LIPOPHILIC ANTIOXIDANTS IN VARIOUS TISSUES OF SELECTED MALAYSIAN FRESHWATER FISH

EZARUL FARADI ANNA BT LOKMAN.

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LIPOPHILIC ANTIOXIDANTS IN VARIOUS TISSUES OF SELECTED MALAYSIAN FRESHWATER FISH

By

EZARUL FARADIANNA BT LOKMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the Requirement for the Degree of Master of Science

January 2006
Specially dedicated to;

My beloved late father, mother
my family and friends
LIPOPHILIC ANTIOXIDANTS IN VARIOUS TISSUES OF SELECTED MALAYSIAN FRESHWATER FISH

By

EZARUL FARADIANNA BT LOKMAN

January 2006

Chairman:  Associate Professor Juzu Hayati Arshad, PhD
 Faculty:  Faculty of Biotechnology and Biomolecular Sciences

The total antioxidant activity in the muscle, liver and intestine of eleven species of Malaysian freshwater fish known as Pangasius polyuranodon, Anabas testudineus, Channa striatus, Clarias batrachus, Labeo rohita, Tilapia mossambica, Leptobarbus hoevenii, Trichogaster pectoralis, Hemibagrus nemurus, Cyprinus carpio carpio and Puntius gonionotus were evaluated using the optimized thiobarbituric acid (TBA) method. The synthetic antioxidant, butylated hydroxytoluene (BHT) was used as positive control. The peroxidation of linoleic acid in the thiobarbituric acid system was markedly inhibited by all the sample extracts compared with the control assay and also showed very low chelating activity with the iron chelator test. This indicated that the tissue samples contained insignificant quantities of iron chelators which would otherwise interfere with the TBA method. All the fish extracts exhibited total antioxidant activity in the order of muscle (61-81%) > liver (51-83%) > intestine (40-70%).

Three fish species identified to have high antioxidant activities in the muscle tissue namely Anabas testudineus, Clarias batrachus and Labeo rohita and a
species with the lowest antioxidant activity, *Hemibagrus nemurus* were selected for determination of the lipophilic antioxidants using the High Performance Liquid Chromatography (HPLC) analysis. The high antioxidant activities found in the muscle of *Anabas testudineus* and *Labeo rohita* were influenced by the presence of relatively high amounts of lipophilic antioxidants namely α-, β-, γ-, δ- tocopherols, retinol and coenzyme Q₁₀. Liver, which was found to have retinol at 0.711 ± 0.09 to 6.05 ± 0.16 µg/g wet weight probably influenced the antioxidant activities obtained. However, the intestine showed the lowest antioxidant activity compared to the other tissues examined. It was found that β-tocopherol (1.316 -3.861µg/g wet weight) was the only lipophilic antioxidant present.

The distributions of the various tocopherol homologues, retinol and coenzyme Q homologues in different tissues of Malaysian freshwater fish indicated that these compounds might be independently regulated in each tissue. The difference in the antioxidant activities in the muscle, liver and intestine of different samples in this study may be influenced by the presence of different types of lipophilic antioxidants in each sample. The three potential Malaysian freshwater fish species with high antioxidant activities identified were *Anabas testudineus*, *Clarias batrachus* and *Labeo rohita*.

In conclusion, the Malaysian freshwater fish species examined which were found to have high antioxidant activities are recommended as part of the diet as they may be able to protect the human body from free radicals and retard the progress of many chronic diseases. The fish extracts found to have lipophilic antioxidants in this study also can be used as accessible source of
natural antioxidants to replace synthetic antioxidants, as possible food supplement as well as in pharmaceutical applications.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANTIOKSIDA LIPOFLIK DALAM BEBERAPA JENIS TISU IKAN AIR TAWAR TERPILIH DI MALAYSIA

Oleh

EZARUL FARADIANNA BT LOKMAN

Januari 2006

Pengerusi:  Professor Madya Juzu Hayati Arshad, PhD
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Jumlah aktiviti antioksida dalam otot, hati dan usus sebelas spesis ikan air tawar di Malaysia dikenalpasti sebagai *Pangasius polyuranodon, Anabas testudineus, Channa striatus, Clarias batrachus, Labeo rohita, Tilapia mossambica, Leptobarbus hoevenii, Trichogaster pectoralis, Hemibagrus nemurus, Cyprinus carpio carpio* dan *Puntius gonionotus* dinilai menggunakan kaedah asid thiobarbiturik (TBA) yang optimum. Antioksida sintetik, butylated hydroxytoluene (BHT) digunakan sebagai kawalan positif.

Pengoksidaan asid linoleik di dalam sistem asid tiobarbiturik nyatanya direncat oleh ekstrak sampel-sampel berbanding dengan asai kawalan dan juga menunjukkan aktiviti pengelat besi yang rendah. Ini menunjukkan bahawa tisu sampel mengandungi kuantiti pengelat besi yang tidak signifikan. Semua ekstrak tisu menunjukkan jumlah aktiviti antioksida mengikut turutan otot (61-81%) > hati (51-83%) > usus (40-70%).

Tiga spesis ikan dikenalpasti mengandungi aktiviti-aktiviti antioksida yang tinggi di dalam otot iaitu *Anabas testudineus, Clarias batrachus* and *Labeo rohita* dan sejenis spesis aktiviti antioksida yang terendah, *Hemibagrus*...
nemurus dipilih untuk menentukan antioksida lipofilik menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC). Aktiviti antioksida yang ditemui tinggi dalam otot Anabas testudineus dan Labeo rohita dipengaruhi oleh kehadiran secara relatif jumlah antioksida lipofilik yang tinggi seperti α-, β-, γ-, δ-tokoferol, retinol and coenzyme Q₁₀. Hati, yang dikenalpasti mengandungi retinol pada 0.711 ± 0.09 to 6.05 ± 0.16 μg/g berat basah berkemungkinan mempengaruhi aktiviti antioksida yang diperolehi. Walaubagaimanapun, usus menunjukkan aktiviti antioksida yang terendah berbanding dengan tisu-tisu lain yang dikaji. Dikenalpasti bahawa hanya β- tokoferol (1.316 – 3.861 μg/g berat basah) sahaja lipofilik antioksida yang hadir.

Taburan homolog tokoferol, retinol dan homolog coenzyme Q di dalam beberapa jenis tisu ikan air tawar di Malaysia menunjukkan bahawa sebatian-sebatian ini berkemungkinan dikawal atur secara tersendiri di dalam setiap tisu. Perbezaan aktiviti antioksida dalam otot, hati dan usus bagi sampel berbeza di dalam kajian ini mungkin dipengaruhi oleh jenis lipofilik antioksida yang berbeza di dalam setiap sampel. Tiga spesis ikan air tawar yang dikenalpasti berpotensi dengan aktiviti antioksida yang tinggi ialah Anabas testudineus, Clarias batrachus and Labeo rohita.

Pada kesimpulannya, ikan air tawar Malaysia yang dikaji mengandungi aktiviti antioksida tinggi digalakkan untuk dijadikan sebagai sebahagian daripada diet disebabkan kebolehan antioksida untuk melindungi badan manusia daripada radikal bebas dan merencat pembentukan peyakit-penyakit kronik. Ekstrak ikan yang mengandungi lipofilik antioksida di dalam kajian ini juga boleh digunakan sebagai sumber antioksida semulajadi untuk...
menggantikan antioksida sintetik, sebagai makanan tambahan serta di dalam aplikasi farmaseutikal.
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I would like to express my deepest gratitude to the Almighty Allah for His infinite mercy and abundant graces.

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Without all of the people mentioned above, who kindly took time to participate and to help me gather information, this project would not have been accomplished.
I certify that an Examination Committee has met on 24th January 2006 to conduct the final examination of Ezarul Faradianna Lokman on her Master of Science thesis entitled "Lipophilic Antioxidants in Various Tissues of Selected Malaysian Freshwater Fish" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory committee are as follows:

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School of Graduate Studies  
Universiti Putra Malaysia

Date: 13 APR 2006
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Date: 20 MARCH 2006

EZARUL FARADIANNA BT LOKMAN
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ix</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>xi</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xix</td>
</tr>
</tbody>
</table>

## CHAPTER

### 1 INTRODUCTION

### 2 LITERATURE REVIEW

2.1 Reactive Oxygen Species

2.2 Mechanism of Free Radicals on Polyunsaturated Fatty Acids (PUFA)

2.3 Lipophilic Antioxidants
   2.3.1 Vitamin E
   2.3.2 Coenzyme Q
   2.3.3 Vitamin A (Retinol)

2.4 Mechanism of Ubiquinone, Vitamin E and Ascorbate on Lipid Peroxidation Process

2.5 Sources of Antioxidants

2.6 Lipophilic Antioxidants in Fish

2.7 Malaysian Freshwater Fish

2.8 Lipid Extraction Methods

2.9 Antioxidant Activity Assay

2.10 Identification of Lipophilic Antioxidants Using High Performance Liquid Chromatography (HPLC)

### 3 MATERIALS AND METHODS

3.1 Materials
   3.1.1 Experimental Design, Fish, Rearing
   3.1.2 Chemicals and Equipments

3.2 Statistical Analysis

3.3 Preparation of Fish

3.4 Lipid Extraction Methods
   3.4.1 Bligh and Dyer Method
   3.4.2 Sodium Dodecyl Sulphate (SDS) Method
   3.4.3 n-hexane/ethanol Method

3.5 Determination of Antioxidant Activity – Thiobarbituric Acid (TBA) Method
3.6 The Optimization of the Thiobarbituric Acid (TBA) Method
3.7 Iron Chelator Test
3.8 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity
3.9 Identification and Quantitation of Lipophilic Antioxidants - High performance Liquid Chromatography (HPLC)
3.9.1 Samples Extraction
3.9.2 Standards Preparation
3.9.3 High Performance Liquid Chromatography (HPLC) Analysis

4 RESULTS AND DISCUSSION
4.1 The Optimization of Thiobarbituric Acid Assay
4.1.1 Incubation Period
4.1.2 Linoleic Acid
4.1.3 FeCl₂ and FeCl₃
4.1.4 Thiobarbituric Acid
4.2 Butylated Hydroxytoluene and α-Tocopherol
4.3 Effect of Different Extraction Procedures (Bligh and Dyer, Sodium Dodecyl Sulphate and n-hexane/Ethanol) on the Total Antioxidant Activity of Fish Extracts
4.4 Effect of Different Amounts of Samples Extracted with Bligh and Dyer (1959) on the Total Antioxidant Activity
4.5 Determination of Total Antioxidant Activities in Various Tissues of Malaysian Freshwater Fish Using the Thiobarbituric Acid Assay
4.6 Iron Chelator Test
4.7 Determination of DPPH Radical Scavenging Activities of Selected Malaysian Freshwater Fish
4.8 Identification and Quantitation of Lipophilic Antioxidants Using High Performance Liquid Chromatography (HPLC)

5 CONCLUSION

REFERENCES 76
APPENDICES 87
BIODATA OF THE AUTHOR 101
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
</tr>
<tr>
<td>7</td>
<td>59</td>
</tr>
<tr>
<td>8</td>
<td>62</td>
</tr>
<tr>
<td>9</td>
<td>64</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The Initiation, Propagation and Termination Steps in Lipid Peroxidation</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Chemical Structures of Vitamin E; (a) Tocopherols and (b) Tocotrienols</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>Absorption, Transport and Metabolism of α- and γ- Tocopherol in the Body.</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Chemical Structures of Coenzyme Q: Ubiquinol (CoQH₂), Semiquinol Radical (CoQH) and Ubiquinol (CoQ)</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>Structures of Vitamin A; Retinyl Ester, Retinol, Retinal and Retinoic Acid</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>Mechanism of Coenzyme Q, Vitamin E and Ascorbate on Lipid Peroxidation</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>Effect of Different Incubation Period Ranging from 0 to 48 Hours on the TBA Assay at 532 nm.</td>
<td>39</td>
</tr>
<tr>
<td>8</td>
<td>Effect of Different Concentrations of Linoleic Acid Ranging from 0.49 to 0.61% on the TBA Assay at 532 nm.</td>
<td>41</td>
</tr>
<tr>
<td>9</td>
<td>Effect of Different Concentrations of FeCl₂ and FeCl₃ Ranging from 2 to 4.8 mM on the TBA Assay at 532 nm.</td>
<td>41</td>
</tr>
<tr>
<td>10</td>
<td>Effect of Different Concentrations of TBA Ranging from 0 to 0.4 % on the TBA Assay at 532 nm.</td>
<td>45</td>
</tr>
<tr>
<td>11</td>
<td>Effect of Different Concentrations of the Positive Controls, ATF and BHT Ranging from 0.2 to 1.4 mM on the TBA Assay at 532 nm.</td>
<td>45</td>
</tr>
<tr>
<td>12</td>
<td>Total Antioxidant Activity of Muscle, Liver and Intestine Tissues of <em>Clarius Batrachus</em> using Different Lipid Extraction Procedures (Bligh and Dyer (BD), Sodium Dodecyl Sulphate (SDS) and n-hexane (HE)) from the Crude Extract Obtained from 5 grams of Tissue Samples.</td>
<td>48</td>
</tr>
</tbody>
</table>
Effect of Different Concentrations of Samples on the TBA Assay Ranging from 0.1 to 0.5 mg/ml of Reaction Mixture at 532 nm Using the Bligh and Dyer Extraction Method.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF</td>
<td>Alpha tocopherol</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing ability of plasma</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>Ferrous chloride</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>MADA</td>
<td>Muda Agricultural Development Authority</td>
</tr>
<tr>
<td>MS</td>
<td>Mass chromatography</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>TAA</td>
<td>Total Antioxidant Activity</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

All aerobic organisms require protection against deleterious effect of oxygen radical produced from metabolic oxidation reaction in the cell. These reactive oxygen species (ROS) are capable of damaging lipids, DNA, nucleic acid and protein (Di Giulio et al., 1986 and Tyrell, 1991). Unsaturated membrane lipids are the main cellular targets of ROS damage, oxidation of which impairs normal metabolic functions (Slater, 1984).

Living organisms are usually protected from reactive oxygen species by several defence mechanisms, including antioxidant enzymes and low molecular weight antioxidants (Pascual et al., 2003). Biochemical defences against reactive oxygen species include hydrophilic (glutathione, ascorbic acids and uric acids) and lipophilic compounds (vitamin E, ubiquinol and retinol) that scavenge radical species. In contrast to these low molecular weight scavengers, antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase can specifically remove active species leading to the initiation of lipid peroxidation and oxidation of other cellular biomolecules (Ahmad, 1995).

Current sources of natural lipophilic antioxidants include vegetables, plants, and animal tissues (Ruperez et al., 2001). The most commonly used synthetic antioxidants to preserve food are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), tert- butylhydroquinone (TBHQ) and α- tocopherol (ATF). However, synthetic antioxidants have been implicated for several diseases such as liver damage and carcinogenesis.
Therefore, the utilization of more effective lipophilic antioxidants of natural origin are needed. Few studies on the lipophilic antioxidants in fish species were reported in the previous studies particularly in the marine fish species (Giardina et al. 1997; Erickson, 1992; Marcon and Filho, 1999, Gieseg et al., 2000; Dunlap et al., 2002). However, no study has been carried out on the antioxidant activity and lipophilic antioxidants in the Malaysian freshwater fish.

Therefore, the objectives of this study were:

1) to screen the antioxidant activity in the muscle, liver and intestine of eleven Malaysian freshwater fish species using the optimized thiobarbituric acid (TBA) assay (via inhibition of lipid peroxidation)

2) to identify and quantify the lipophilic antioxidants in the selected fish using High Performance Liquid Chromatography (HPLC) analysis.

The Malaysian freshwater fish selected in this study were patin (*Pangasius polyuranodon*), puyu (*Anabas testudineus*), haruan (*Channa striatus*), keli (*Clarias batrachus*), rohu (*Labeo rohita*), tilapia (*Tilapia mossambica*), jelawat (*Leptobarbus hoevenii*), sepat (*Trichogasfer pectoralis*), baung (*Hemibagrus nemurus*), lee koh (*Cyprinus carpio carpio*) and lampam jawa (*Puntius gonionotus*). The fish species used in this study were chosen based on its popularity amongst Malaysian.
2.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive chemicals, containing oxygen that can react easily with other molecules resulting in potentially damaging modifications. Reactive oxygen species are formed by several different mechanisms, which include cellular respiration and the interaction of ionizing radiation with biological molecules.

Reactive oxygen species are free radicals, also known as radicals (molecules having an unpaired electron in the outer orbit and unstable), which include hydroxyl, alkoxyl, hydroperoxyl, peroxyl, nitric oxide and superoxide. The non-radical oxygen species include peroxynitrite, hypochlorite, hydroperoxide, singlet oxygen and hydrogen peroxide (Abuja and Albertini, 2001). Hydroperoxides (a non-radical species) will lead to the formation of alkoxyl and peroxyl radicals in the presence of transition metal ions. The lists of radical and non-radical species are shown in Table 1.

Radicals can give their unpaired electron to other compounds and may cause chain reactions, polymer breakage and lipid peroxidation (Özben, 1997). Free radicals may cause oxidative damage to lipid, protein DNA and nucleic acids. Unsaturated membrane lipids are the main cellular targets of ROS damage, oxidation of which impairs normal metabolic functions (Slater, 1984) which may lead to many biological complications including carcinogenesis, mutagenesis, aging and artherosclerosis (Halliwell and Gutteridge, 1989).
Table 1: Reactive oxidant species (Abuja and Albertini, 2001)

<table>
<thead>
<tr>
<th>Reactive</th>
<th>Symbol</th>
<th>Non-radical</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl</td>
<td>*OH</td>
<td>Peroxynitrite</td>
<td>ONOO&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alkoxy</td>
<td>L(R)O&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Hypochlorite</td>
<td>OCl&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hydroperoxyl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HOO&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Hydroperoxide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>L(R)OOH</td>
</tr>
<tr>
<td>Peroxyl</td>
<td>L(R)OO&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Singlet oxygen</td>
<td>¹&lt;sup&gt;O&lt;/sup&gt;₂</td>
</tr>
<tr>
<td>Nitric oxide&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NO&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Hydrogen peroxide&lt;sup&gt;d&lt;/sup&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Superoxide</td>
<td>O₂&lt;sup&gt;-&lt;/sup&gt;</td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Hydroperoxyl radical is the conjugated acid of superoxide anion and it is present in aqueous solution at concentrations dependent on pH.

<sup>b</sup>In the presence of transition metal ions, hydroperoxides will lead to the formation of alkoxy and peroxyl radicals.

<sup>c</sup>NO<sup>*</sup> itself is rather unreactive and is often regarded as an antioxidant in lipid peroxidation processes. In the presence of superoxide, it forms peroxynitrite, which is strongly oxidizing.

<sup>d</sup>Superoxide is not a good oxidant, rather it is a reductant. It’s importance in biological oxidation is a consequence of its ability to both oxidize and reduce transition metal ions, which leads to the formation of H<sub>2</sub>O<sub>2</sub> leading to the production of *OH in the presence of reduced transition metal ions.
2.2 Mechanism of Free Radicals on Polyunsaturated Fatty Acids (PUFA)

The polyunsaturated lipid peroxidation process initiates by reactive oxygen species can be divided into initiation, propagation and termination phases as shown in Figure 1. Initiation takes place through a transition metal-induced (or radiation-induced) abstraction of a hydrogen atom from a methylene group of a fatty acid containing two or more separated double-bonds, forming a carbon-centered alkyl radical (L\(^*\)), with a simultaneous rearrangement of the double-bonds to become conjugated ("diene conjugation"). The L\(^*\) formed reacts with O\(_2\) rate giving rise to a peroxyl radical (LOO\(^*\)).

Propagation involves the abstraction of a hydrogen atom from an adjacent unsaturated fatty acid by LOO\(^*\), resulting in the formation of a lipid hydroperoxide (LOOH) and a new L\(^*\) radical. LOOH can react with Fe\(^{2+}\), producing the alkoxyl radical (LO\(^*\)). This radical, which is more reactive than LOO\(^*\), can again reinitiate lipid peroxidation by hydrogen abstraction from an adjacent polyunsaturated fatty acids, with the formation of L\(^*\) and alcohol (LOH) at the end product.

LOOH can also undergo degradation into hydrocarbons (ethane, pentane), alcohols, ethers, epoxides and aldehydes. Among the latter, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are of special importance since they can cross-link phospholipids, proteins and DNA. Termination of the lipid peroxidation process is generally believed to take place by interaction between two radicals, to form a non-radical product (Özben, 1997).