luminol chemiluminescent reaction in aqueous solution occurs according mechanism given by Miyazawa, Fujimoto, Suzuki, and Yasuda (1994), however, the exact mechanism is not known. Based on this mechanism we may assume that LCL observed from lipid hydroperoxides/luminol system will follow the mechanism given bellow. In basic solution the formation of mono- and dianion of luminol occurs, Lum^{2-}. Next, hemin catalyzed decomposition of hydroperoxide occur. LOOH + hemin → $LO^{\cdot}$ + $LO^{\cdot}$ + Lum^{2-}. Luminol anions react with $LO^{\cdot}$ radicals. This leads to formation of luminol-derived free radical (diazamiquinone radical) $Lum^{\cdot}$ and LOH. Interaction of oxygen O_2 with luminol radical leads to formation of superoxide radical, $O^{\cdot 2-}$, which interacting with $Lum^{\cdot}$ leads to formation of transient luminol endoperoxide, Lum–O–O$^{\cdot}$. Finally, endoperoxide decomposes to give light emission (CL) and products, aminophthalate and N_2. Hemin in this reaction plays a role of catalyst and cooxidant.

LCL method has frequently been used to monitor on-line detection for lipid hydroperoxides in connection with HPLC what has allowed the quantification of various hydroperoxides at picomole level (Chan, Cheng, Tsao, Niu, & Hong, 1996; Henderson, Slickman, & Henderson, 1999; Kondo, Kawai, Miyazawa, & Mizutani, 1993; Miyazawa, 2000; Ohba, Kuroda, & Nakashima, 2002; Sanchez-Moreno & Larrauri, 1998). In all reported cases the application of LCL shifted the detection limits of hydroperoxide to the level of single picomoles, the sensitivity unreachable by the other analytical methods (Navas & Jimenez, 1996).

Several LCL methods for lipid hydroperoxide detection are reported in the literature like stationary (Matthaeus, Wiezorek, & Eichner, 1995) or flow injection method (Bunting & Gray, 2003), or providing only qualitative data (Burkow, Moen, & Overbo, 1992; Pettersen, 1994; Saito & Nakamura, 1989), or post-column quantitative data (Miyazawa, 2000; Miyazawa, Kunika, Fujimoto, Endo, & Kaneda, 1995; Yasaei, Yang, Warner, Daniels, & Yuoh, 1996). However, no report is available which directly measures the content of hydroperoxides during oxidation process. The objective of this study was to report a new procedure for quantitative determination of lipid hydroperoxide formation using luminol-enhanced CL in the phospholipids and oil emulsions. The use of a detergent increases the emulsion stability and efficiency of the system due to preventing the side reactions. In order to validate our studies we have calibrated the chemiluminescence scale with linoleic acid hydroperoxide 13-HPODE (13-(S)-hydroperoxy-9Z,11E-octadecadienoic acid) as external standard. The LCL intensity is dose-dependent and increases with increasing hydroperoxides concentration. In this work we present a rapid, reliable LCL method to directly measure the amount of formed hydroperoxides in lipid emulsions during oxidation.

2. Materials and methods

2.1. Chemicals

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), TRITON X-100 (t-octylphenoxypoly-ethoxyethanol), buffer CAPS (3-[cyclohexylamino]-1-propansulfonic acid, methanol, hemin, linoleic acid and phosphaditylcholine (PC) were purchased from SIGMA. Linseed oil (Linolia) was purchased from Natural Fiber Institute (Poznan, Poland). Linseed oil containing essential fatty acids, phytosterols, phospholipids, vitamins and content of α-linolenic acid was estimated to 60%. Peroxide value (PV) was determined according to the standard ISO 3960:1977 (E). The method is based on the iodometric titration, which measures the iodine produced from potassium iodide by the peroxides present in the oil. The result is reported in milli-equivalents of oxygen per kilogram of fat. For PV measurement the volume of 50 ml of linseed oil was heated and every 30 min appropriate amount of oil was taken for analysis.

Doubly distilled water and the other reagents used were of HPLC grade. Pure 13-HPODE needed for chemiluminescence calibration was obtained by enzymatic oxygenation of linoleic acid (Nogala-Kalucka, Kupczyk, Polewski, Siger, & Dwiecki, 2007).

2.2. Methods

The synthesis of free fatty acid hydroperoxides (FAOOHs) standards was carried out as described by Spiteller, Kern, Reiner, and Spiteller (2001) and Spiteller and Spiteller (1997) but their procedures were modified for the synthesis of 13-hydroperoxy-octadecadienoic acid (13-HPODE) standards and details are given elsewhere (Nogala-Kalucka et al., 2007).

2.3. Oxidation of linoleic acid and lipids

Weighted amount of linoleic acid, linseed oil or 2 mg/ml of PC suspended in water – sonicated until clear solution was obtained – were incubated at 60 ± 1 °C for maximum 24 h without light in the presence of air. The hydroperoxide content was determined every 30 min. Oxidized solution (100 μl) was added to 0.1 M CAPS buffer with 5% methanol and 0.025% TRITON X-100. The samples were sonicated (IS-K1, InterSonic, Poland) for 2–3 min in ice-cold water to form emulsion. After sonication the solution cooled down to room temperature and was used for LCL analysis by injecting luminol and hemin. For temperature dependent measurements the heating temperature was varied.

UV absorption of conjugated diene hydroperoxides during lipid oxidation was measured using Shimadzu UV 1200 spectrophotometer (Shimadzu Ltd.)

2.4. Chemiluminescence measurements

The chemiluminescent solution consisted of: luminol 0.1 mM, 5% methanol, 0.025% TRITON X-100, buffer CAPS 0.1 M pH 10, hemin 5×10^{-6} M and source of hydroperoxides (standard, linoleic acid, linseed oil or PC). Chemiluminescent reaction started by adding hemin to the mixture containing thermally oxidized lipid emulsion. A 3 ml round, flat cuvette was placed directly before a photocathode of an RCA EMI 9558 QB photomultiplier connected to data collecting board in PC computer. As a measure of CL the integrated area under registered signal was used, I_0. In order to obtain blank level of CL emission the registration started before any component was added to the cuvette.

2.5. Statistical analysis

Experiments were repeated several times and when appropriate the SD, errors and correlation coefficients were calculated. Statistical calculation and graphs were done in program ORIGIN (OriginLab Corp., MA, USA).