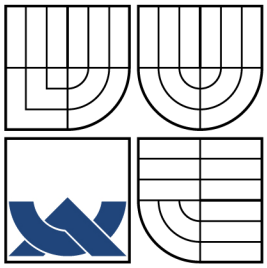

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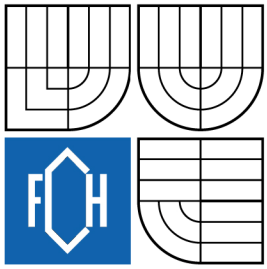

FACULTY OF CHEMISTRY
INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

EVALUATION OF ANTIOXIDANT EFFECT USING DIFFERENT ANALYTICAL METHODS

ZHODNOCENÍ ANTIOXIDAČNÍHO PŮSOBENÍ ZA POUŽITÍ RŮZNÝCH ANALYTICKÝCH METOD

DIPLOMA THESIS
DIPLOMOVÁ PRÁCE

AUTHOR
AUTOR
VĚRA KRISTINOVÁ

SUPERVISOR
VEDOUCÍ PRÁCE
DR.ING. TURID RUSTAD

BRNO 2008

Brno University of Technology
Faculty of Chemistry
Purkyňova 464/118, 61200 Brno 12

Diploma Thesis Assignment

Number of diploma thesis:	**FCH-DIP0163/2007**	Academic year:	**2007/08**
Institute:	Institute of Food Science and Biotechnology		
Student:	**Kristinová Věra**		
Study programme:	Chemistry and Technology of Foodstuffs (M2901)		
Study field:	Food Science and Biotechnology (2901T010)		
Head of diploma thesis:	**Dr.Ing. Turid Rustad**		
Supervisors of diploma thesis:	RNDr. Milena Vespalcová, Ph.D.		

Title of diploma thesis:

Evaluation of antioxidant effect using different analytical methods

Diploma thesis assignment:

Bibliographic search:
a) Summary of how different antioxidants work
b) Summary of methods to determine antioxidant effect
c) Literature search of comparison of different antioxidants using different methods and model systems

Experimental part:
1) Determination of antioxidant activity of caffeic acid, ferulic acid, coumaric acid, ascorbic acid and propyl gallate using
a) ABTS assay
b) FRAP assay
c) DPPH assay
d) Folin Ciocalteau assay
2) Assesment of the experimental working range for these assays
3) Study of the antioxidant effect of the same antioxidants in a liposome model system with cod roe phospholipids measuring oxygen uptake, using Fe2+/Fe3+ and hemoglobin as prooxidants. Assesment of good experimental conditions for this assay.

Deadline for diploma thesis delivery: 16.5.2008

Diploma thesis is necessary to deliver to a secretary of institute in three copies and in an electronic way to a head of diploma thesis. This assignment is enclosure of diploma thesis.

Kristinová Věra Student	Dr.Ing. Turid Rustad Head of thesis	doc. Ing. Jiřina Omelková, CSc. Head of institute

In Brno, 1.9.2007

doc. Ing. Jaromír Havlica, CSc.
Dean

ABSTRACT

The objective of this work was to evaluate antioxidant effects of five different compounds that have a potential as food antioxidants [propyl gallate (PG), caffeic acid (CaA), ferulic acid (FeA), *p*-coumaric acid (CoA) and L(+)-ascorbic acid (AsA)] by means of four antioxidant capacity (AOC) assays (FC, FRAP, DPPH, ABTS), and in a liposome model system using free iron (Fe^{2+}, Fe^{3+}) and bovine hemoglobin (Hb) as prooxidants, with focus on different concentration levels of the tested compounds. The oxygen uptake was used for continuous monitoring lipid oxidation at pH 5.5 and 30°C.

The orders of AOC obtained by the FC, FRAP and DPPH assay had a similar trend: PG > CaA > AsA > FeA > CoA. However, the degree of the antioxidant activity differed for the same compound in the different assays. The AOC order obtained by the ABTS assay differed substantially from the other orders: PG > CoA ~ FeA > CaA > AsA. Only PG showed the highest capacity in all the assays. The inconsistencies in the orders and degrees are discussed in relation to the methodology and chemistry of the assays, and in relation to the chemical properties of the tested compounds. The comparative study showed that the interpretation of the results obtained by these assays must be done with care taking into consideration drawbacks and limitations of each assay, and the use of only one assay to evaluate AOC may result in misleading information.

In the liposome model system the type of oxidation promoter, the interactions of the tested compounds with the prooxidants and the molar antioxidant-to-prooxidant ratio were found to be highly important factors. Other factors, such as structure of the molecule and location of the antioxidant in the system, also influenced the efficacy of the compounds. PG, CaA and FeA inhibited Hb-induced oxidation at all tested concentrations; the efficacy increased with increasing number of hydroxyl groups on the aromatic ring and with increasing concentration, and also correlated with reducing capacity of the compounds. CoA did not exhibit any activity at the tested concentrations. PG and FeA inhibited Fe-induced oxidation when the ratios between the antioxidant and Fe were ≥ 1. When the ratio was 0,1, PG slightly promoted oxidation. CaA strongly promoted Fe-induced oxidation at the ratios ≥ 0,1 by reduction of Fe^{3+} to Fe^{2+} *via* so called intra-molecular electron transfer, but did not exhibit any effect when the ratio was 0,01. CoA was completely inactive at all tested concentrations. AsA itself promoted oxidation, presumably *via* breaking down pre-formed lipid hydroperoxides and reduction of endogenous transition metals. After addition of Fe, the prooxidative effect was further intensified due to reduction of Fe^{3+} to Fe^{2+} facilitated by AsA. The effects of AsA on Hb-induced oxidation varied in a concentration range 1 – 100 μM, and above a concentration of 100 μM a prooxidative effect was observed.

The AOCs determined in the assays only partially matched with the effectivity of the compounds in the *in vitro* liposome model system. Therefore, potential food antioxidants should preferentially be evaluated in biologically relevant model systems with food-related conditions, and information achieved by the AOC assays could serve as a tentative or preliminary estimation of antioxidant potentials.

The outcomes of this work contribute to better understanding the basic pro- and antioxidant mechanisms and factors influencing oxidation of cell membranes, liposome solutions, and oil-in-water emulsions stabilized by phospholipids.

KEYWORDS

Antioxidants, antioxidant capacity assays, liposomes, lipid oxidation, iron, hemoglobin, oxygen uptake.

ABSTRAKT

Cílem této práce bylo zhodnotit antioxidační účinky pěti různých sloučenin s potenciálním využitím jako antioxidanty v potravinách [propylgalát (PG), kávová kyselina (CaA), ferulová kyselina (FeA), *p*-kumarová kyselina (CoA) a L(+)-askorbová kyselina (AsA)], a to prostřednictvím čtyř testů antioxidační kapacity (AOK) (FC, FRAP, DPPH, ABTS) a v modelovém systému liposomů za použití volného železa (Fe^{2+}, Fe^{3+}) a hovězího hemoglobinu (Hb) jako prooxidantů se zaměřením na různé koncentrace testovaných sloučenin. K nepřetržitému monitorování oxidace lipidů při pH 5,5 a teplotě 30 °C bylo použito spotřeby kyslíku.

Pořadí AOK stanovené FC, FRAP a DPPH testem mělo podobný trend: PG > CaA > AsA > FeA > CoA. Nicméně, míra antioxidační aktivity se u té same sloučeniny v jednotlivých testech lišila. Pořadí AOK stanovené ABTS testem se od ostatních lišilo podstatně: PG > CoA ~ FeA > CaA > AsA. Pouze PG vykazoval nejvyšší kapacitu ve všech testech. Rozdíly v pořadí a míře AOK jsou blíže rozebrány vzhledem k metodologii a chemii testů a vzhledem k chemickým vlastnostem testovaných sloučenin. Komparativní studie ukázala, že interpretace výsledků získaných těmito testy by měla být provedena obezřetně, v úvahu by měly být brány nevýhody a omezení každého testu, a využití pouze jednoho testu k posouzení AOK může mít za následek zavádějící informace.

V modelovém systému liposomů se jako vysoce důležité faktory ukázaly být typ prooxidantu, interakce testovaných sloučenin s prooxidanty a molární poměr mezi antioxidantem a prooxidantem. Další faktory, jako struktura molekuly a umístění antioxidantu v systému, také ovlivňovaly účinnost testovaných látek. PG, CaA a FeA utlumily oxidaci vyvolanou Hb při všech testovaných koncentracích; účinnost stoupala s vyšším počtem hydroxylových skupin na aromatickém jádře a s vyšší koncentrací, a korelovala také s redukční kapacitou sloučenin. CoA nejevila žádnou aktivitu při testovaných koncentracích. PG a FeA utlumily oxidaci vyvolanou Fe, když poměr mezi antioxidantem a Fe byl ≥ 1. Když byl poměr 0,1, PG mírně urychlil oxidaci. CaA silně urychlila oxidaci vyvolanou Fe při poměru ≥ 0,1 následkem redukce Fe^{3+} na Fe^{2+} označované jako intra-molekulární přenos elektronů, ale nejevila žádný účinek, když byl poměr 0,01. CoA byla zcela neaktivní při všech koncentracích. AsA urachlila oxidaci sama o sobě, pravděpodobně rozkladem již existujících lipidových hydroperoxidů a redukcí endogenních přechodných kovů. Po přidání Fe se tento prooxidační efekt ještě více zintenzívnil následkem redukce Fe^{3+} na Fe^{2+}, kterou AsA zprostředkovává. Účinky AsA na oxidaci vyvolanou Hb se měnily v rozmezí koncentrace 1 – 100 μM a nad koncentrací 100 μM byl pozorován prooxidační efekt.

Antioxidační kapacity stanovené v testech se jen částečně shodovaly s účinností sloučenin v *in vitro* modelovém systému liposomů. Proto by látky s potenciálním využitím jako antioxidanty v potravinách měly být přednostně posuzovány v biologicky významných modelových systémech s podmínkami blížícími se potravinám a informace získané testy AOK by mohly sloužit jako přibližný nebo předběžný odhad antioxidačního potenciálu.

Výsledky této práce přispívají k lepšímu pochopení základních pro- a antioxidačních mechanismů a faktorů ovlivňující oxidaci buněčných membrán, liposomálních roztoků a emulzí typu olej ve vodě stabilizovaných fosfolipidy.

KLÍČOVÁ SLOVA

Antioxidanty, testy antioxidační kapacity, liposomy, oxidace lipidů, železo, hemoglobin, spotřeba kyslíku.

KRISTINOVÁ, V. *Evaluation of antioxidant effect using different analytical methods.* Brno: Brno University of Technology, Faculty of Chemistry, 2008. 107 p. Supervisor of the diploma thesis: Dr. Ing. Turid Rustad, Professor at Dep. Biotechnology, NTNU in Trondheim, Norway.

The experimental part of the diploma thesis assignment had been carried out partly at the Department of Biotechnology of the Norwegian University of Science and Technology (NTNU) in Trondheim, and partly in SINTEF Fisheries and Aquaculture in Trondheim, Norway from September to December 2007.

DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

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signature

Acknowledgements

My biggest thanks belongs to my supervisor, Prof. Turid Rustad, for her good guidance and patient supervision of the thesis, and for the opportunity she gave me to work on the thesis in the laboratories of NTNU and SINTEF Fisheries and Aquaculture, where I was introduced to the very interesting field of lipid oxidation and antioxidants.

A special thanks belongs also to the researchers at SINTEF, namely Dr. Revilija Mozuraityte and Dr. Ivar Storrø, for the professional help, advice and valuable discussions they gave me during the work on the thesis, and for creating a friendly working environment.

Last, but not least, I would like to thank to my family and friends for their encouragement and support.

Věra Kristinová
Brno, 8th May 2008

CONTENTS

1 INTRODUCTION

Evaluation of antioxidant capacity of various matrices, such as beverages, plants, vegetables and fruits, as well as of pure compounds (e.g. phenols, vitamins), has lately become an important issue. [24] Many epidemiological studies have demonstrated an inverse correlation between the intake of natural antioxidants and the occurrence of oxidative stress related diseases. [11] Compounds of natural origin and certain plant materials with high antioxidant capacity have been of high interest for food industry as additives into fatty foods for protection against oxidative deterioration due to efforts to replace synthetic antioxidants with natural ones. [3] One way to evaluate antioxidant capacity is indirectly by means of spectrophotometric assays, other possibility is by the use of a lipid model system. [13]

Foods containing *n*-3 polyunsaturated fatty acids (PUFAs) are highly susceptible to oxidation, which causes undesirable flavours, rancid odours, and loss of the health beneficial fatty acids. To prevent oxidation of PUFAs antioxidants can be added. [2] To achieve the protective effects, an intimate knowledge of the factors that influence lipid oxidation is essential in order to select both the right antioxidant and the effective antioxidant concentration for a given application.

Foods are very complex systems. Therefore, the use of a simpler model system with defined and controllable variables can provide better conditions for investigations of the mechanisms of lipid oxidation, the effects of antioxidants and factors that influence lipid oxidation.

Marine phospholipid liposomes represent a convenient lipid model system, because they provide the oxidizable lipid substrate that is rich in PUFAs, and mimic both biological membranes and lipid emulsions. [63] Moreover, food-related conditions can be easily simulated in a liposome solution.

The presence and effects of lipid oxidation promoters, such as transition metals or heme pigments, are often neglected when different lipid model systems are used, yet they are of great importance. Traces of hemoglobin and iron are naturally present in many foods, both of fish and meat origin, and can be responsible for significant decrease in shelf-life of foodstuffs. Therefore, knowledge of how antioxidants affect activity of these promoters in a given system is desirable.

Screening of liposome oxidation by the oxygen uptake enables measuring the rate and kinetics of lipid oxidation. Moreover, the duration of one measurement takes less than one hour compared to days in conventional methods, and the effects of antioxidants can be observed virtually instantly, which reduces time and costs of the analysis.

The objective of this work is to evaluate antioxidant effects of five different compounds that have a potential as food antioxidants (propyl gallate, caffeic acid, ferulic acid, *p*-coumaric acid and L(+)-ascorbic acid) by means of four commonly used spectrophotometric antioxidant capacity assays (FC, FRAP, DPPH, ABTS), and in a marine phospholipid liposome model system with free iron (Fe^{2+}, Fe^{3+})- and bovine hemoglobin-catalyzed oxidation at pH 5.5 and 30°C. The latter study is focused on different concentration levels of the tested compounds relative to fixed concentrations of phospholipids, free iron, and hemoglobin.

2 THEORY

2.1 Lipids and lipid oxidation

2.1.1 Lipids

Lipids are a broad group of chemically diverse compounds that are soluble in non-polar solvents such as hydrocarbons or alcohols. They are classified as non-polar (e.g. triacylglycerol and cholesterol) and polar lipids (e.g. phospholipids). Polar lipids contain a hydrophilic "head" group that has a high affinity for water attached to a lipophilic "tail" group that has a high affinity for oil. [5]

The main components of lipids are *fatty acids* – compounds consisting of an aliphatic unbranched carbon chain and a carboxylic acid group attached to one end of the chain. The majority of fatty acids in nature contain 14 – 20 carbons (so called long-chained fatty acids). They can be either saturated or unsaturated. [5] The latter contain at least one double bond which is almost invariably *cis*.

n-3 and *n*-6 polyunsaturated fatty acids (PUFAs) belong to the fatty acids that are essential for human and have a documented beneficial effect on human health, hence they are important components of human diet. Figure 2–1 shows some important essential PUFAs. The highest proportions of long chain PUFAs are found in fish oil.

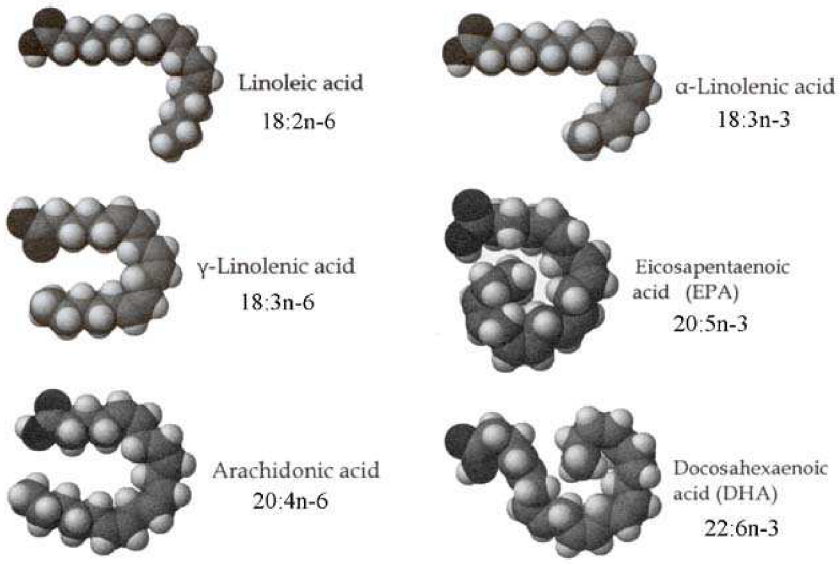
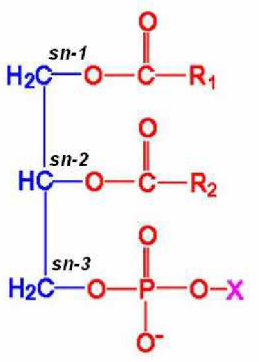
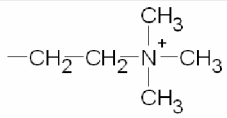
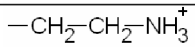
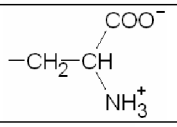
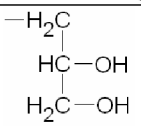
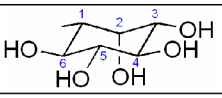


Figure 2–1 *Some essential n-6 (left) and n-3 (right) poly unsaturated fatty acids (PUFAs)* [98]

Over 99 % of fatty acids found in plants and animals are esterified to glycerol. Triacylglycerols are the most abundant group of acylglycerols followed by phospholipids (phosphoglycerides), where one of the fatty acid chains, typically in the *sn*-3 position, is replaced by a phosphate group. Some basic types of phospholipid molecules are shown in Table 2.1. [5]

Table 2.1 *Types of phospholipids (adapted from* [4]*)*

Basic structure	X		Name of phospholipid	
 **R_1, R_2** … fatty acids	Hydrogen atom	–H	*Phosphatidic acid*	PA
	Choline		*Phospahtidylcholine*	PC
	Ethanolamine		*Phosphatidylethanolamin*	PE
	Serine		*Phosphatidylserine*	PS
	Glycerol		*Phosphatidylglycerol*	PG
	Inositol		*Phosphatidylinositol*	PI

2.1.1.1 *Marine phospholipids and liposomes*

Marine phospholipids contain a high amount of *n*-3 PUFAs, mainly eicosapentaenoic acid (EPA, 20:5, *n*-3) and docosahexaenoic acid (DHA, 22:6, *n*-3). Antarctic krill and fish roe are examples of raw materials rich in marine phospholipids. [4] Phospholipids are the main constituents of biological membranes; phospholipids (soybean lecithin, egg yolk) are often added to food as emulsifiers because of their ability to stabilize emulsions.

Liposomes are microscopic spherical structures of one or more concentric lipid bilayers of phospholipids enclosing an equal number of aqueous compartments (a unilamellar liposome is shown in Figure 2–2). The vesicles can range in size from tens of nanometers to tens of micrometers in diameter and can be formed by variety of methods so as to control the size and also the number of bilayers. [62]

Liposomes made from marine phospholipids have a high potential as an oral supplement for PUFAs due to the observed higher lipid bioavailability from liposomes compared to fish oil. They also have a potential as an α-tocopherol supplement and as a delivery system in pharmacology. [4]

The use of liposomes as a model system for study of lipid peroxidation has several advantages. Primarily, the liposome system allows manipulation of lipid composition, pH, temperature, and contents of various agents in a defined way. [62] Due to the bilayer vesicular structure liposomes strongly resemble cell membranes; liposomes can also mimic emulsions stabilized by phospholipids. The only lipids in lean fish muscle are phospholipids of cell membranes [4]; therefore, marine phospholipids can be used for study of oxidation of fish meat matrices.

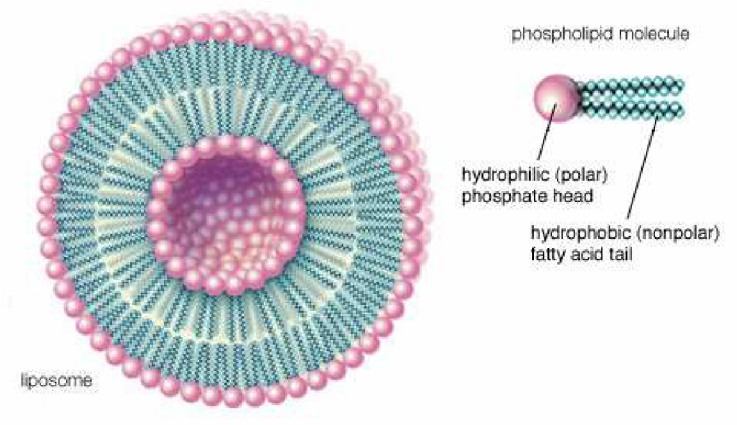


Figure 2–2 *Structure of a unilamellar liposome composed of phospholipid molecules* [94]

2.1.2 Mechanisms of lipid oxidation

Lipid oxidation is a complex phenomenon induced by oxygen in the presence of initiators such as heat, free radicals, light, photosensitizing agents and metal ions. It occurs *via* three reaction pathways:

a) nonenzymatic chain autoxidation mediated by free radicals,
b) nonenzymatic and nonradical photooxidation, and
c) enzymatic oxidation.

The first two types of oxidation consist of reactions involving triplet oxygen ($^3O_2$), the common oxygen that we breathe, and singlet oxygen ($^1O_2$), the excited form of the common oxygen. Singlet oxygen is short-lived and highly reactive and can react directly with the double bonds of fatty acids, while triplet oxygen can not.

Singlet oxygen is most often formed in the presence of triplet oxygen, UV light and type II photosensitizers (Sen), such as chlorophylls, hematoporphyrin or erythrosine. Type I photosensitizers, such as riboflavin, do not generate singlet oxygen. [7, 9]

When a ground singlet state photosensitizer (^{1}Sen) is exposed to light of a specific wavelength, it becomes an excited singlet state photosensitizer ($^1Sen^*$), which returns to the ground state *via* emission of light, internal conversion, or intersystem crossing (ISC). The latter produces an excited triplet photosensitizer ($^3Sen^*$) (1). The excited triplet photosensitizer may accept hydrogen from the substrate or donate an electron to the substrate and produce radicals (type I) (2). The excitation energy of the triplet sensitizer can be transferred to triplet oxygen to produce singlet oxygen or superoxide anion (type II) (3, 4). The excited triplet sensitizer returns to its ground state. [7, 9]

$$^1Sen \xrightarrow{h\nu} {}^1Sen^* \xrightarrow{ISC} {}^3Sen^* \quad (1)$$

$$\text{Type I:} \quad {}^3Sen^* + LH \rightarrow L^\bullet + H^\bullet + {}^1Sen \quad (2)$$

$$\text{Type II:} \quad {}^3Sen^* + {}^3O_2 \rightarrow \mathbf{{}^1O_2} + {}^1Sen \quad (3)$$

$${}^3Sen^* + {}^3O_2 \rightarrow O_2^{\bullet-} + {}^1Sen^{\bullet+} \quad (4)$$

The singlet oxygen formed through reaction (3) is highly electrophilic and can thus bind directly to C=C double bonds of fatty acids leading to hydroperoxide formation (LOOH) (5).

$$LH + {}^1O_2 \rightarrow LOOH \quad (5)$$

Nonradical photooxidation is believed to be an important mechanism, but not the only one, responsible for the onset of lipid autoxidation (see below) because the generated hydroperoxides may break down into free radicals and these radicals can initiate $^3O_2$-induced radical chain *autoxidation*.

Autoxidation is a key mechanism in lipid oxidation. It usually proceeds by a three-phase process: (i) initiation, (ii) propagation, and (iii) termination. [7]

The *initiation phase* involves homolytic breakdown of C–H bond, while the C atom is in α position relative to the fatty acid chain (LH) double bond. The reaction can be initiated *via* external physical agents, such as heat, ionizing radiation or UV light, and also by chemical agents such as metal ions, free radicals and metalloproteins (sensitizers). (6) However, the exact mechanism is still unknown.

$$LH \xrightarrow{initiator} L^{\bullet} + H^{\bullet} \quad (6)$$

In the *propagation phase*, the $L^{\bullet}$ radicals formed during the initiation phase react very quickly with triplet oxygen to generate peroxyl radicals ($LOO^{\bullet}$). (7) The peroxyl radical then abstracts a hydrogen atom from another unsaturated lipid molecule to form hydroperoxide (LOOH) (primary oxidation product) and another $L^{\bullet}$ (8), which can react in reaction (7). [7]

$$L^{\bullet} + {}^3O_2 \rightarrow LOO^{\bullet} \quad (7)$$
$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (8)$$

In the *termination phase*, free radicals react with each other to form stable non-radical end-products (secondary oxidation compounds). These reactions lead to the formation of hydrocarbons, aldehydes, alcohols and volatile ketones. Other nonvolatile compounds are also formed, such as nonvolatile aldehydes, oxidized triacylglycerols and their polymers. [7]

2.1.3 Prooxidants

Lipid oxidation can be promoted by *transition metals* with two or more valence states (Fe, Cu, Mn, Cr, Ni, V, Zn, Al). Such metals can oscillate between their reduced and oxidized states transferring electron (redox cycling), which catalyzes peroxide breakdown (9 – 11). Three mechanisms of oxidation promotion by metals have been proposed (further shown in example with iron):

1) interaction with unsaturated fatty acids:

$$Fe^{2+} + LH \rightarrow Fe^{3+} + H^{+} + L^{\bullet} \quad (9)$$

2) interaction with hydroperoxides (so called *Fenton-type reactions*) – two reaction pathways are possible [2]:

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + H^{+} + LOO^{\bullet} \quad (10)$$
$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + OH^{-} + LO^{\bullet} \quad (11)$$

3) activation of ground state molecular oxygen to its excited state, singlet oxygen [3]:

$$Fe^{2+} + {}^3O_2 \rightarrow Fe^{3+} + O_2^- \xrightarrow{-e^-} {}^1\mathbf{O_2} \quad (12)$$

$$\xrightarrow{+H^+} HO_2^- \quad (13)$$

Because of thermodynamic constrains, spin barriers, and an extremely low reaction rate, the direct interaction of metals with lipid molecules (9) is not considered to be the main mechanism of metal catalysis. [5]

The main mechanism is believed to be the interactions with lipid hydroperoxides (10 – 11). It has been generally accepted that a metal-hydroperoxide complex is formed and subsequently decomposed producing lipid radicals. Even trace amounts of these metals promote electron transfer from lipid hydroperoxides because the reaction (10) and (11) can ran cyclically with regeneration of the lower oxidation state of the metal. [5] Metals in their lower oxidation states catalyze hydroperoxide degradation to a larger degree and faster than metals in their higher oxidation states. The presence of a pre-existing lipid hydroperoxides has been found to be an essential condition for these reactions. [59]

The mechanism of metal catalyzed lipid peroxidation is shown in Figure 2–3.

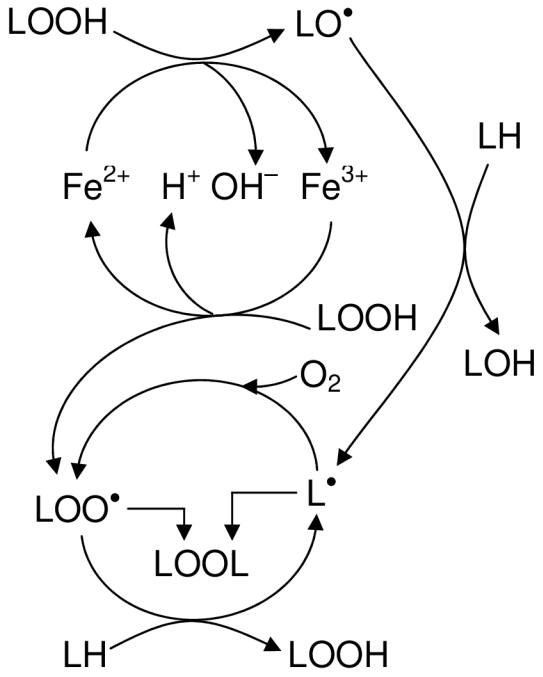


Figure 2–3 *Proposed mechanism of lipid peroxidation promoted by transition metals* [4]

Hemoglobin (Hb) is an iron-containing oxygen-transport metalloprotein present in the red blood cells of almost all vertebrates. The most common type of Hb in mammals consists of four subunits of the globular protein *globin* with an embedded *heme* group. The heme group consists of a porphyrin ring with a central iron atom, and is responsible for reversible binding of oxygen through ion-induced dipole forces (Figure 2–4). [5, 6, 95]

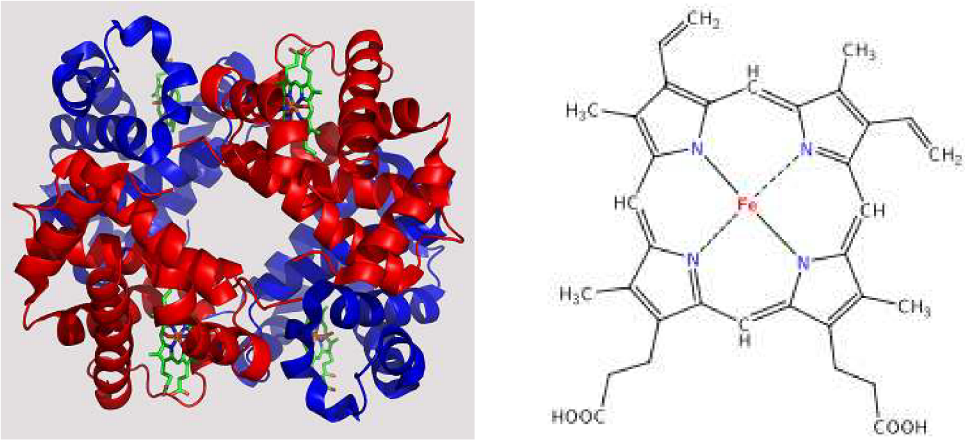


Figure 2–4 *Three-dimensional model of hemoglobin consisting of four globulin subunits, each with embedded heme group (left), and a structure of heme group – a porphyrin ring with iron (right)* [95]

Hemoglobin can exist in several different forms:

In its *reduced state* (Fe^{2+}), the O_2 molecule can be bound to the iron (red oxyhemoglobin) which is stabilized *via* hydrogen bonding by the nearby distal histidine, or it can be without the oxygen (blue deoxyhemoglobin), which occurs at low pH or at low oxygen tension.

Under the right conditions the iron can oxidize to form the *oxidized state* of hemoglobin (Fe^{3+}) (brown methemoglobin), which is not able to bind O_2. [74]

In the presence of strong oxidizing agents, such as hydrogen peroxide or lipid hydroperoxides, hemoglobin oxidizes to ferrylhemoglobin (Fe^{4+}). [51] Both the oxidized and the reduced forms can be prooxidative. The relationship between the individual forms of Hb is shown in Figure 2–5.

Several different mechanisms of the prooxidative activity of Hb have been proposed; they are summarized in Figure 2–6.

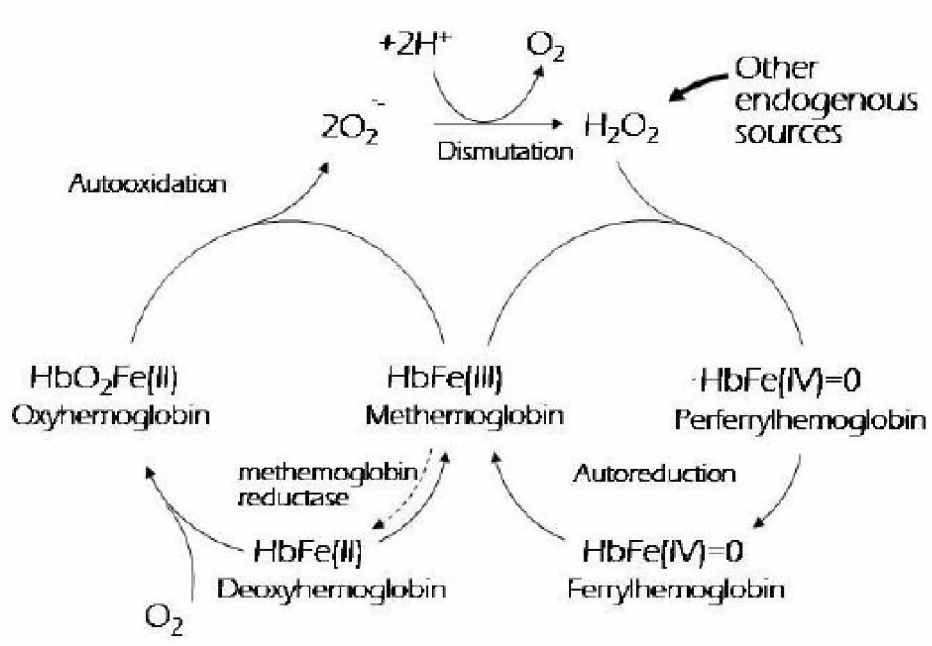


Figure 2–5 *Reaction mechanisms of hemoglobin autoxidation and autoreduction* [92]

Oxy-Hb (Fe^{2+}–O_2) can *autoxidize* to met-Hb (Fe^{3+}) releasing oxygen as a superoxide anion radical ($O_2^{\bullet-}$). This radical can further transform to hydrogen peroxide (H_2O_2), which can activate met-Hb to form a hypervalent ferryl-Hb (Fe^{4+}=O). Although this form is only transient in nature and has a short half-life, it is capable of peroxidizing lipids and is thought to be the main form responsible for Hb-catalyzed oxidation of lipids. [63]

The ferryl-Hb exists as a protein radical form and exerts its action by abstracting an electron from the lipid substrate leaving lipid radicals, which can cause further oxidation. The superoxide released on autoxidation can also lead to the formation of other ROS ($HOO^{\bullet}$, $HO^{\bullet}$) that are prooxidative.

The prooxidative activity of Hb is highly influenced by pH. At acidic pH the conformation of Hb is less stable. The lower the pH, the more unfolded is the Hb structure and the more exposed is the heme group, which leads to an increase in the prooxidative activity. On contrary, at alkaline pH the conformation of hemoglobin is much more stable, and the prooxidative activity of hemoglobin is greatly suppressed compared to the activity of native Hb at pH 7 or lower. [74]

The autoxidation reaction is enhanced by a low pH while it is reduced at an alkaline pH as interactions with the distal histidine become stronger. Part of this enhancement of autoxidation at low pH comes from the increased dissociation of the tetramer to dimers for mammalian hemoglobins and possibly full dissociation of fish hemoglobin to monomers. Dissociation is also accomplished when the protein is diluted. The dissociated form is also more prooxidative and has an increased tendency to lose the heme group. The presence of pre-formed lipid hydroperoxides and other oxidation products may also increase the autoxidation of hemoglobin. [74]

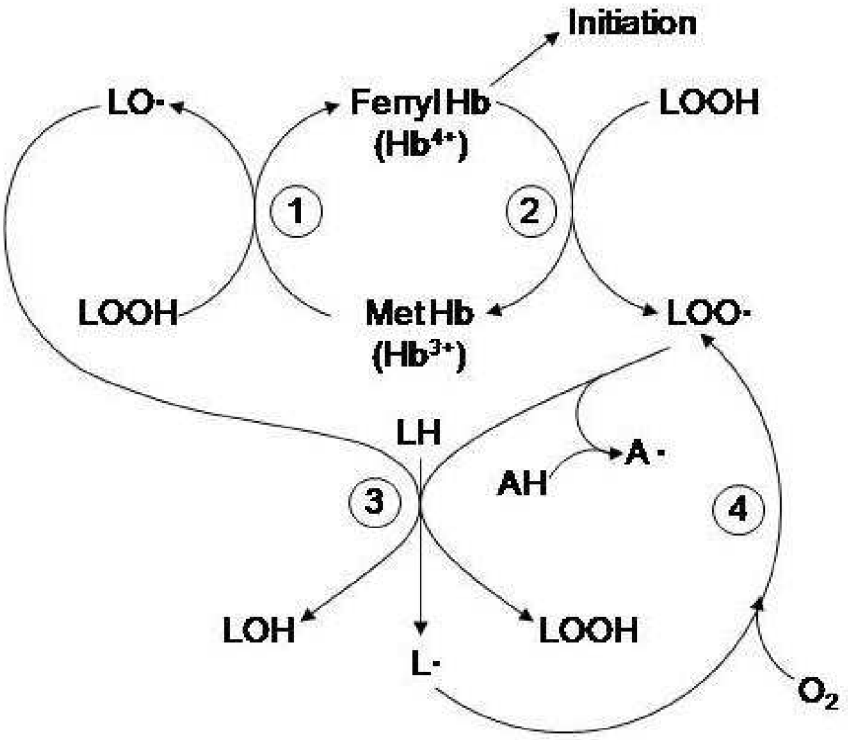


Figure 2–6 *Mechanism of hemoglobin promoted lipid oxidation* [92]

2.2 Antioxidants

An antioxidant is defined as any compound that can prevent biomolecules (proteins, nucleic acids, polyunsaturated lipids, sugars, etc.) from undergoing oxidative damage through free radical mediated reactions, when present at low concentrations compared to those of the oxidizable substrates. [1] This definition encompasses a wide array of mechanisms by which antioxidants can act and subsequently a wide array of compounds that can be classified as antioxidants.

According to the mechanism of action antioxidants can be broadly classified as *primary antioxidants* and *secondary antioxidants*. Some antioxidants exhibit more than one mechanism and are often referred to as multiple-function antioxidants. [2]

2.2.1 Primary antioxidants

Primary, or type I, antioxidants are free radical scavengers (FRS). [2] The ability of a compound to scavenge free radicals that participate in lipid peroxidation is commonly associated with the term *antioxidant*. Donation of antioxidant's hydrogen atom to the free radicals is the reaction mechanism involved here.

Free radical scavengers can slow lipid oxidation by inhibiting the initiation phase of lipid peroxidation (so called *preventive primary antioxidants*) by scavenging free lipid radicals ($L^{\bullet}$), or by inhibiting the propagation phase of lipid peroxidation by scavenging lipid alkoxyl ($LO^{\bullet}$) (14) and/or lipid peroxyl radicals ($LOO^{\bullet}$) (so called *chain-breaking antioxidants*) (15).

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet} \quad (14)$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet} \quad (15)$$

FRSs are considered to interact mainly with peroxyl radicals. Low energy state of peroxyl radicals makes them less reactive and extends their lifetime, and thus they have a greater chance of reacting with FRSs. This is in contrast with high energy free radicals (e.g. $OH^{\bullet}$) which are so reactive that they interact with the molecules closest to their sites of production. Since antioxidants are generally found in substrates at low concentrations, they would be less likely to react with the high energy free radicals. [5]

Antioxidant efficiency is dependent on the ability of the compound to donate a hydrogen atom to a free radical. This ability can be predicted with the help of standard one-electron reduction potentials ($E°$). Any compound that has a reduction potential lower than the reduction potential of a reactive oxygen species (ROS) is capable of donating its hydrogen to that ROS (free radical) unless the reaction is kinetically unfeasible. [5] The standard one-electron reduction potentials of reactive oxygen species and selected antioxidants are shown in Table 2.2.

The efficiency is also dependent on the energy of the resulting antioxidant radical ($A^{\bullet}$). The likelihood that this radical will abstract an H-atom from an unsaturated fatty acid, thus catalyze the oxidation, decreases with the decreasing energy of the antioxidant radical. Effective FRSs form low energy radicals owing to resonance delocalization of the unpaired electron, and also produce radicals that do not react rapidly with oxygen ($^3O_2$) to form hydroperoxides that could undergo decomposition reactions producing additional free radicals. $A^{\bullet}$ may participate in termination reactions with other $A^{\bullet}$ or lipid radicals to form nonradical compounds (16 – 18). [5]

$$LOO^{\bullet} + A^{\bullet} \rightarrow LOOA \quad (16)$$
$$LO^{\bullet} + A^{\bullet} \rightarrow LOA \quad (17)$$
$$A^{\bullet} + A^{\bullet} \rightarrow AA \quad (18)$$

Table 2.2 *Standard reduction potentials (E°) of reactive oxygen species* [9] *and selected antioxidants* [43]

Reactive oxygen species (half-cell)	**E°** (mV)
O_2, H^+ / $HO_2^{\bullet}$	– 460
O_2 / $O_2^{\bullet-}$	– 330
H_2O_2, H^+ / H_2O, $HO^{\bullet}$	320
$O_2^{\bullet-}$, H^+ / H_2O_2	940
$ROO^{\bullet}$, H^+ / ROOH	1000
$HO_2^{\bullet}$, H^+ / H_2O_2	1060 ~ 1500
$RO^{\bullet}$, H^+ / ROH	1600
$HO^{\bullet}$, H^+ / H_2O	2310
PUFA ($LOO^{\bullet}$, H^+ / LOOH)	~ 600
Antioxidants	**E°** (mV) *vs* Ag/AgCl
ascorbic acid	167
caffeic acid	212
ferulic acid	430
p-coumaric acid	583

Effective FRSs are *phenolic compounds*. [5] The mechanism of action of phenolic antioxidants will be explained in section 2.2.4.2.

Carotenoids can act as scavengers of lipid peroxyl radicals in the absence of singlet oxygen or at low oxygen partial pressure. [2] The conjugated double bonds of carotenoids are capable of reacting with peroxyl radicals to form a resonance-stabilized radical due to delocalization of electrons in the unsaturated structure. These radicals are unable to initiate lipid peroxidation and can participate in termination reactions with lipid radicals. [2, 5] β-carotenes are most active at a concentration of 5×10^{-5} mol/L while at higher concentrations the prooxidative effect is predominant. [6]

Ascorbic acid (Vitamin C) is active as a radical scavenger in aqueous media, but only at higher concentrations (~ 10^{-3} mol/L). A prooxidative activity has been observed at lower levels (10^{-5} mol/L), especially in the presence of heavy metal ions. [6]

2.2.1.1 *Reaction mechanisms of hydrogen donation*

Two distinct reaction mechanisms, by which the hydrogen atoms of antioxidants are transferred to a free radical, are generally accepted [42]; they are referred to as

- hydrogen-atom transfer (HAT), and
- single-electron transfer (SET) or proton-coupled electron transfer (PCET). [41, 42]

In the HAT mechanism, a whole hydrogen atom ($H^{\bullet}$) is abstracted from an antioxidant (ArOH) by the free radical using the same sets of orbitals. The antioxidant itself becomes a radical (19):

$$R^{\bullet} + ArOH \Rightarrow RH + ArO^{\bullet} \quad (19)$$

In the SET mechanism, the hydrogen atom ($H^{\bullet}$) of an antioxidant is transferred as a proton (H^{+}) and an electron to the free radical using different sets of orbitals. This means that the electron is transferred to the free radical turning it into an anion while the antioxidant turns itself into a radical cation ($ArO^{\bullet+}$) (20). In aqueous media, a rapid and reversible deprotonation of the radical cation (21) and a neutralization of the anion (22) follow. [41]

$$R^{\bullet} + ArOH \Rightarrow R^{-} + ArO^{\bullet+} \quad (20)$$
$$ArO^{\bullet+} + H_2O \Leftrightarrow ArO^{\bullet} + H_3O^{+} \quad (21)$$
$$R^{-} + H_3O^{+} \Leftrightarrow RH + H_2O \quad (22)$$

In the HAT mechanism, the bond dissociation enthalpy (BDE) of the O–H bonds is an important parameter in evaluating the antioxidant mechanism, because the weaker the O–H bond, the easier will be the free radical inactivation. In the SET mechanism the ionization potential (IP) is the most important energetic factor for evaluation of the scavenging ability. The lower the ionization potential, the easier is the electron abstraction. [42]

Mechanistically, electron transfer and hydrogen atom transfer can be difficult to distinguish, because the net result is the same ($R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet}$). [11] It is presumed that both HAT and SET mechanism must always occur in parallel, but at different rates. [42] Wright et al. investigated the BDE and IP values for a number of phenolics in the gas phase and concluded that the HAT mechanism is predominant for most of the phenolics. They also assumed that the IP values in solutions will be highly correlated with the IP values in gas, since solution-phase enthalpies of bond dissociation or electron transfer appear to follow the same trends as in the gas phase. [42]

However, in the solution-phase, several factors that can influence which mechanism prevails must be taken into consideration, they are:

- nature of solvent (polar × non-polar),
- pH of solvent,
- redox potentials of the antioxidants,
- presence of bulky groups near the OH group, or
- solubility of the antioxidant in medium. [42]

One of the important factors that influence the ratio between the HAT and SET mechanism is the *hydrogen-bonding characteristics* of the solvent (S). [42]

It is expected that the SET mechanism prevails in polar solvents (e.g. alcohols) due to solvent stabilization of the charged molecules (antioxidants) and therefore is strongly solvent dependent, whereas HAT mechanism is predominant in non-polar solvents (e.g. hexane) and therefore is only weakly solvent dependent. [27, 42]

The observations that the polar solvents reduce the rate of HAT reactions have been explained by considering that most of the molecules of antioxidants are hydrogen-bonded to the solvent (ArOH --- S), and these species are unable to react by HAT with free radicals. Two parameters are important for the strength of the hydrogen bond in the ArOH --- S complex and for the stability of the complex: hydrogen-bond basicity of the solvent and hydrogen-bond acidity of the antioxidant. [27]

2.2.2 Secondary antioxidants

Secondary, preventive, or type II antioxidants slow the rate of lipid oxidation by several different actions, but they do not convert free radicals to more stable products. They can

- chelate prooxidant metals and deactivate them,
- replenish hydrogen to primary antioxidants,
- decompose hydroperoxide to nonradical species,
- deactivate singlet oxygen,
- absorb ultraviolet radiation, or
- act as oxygen scavengers.

These antioxidants are often referred to as synergists because they promote the antioxidant activity of primary antioxidants (e.g. ascorbic acid, citric acid, lecithin, etc.). [2]

2.2.2.1 *Metal chelators*

Transition metals with two or more valence states (Fe, Cu, Mn, Cr, Ni, Al) are important promoters of lipid oxidation (see section 2.1.3 for the mechanisms). [2] The prooxidative activity of metals can be altered by chelators or sequestering agents. Chelators can inhibit the activity of these metals (the metal redox cycling) by one or more of the following mechanisms:

- occupation of all metal coordination sites,
- formation of insoluble metal complexes, and
- steric hindrance of interaction between metals and lipids or oxidation intermediates (e.g. hydroperoxides). [5]

Some metal chelators can increase oxidative reactions by increasing metal solubility or altering the redox potential of the metal. The tendency of chelators to inhibit or accelerate prooxidative activity depends on metal-to-chelator ratio. The typical example is EDTA (ethylenediamine tetraacetic acid): EDTA is ineffective or prooxidative when EDTA : iron ratio is ≤ 1 and antioxidative when EDTA : iron ratio is > 1. [5]

Chelators must be ionized to be active. Therefore their activity decreases at pH value below the pK_a of their ionizable groups.

The main metal chelators found in foods contain multiple carboxylic acid groups (e.g. EDTA, citric acid) or phosphate groups (e.g. polyphosphates and phytates). Prooxidant metals can also be controlled by metal binding proteins, such as transferrin, ferritin, phosvitin, lactoferrin, albumin and casein. [5, 7] Phenolic acids containing catechol and pyrogallol moiety, and flavonoids containing 3'4'-dihydroxy group in the B or C ring, and ketol structures 4-keto, 3-hydroxy or 4-keto, 5-hydroxy in the C ring, under favorable conditions also exhibit chelating abilities (Figure 2–7). [7, 15, 47, 46]

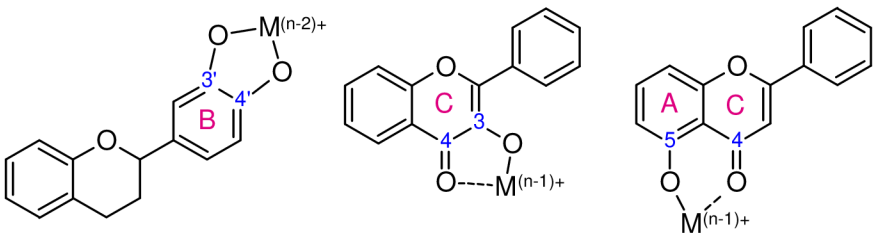


Figure 2–7 *Metallic ion complexation by flavonoids via the 3'-4'-o-diphenolic group in the B ring (left) and ketol structures 4-keto, 3-hydroxy in the C ring (middle) or 4-keto, 5-hydroxy in the C and A rings (right).*

2.2.2.2 *Oxygen scavengers and reducing agents*

Oxygen scavengers and reducing agents function by donating hydrogen atoms. Typical examples are ascorbic acid, ascorbyl palmitate, erythorbic acid, sodium erythorbate, and sulfites. Oxygen scavenging is useful in products with dissolved oxygen. [2]

Sulfites (SO_2, Na_2SO_3 and metabissulfites) react with molecular oxygen to form sulfates. They also act as reducing agents by promoting the formation of phenols from quinines. [2]

2.2.2.3 *Singlet oxygen quenchers*

Carotenoid pigments, such as carotenes (β-carotene, lycopene, lutein, etc.) and xanthophylls (isozeaxanthin, astaxanthin, etc.) represent the most active singlet oxygen ($^1O_2$) quenchers. [2] It is estimated that one carotenoid molecule is able to quench around 1000 $^1O_2$ molecules. [7] In the presence of a carotenoid, $^1O_2$ preferentially transfers its energy to the carotenoid producing a triplet state carotenoid and triplet oxygen. (23) Triplet state carotenoid dissipates the energy in the form of heat into the environment returning itself to the ground state. (24) [2]

$$^1O_2 + \text{carotenoid} \Rightarrow {}^3\text{carotenoid} + {}^3O_2 \quad (23)$$

$$^3\text{carotenoid} \Rightarrow \text{carotenoid} + \text{heat} \quad (24)$$

Carotenoids with nine or more conjugated double bonds are more efficient as $^1O_2$ quenchers than carotenoids with less unsaturated hydrocarbon structure or the ones with some functional groups attached to the hydrocarbon structure. [2] Beside quenching $^1O_2$, carotenoids can act also as chain breaking antioxidants (see section 3.2.1 for more details). [7]

Tocopherols, some phenolics, urate and ascorbate can also act as $^1O_2$ quenchers. [2, 7]

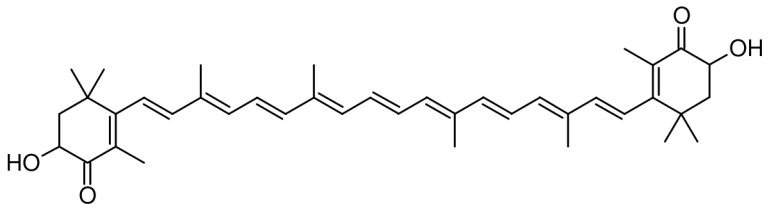


Figure 2–8 *Chemical structure of a carotenoid antioxidant astaxanthin* [7]

2.2.2.4 *Enzymatic antioxidants*

Almost all living systems have their own defensive system against ROS in the shape of endogenous enzymatic antioxidants. One of the most important enzymes is *superoxide dismutase* (SOD), a metalloenzyme that is omnipresent in eukaryotic organisms, and catalyzes superoxide dismutation into hydrogen peroxide and molecular oxygen (25). [7]

$$2\,O_2^{\bullet -} + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (25)$$

Another notable enzyme is *glutathion peroxidase* (GSH-Px), a selenocystein-dependent enzyme that has deactivation activity concerning three reactive species – hydrogen peroxide, lipid hydroperoxides and peroxylnitrite. [7]

A third enzyme of great importance is *catalase* (CAT), a heminic enzyme that mainly occurs in peroxisomes and erythrocytes, and catalyzes reduction of hydrogen peroxide to water and molecular oxygen. (26). [7]

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2 \qquad (26)$$

It is assumed that a direct cooperation exists between different enzymes *in vivo*. For example, SOD activity leads to the formation of hydrogen peroxide, which in turn is detoxified by CAT and/or GSH-Px. [7]

2.2.3 Physical location of antioxidants

The effectiveness of antioxidants depends apart from other factors on the physical nature of the lipid and the polarity of antioxidants. Hydrophilic antioxidants are often less effective in emulsions than lipophilic antioxidants, whereas lipophilic antioxidants are less effective in bulk oils than hydrophilic antioxidants. This phenomenon has been called “the polar paradox”. [5] (Figure 2–9)

Differences in the effectiveness of the antioxidants in bulk oils and emulsions are due to their physical location in the two systems. Polar antioxidants are more effective in bulk oils because they can accumulate at the air-oil interface or in reverse micelles within the oil, the location where lipid oxidation reaction would be greatest owing to high concentrations of oxygen and prooxidants. Non-polar antioxidants are more effective in emulsions because they are retained in the oil droplets and and/or may accumulate at the oil-water interface (created by emulsifiers, e.g. lecithin), the location with the occurrence of interactions between hydroperoxides at the droplet surface and prooxidants in the aqueous phase. On the other hand, in emulsions, polar antioxidants would tend to partition into the aqueous phase where they would be less able to protect the lipid. [5]

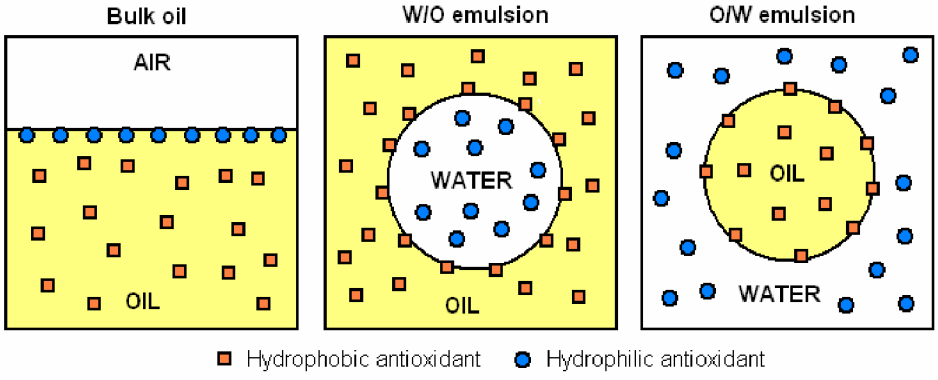


Figure 2–9 *Effects of antioxidant polarity in bulk oil and emulsions*

2.2.4 Natural antioxidants

Antioxidants in foods may originate from components that occur naturally in the food ingredients. Natural antioxidants are primarily plant phenolics (flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols, etc.), than carotenoids or vitamin C, and may occur in different parts of the plant.

This thesis deals mainly with phenolic antioxidants (concretely simple phenolics – caffeic, ferulic, *p*-coumaric acid and propyl gallate), thus further overview of antioxidants will be focused mainly on these substances.

2.2.4.1 *Phenolic compounds*

Phenolic compounds (phenolics) are a group of approximately 8000 naturally occurring compounds, all of which possess one common structural feature – a phenol (an aromatic ring bearing at least one hydroxyl group). [3]

Current classification divides the broad category of phenolic compounds into three major groups according to the number of phenol subunits in the molecule:

a) *simple phenols* – phenolics consisting of one phenol unit (see section 2.2.4.2),
b) *flavonoids* – phenolics consisting of two phenol subunits, and
c) *tannins* – phenolics consisting of at lest three phenol subunits.

Flavonoids and tannins are referred to as *polyphenols* (PP). All the three groups can be further sub-divided according to various structural features. The main groups of flavonoid antioxidants are shown in Figure 2–10.

Phenolic antioxidants are widely spread throughout the plant kingdom as secondary plant metabolites. They are present either in free form or, more typically, conjugated to various molecules (quinic acid, sugars). [73]

Plants rich in phenolics are for example soybean (tocopherols, isoflavones, phenolic acids), peanuts and cottonseed (quercetin, rutin), mustard and rapeseed (phenolic acids, condensed tannins – cyanidin, pelardonidin, kaempferol), rice (isovitexin), sesame seed (sesamin, sesamolin, sesamol, sesamolinol), tea leaves (catechins), herbs and spices – rosemary and sage (carnasol, rosmanol, rosmaridiphenol, rosmaric acid), oregano, mace, black pepper (phenolic acid amides), turmeric (tetrahydrocurcumin), olives (phenolic acids), onion (quercetin), sweet potato (chlorogenic acids, caffeic acid), oats (dihydrocaffeic acid), filamentous fungi (curvulic acid, tocatechuic acid, citrinin), berry fruits, coffee and cocoa bean (caffeic acid), most fruits (apples, grapes, pears, pineapple, citrus and stone fruits, etc.) and some green vegetables (spinach, broccoli etc.). [2, 73, 81] Consequently, beverages made of these plants, such as red wine, juices, tea or coffee, show high antioxidant potency. [82]

2.2.4.2 *Phenolic acids*

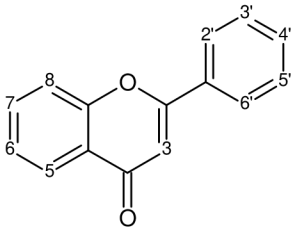
The term *phenolic acids* describes phenols that possess one carboxylic acid group. However, when describing plant metabolites, it refers to a distinct group of organic acids.

These acids contain two distinguishing constitutive carbon frameworks:

- the hydroxycinnamic, and
- hydroxybenzoic structures.

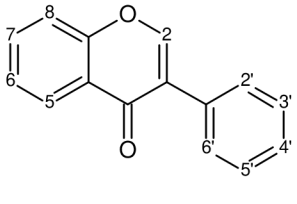
Although the basic skeleton remains the same, the number and position of the hydroxyl groups on the aromatic ring create a variety of compounds (Figure 2–11). In many cases, aldehyde analogues are also grouped in with, and referred to as, phenolic acids (e.g. vanillin).

Flavones



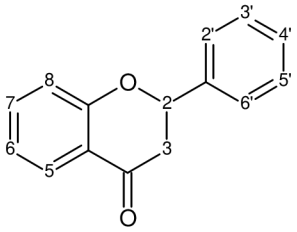
Apigenin: 5 = 7 = 4′ = OH
Luteolin: 5 = 7 = 3′ = 4′ = OH
Diosmetin: 5 = 7 = 3′ = OH, 4 = OCH_3
Isovitexin: 5 = 7 = 4′ = OH, 6 = glucose

Isoflavones



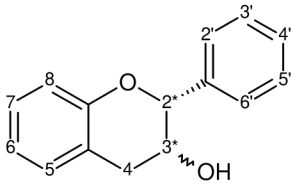
Diadzein: 7 = 4′ = OH
Genistein: 5 = 7 = 4′ = OH

Flavanones



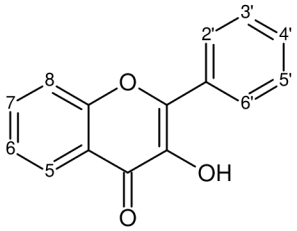
Naringenin: 5 = 7 = 4′ = OH
Hesperitin: 5 = 7 = 3′ = OH, 4′ = OCH_3

Flavanols



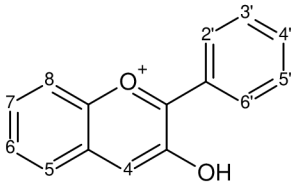
Catechin (2*R, 3*S):
5 = 7 = 3′ = 4′ = OH
Epicatechin (2*R, 3*R):
5 = 7 = 3′ = 4′ = OH
Epigallocatechin (2*R, 3*R):
5 = 7 = 3′ = 4′ = 5′ = OH

Flavonols



Kaempferol: 5 = 7 = 4′ = OH
Quercetin: 5 = 7 = 3′ = 4′ = OH
Morin: 5 = 7 = 2′ = 4′ = OH
Fisetin: 7 = 3′ = 4′ = OH
Myricetin: 5 = 7 = 3′ = 4′ = 5′ = OH

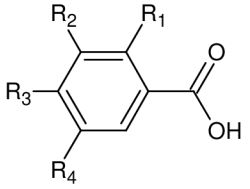
Anthocyanidins



Pelargonidin: 5 = 7 = 4′ = OH
Cyanidin: 5 = 7 = 3′ = 4′ = OH
Delphinidin: 5 = 7 = 3′ = 4′ = 5′ = OH
Malvidin: 5 = 7 = 4′ = OH,
3′ = 5′ = OCH_3

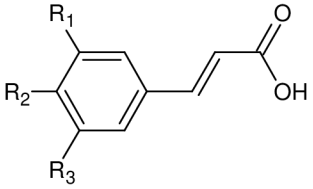
Figure 2–10 *Main flavonoid antioxidants found in the plant kingdom* [7]

Hydroxybenzoic acids



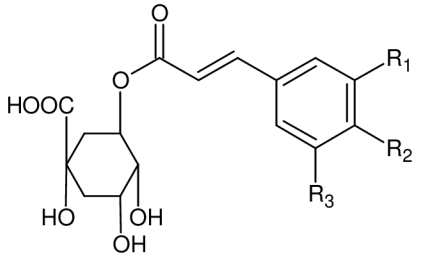
$R_1 = R_2 = R_3 = R_4 = H$	Benzoic acid (non phenolic)
$R_1 = R_4 = H$, $R_2 = R_3 = OH$	Protocatechic acid
$R_1 = H$, $R_2 = R_3 = R_4 = OH$	Gallic acid
$R_1 = OH$, $R_2 = R_3 = R_4 = H$	Salicylic acid
$R_1 = R_4 = OH$, $R_2 = R_3 = H$	Gentisic acid
$R_1 = R_2 = H$, $R_3 = OH$, $R_4 = OCH_3$	Vanillic acid
$R_1 = H$, $R_3 = OH$, $R_2 = R_4 = OCH_3$	Syringic acid

Hydroxycinnamic acids



$R_1 = R_2 = R_3 = H$	Cinnamic acid (non-phenolic)
$R_1 = R_3 = H$, $R_2 = OH$	***p*-Coumaric acid**
$R_1 = R_2 = OH$, $R_3 = H$	**Caffeic acid**
$R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$	**Ferulic acid**
$R_1 = R_3 = OCH_3$, $R_2 = OH$	Sinapic acid

Hydroxycinnamates (Chlorogenic acids)



$R_1 = R_2 = OH$, $R_3 = H$	Caffeoyl quinic acid (Chlorogenic acid)
$R_1 = R_3 = H$, $R_2 = OH$	*p*-Coumaroyl quinic acid
$R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$	Feruloyl quinic acid
$R_1 = R_3 = OCH_3$, $R_2 = OH$	Sinapoyl quinic acid

Figure 2–11 *Main phenolic acids and esters found in the plant kingdom* [7]

Phenolic compounds (ArOH) donate hydrogen from their hydroxyl groups and the formed phenolic radical ($ArO^{\bullet}$) has low energy as the unpaired electron is delocalized throughout the phenolic ring structure (Figure 2–12). The effectiveness of phenolics is often increased by substitution groups on the phenolic ring. These substituents increase the ability of ArOH to donate hydrogen and/or increase the stability of the $ArO^{\bullet}$. [5]

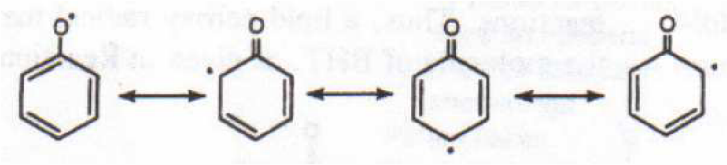


Figure 2–12 *Delocalization of unpaired electrons around the aromatic ring of a phenoxy radical* [3]

Phenol itself is inactive as an antioxidant. Substitution of the hydrogen atoms in the *ortho*- and *para*-positions with alkyl groups (e.g. ethyl, *n*-butyl; propenoic acid in the *para*-position in the case of hydroxycinnamic acids) increases the electron density of the OH moiety by an inductive effect and thus enhances its reactivity toward lipid radicals. The stability of the phenoxy radical is increased by bulky groups at the *ortho*-position since these substituents increase the steric hindrance in the region of the radicals. They further reduce the rate of possible propagation reactions mediated by $ArO^{\bullet}$ that may occur. [3]

The introduction of a second hydroxy group at the *ortho*- or *para*-position of the hydroxy group of a phenol increases its antioxidant activity. The effectiveness of a 1,2-dihydroxybenzene derivative (catechol, e.g. caffeic acid) is increased by the stabilization of the phenoxy radical through an intramolecular hydrogen bond (Figure 2–13). The increased antioxidant activity of dihydroxybenzene derivatives is partly due to the fact that the initially produced semiquinoid radical can be further oxidized to a quinone by reaction with another lipid radical or another $ArO^{\bullet}$ (Figure 2–14).

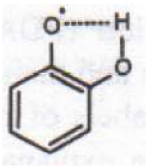


Figure 2–13 *Stabilization of the phenoxy radical through an intramolecular hydrogen bond in 1,2-dihydroxybenzene derivatives (catechols)*

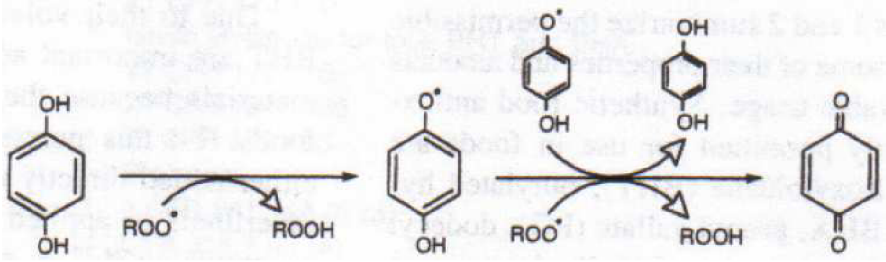


Figure 2–14 *Oxidation of a dihydroxybenzene derivative leading to the formation of a quinone*

The antioxidant activity of 2-methoxyphenol (e.g. ferulic acid) is lower than that of catechol, because 2-methoxyphenols are unable to stabilize the phenoxy radical by hydrogen bonding. [3]

Antioxidants are more effective when added to lipids with a low degree of oxidation; they are poorly effective in retarding oxidation in highly oxidized lipids. [3]

2.2.4.3 *Ascorbic acid*

Ascorbic acid represents a multifunctional antioxidant, it is capable of:

- quenching singlet oxygen,
- reduction of free radicals and primary antioxidant radicals, and
- removal of molecular triplet oxygen in the presence of metal ions as catalysts. [2]

In the latter, ascorbate and molecular oxygen form a ternary complex with the metal catalyst and two π electrons from ascorbate shift to oxygen through the transition metal yielding water and the oxidized more stable form of ascorbic acid (dehydroascorbic acid). [2]

Ascorbic acid and its salts (ascorbates) belong to a group of widely used food additives of natural origin, registered under the E numbers E 300 – E 303. They are used to protect aqueous matrices. The fatty acids esters (ascorbyl palmitate or stearate, E 304) of ascorbic acid are synthesized and used for protection of lipids. Vitamin C is an essential compound for humans, has remarkably low toxicity, and its use as a food additive is considered to be beneficial for consumers. [85, 97]

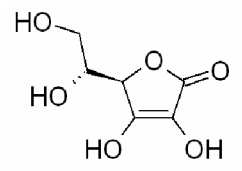


Figure 2–15 *Ascorbic acid*

2.2.5 Synthetic antioxidants

Synthetic antioxidants are very effective inhibitors of lipid oxidation in a wide variety of food products After the interaction of the antioxidant with lipid radicals, the bulky substituents (such as *tert*-butyl or methoxy groups) in *ortho*- and *para*- positions relative to OH group on a benzene ring provides the formation of a very low energy resonance-stabilized phenolic radical. Due to its low energy, this radical does not rapidly catalyze the oxidation of unsaturated fatty acids. In addition, synthetic antioxidants do not react readily with oxygen to form unstable antioxidant hydroperoxides, which may decompose into high energy free radicals that could promote oxidation. Instead they tend to react in radical-radical termination reactions. [5]

The main reason for using synthetic antioxidants is to extend the shelf-life of foodstuffs and to reduce wastage and nutritional losses by inhibiting and delaying oxidation. [3]

Synthetic food antioxidants currently permitted for use in foods in the Czech Republic are butylated hydroxytoluene (BHT; E 321), butylated hydroxyanisole (BHA; E 320), propyl gallate (PG; E 310), octyl gallate (OG; E 311), dodecyl gallate (DG; E 312), tertiary-butylhydroquinone (TBHQ; E 319) and 4-hexylresorcinol (E 586). [85] Each country has its own restrictions and regulations given by law for the use of the synthetic antioxidants. The major criterion for the acceptability of these compounds is their potential toxicity, which has been studied extensively and is still not clear. [3]

2.2.5.1 *Propyl gallate*

Propyl gallate, an *n*-propyl ester of 3,4,5-trihydroxybenzoic acid (Figure 2–16), is a synthetic phenolic compound approved for use as an antioxidant in food [1] and cosmetic industry to protect fats and oils against oxidation.

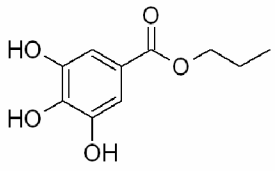


Figure 2–16 *Propyl gallate*

As a food additive it is used under the number E 310. [85] GSFA (Codex General Standard for Food Additives) provisions for propyl gallate range from 50 – 1000 mg/kg depending on a food category; for instance lard, tallow, fish oil, and other animal fats, emulsions containing less than 80 % fat and products based on fat emulsions may contain max 200 mg/kg (fat or oil basis). [90] Acceptable daily intake (ADI) of propyl gallate for man is estimated 1,4 mg/kg of body weight (1993). [91] Commercially, propyl gallate is used in substantial quantity [1] despite the efforts of recent years to replace synthetic antioxidants with natural ones.

Within the group of synthetic antioxidants, propyl gallate is the compound with the lowest polarity [5] due to the esterification of the acid group with aliphatic chain. Propyl gallate is available as a white crystalline powder and is sparingly soluble in water, fats, oils and glyceryl monooleate, and soluble in alcohols, glycerol and propylene glycol. [2, 3] Propyl gallate can chelate iron ions forming a blue-black complex. [3]

2.3 Evaluation of antioxidant activity

2.3.1 Introduction

By definition, the *antioxidant activity* (AOA) is the capability of a compound (or a mixture) to inhibit oxidative deteriorations, e.g. lipid peroxidation. [13] The terms *antioxidant capacity* (AOC) or *antioxidant potential* is also frequently used in the literature.

Evaluation of AOC of various matrices, such as plasma, beverages, vegetables and fruits, as well as of pure compounds (e.g. phenols, vitamins), has received much attention during the past two decades, for many studies have demonstrated an inverse correlation between the intake of natural antioxidants and the occurrence of oxidative stress related diseases, such as inflammation, cardiovascular disease, cancer and aging-related disorders. [11, 24] Pure antioxidants of natural origin or some specific plant materials (e.g. grape pomace, rosemary or berry extracts) are of great interest for food industry as additives to foods for protection against oxidative deterioration, thus evaluation of their antioxidant capacity has also been an important issue.

Even if there is a large demand for information about antioxidant properties of various substrates, the evaluation of these properties is not an easy task, probably due to the fact that the area of antioxidants is such a complex issue. The large number of papers devoted to assessment of antioxidant properties of various antioxidants is a clear proof of this.

Up to date, there are numerous published methods claiming to measure total antioxidant capacity *in vitro*, however the lack of a validated assay that could reliably measure the AOC still remains a big problem. Several reviews have been published on this topic. [11, 12, 13, 18] The problems with reliability of AOC assays are more closely commented in this thesis.

2.3.2 Approaches to AOC evaluation

An increased interest in information about antioxidant potentials of phenolic rich matrices has led to the development of a wide array of assays for determination of antioxidant capacity.

There are two main approaches to AOC evaluation that are generally applied in these methods/assays: direct, and indirect. [13]

When the *indirect approach* is applied, the ability of antioxidants to scavenge some stable coloured synthetic free-radicals is most often evaluated. This has little in common with real

biological oxidative degradation mediated by highly reactive radical oxygen species, or by the effects of transition metals. The ability to donate an electron or a hydrogen proton under conditions that are very different to those *in vivo* is usually measured. [13]

Direct methods are based on studying the antioxidant effect of antioxidants on the oxidative degradation of a system of biological relevance (individual lipids, lipid mixtures – oils, lipid membranes, low density lipoprotein, DNA, blood, plasma, etc.). [13]

The direct approach of evaluation that utilizes various lipid model systems has been suggested as being superior to the indirect approach where the antioxidant activity is evaluated more or less artificially by means of so called one-dimensional AOC assays. [7, 18] The type of oxidative substrate in the model systems and the overall system conditions play an essential role. It is expected that the closer the conditions are to the real lipid systems, both *in vivo* (such as cell biomembranes) and *in vitro* (lipid-containing foods) the more valid information about antioxidant potentials is likely to be achieved.

Preferences should be given to the direct methods, however these methods are often time consuming, which does not fulfill the demand for quick and easy assessments important mainly for food and nutraceutical industry.

The use of more than only one and combination of various analytical methods for evaluation of antioxidant activity has been recommended to obtain more objective information about antioxidant potentials of various compounds. [7, 18, 33]

2.3.3 Indirect methods

The majority of the indirect methods is based on spectrophotometric measurements since such measurements are fast, easy to perform and provide reproducible data.

The spectrophotometric methods themselves (their methodology, chemistry, ways of quantification and interpretation of results, and most importantly biological relevance) as well as the information about the antioxidant capacity of a tested material provided by them have been constantly debated among researches. [11, 12, 13, 15, 18, 24]

Because the results of these *in vitro* assays often serve to extrapolate a potential *in vivo* activity, the biological relevance has been frequently and strongly questioned. The following aspects have been criticized: The assays are strictly based on chemical reactions *in vitro* that bear no similarity to biological systems. Moreover, the assays do not measure bioavailability, *in vivo* stability, or retention of antioxidants by tissues, and reactivity *in situ*. [11] The recent study of Serrano et al. suggests that determination of AOC in food extracts by the AOC assays may even underestimate the real physiological antioxidant capacity. [89]

Principles, methodology and drawbacks of the most popular AOC assays in current use within the food research and industry are briefly summarized in section 2.3.8. Special attention is paid to the Folin-Ciocaltau, FRAP, DPPH and ABTS assay as these were chosen for evaluation of antioxidant capacity of compounds in this study.

2.3.4 Comparison of AOC results

Although a large variety of assays are available for AOC assessment, the information provided is often rather conflicting. The results obtained for a particular substrate vary significantly with each method and even within a method itself evoking a lot of debates among researchers. As a consequence of these divergences, interpretation and comparison of the results from different assays is very difficult. [12, 13, 18, 24]

According to the authors of the critical papers on AOC assays, following factors are responsible for differences in results:

(1) Many assays have *variable end points* and different laboratories use different criteria to define the end point. This results in different antioxidant capacity values for identical foods (or compounds) assessed by different laboratories using the same method.

(2) In addition to variable endpoints, *variable concentrations* of reagents and *doses* of reactants are frequently used, which contributes to different values within the same method and the same substrate.

(3) Another issue that makes comparison between antioxidant capacity measurements difficult is the *choice of standard* (or reference compound) used to calibrate the assay. This affects the final value obtained for antioxidant capacity, which again makes comparisons between different substrates problematic, even if they were assayed by the same type of method under identical assay conditions.

In many studies it was pointed out that without assay standardization it is impossible to compare antioxidant capacity values assessed at different laboratories. However, establishing standardized procedures is a complicated task and it is difficult to achieve this.

2.3.5 New trends in evaluation of antioxidant capacity

Besides conventional AOC assays (see section 2.3.8 for examples), new methods to evaluate AOC have been recently introduced – for instance, a method based on protein structural change [83], on spectrophotometric measurement of Ce(IV) reducing capacity [87], or on bleaching of pyranine and pyrogallol red induced by free radicals generated from an azo initiator [93]. The creators of these assays have given thorough discussions on the use and the merits and weaknesses of the methods, and on the interpretation of the information these new assays provide.

Special attention has been also paid to the measurement of AOC of insoluble food components. This aspect has often been forgotten when measuring the AOC of various matrices. It was shown that the contribution of insoluble matter to total AOC was highly relevant for cereal-based foods and for dietary-fiber-rich ingredients. [84]

So called *integrated approach* has been proposed as a novel method for evaluation of antioxidant capacity. This approach binds together values obtained by different AOC assays by means of so called radar charts, as shown in the work of Terashima et al. [83], or by a calculated value (e.g. relative antioxidant capacity index, antioxidant potency composite index), as shown in the work of Sun et al. [86] and Seeram et al. [82].

2.3.6 Antioxidant activity in lipid systems

Although AOC assays provide some information about the potentials of an antioxidant to protect matrices susceptible to oxidative damage, they may not reflect this ability in real systems. In fact, the antioxidants may behave quite differently than they do in the AOC assays. [11]

It has been shown in different studies that the relative effectiveness of antioxidants in lipid systems is dependent on a number of factors [14, 30, 33, 35], mainly:

- the type of lipid substrate (triacylglycerols, free fatty acids, phospholipids, LDL, etc.),
- the nature of lipid system (bulk oils, minces, emulsions, micelles, LDL, membrane structures – liposomes, microsomes, fibroblasts, etc.),

- the structure of antioxidant (number of OH groups, extent of conjugated double bond systems, substituents on aromatic rings, etc.),
- the concentration of antioxidant,
- the presence and the type of prooxidants (free metals, photosensitizers, metalloproteins, stable free radicals, etc.),
- the antioxidant-to-prooxidant ratio,
- the presence of other interfering substances (endogenous antioxidants, enzymes, metals, sugars, proteins, etc.),
- other variables such as temperature, pH, time of oxidation, light, etc.

In addition, the method used to measure the degree of lipid oxidation is very important. Both rapid and time-consuming methods exist; methods based on detecting different products of lipid oxidation or methods utilizing changes in concentration of oxidation reactants. These methods have been recently summarized in the review of Laguerre et al. [7]

2.3.7 Evaluation of effectivity of antioxidants in different model systems

Many studies have shown that phenolic compounds have different antioxidant activity in different lipid model systems. A wide choice of lipid model systems is available for studies of lipid peroxidation, for instance:

- bulk oils or emulsions of triacylglyceroles, free fatty acids or their esters,
- low-density-lipoprotein (LDL),
- tissue homogenates, minces,
- liposomes, and
- other membrane structures (e.g. erythrocytes, microsomes, fibroblasts). [1]

The variability of these systems is further enlarged by the choice of the oxidizable substrate. These systems can be either *bulk oils*, for example bleached and deodorized olive oil, purified sun flower oil, lard [37], or *dispersed systems*, such as oil-in-water and water-in-oil emulsions [33, 34], micellar systems [31], LDL [37], liposomes [14], microsomes [35] etc. Antioxidant behaviour is more complex when evaluated in dispersed systems than in bulk oils because more variables can influence lipid oxidation, such as emulsifiers, pH, and buffers.

Linoleic acid is not considered to be a representative model system anymore, because foods contain mainly triacylglycerols, which have a behaviour significantly different from that of free linoleic acid both as a bulk and in micelles. [33]

Early studies on evaluation of effectivity of antioxidants were focused on classical antioxidants, such as α-tocopherol, ascorbyl palmitate, methyl carnosoate, or propyl gallate, and their water-soluble analogs, Trolox, ascorbic acid, gallic acid and carnosic acid, respectively. Simple bulk oils and emulsions made of purified triglycerides, methyl linoleate or linoleic acid were used as lipid model systems. The study of Frankel et al. [88] proposed the so called *interfacial phenomena* or *polar paradox* of antioxidants (explained in section 2.2.3). Later studies proved the validity of this phenomenon for other compounds, namely some phenolic acids [36], and pointed out also the importance of emulsifiers for the effectivity of antioxidants in dispersed systems, e.g. the works of Huang et al. [33] and Schwarz et al. [34], because antioxidants partition in the interface and may interact with the emulsifiers. It has been presumed that emulsifiers increase the solubility of polar antioxidants but may decrease the activity due to hydrogen bonding. [34, 36]

The antioxidant activity of selected representatives of flavonoids, coumarins, and cinnamic acids was examined by Foti et al. by measuring their protective action toward linoleic acid peroxidation in micelles of sodium dodecyl sulfate (SDS) in buffer solution, pH 7.4 and 50°C. The best values were observed for flavonoids, followed by coumarins and cinnamic acids. Due to measurements with many structurally similar compounds and their water- or oil-soluble analogues the structure-activity relationship was deduced with relation to hydrophilicity of the compounds. [31] Micellar systems can be used for a rapid evaluation of antioxidant activity; however, neither linoleic acid nor SDS micelles represent physiologically relevant or food-related conditions.

Tea catechins represent an important group of phenolic antioxidants because of their high proportions in green tea, which belongs to traditional and broadly consumed beverages. Antioxidant activity of selected tea catechins in different lipid model systems were studied by Hunag et al. [30] The study demonstrated that whether tea catechins, gallic acid and propyl gallate act as antioxidants or prooxidants is dependent on the lipid system and the presence of metal catalysts. Tea catechins, gallic acid, and propyl gallate were all antioxidants in corn oil triacylglycerols and in liposomes without added copper ions, whereas in oil-in-water emulsions and in liposomes with added copper catalyst these compounds were all prooxidants. The antioxidant concentration, the temperature of oxidation, the oxidation state and the methods used to measure lipid oxidation were limiting factors. [30] However, the study lacks deeper explanation of the effects that were observed, mainly on a molecular basis.

Some commonly used antioxidants (BHA, BHT, TBHQ, α-tocopherol and caffeic acid) were studied in phosphatidylcholine liposomes with Cu-catalyzed oxidation and in o/w emulsions at 37 °C and pH ~ 5.8 by Nenadis et. al. [14] Synthetic antioxidants were the most effective compounds both in liposomes and emulsions; lower levels of addition compared to that of α-tocopherol and caffeic acid were efficient to retard oxidation during the monitoring period. α-Tocopherol was less effective and the behaviour of caffeic acid was concentration dependent – at low levels of addition it was ineffective or promoted oxidation. Interestingly, caffeic acid promoted the oxidation even in the emulsions where no metal initiator was present. A hypothesis that caffeic acid could induce the generation of hydrogen peroxide in aqueous solution, which then promotes the oxidation of the emulsions have been suggested, however no verification experiments were performed.

Microsomes represent a more complex lipid model system, because the phospholipid bilayers contain endogenous enzymes, tocopherols and a variety of other lipids than phospholipids. [1] Evaluation of antioxidant effects of a grape extract, grape procyanidins, hydroxytyrosol obtained from olive-oil byproducts, and of propyl gallate on inhibition of hemoglobin, enzymatic-NADH iron, and iron-ascorbate promoted oxidation of fish microsomes was done by Pazos et al. [35] All compounds delayed lipid oxidation promoted by the three prooxidants except for hydroxytyrosol in iron-ascorbate promoted oxidation.

The correlations between physicochemical properties of the phenolics, such as polarity, reducing capacity, chelating properties, affinity for being incorporated into the microsomal membranes, and inhibitory activity against the hemoglobin autoxidation, and their protective effects were also investigated in the study. The most decisive factor has been found to be the affinity, which seems to be ruled by interaction between phenolics and the phospholipid headgroups or membrane proteins rather than by polarity of the compounds. [35]

Low-density-lipoprotein (LDL) represents one of the most complex lipid model systems of natural origin owing to the content of various molecules (Figure 2–17). A key substance in the LDL particle is endogenous α-tocopherol, which can be regenerated by reducing agents, such as ascorbic acid, but also by many phenolics, and so provide the protective action. [3]

Moon et al. evaluated the antioxidant activity of caffeic acid and dihydrocaffeic acid in lard, as a representative of edible fat, and human plasma LDL oxidized with copper ions at 37°C. Dihydrocaffeic acid was more effective in suppressing the induction period of lard at 60 °C, while caffeic acid was more effective in protection of LDL. This phenomenon has been attributed to the nature of oxidizable substrate rather than the 2,3-double bond that differentiates the two compounds.

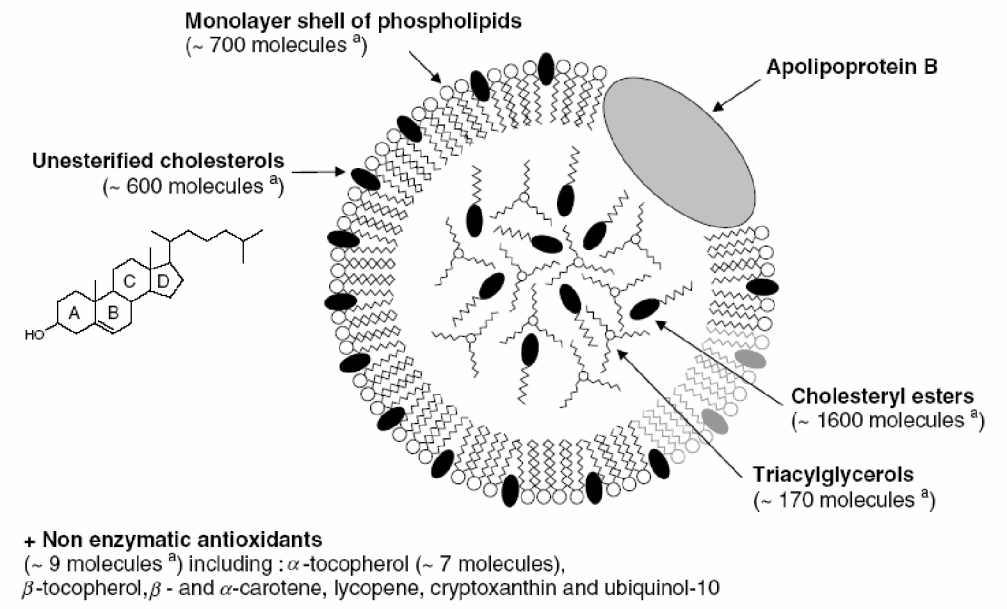


Figure 2–17 *A simplified structure of LDL particle* [7]

Inhibition of ferrylmyoglobin-induced LDL peroxidation by phenolic acids was studied in the work of Laranjinha et al. [51] The effectivity of the compounds turned out to be strongly dependent on the substitution pattern on the phenol ring; *o*-dihydroxy derivatives of cinnamic and benzoic acids were more efficient than when one of the OH groups was replaced with H or methoxy group. The protection of LDL against oxidation was assigned to the ability of the compounds to reduce the oxoferryl moiety of the ferrylmyoglobin (Fe^{4+}) to the ferric form (Fe^{3+}) of metmyoglobin. The lipid-radical scavenging abilities and regeneration of α-tocopherol were considered to be minor contributors to the protective effects.

A less traditional model system is intact cells, for example fibroblasts. The effects of ferulic acid, α-tocopherol, β-carotene, and ascorbic acid dosed at micromolar concentrations alone or in combination on peroxidation of rat liver microsomes and murine fibroblasts induced by *tert*-BOOH and AAPH were studied by Trombiono et al. [68] In these models, ferulic acid acted as a potent antioxidant being even more active than the other compounds which also had protective effects; synergistic effects were observed when ferulic acid was used in combination with the other antioxidants. This study highlights the antioxidant potentials of ferulic acid; however, the authors admit that the choice of a prooxidant may

influence the antioxidant properties of ferulic acid. Unfortunately, the prooxidants used in this study are not biologically relevant because of their synthetic origin, also the mechanism of oxidation promotion occurs *via* different pathways compared to more biologically relevant promoters, such as metal ions or metalloproteins.

Real foods systems, such as tissue homogenates, meat minces, mayonnaise and other dairy products, represent natural model systems; yet, such systems are highly complex. It is therefore not convenient to use these systems for studying the influence of specific factors on lipid oxidation of foodstuff. However, they are ideal model systems for studying the applicability of antioxidants.

Chilled minced fatty fish muscle was used for evaluation of effectiveness of hydroxycinnamic acids and catechins in the study of Medina et al. [32] Caffeic acid, propyl gallate and catechin supplemented at 10 ppm (0.001 %) showed a potent effectivity in retarding the development of rancidity in fish muscle. The inhibitory activity correlated with the reducing capacity of the compounds rather than with their chelating abilities and their distribution between oily and aqueous phase of an emulsion (O:W = 1:1, v/v); properties that were also measured in the study.

It is clear that a universal lipid model system does not exist. Every system is somehow specific, and the effectivity of antioxidants therefore differs in these systems. The choice of a model lipid system should primarily be made with respect to the purposes of the tested antioxidants or with respect to the knowledge that needs to be gained.

2.3.8 Antioxidant Capacity Assays

Many antioxidant capacity (AOC) assays have been developed and the merits and disadvantages of them have been fully discussed in several reviews. [11, 12, 13, 18, 24]

On the basis of the chemical reaction mechanisms involved (these mechanisms are described in section 2.2.1.1) the assays can be roughly divided into two categories:

- hydrogen atom transfer (HAT) reaction based assays, and
- single electron transfer (SET) reaction based assays.

In general, the SET-based assays measure an antioxidant's reducing capacity, and the HAT-based assays quantify hydrogen atom donating capacity. [11]

2.3.8.1 *HAT-based assays*

HAT-based assays measure the ability of an antioxidant to quench free-radicals by hydrogen atom donation. HAT-based assays are generally composed of a synthetic free-radical generator, an oxidizable probe, and an antioxidant. In most HAT-based methods, antioxidants and a probe compete for thermally generated peroxyl radicals ($ROO^{\bullet}$) and the quantification is derived from the kinetic curves after monitoring the competitive reaction kinetics. [86]

The most biologically relevant HAT-based assays are considered:

- oxygen radical absorbance capacity (ORAC),
- total radical-trapping antioxidant parameter (TRAP), and
- inhibition of autoxidation of induced low-density lipoprotein (LDL) oxidation. [12]

It has been assumed that the AOC from the HAT-based *in vitro* assays may more closely reflect *in vivo* action, because hydrogen atom transfer is a key reaction mechanism in the radical chain reactions. [11, 12]

The ORAC assay was recommended as a standard method for a routine quality control and measurement of food AOC because it is a method that uses a controllable source of peroxyl radicals and can detect both hydrophobic and hydrophilic antioxidant. [12, 86]

2.3.8.2 *SET-based assays*

SET-based assays measure the ability of a compound (antioxidant) to transfer one electron to reduce radicals, metals or carbonyls (oxidant). The oxidant serves also as a probe for monitoring the reaction and as an indicator of the reaction endpoint. [86]

SET-based assays resemble the redox titration in classical chemical analysis and can be described by the following electron-transfer (redox) reaction:

$$\text{oxidant (probe)} + \text{e (from antioxidant)} \longrightarrow \text{reduced probe} + \text{oxidized antioxidant}$$

The oxidant (probe) itself is a substance of a specific colour which has the ability to absorb light in the visible spectrum (VIS) with a specific wavelength. When abstracting an electron from the antioxidant the colour characteristically changes. The degree of the colour change is proportional to the antioxidant concentration. The reaction endpoint is reached when the colour change stops.

Typically, the change of absorbance (ΔA) is plotted against the antioxidant concentration to give a linear curve. The slope of the curve reflects the antioxidant's reducing capacity, which is mostly expressed as equivalents of a chosen standard compound (Trolox, gallic acid, etc.).

Because there is not a competitive reaction involved and there is no oxygen radical in these assays, it has often been argued how the results relate to the radical scavenging capacity of a sample. It has therefore been assumed that the AOC expressed by these assays is equal to the reducing capacity. [11]

The most popular SET-based methods include:

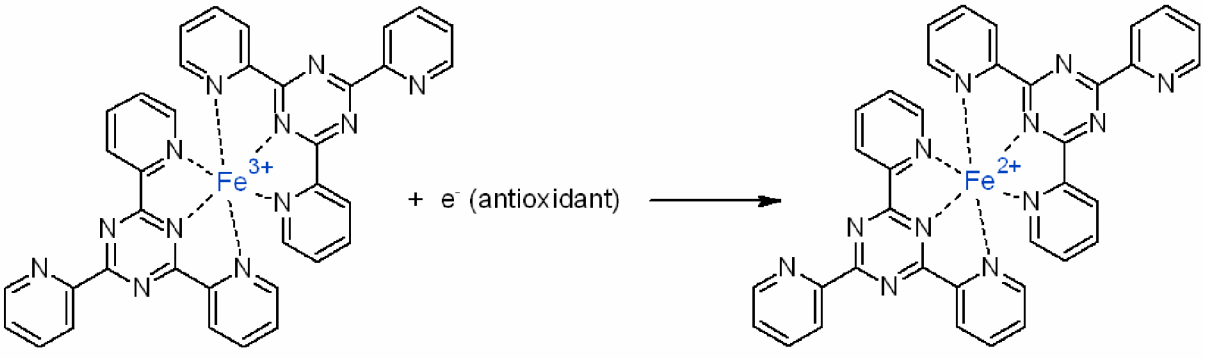
- 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay,
- 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay,
- ferric reducing antioxidant power (FRAP) assay,
- Folin-Ciocaltau (FC) assay,
- cupric reducing antioxidant capacity (CUPRAC). [12]

2.3.8.3 *Ferric Reducing Antioxidant Power (FRAP) Assay*

The FRAP assay was originally developed by Benzie and Strain in 1996 to measure reducing power in plasma [10]. Subsequently, the assay has been adapted and used for the assessment of reducing capacity of a wide array of substrates [23, 24, 82] and of pure compounds [15, 16, 28].

The FRAP assay measures the ability of a compound to reduce a ferric salt, $Fe(III)(TPTZ)_2Cl_3$ (TPTZ = 2,4,6-tripyridyl-*s*-triazine), to a ferrous coloured product (Figure 2–18). The reaction mechanism is totally electron transfer rather than mixed SET and HAT, thus the *reducing capacity* of the compound is evaluated. The FRAP assay is carried

out under acidic conditions (pH 3.6) in order to maintain iron solubility, and detects only compounds with redox potentials of < 0.7 V (the redox potential of the ferric salt). [12, 24]



[Fe(III)(TPTZ)$_2$]$^{3+}$
yellow

[Fe(II)(TPTZ)$_2$]$^{2+}$
intense blue at 593 nm

Figure 2–18 *Reaction for the FRAP assay*

For the FRAP reagent preparation and the assay procedure see section 3.1.2.

The FRAP assay rely on the hypothesis that the redox reaction proceeds so rapidly that all reactions are completed within 4 minutes, but it has been shown that this is not always true. Fast-reacting phenols are best analyzed with short reaction times, for example, 4 min. However, some polyphenols react more slowly and require longer reaction times for detection, for example 30 min. This may cause shifts in the order of reactivity of a series of antioxidants or matrices, and even the FRAP values of the same substance can vary significantly depending on the end-point of analysis. [12]

The FRAP assay cannot detect compounds that act by radical quenching (H atom transfer), particularly thiols (such as glutathione) and proteins. This may lead to underestimation of antioxidant activity of some complex matrices. The assay measures only the reducing capability based upon the ferric ion in the environment of a polar solvent, which has poor relationship to the radical quenching process mediated by most antioxidants *via* the HAT mechanism in a lipid (non-polar) substrate. [12]

The advantage of the FRAP assay is that it is a simple, rapid, inexpensive and robust assay, and it does not require specialized equipment. It can be performed using automated, semiautomatic, or manual methods. [12]

2.3.8.4 *2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay*

The DPPH assay was first introduced by Brand-Williams et al. [22] as a convenient free radical method for evaluating antioxidant capacity of pure compounds. Since then it has become, most probably due to its simplicity, a popular and routine method for AOC assessments of a wide array of food matrices.

The DPPH radical (DPPH$^{\bullet}$) (Figure 2–19) is a stable organic nitrogen-radical having a deep purple colour with a UV-VIS absorption maximum at 515 nm. It is commercially available and does not have to be generated before the assay as for example the ABTS radical monocation (see section 2.3.8.4). [12]

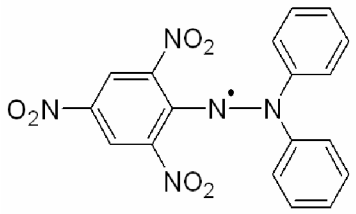


Figure 2–19 *Chemical structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical*

The DPPH assay is based on the measurement of the loss of the radical's colour after reaction with an antioxidant, while the radical can be deactivated either by the HAT reaction mechanism or by the SET reaction mechanism. The latter is considered to be predominant in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. The reaction progress is monitored by a spectrophotometer and the reaction is pH-dependent. [11, 12, 24]

Two versions of the DPPH assay can be used: 1) dynamic, and 2) static. In the dynamic version, the rate of $DPPH^{\bullet}$ decolorization is measured after the addition of a phenolic-containing sample; in the static version, the amount of $DPPH^{\bullet}$ scavenged by a defined amount of sample is measured. [13] The way the results are expressed differ in published works, mainly depending on the version of the method that was used for analysis. The most frequent expression in the static version is by calculating the percentage of the initial amount of $DPPH^{\bullet}$ scavenged by a defined amount of sample/antioxidant, or by means of EC_{50} values, which is the amount of antioxidant needed (efficient concentration) to decrease the initial $DPPH^{\bullet}$ concentration by 50 %. The time needed to reach the steady state with EC_{50} is calculated from the kinetic curve and is defined as T_{EC50}. [11]

For the reagent preparation and the assay procedure see section 3.1.3.

Although the DPPH assay is technically very simple and rapid and only a UV-VIS spectrophotometer is needed to perform it, many drawbacks of the assay have been found which limits the application of the assay making it less valid for AOC measurements. [11, 25]

The assay is not a competitive reaction because $DPPH^{\bullet}$ is both radical probe and oxidant. $DPPH^{\bullet}$ can be decolorized either by reducing agents (SET) or H-donation (HAT) as well as by some unrelated reactions. Steric accessibility is a major determinant of the reaction. Thus, small molecules that have better access to the radical site have higher apparent AOC with this test. [12]

$DPPH^{\bullet}$ is a stable nitrogen radical that bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to $DPPH^{\bullet}$ due to steric inaccessibility. [12]

Interpretation is complicated when the test compounds have spectra that overlap $DPPH^{\bullet}$ at 515 nm (e.g. carotenoids). [12]

2.3.8.5 *2´-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay*

The ABTS assay, also known as TEAC (Trolox Equivalent Antioxidant Capacity) assay, when calibrated relative to Trolox, measures the ability of antioxidants to neutralize the pre-formed strongly absorbing turquoise ABTS radical monocation ($ABTS^{\bullet+}$) (Figure 2–20). The radical reacts with electron/hydrogen donors that has a redox potential lower than 0.68 V (redox potential of ABTS) to form a colourless product. The radical is generated by an

oxidation reaction of ABTS with potassium persulfate prior the deactivation reaction. The reaction is pH-independent and is not affected by ionic strength. A decrease of the $ABTS^{\bullet+}$ concentration is linearly proportional to the antioxidant concentration. [12, 20, 24]

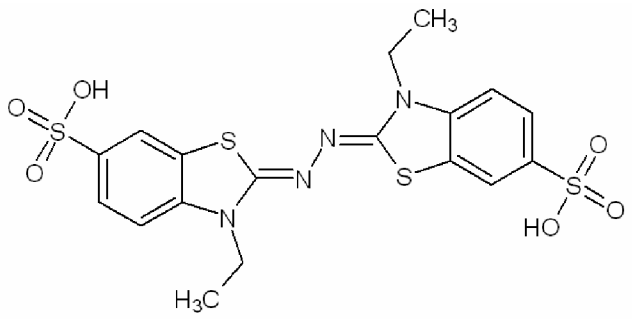


Figure 2–20 *Chemical structure of 2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)*

For the reagent preparation and the assay procedure see section 3.1.4.

The advantage of the ABTS assay is that the $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents, so it can be used to determine both hydrophilic and lipophilic antioxidants in various matrices (food extracts, body fluids, etc.). Changeable mechanism of $ABTS^{\bullet+}$ deactivation (HAT or SET) has been considered to be one of the most important weaknesses of the assay – the mechanism may shift with pH and may change during reactions of slowly reacting antioxidants. [12] Poor selectivity of $ABTS^{\bullet+}$ to H-atom donors is another limitation of the assay. It has been found that $ABTS^{\bullet+}$ reacts with OH-groups of hydroxylated aromatics which do not contribute to the antioxidation. [13] A short incubation time (usually 4 – 6 min) has been the most criticized aspect in methodology of the assay because it may not provide long enough period for the reaction to be completed. [12, 13]

However, the ABTS assay is operationally very simple which makes it a popular and routine test for AOC assessment. [12]

2.3.8.6 *The total phenol assay by Folin-Ciocaltau reagent*

The total phenol(ics) assay by Folin-Ciocaltau reagent (further referred to as FC assay) belongs to the oldest and commonly accepted assays in food research laboratories. The basic mechanism of the assay is an oxidation-reduction reaction between the Folin-Ciocaltau reagent (FCR) containing molybdenum (Mo), and a phenolic compound (27), thus *reducing capacity* of a sample is measured. Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. Basic conditions (pH ~ 10) are required for the proton dissociation; this is facilitated by the use of a sodium carbonate solution. [11, 12]

$$Mo^{VI}\,(\text{yellow}) + e^{-} \longrightarrow Mo^{V}\,(\text{blue}) \qquad (27)$$

The method has been altered several times since its development in 1927. Originally, it was developed for determination of proteins, taking advantage of the reagent's activity toward tyrosine (an amino acid containing phenol group). Later, Singleton at el. improved the method by changes in composition of the FCR and extended the assay to the analysis of total phenols in wine. [11] The improved method reduces phenols more specifically and provides also mandatory steps and conditions to obtain reliable data. Since then, the assay found many other

applications; one of them is a measure of total phenolics in natural products (e.g. teas, juices). In literature, the alternative term *FCR reducing capacity* is sometimes used for this method.

For the reagent preparation and the assay procedure see section 3.1.1.

The total phenols assay by FCR is carried out in water (aqueous phase), thus for lipophilic antioxidants this assay is not applicable. A significant weakness of this method is that the FCR is nonspecific to phenolic compounds and it can be reduced by many nonphenolic compounds (e.g. vitamin C, Fe^{2+}, Cu^{+}). [11, 12]

3 EXPERIMENTAL

3.1 Antioxidant capacity assays

For the AOC assays, a 10 mM methanolic stock solution of each tested compound was prepared and stored in dark at 4°C for a maximum of 14 days. Working solutions were prepared daily by dilution of suitable aliquots of a stock solution with 99 % methanol.

3.1.1 Folin-Ciocaltau Assay

A series of 0 – 3 mM working methanolic solutions for PG, CaA and AsA, and 0 – 5 mM for FeA and CoA was prepared. Deionized water (10 mL), antioxidant solution (1 mL) and 2.0 M Folin-Ciocaltau phenol reagent (1 mL) were transferred to a 20-mL volumetric flask, the reaction mixture was mixed by shaking, and after 3 min exactly 2 mL of 25 % Na_2CO_3 solution (75 g/L) was added. The volume was brought up with deionized water. The absorption at 725 nm was read after 1 h incubation at room temperature against water.

Graphs of antioxidant concentration *vs* absorbance were then constructed. The FC value was considered the slope of the linear curve derived from the constructed graphs. [15, 19, 29]

3.1.2 FRAP assay

The FRAP reagent was prepared freshly before analysis by mixing 2,5 mL of a 10 mM TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine) solution in 40 mM HCl, 2,5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL of 0,3 M acetate buffer, pH 3.6 (prepared by mixing 3,1 g of sodium acetate with 16 mL of glacial acetic acid per liter). The FRAP reagent was then pre-warmed at 37°C in water bath.

A series of 0 – 150 μM working methanolic solutions for PG and CaA, 0 – 120 μM for AsA, 0 – 200 μM for FeA, and 0 – 2700 μM for CoA was prepared.

1680 μL of pre-warmed FRAP reagent was mixed with 120 μL of a sample or methanol (control) and the absorption at 593 nm was recorded against water after 4 min incubation at 37°C. The final concentration of the tested compounds in the reaction mixture was 0 – 10 μM for PG and CaA, 0 – 8 μM for AsA, 0 – 13,3 μM for FeA, and 0 – 180 μM for CoA (final dilution 1/15).

Graphs of final concentration of antioxidants *vs* ΔA ($\Delta A = A_{AH} - A_{cont}$) were then constructed. The FRAP value was considered the slope of the linear curve derived from the constructed graphs. [15]

3.1.3 DPPH assay

The day before analysis, 0,1 mM methanolic $DPPH^{\bullet}$ working solution was prepared and kept on a magnetic stirrer overnight at 4°C. A series of 0 – 750 μM methanolic working solutions for PG, 0 – 1200 μM for CaA and AsA, and 0 – 2400 μM for FeA was prepared fresh from stock solutions.

An aliquot of 0,1 mM methanolic $DPPH^{\bullet}$ solution (2,9 mL) was mixed with 0,1 mL of an antioxidant solution or methanol (blank) and vortexed well. The final concentration of the tested compounds in the reaction mixture was 0 – 25 μM for PG, 0 – 40 μM for CaA and AsA, and 0 – 80 μM for FeA (final dilution 1/30).

After 20 minutes of incubation at room temperature, the absorbance at 515 nm of was recorded against water.

Graphs of inhibition (%) of initial absorbance of the $DPPH^{\bullet}$ solution *vs* antioxidant final concentration were then constructed and EC_{50} values were calculated from the linear curves derived from the constructed graphs.

The inhibition of initial absorbance of the $DPPH^{\bullet}$ solution was calculated according to the following equation:

$$I(\%) = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100$$

where A_{sample} and A_{blank} are absorbance values of the reaction mixture with and without sample, respectively. [15, 24, 69]

Each measurement was performed in duplicate.

3.1.4 ABTS assay

The $ABTS^{\bullet+}$ solution was prepared by reaction of 25 mL of 7 mM aqueous ABTS solution and 440 µL of 140 mM $K_2S_2O_8$ solution, and the mixture was stored in the dark at laboratory temperature for 16 hours. After that the radical cation solution was further diluted with methanol until the initial absorbance value reached 0,75 ± 0,05 AU at 734 nm (against water).

A series of 0 – 55 µM methanolic working solutions for PG, and 0 – 110 µM for AsA, CaA, FeA and CoA was prepared fresh from stock solutions.

An aliquot of the $ABTS^{\bullet+}$ solution (2,0 mL) was mixed with 200 µL of an antioxidant solution or methanol (blank) and vortexed well. The final concentration of the tested compounds in the reaction mixture was 0 – 5 µM for PG, and 0 – 10 µM for AsA, CaA, FeA and CoA (final dilution 1/11).

After 6 minutes of incubation at room temperature, the absorbance at 734 nm of the samples was recorded against water.

Graphs of inhibition (%) of initial absorbance of the $ABTS^{\bullet+}$ solution *vs* antioxidant final concentration were then constructed and EC_{50} values were calculated from the linear curves derived from the constructed graphs. [15]

The inhibition of initial absorbance of the $ABTS^{\bullet+}$ solution was calculated according to the following equation:

$$I(\%) = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100$$

where A_{sample} and A_{blank} are absorbance values of the reaction mixture with and without sample, respectively. [15, 20, 21]

3.2 Isolation of phospholipids

The liposomes were prepared from marine phospholipids that were isolated from cod (*Gadus macrocephalus*) roe. Before isolation, the cod roe was kept at –40°C. The whole isolation procedure consisting of extraction of total lipids and isolation of phospholipids from the total lipids was done as described by Mozuraityte et al. [4]

The extraction of *total lipids* from cod roe was performed according to the method of Bligh and Dyer [75]. A portion of cod roe (ca. 150 g) was homogenized using an Ultra Turrax homogenizer in 100 mL of distilled water, 400 mL of methanol and 200 mL of chloroform for 2 min. After that, 200 mL of chloroform was added and the mixture was homogenized for another 1 min. Finally, 100 mL of distilled water was added and the suspension was homogenized for another 1 min. The mixture was centrifuged (9000 rpm, 15 min), and the chloroform (bottom) phase containing lipids was pipetted and collected. All the procedure was carried out on ice. Total lipids in chloroform were concentrated by evaporation of chloroform with rotor evaporator and stored in chloroform at –20°C.

Phospholipids were isolated from the total lipids using the acetone precipitation method as described by Kates [76], and modified by Mozuraityte et al. [55]. The method is based on the insolubility of phospholipids in cold acetone. An aliquot of total lipids, 4 g in 10 mL of chloroform, was mixed with 200 mL of acetone and stored at –20°C overnight. The acetone phase was decanted and the precipitated phospholipids were dissolved in chloroform and collected. The collected phospholipids were then concentrated by means of rotary evaporator and the isolation procedure was repeated once more. The final phospholipids dissolved in chloroform (0,4 g of phospholipids in 1 mL) were stored at –20°C until needed.

Five products were obtained during the extraction and isolation, named OIL (total lipids), OIL1, PL1, OIL2, PL2 (final phospholipids) (Figure 3–1) and these were further subjected to analysis of purity and degree of oxidation (TLC, NMR, PV, TBARS).

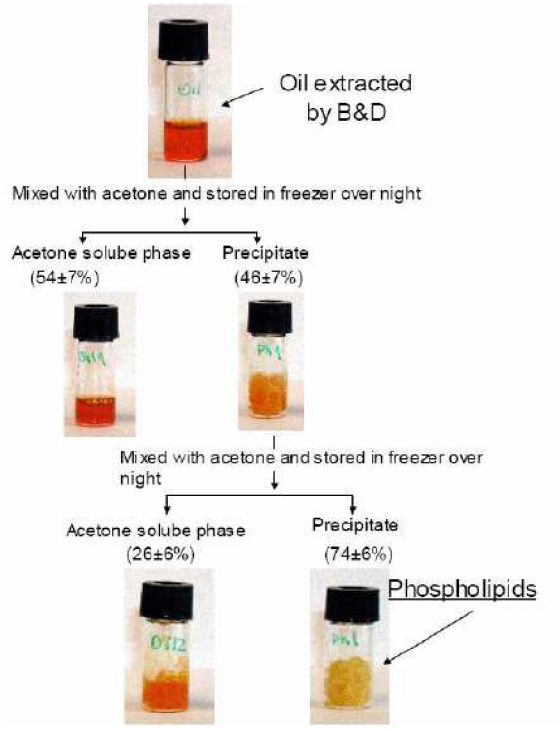


Figure 3–1 *Isolation of phospholipids from total lipids* [4]

3.3 Preparation of liposomes

The liposome solution was prepared according to Mozuraityte et al. [4, 55]. The solvent (chloroform) was removed from an aliquot of phospholipid solution with a stream of nitrogen gas (99,99 %). After solvent removal the phospholipids were kept in a vacuum exsiccator for 2 h to evaporate the residual solvent. The dried mass of phospholipids was then dissolved in a 5 mM MES buffer (pH 5.5) to a concentration of 30 mg/mL (3 % (w/v)). MES buffer was used, since this buffer does not bind iron, has a very low solubility in non-polar solvents and the effective pH is given as 5.5 – 6.4 (pK_a = 6.1). [4] The solution was then sonicated with 50 % amplitude 25 times for 6 s (total sonication time: 2,5 min) with Vibra Cell (Sonics & Materials Inc., USA). During and after the sonication the phospholipids were kept in an ice-bath to avoid temperature increase and prevent oxidation. Fresh liposome solution was prepared daily before measurements (Figure 3–2). Before each experiment the liposome solution was diluted with a MES buffer to a concentration of 15 mg/mL (1,5 % (w/v)).

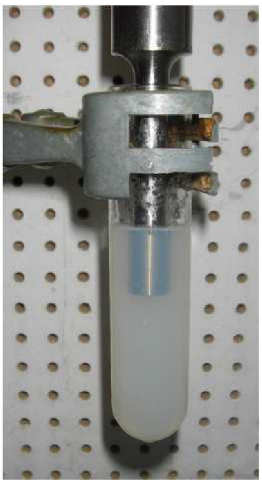


Figure 3–2 *Liposome solution prepared by sonication of phospholipids dispersed in MES buffer* [92]

3.4 Oxygen uptake measurements

If a promoter of lipid oxidation is added into a lipid system the early stage of lipid oxidation proceeds very quickly and can be conveniently monitored by recording oxygen consumption. Simultaneously, if an antioxidant is present in the system, the effect of its action can be observed practically instantly, as the oxygen consumption is altered – inhibited or accelerated – or unchanged. The consumption of dissolved oxygen by liposomes was therefore used as a measure of lipid oxidation.

The concentration of dissolved oxygen in the reaction mixture was measured continuously by a polarographic oxygen electrode that is a pivotal part of the Oxygraph system (Hansatech Instruments Ltd., Norfolk, UK).

The *Oxygraph system* (Figure 3–3) consists of an electrode unit (a) mounted on a control unit (b). The electrode disk (c) forms the floor of the reaction cell (d), into which liposomes or other reaction mixtures can be added. A magnet (e) mixes the reaction mixture to maintain equally distributed oxygen throughout the whole volume. A plunger (f) prevents oxygen diffusion from the atmosphere. The capillary hole in the plunger enables injection of reactants during measurements. The reaction cell is water jacketed; the outlets of a water jacket are

connected to a circulating water bath, so that it enables oxidation experiments to be performed at different temperatures.

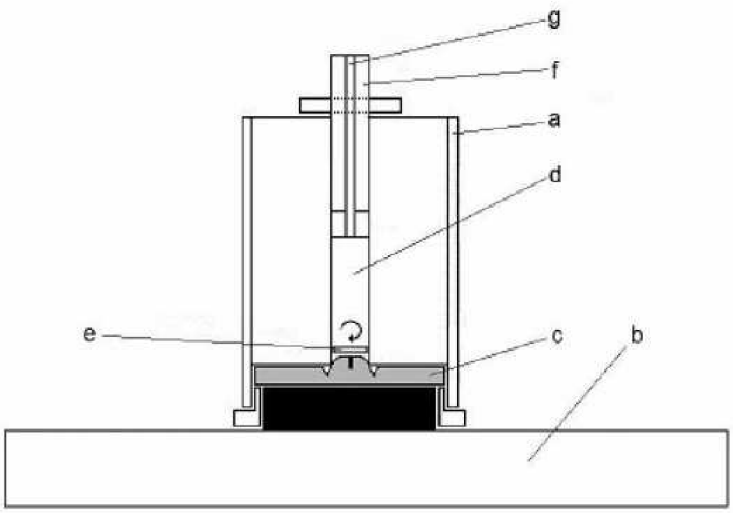


Figure 3–3 *Oxygraf* [4]

The *oxygen electrode* itself (Figure 3–4) consists of a platinum cathode and a silver anode. An electrolyte bridge between the electrodes is established by placing a small amount of electrolyte solution (3 M KCl) on the dome area of the electrode disk which is held by a paper spacer and a polytetrafluorethylene membrane. Application of a stable polarizing voltage across the electrodes from the electrode control box results in ionization of the electrolyte and a flow of current through the electrolyte. The magnitude of this current flow is proportional to the concentration of oxygen dissolved in the electrolyte which in turn is proportional to the concentration of oxygen in the surrounding media (solution in the reaction cell). [4]

Figure 3–4 *Electrode disk* [96]

The conditions in the reaction cells used in all our experiments were:

- concentration of phospholipids: 1,5 mg/mL or 1,5 % (w/v),
- volume of reaction mixture (liposomes dispersed in a 5,5 mM MES buffer): 1 mL,
- pH 5.5 (this pH was chosen because a maximum OUR for Fe-induced oxidation was observed at this pH [55]),
- temperature: 30°C.

Stock solutions of propyl gallate, caffeic, ferulic and *p*-coumaric were prepared in 96 % ethanol and stored at 4°C for a maximum of 14 days. Working solutions (10 mM) of these compounds were prepared daily by diluting an appropriate aliquot of the stock solution with

50 % ethanol in MES buffer (pH 5.5). Working solution of ascorbic acid (10 mM) was prepared in 5 mM MES buffer (pH 5.5) freshly before experiments.

Stock solutions of Fe^{2+} ($FeSO_4 \cdot 7H_2O$) and Fe^{3+} ($FeCl_3$) in 0,5 M HCl were prepared monthly. Working solutions (0,5 mM) were prepared daily by diluting an appropriate aliquot of the stock solution with 5 mM MES buffer. Working solution of bovine hemoglobin (0,001 g/mL) was prepared freshly before experiments by dissolving an appropriate amount of Hb in 5 mM MES buffer (pH 5.5). The concentrations of prooxidants used in all our experiments were (calculated as a final concentration in the reaction mixture):

- $C(Fe^{2+}) = 10$ µM,
- $C(Fe^{3+}) = 10$ µM,
- C(Hb) = 20 µg/mL, corresponding to an amount of iron of C(Hb-Fe) = 1,24 µM.

When measuring concentration of dissolved oxygen, a background oxygen uptake rate (OUR) was observed for 2 – 4 minutes before addition of an antioxidant or ethanol (blank) into the system. After the addition of an antioxidant (or ethanol), a background OUR was observed again until it became constant for at least 2 minutes. When a constant background OUR (r_2) was reached a prooxidant (Fe^{2+}, Fe^{3+} or Hb) was added into the system. After this a fast decrease in concentration of dissolved oxygen was observed. This faster decrease differed and was characteristic for each of the prooxidants and is discussed in section 4.3.1. The OUR of total oxidation (r_2) was measured after addition of a prooxidant. In the cases where the oxygen consumption was not constant after addition of a prooxidant, the initial OURs was measured, i.e. the oxygen consumption during first 2 – 4 minutes after addition of a prooxidant. The rate of oxidation (r) was found by subtracting the background OUR from the total OUR (28). An example of an OUR measurement is shown in Figure 3–5. The duration of experiments for oxygen uptake ranged 20 – 40 min.

The OURs were measured using Oxygraph software "oxyg32". To evaluate the antioxidant effect, the rate of inhibited oxidation was compared with the rate of non-inhibited oxidation (appropriate blank). Two or three parallel measurements were run for each concentration of each antioxidant.

$$r = r_2 - r_1 \tag{28}$$

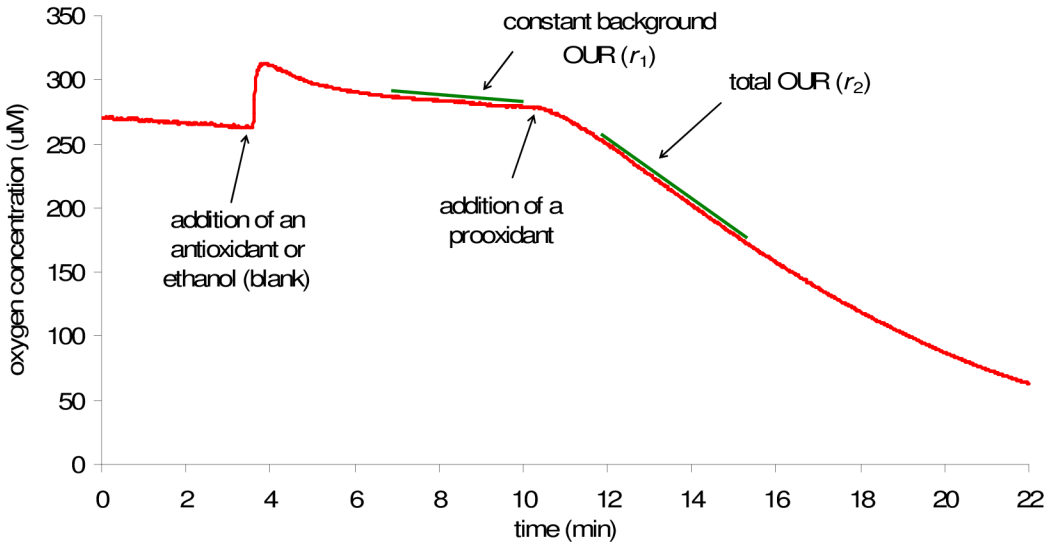


Figure 3–5 *A model example of an oxygen uptake rate measurement by polarographic oxygen electrode*

This part of the thesis is connected to the works of Mozuraityte et al. who studied free iron catalyzed oxidation of cod roe phospholipids by means of the oxygen uptake method [4, 55] and established working conditions for this system and method, and Carvajal et al. who similarly investigated hemoglobin catalyzed oxidation of liposomes and evaluated antioxidant effect of astaxanthin in the same system. [92]

3.5 Analysis of cod roe oil and phospholipids

3.5.1 Peroxide value

Peroxide value (PV) of extracted cod roe oil and isolated and purified phospholipids was analyzed by the ferric thiocyanate method as described by the International Dairy Federation [78], and modified by Ueda et al. [79] and Undeland et al. [80]. Each material was analyzed in triplicate.

3.5.2 Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) in cod roe oil and isolated and purified phospholipids were determined by the spectrophotometric method as described by Ke et al. [77] All the reaction amounts were reduced to one-half relative to the original amounts. The absorbance values of samples were compared to a standard curve prepared with 1,1,3,3-tetraethoxypropane for the calculation of TBARS concentrations (μM/g fat). The analysis was performed at least with five samples for each material.

3.5.3 Classes of isolated phospholipids

Classes of isolated phospholipids were analyzed by P-31 NMR. 50 mg of phospholipids was dissolved in 0.6 mL solution of chloroform–d, methanol–d (2:1, v/v) containing the internal standard (triethylphosphate) in 5 mm NMR-tubes. NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer with QNP probe operating at P-31 frequency of 121.49 MHz at ambient temperature (25 ^{o}C). The acquisition parameters used were: spectral width of 30 ppm, 20k time-domain data points, zero-filled to 64k, acquisition time 2.8 s, relaxation delay 50 s, 90^{o} acquisition pulse. Chemical shifts were referred to triethylphosphate (δ = 0 ppm).

The analysis was performed as described above by Ph.D. Revilija Mozuraityte, in SINTEF Fisheries and Aquaculture in Trondheim, Norway.

3.5.4 Composition of cod roe lipids and purity of isolated phospholipids

The composition of total lipids (OIL), intermediate products of phospholipid isolation (OIL1, OIL2, PL1), and final isolated phospholipids (PL2) was analyzed by Iatroscan TH10 MK-4 Thin Layer Chromatography – Flame Ionization Detector (TLC-FID) analyzer (Iatron, Japan).

Briefly, the standards or lipid samples dissolved in chloroform were spoted by 10 μL Hamilton syringe on the starting points of cleaned and dried chromarods SIII, the solvent was removed by a stream of N_2 gas. The chromarods in their holder were placed into the development tank for separation of the substances by the solvent mixture consisting of *n*-hexane–diethylether–formic acid (85:15:0.04, v/v/v). After exactly 28 min the rods were taken out of the chamber and allowed to dry for 5 min in the air. The holder with the rods was

brought into the Iatroscan and scanned for analysis. After the analysis the chromarods were reactivated by blank scanning. Each sample was analyzed in triplicate. The components of the sample were identified by comparison of the retention times with those of the reference solution, and the quantitative results were expressed in area (%) as the mean value ± SD.

Microsof Excel was used for data processing and statistical analysis.

3.6 List of chemicals

The following chemicals were purchased at Merck KGaA (Darmstadt, Germany):

- Methanol
- Chloroform, p.a.
- Acetone
- Hydrochloric acid (37 %), p.a.
- Acetic acid, glacial
- KCl, p.a.
- Formic acid (98 – 100 %), p.a.
- Diethylether, p.a.
- $FeCl_3 \cdot 4H_2O$, p.a.
- *n*-Hexane, p.a.
- Ethanol, 96 %
- Ammonium thiokyanate (NH_4SCN), p.a.
- Titrisol [$(NH_4)_2Fe(SO_4)_2$] – iron standard 1000 mg Fe
- $FeSO_4 \cdot 7H_2O$, 99,5 %

The following chemicals were purchased at Riedel de Haën (Seelze, Germany):

- L(+)-Ascorbic acid, puriss
- Iron(III) chloride anhydrous ($FeCl_3$), ≥ 98 %

The following chemicals were purchased at Sigma-Aldrich Chemie GmbH (Steinheim, Germany):

- Propyl gallate, 97 %
- Caffeic acid, ≥ 98 %
- Ferulic acid, 99 %
- *p*-Coumaric acid, ≥ 98 %, predominantly trans isomer
- 2.0 M Folin-Ciocaltau phenol reagent, suitable for determination of total protein by Lowry method
- MES (2-(N-Morpholino)ethanesulfonic acid)
- 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ≥ 98 %
- 2,2-Diphenyl-1-picrylhydrazyl (DPPH)
- 2-thiobarbituric acid (TBA), 98 %
- 2-methylpentane, +99 %
- 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ~ 98%
- Sodium carbonate, anhydrous
- Sodium acetate, ≥ 99 %, anhydrous
- Hemoglobin from bovine blood, powder
- Sodium sulfite, ≥ 98 %
- trichloroacetic acid (TCA), ≥ 99 %
- 1,1,3,3–tetraethoxypropan (TEP), ~ 97 %
- Sodium dithionite $(NaO_2S)_2$, ≥ 85 %
- Potassium perulfate ($K_2S_2O_8$), ≥ 99 %

The following chemicals were purchased at Fluka Chemie (Buchs, Germany):

- NaOH
- Iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), puriss. p.a., ≥ 99 %

N_2 gas (99,999 %) was purchased at AGA AS, Oslo.

3.7 List of instrumental equipment

The following instrumental devices were used:

- Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech Inc., Sweden)
- Vibra Cell (Sonics & Materials Inc., USA)
- Oxygraph System (Hanstech Instruments Ltd., Norfolk, UK)
- Iatroscan TH-10 model MK4 TLC-FID analyser (Iatron Laboratories Inc., Tokyo, Japan)
- Ultra-Turrax homogenizer (IKA Works Inc., Germany)
- Digital Thermometer, Model 52 Series II (Fluke Corporation, USA)
- MP220 Basic pH/mV/°C Meter (Mettler – Toledo International Inc., USA)
- Analytical balances AB-S (Mettler – Toledo International Inc., USA)
- Mettler AE200 Electronic Balance (Mettler – Toledo International Inc., USA)
- Bruker Avance DPX spectrometer (BRUKER BioSpin AG, Fällanden, Switzerland)
- Boeco Vortex Mixer V 1 Plus (Boeco, Germany)
- Single channel adjustable/fixed-volume pipettes – Finnpipette (Thermo Fisher Scientific Inc., Waltham, MA, USA), Calibra digital (Socorex Isba S.A., Switzerland), Eppendorf research (Eppendorf, Germany)
- Mini MR standard IKAMAG magnetic stirrer (IKA Works Inc., Germany)
- Sorvall RC-5C Superspeed centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA)
- Thermostatic Bath Comfort CB 8-30 (Heto, Allerod, Denmark)
- Büchi Rotavapor R-210 evaporator (BÜCHI Labortechnik AG, Switzerland)

4 RESULTS AND DISCUSSION

4.1 Characterization of cod roe lipids

4.1.1 Composition of total cod roe lipids and purity of isolated phospholipids

Marine phospholipids used for preparation of liposomes were isolated from cod roe lipids (total lipids) that were extracted from fresh cod roe. The aim of the isolation was to obtain marine phospholipids containing a minimum of other compounds, such as cholesterol, triacylglycerols or free fatty acids, naturally occurring in oils. The composition of total lipids (OIL) and purity of the final isolated phospholipids (PL2) as well as of the intermediate products of the isolation (OIL1, OIL2, PL1) was analyzed by Iatroscan TLC-FID.

The compositions of the different products are given in Table 4.1. The extracted oil contained 85,9 ± 0,8 % phospholipids.

However, a substantial part of the total phospholipids was lost during the isolation and purification of phospholipids by the acetone precipitation method, since the amount of phospholipids in OIL1 and OIL2 was relatively high, 84,5 ± 1,9 % and 83,0 ± 1,3 %, respectively. Phospholipids were not further isolated from these products. From ca. 12 kg of fresh cod roe, ca. 85 g of phospholipids was isolated.

The final phospholipids contained 97,9 ± 1,2 % of phospholipids, and traces of free fatty acids, cholesterol and monoacylglycerols (< 1 %); other compounds such, as cholesterols esters, di- and triacylglycerols were not detected in PL2. The data are in good agreement with analysis of cod roe phospholipids isolated by Mozuraityte et al. [55]

Table 4.1 *Analysis of composition of the different products obtained during the isolation of phospholipids*

% / Lipids	**Cholesterol esters**	**Triacyl-glycerols**	**Free fatty acids**	**Cholesterol**	**Monoacyl-glycerols**	**Phospholipids**
OIL (total lipids)	0,39 ± 0,02	5,3 ± 0,5	1,6 ± 0,4	6,9 ± 0,3	0,49 ± 0,05	85,9 ± 0,8
OIL1	0,9 ± 0,0	4,0 ± 0,4	4,0 ± 0,9	7,2 ± 0,1	nd	84,5 ± 1,9
PL1	nd[1]	nd	0,8 ± 0,1	2,0 ± 0,3	0,8 ± 0,1	97,0 ± 0,4
OIL2	nd	0,8 ± 0,1	3,9 ± 0,5	12,0 ± 0,5	1,3 ± 0,0	83,0 ± 1,3
PL2 (final PL)	nd	nd	0,4 ± 0,1	1,0 ± 0,5	1,0 ± 0,7	97,9 ± 1,2

[1] not detected; Results are means ± standard deviation (SD).

4.1.2 Classes of phospholipids

The classes of isolated phospholipids were analyzed by P-31 NMR spectroscopy. The quantitative NMR analysis revealed that the isolated phospholipids consist of mainly phosphatidylcholine (PC) (69 mol %) and phosphatidyl-ethanolamine (PE) (23 mol %). Small amounts of lyso PC (5 mol %) and lyso PE (3 mol %) were present. Trace amounts of other substances, presumably cardiolipin, were detected.

4.1.3 Peroxide value and TBARS

In order to estimate the degree of lipid autoxidation in the extracted oil (OIL) and the final phospholipids (PL2), and to see whether the isolation of phospholipids and storage conditions influenced the degree of lipid oxidation, PV and TBARS in OIL and PL2 were determined. The obtained values are given in Table 4.2.

Table 4.2 *Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) of extracted oil and isolated phospholipids*

Lipids	PV (meq H_2O_2 / kg fat)	TBARS (μmol / g fat)
OIL (total lipids)	6,8 ± 0,6	2,4 ± 0,5
PL2 (final phospholipids)	6,6 ± 1,3	2,4 ± 0,2

Results are means ± standard deviation (SD) of five and six determinations for PV and TBARS, respectively

The PVs, characterizing formation of primary oxidation products, determined in OIL and PL2 were not significantly different. This was also the case for TBARS, characterizing formation of secondary oxidation products. This shows that the oxidation of phospholipids did not proceed to any larger degree during their isolation and storage. Thus, keeping the isolated phospholipids dissolved in chloroform and storage at low temperatures does not provide conditions for development of oxidation. The PV for PL2 is consistent with the values reported by Mozuraityte et al. [55]

4.2 Antioxidant capacity assays

The antioxidant capacity (AOC) of the five different compounds (propyl gallate, caffeic, ferulic, *p*-coumaric and L-ascorbic acid) that were studied in this work was evaluated by the following one-dimensional antioxidant capacity assays:

a) Folin-Ciocaltau Assay (FC assay)
b) Ferric Reducing/Antioxidant Power Assay (FRAP assay)
c) 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Assay (DPPH assay)
d) 2,2'-Azinobis-3-ethylbenzotiazoline-6-sulfonic acid Assay (ABTS assay)

To perform the assays, the range of working (effective) concentration for each compound and each assay, with respect to conditions under which each assay was performed, needed to be established. The order of AOC of the studied compounds was established in each assay and the orders that were found were compared with one another and with data of other studies. The measured data are expressed both in absolute values and indirectly with regard to a reference substance for purposes of comparison. Propyl gallate was chosen as a reference substance due to its highest efficiency in all the assessments.

As described in section 2.2.1.1, antioxidants deactivate free radicals involved in lipid peroxidation by donation of a hydrogen atom. Two reaction mechanisms can participate in this reaction, so called *hydrogen-atom transfer* (HAT) and *single-electron transfer* (SET).

The first two assays represent purely a SET based reaction mechanism. Because an electron transfer is a basis of redox reactions [6], the reducing capacity of antioxidants is determined by these assays. It is important to emphasize that these assays do not characterize the tested compounds as free radical scavengers.

In the latter two assays the SET mechanism is presumed to be dominant, because the HAT mechanism occurs as well, but only as a marginal reaction pathway. The reason for this will be explained in discussions to the respective assays. In these assays, the ability of antioxidants to act as free-radical scavengers is evaluated. However, the radical compounds that are scavenged are not biologically relevant, because their physicochemical properties differ substantially from the properties of free lipid radicals found both *in vivo* and *in vitro* lipid systems.

A large number of factors can influence the antioxidant activity assessed by these methods. Some of them are connected to the nature and composition of testing matrices. Since our tested compounds are pure and of standard quality, interferences caused by e.g. other substances present in the sample, as in the case of food extracts, are reduced to a minimum. This increases the reliability of measured data. On the other hand, under- or overestimation of the results can be caused by the chemistry and methodology of the assays themselves. Special attention is paid to these aspects in the discussions to the assays.

4.2.1 Folin-Ciocaltau Assay

The Folin-Ciocaltau (FC) assay measures the electron donating ability of a compound, in other words its *reducing capacity* or relative *redox potential*. [29].

The results obtained by FC analysis of the compounds in this study are summarized in Table 4.3. The reducing capacity is expressed as the slope value of a linear curve describing the dependence of absorbance as a function of antioxidant concentration ($A_{725} = f(C_{AO})$). On the basis of the obtained values, the following order of antioxidant (reducing) capacity was established:

Propyl gallate > Caffeic acid > Ascorbic acid > Ferulic acid >> *p*-Coumaric acid

Propyl gallate and caffeic acid gave the highest values, thus the highest ability to donate an electron. Ferulic acid exhibited lower reducing capacity and *p*-coumaric acid was by far the least active compound. Its reactivity with the FC reagent was very low. Ascorbic acid was found to have a reducing capacity higher than that of ferulic acid and lower than that of caffeic acid.

Expressing results indirectly with regard to a reference compound is also possible and is commonly used. When assessing total phenolics in food samples, it is even necessary. In the improved method by Singleton et al., gallic acid is recommended as a suitable reference compound. [12] Some other studies suggest caffeic acid. [15, 24] Our results support this suggestion, for caffeic acid exhibited good reactivity with the FC reagent and relatively low absorbance values. A number of papers replaced the recommended gallic acid with catechin, tannic acid, chlorogenic acid, vanillic acid or ferulic acid. [12] The reference compound should be chosen with care. If the reference compound is highly reactive with the FC reagent giving high absorbance values, then the measured values of the samples might seem to be low unless the sample is highly reactive with the reagent too. On contrary, if the reference compound gives low absorbance values, then the reducing capacity of the samples might seem to be too high. Therefore the reference compound influences the extent of the total values.

Transformation of absolute values into equivalents of a reference compound has been proposed as a part of a standardized protocol [18], because it allows easier comparison between different substances. However, a universal type of a reference compound has not yet been agreed upon by researchers. In our study, conversion of absolute values into propyl gallate equivalents was done to make a comparison of antioxidant activities determined by different antioxidant capacity assays easier.

Table 4.3 *Overview of the results obtained by the Folin-Ciocaltau assay: The reducing capacity is expressed as the slope value ± SD of a linear curve derived from the dependence AU = f(C), and in propyl gallate (PG) equivalents; the effective concentration range represents a range of concentrations in the samples that under the assay conditions gives a linear response in range up to 0,9 AU.*

antioxidant	slope ± SD	PG equivalents	effective concentration range (mM)
Propyl gallate	0,34 ± 0,02[1]	1,00	0 – 3,0
Caffeic acid	0,33 ± 0,02[1]	0,96	0 – 3,0
Ferulic acid	0,10[2]	0,30	0 – 5,0
p-Coumaric acid	0,04[2]	0,11	0 – 5,0
Ascorbic acid	0,22 ± 0,01[1]	0,64	0 – 3,0

[1] Each value is the mean of two determinations ± standard deviation (SD)

[2] Each value is the absolute value of a single determination

The obtained values for the phenolic compounds imply that the FC results might correlate with the structure-antioxidant relationship (SAR) principles. A higher number of available hydroxyl groups in the aromatic ring increased reducing capacity as well as did the presence of other substituents attached to the ring, such as a methoxy group. [17] A similar trend was found also by other studies [15, 24] when testing pure phenolic compounds (Table 4.4).

This might be the explanation for a very weak reaction of *p*-coumaric acid with the FC reagent. The compound has a single hydroxyl group and lacks other types of substituents, such as a methoxy group, that could further enhance the reducing ability. Indeed, ferulic acid, a mono-phenol having one methoxy substituent, presented higher reducing capacity.

Table 4.4 *AOC of the tested compounds as analyzed with the ABTS assay in different studies*

antioxidant	slope [1]	slope [2]
caffeic acid	0,0201	0,84 ± 0,06
ferulic acid	0,0145	×
ascorbic acid	0,0128	0,83 ± 0,01

[1] Stratil et al. (2005) [24]; [2] Nenadis et al. (2007) [15]

Antioxidant capacity is expressed as the slope value of a calibration equation A = a × C + b (A – absorbance, C – antioxidant concentration).

If the single electron transfer or red-ox reactions is one of the antioxidant mechanisms, on the basis of the results obtained by the FC method, PG and CaA seem to give a good protection. However, the conditions of the assay have nothing in common with quenching of lipid radicals by phenolic antioxidants – a process, where the hydrogen atom is transferred by the HAT mechanism. [3] A positive reducing capacity also signals possible redox reactions with transition metals (Fe, Cu) and their reduction into a more prooxidative valence status. On the other hand, the chemical structure of PG and CaA is favorable also for chelation of metal ions, which is one of the indirect antioxidant mechanisms. [5]

Although explicit conditions and procedure for performing the FC assay are given in the improved FC assay by Singleton et al., it is not followed in the majority of recently published

papers where this method has been used. Procedures vary considerably with regard to reagent concentrations and ratios, timing of additions and length of incubation. [15, 19, 24] The proposed improved procedure was not followed in our study either, and some modifications were made. In the proposed procedure, a saturated Na_2CO_3 solution is used in order to create basic conditions in the reaction mixture. In our experiments, 25 % Na_2CO_3 solution was used; this was also used in the work of Miliauskas et al. [19] where the FC assay was applied for determination of total phenolics in some plant extracts. Using saturated solution caused precipitation in the reaction mixture that made it impossible to measure absorbance due to dispersed particles. The incubation time was reduced to 1 hour from the proposed 2 hours. 1 hour reaction time has been reported to be sufficient for the completion of the reaction. Moreover, longer reaction time may cause instability of the reaction products. [24, 29]

The FC method has been standardized for analysis of total phenolics in wine [2] and in wine viniculture it is an approved test for assessing total phenolics. Standardization of the method for analyzing other food extracts or pure phenolics is still needed and this has recently been a matter of discussion. Some suggestions for standardized protocol have already been proposed. [12, 18]

The FC reagent is non-specific to phenolic compounds. [11] A large number of interfering substances (particularly sugars, aromatic amines, sulfur dioxide, enediols and reductons, organic acids and ferrous (Fe^{2+}) ions; also many non-phenolic and inorganic substances) reacts with the FC reagent. [12]

Laboratories frequently either modify the procedure and conditions, neglect some important interfering species present in tested matrices (e.g. ascorbic acid and proteins), or use different reference compounds. These factors, alone or in combination, make it problematic to compare data published in literature; moreover this has lead to providing rather controversial information. For example, reported values for total phenolics in blueberries ranged from 22 – 4180 mg/100 g of fresh weight depending mostly on assay conditions [12].

The FC method, as an assay for assessing total phenolics and as a rough estimate of antioxidant activity of food matrices, is simple and rapid, and therefore a popular method among researchers. Repeatability of the data is considered to be quite acceptable. [13] However, it is distinct from the published data that over- or underestimation of results can be easily achieved without properly controlled steps and limitations of the method, which should be know to analysts.

The effective concentration range for each compound representing a range of concentrations in the samples that gives a linear dose-response dependence under the assay conditions in range up to 0,9 AU is summarized in Table 4.3. A linear dependence was observed for concentrations of samples up to 5,0 mM for all the tested compounds. The graphs of dose-response dependence with equations of linear regression can be found in Attachments (A1).

The reaction of *p*-coumaric acid with the FC reagent was very weak. Absorbance values within the concentration range of 0 – 5,0 mM reached a maximum at ca. 0,250 AU. Above this concentration range, the absorbance slowly and unlinearly increased up to values of ca. 0,450 AU and it did not further increase, moreover a formation of some precipitates in the reaction mixture was observed (Figure 4–1) that made the spectrophotometric measurements impossible due to unstable absorbance values. A similar trend was also observed with ferulic

acid. The low solubility of ferulic and *p*-coumaric acid in aqueous solutions is probably responsible for this phenomenon since the major solvent in the FC assay is water.

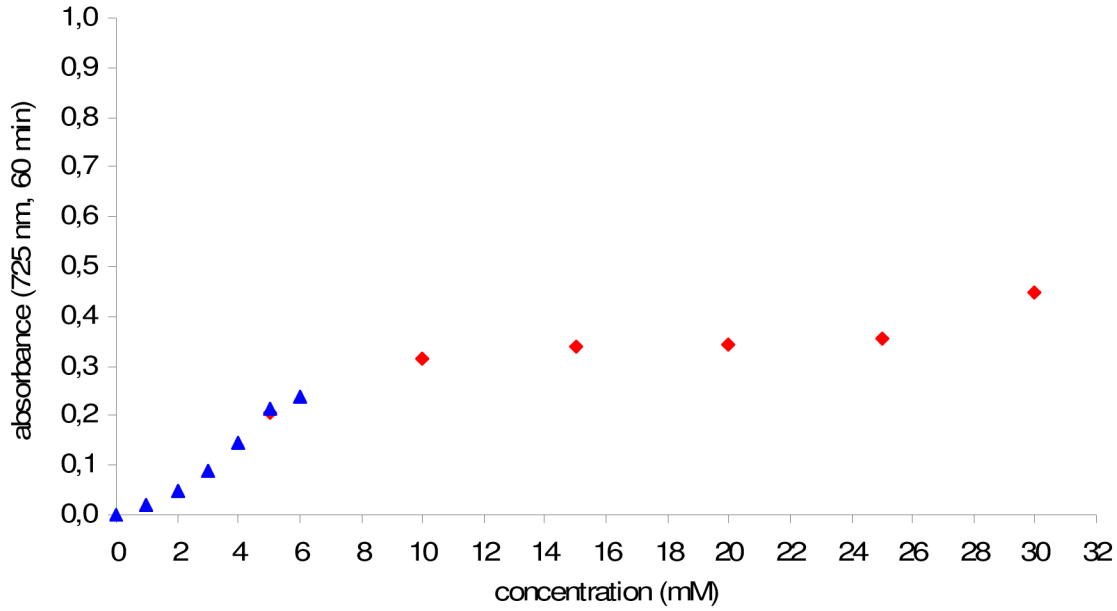


Figure 4–1 *Reaction of p-coumaric acid with the Folin-Ciocaltau reagent illustrating non-linear dose-response dependence at concentrations above 5 mM.*

4.2.2 FRAP Assay

The FRAP assay measures the ability of a compound to reduce a ferric salt to a ferrous coloured product, thus the *reducing capacity* of the compound is evaluated. The reaction mechanism is the same as in the Folin-Ciocaltau assay (single electron transfer); the main difference between the two assays resides in the pH conditions; the FRAP assay is carried out under acidic conditions (pH 3.6) in order to maintain iron solubility, while the FC assay is performed under alkaline conditions (pH ~ 10). [24]

The results obtained for all the tested compounds are summarized in Table 4.5. The reducing capacity is expressed as the slope value of a linear curve describing the dependence of absorbance as a function of antioxidant concentration in the reaction mixture ($A_{593} = f(C_{AO})$). To make evaluation and comparison of antioxidant capacities easier transformation of absolute values into propyl gallate equivalents was made as well. On the basis of the obtained values, the following order of antioxidant (reducing) capacity was established:

Propyl gallate > Caffeic acid > Ascorbic acid ~ Ferulic acid > *p*-Coumaric acid

The assay showed that all the compounds possess electron donating ability under acidic conditions (pH 3.6). However, the degree of this ability varied considerably. Propyl gallate, bearing a pyrogallol moiety (3 hydroxyl groups attached to a benzene ring), was by far the most powerful compound. Caffeic acid having a catechol moiety (2 hydroxyl groups attached to a benzene ring) followed in activity, but the ferric reducing power was significantly lower than that of propyl gallate. Ferulic acid having one available hydroxyl and one methoxy group was found to be less active than caffeic acid. The least active compound of all was *p*-coumaric acid with one hydroxyl group. The presence of two additional hydroxyl groups in propyl gallate as well as one hydroxyl group in caffeic acid or a methoxy group in ferulic acid increased the reducing ability. Ascorbic acid, a compound well known for its reducing properties, presented the FRAP value slightly lower than that of caffeic acid; however the values for caffeic and ferulic acid were not significantly different at 5 % level. The obtained order is in fair agreement with data reported in the literature (Table 4.6).

Table 4.5 *Overview of the results obtained by the FRAP assay: The reducing capacity is expressed as the slope value ± SD of a linear curve derived from the dependence AU = f(C), and in propyl gallate (PG) equivalents; the effective concentration range represents a range of concentrations in the reaction mixture that under the assay conditions gives a linear response in range up to 0,9 AU.*

antioxidant	slope ± SD	PG equivalents	effective concentration range (μM)
Propyl gallate	0,119 ± 0,005[1]	1,00	0 – 10
Caffeic acid	0,078 ± 0,003[1]	0,65	0 – 10
Ferulic acid	0,065 ± 0,004[2]	0,54	0 – 15
p-Coumaric acid	0,0051 ± 0,0004[2]	0,04	0 – 100
Ascorbic acid	0,068 ± 0,002[1]	0,57	0 – 10

[1] The value is the mean of tree determinations ± standard deviation (SD)

[2] The value is the mean of two determinations ± SD

The results for the phenolics correlate with the structure-activity relationship (SAR) principles. A good correlation between SAR principles and FRAP values of simple phenolics (i.e. hydroxybenzoic and hydroxycinnamic acids) was reported. [28] However, in the case of some polyphenols SAR principles cannot be applied when evaluating the FRAP values due to some subsequent dimerization or polymerization reactions. These reactions yield additional electrons which contribute to the reduction of iron and increase the FRAP values. [12, 15]

The obtained reducing capacity order is identical with the one established in the FC assay, which is not unexpected as both the methods utilize the same reaction mechanism (single electron transfer). Thus some similar conclusions can be made as in the FC assay: propyl gallate with caffeic acid seem to be the best candidates for prevention of lipid peroxidation. However, the ability to reduce iron bears no similarities to the radical quenching by antioxidants in lipids, nor the assay conditions (low pH, polar nature of solvents, and absence of lipid substrate) resemble lipid environment [18]. Most antioxidants quench lipid free radicals by hydrogen donation (HAT mechanism) [12] and lipid environment is in principle non-polar and of neutral pH. Thus such conclusion is not supported enough. On the other hand, high FRAP values show plainly that these acids may act as potent metal reductants, or possibly metal chelators. Medina et al. [32] reported strong chelating capacity of these two compounds toward ferrous iron.

Even if the orders of antioxidant activity determined by the FC assay and the FRAP assay are the same, indirect expression by means of propyl gallate equivalents reveals differences in the degree of activity. While in the FC assay caffeic acid and propyl gallate showed similar activity (the ratio between them is 0,96), in the FRAP assay propyl gallate turned out to be more efficient in reduction of ferric salt (the same ratio is 0,65).

A large number of electrons involved in redox reactions of some phenolic compounds is attributed to subsequent chemical reactions (dimerization, polymerization). [15] Such chemical reactions may explain the relatively high FRAP value of propyl gallate compared to the rest of the phenolics and ascorbic acid.

Table 4.6 *AOC of the tested compounds as analyzed with the FRAP assay in different studies*

FRAP values	μM e^-/ mg AO *	EC_1 (μmol/L) **	slope ***	slope × 10^3 ****
propyl gallate	18,9	×	×	×
caffeic acid	12,2	196	0,0715	1,43 ± 0,06
ferulic acid	9,8	390	0,0471	×
coumaric acid	0,6	×	×	×
ascorbic acid	×	392	0,0282	1,37 ± 0,06

* Medina et al. (2007) [32]; reducing capacity is expressed as μmol of donated electrons per mg of antioxidant
** Pulido et al. (2000) [28]; EC_1 means a concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4 \cdot 7H_2O$
*** Stratil et al. (2006) [24], **** Nenadis et al. (2007) [15]; in both studies, the reducing capacity is expressed as the slope value of a calibration equation A = a×C + b (A – absorbance, C – antioxidant concentration)

The FRAP assay was introduced in 1996 by Benzie and Strain as a novel method for determination of reducing capacity in plasma [10]. In later years, the method has been adapted for various food extracts and several studies have pointed out some weaknesses in the method. [16, 24, 28] The most important of these seems to be the end-point for spectrophotometric measurements. Pulido et al. [28] reported that some phenolics still react

after 4 min incubation time; caffeic, ferulic and ascorbic acids were among them. The 4 min incubation time was applied also in our measurements. This may lead to a certain degree of underestimation in our results. In the same study phenolics dissolved in methanol (also our solvent) provided lower values than the same compounds dissolved in water. Composition of the sample solvent, as another factor significantly affecting measured data, was studied by Pérez-Jiménez et al. [16]. A shift in the reducing capacity order can occur when applying different solvents. The shift may occur also due to the 4 min end-point time [16, 28]. In the study of Pérez-Jiménez, the effect of the solvent on the results of AOC assays was lowest in the FRAP assay, compared to the ABTS and DPPH assay. Another important factor that must be taken into account when testing food matrices is the presence of interfering substances, such as sugars and amino acids. [16]

As in the case of many antioxidant capacity assays, a standardized protocol for the FRAP assay is needed. Alterations in the original method are frequently done in recently published papers and the ways of expressing the results vary as well (shown in Table 4.6). Thus, direct comparison of measured data with data reported in various works is problematic.

Dose-response dependence for individual compounds was reported to be linear over a wide range of concentrations [10, 28]. The effective range of concentration for each compound (expressed as a final concentration in the reaction mixture), with respect to the conditions under which the assay was performed, is presented in Table 4.5. These concentrations are chosen to reach absorbance up to 0.9 AU. In the case of *p*-coumaric acid, a non-linear trend in the absorbance range was observed when higher concentrations (approximately above 100 µM) were used (Figure 4–2). The graphs of dose-response dependence with equations of linear regression can be found in Attachments (A.2).

The FRAP assay is a fast, easy-to-handle and inexpensive spectrophotometric method. Because of purely SET reaction mechanism, the method can be useful, when combined with other antioxidant activity assays, in distinguishing which protective mechanism is dominant with different antioxidants. [12]

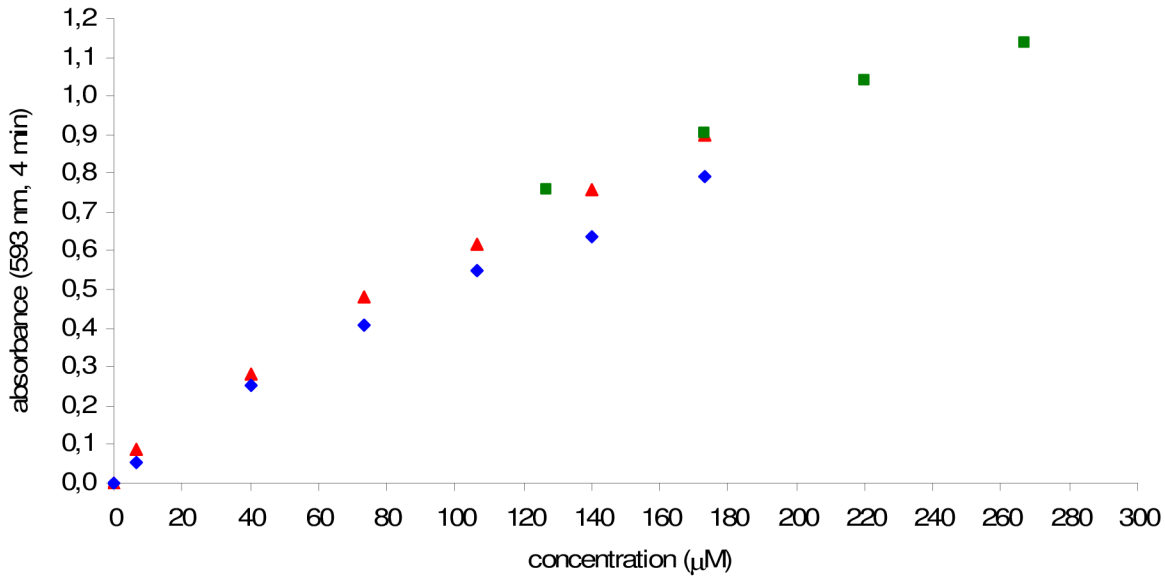


Figure 4–2 *Reaction of p-coumaric acid with the FRAP reagent illustrating non-linear dose-response dependence at concentrations above 100 µM.*

4.2.3 DPPH Assay

The DPPH method is based on the capability of an antioxidant to donate a hydrogen radical or an electron to $DPPH^{\bullet}$, which has a deep purple colour [11]. The radical's colour fades upon the reaction. The loss of the colour is measured by a spectrophotometer and is proportional the antioxidant concentration. [11, 12] In this study, the static version of the assay was applied, and the results are expressed by means of EC_{50} (for more details see section 2.3.8.4).

The results obtained for all the tested compounds are summarized in Table 4.7. The EC_{50} values were calculated from the linear curves describing the dependence of the inhibition (%) of the initial absorbance of the $DPPH^{\bullet}$ solution as a function of antioxidant concentration in the reaction mixture. On the basis of the obtained values, the following order of antioxidant capacity was established:

Propyl gallate > Caffeic acid > Ascorbic acid > Ferulic acid >> *p*-Coumaric acid

Among the tested compounds, *p*-coumaric acid was the only one that exhibited very weak reaction with $DPPH^{\bullet}$. It was reported previously [13] that $DPPH^{\bullet}$ does not react with flavonoids containing no OH-groups in the B-ring nor with monophenols containing only one OH-group attached to the benzene ring. The most reactive compounds were propyl gallate followed by caffeic acid and ascorbic acid. The achieved order is in fair agreement with published data (Table 4.8).

The reaction mechanism of hydrogen-atom abstraction by the $DPPH^{\bullet}$ cannot be strictly classified. Studies devoted to elucidate this matter agreed that both HAT and SET mechanisms are involved at the same time. [27] However, one of the mechanisms is usually marginal while the other prevails. One of the key factors influencing the ratio between the two mechanisms is the hydrogen-bonding characteristics of the sample solvent (see section 2.2.1.1 for explanation). Kinetic analysis of reaction of phenolics (ArOH) with $DPPH^{\bullet}$ in alcohols done by Foti et al. [27] has suggested that the reaction is actually driven by the trace presence of phenoxide anions and a subsequent cascade of SET reactions (29 – 31). The hydrogen-atom abstraction from neutral ArOH by $DPPH^{\bullet}$ becomes a marginal reaction path because in strong hydrogen-bond-accepting solvents it occurs both to a minimal extent and very slowly. The authors also found that the presence of acids or bases (even in trace amounts, such as impurities of the solvent) may dramatically influence the ionization equilibrium of phenols and cause a reduction or an enhancement of the reaction rate.

$$ArOH \Leftrightarrow ArO^{-} + H^{+} \quad (29)$$

$$ArO^{-} + DPPH^{\bullet} \Rightarrow ArO^{\bullet} + DPPH^{-} \quad (30)$$

$$DPPH^{-} + H^{+} \Rightarrow H\text{–}DPPH \quad (31)$$

All our substances were dissolved in 99 % methanol – a protic polar solvent. Thus, an evaluation of antioxidant activity based predominantly on SET mechanism (reduction) was carried out in our study. Some unrelated reduction reactions can occur as well. [12] For instance, products of the reaction can further react with $DPPH^{\bullet}$ leading to its additional quenching. [13] Phenols usually react with highly reactive and transient radicals involved in lipid peroxidation by the HAT mechanism. [1] This fact along with the fact that $DPPH^{\bullet}$ is a

synthesized stable nitrogen radical, are the main objections in several critical appraisals on the DPPH assay. [11, 12, 13]

A direct comparison of the data from other studies is problematic, since the results are based on different interpretation given by the assay version and assay conditions, the latter being frequently altered (different reagent ratios, solvents, incubation time etc.). Even within the same version the comparison is not possible since several ways of expressing the results are possible. Even results expressed in the same way can vary. For example, comparison of EC_{50} cannot be done, as this value is dependent on the initial concentration of DPPH$^{\bullet}$ in the reaction mixture, and on the ratio between the amount of DPPH$^{\bullet}$ and the amount of antioxidant.

Table 4.7 *Overview of the results obtained by the DPPH assay: The antioxidant capacity is expressed as EC_{50} values ± SD, and in propyl gallate (PG) equivalents; the effective concentration range represents a range of concentrations in the reaction mixture that under the assay conditions gives a linear response in range up to 0,9 AU.*

antioxidant	**EC_{50}** (μM)[1]	**PG equivalents**	**effective concentration range** (μM)
Caffeic acid	22,7 ± 1,1	0,47	0 – 40
Ascorbic acid	27,3 ± 1,1	0,39	0 – 40
Propyl gallate	10,6 ± 0,3	1,00	0 – 25
Ferulic acid	48,0 ± 1,9	0,22	0 – 80
p-Coumaric acid	nd[2]	nd	nd

[1] The efficient antioxidant concentration for scavenging 50 % of DPPH$^{\bullet}$ concentration (100 μM). Each value is a mean of duplicate determinations ± standard deviation (SD)
[2] Not determined

The correlation between the ability of simple phenolics to decolorize DPPH$^{\bullet}$ and the number and position of hydroxyl groups on the benzene ring (SAR) was reported to be very good [8, 21, 24]; the level of the activity was found to be highly variable within this group of compounds as well, which was also shown in our experiments. Propyl gallate with 3 OH-groups exhibited a very high activity compared to the hardly detectable activity found for *p*-coumaric acid with 1 OH-group. The addition of the methoxy group in ferulic acid increased the activity.

Apart from the apparent non-relevance to the process of lipid peroxidation, the method suffers from a number of other weaknesses. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH$^{\bullet}$ due to steric inaccessibility. This results in seemingly better antioxidant activity of smaller molecules than of larger molecules (phenolic acids *vs* flavonoids). [12]

The chosen end-point for the reaction is another criticized aspect of the DPPH assay. Many substances react very slowly with DPPH$^{\bullet}$, for instance in range up to 1 hour. [8] Therefore, a short incubation period can lead to low values and underestimated results. A 20-min incubation time was applied in our measurements; however this might still not be enough in the case of caffeic acid, reaction of which with DPPH$^{\bullet}$ has been reported to be very slow.

Reaction kinetics between DPPH$^{\bullet}$ and most antioxidants is not linear. Expressing antioxidant capacity using the EC_{50} or AE (antiradical efficiency) value is therefore inadequate; however this is the most common way of expressing the antioxidant capacity. [11]

The influence of the solvent used to dissolve the sample on antioxidant activity was examined by Pérez-Jiménez et al. [16]. They found that the influence of the solvent was relatively low in the DPPH assay compared to the ABTS, FRAP and ORAC assay; the greatest difference was found between water and methanol (ca. 20 %). They also reported that the acidity greatly influenced the kinetics of the reaction which is in accordance with data published by Foti et al.

Finally, it was reported that the reaction of DPPH$^{\bullet}$ with eugenol was reversible. [11] This might result in false antioxidant capacity of samples containing antioxidants bearing a similar structure (*o*-methoxyphenol). Ferulic acid is such kind of substance.

The effective range of concentration for each compound (expressed as a final concentration in the reaction mixture) with respect to the conditions under which the assay was performed is presented in Table 4.7. When the inhibition reached ca. 80 %, non-linearity in dose-response dependence was observed for all the compounds. The graphs of dose-response dependence with equations of linear regression can be found in Attachments (A.3).

Easy and rapid performance of the DPPH assay makes it a popular method for evaluating antioxidant effects of single compounds as well as of food extracts and physiological fluids. However, its many weaknesses should be considered when applying this method.

Table 4.8 *AOC of the tested compounds as analyzed with the DPPH assay in different studies*

DPPH values	RSA (%) [1]	slope [2]	% inhibition [3]	% inhibition [4]	slope [5]	EC_{50} [6]	RSA (%) [7]
propyl gallate	×	×	×	87,1 ± 2,3	×	×	×
caffeic acid	76,6	–4,49 ± 0,24	51,5 ± 2,44	49,6 ± 0,6	–0,0279	0,20 ± 0,01	30,5 ± 0,31
ferulic acid	30,9	–1,34 ± 0,05	24,8 ± 1,06	27,3 ± 0,8	–0,0244	×	15,7 ± 0,62
p-coumaric acid	3,6	–0,33 ± 0,06	×	7,0 ± 0,8	×	×	×
ascorbic acid	×	–1,83 ± 0,07	×	×	–0,0147	0,20 ± 0,01	×

[1] Nenadis et al. (2004) [21]; RSA (Radical Scavenging Activity) = $[(A_0 - A_t)\times100]/A_0$ (A_0 – absorbance at start, A_t – absorbance after 20 min reaction time)

[2] Fukumoto et al. (2000) [8]; mean value ± SD of slope coefficients of the dependence A = *f*(C) calculated by linear regression

[3] Chen et al. (1997) [26]; % inhibition of 100 μM ethanolic DPPH$^{\bullet}$ solution by a 20 μM solution of antioxidant

[4] Kikuzaki et al. (2002) [69]; values are expressed in the same way as [3]

[5] Stratil et al. (2006) [24]; the slope value of a calibration equation A = a×C + b (A – absorbance, C – antioxidant concentration)

[6] Nenadis et al. (2007) [15]; EC_{50} = efficient antioxidant concentration for scavenging 50 % of the initial DPPH$^{\bullet}$ concentration

[7] Pekkarinen et al. (1999) [36]; the percentage ± SD of DPPH$^{\bullet}$ scavenged in 0,3 mM ethanolic DPPH$^{\bullet}$ solution by antioxidants (13,3 μM in the reaction mixture) after 10 min incubation time

4.2.4 ABTS Assay

The ABTS assay measures the ability of an antioxidant to neutralize a synthetic pre-formed radical, ABTS radical monocation ($ABTS^{\bullet+}$). [12] A decrease of the $ABTS^{\bullet+}$ concentration is linearly proportional to the antioxidant concentration. [24]

The results obtained for all the tested compounds are summarized in Table 4.9. The antioxidant activity (ABTS value) is expressed as the slope value of the dependence $I = f(C)$ calculated by linear regression; I is inhibition (%) of the initial absorbance of the $ABTS^{\bullet+}$ solution, and C (μM) is antioxidant concentration in the reaction mixture. The results are also expressed by means of EC_{50} values, and the relative value to that of propyl gallate was calculated for purposes of comparison. On the basis of the obtained values the following order of antioxidant capacity was established:

Propyl gallate > *p*-Coumaric acid ~ Ferulic acid > Caffeic acid > Ascorbic acid

All the tested compounds were able to scavenge $ABTS^{\bullet+}$ at neutral pH. The most potent one was propyl gallate. *p*-Coumaric and ferulic acids exhibited similar antioxidant activities (the values are not significantly different at $p < 0,05$), but considerably lower than that of propyl gallate. Caffeic acid was the least active compound of all the phenolics. Ascorbic acid was the least active compound of all. The achieved order is in fair agreement with published data (Table 4.10).

Table 4.9 *Overview of the results obtained by the ABTS assay: The antioxidant capacity is expressed as the slope value ± SD of a linear curve derived from the dependence AU = f(C), in propyl gallate (PG) equivalents, and as EC_{50} values; the effective concentration range represents a range of concentrations in the reaction mixture that under the assay conditions gives a linear response in range up to 0,9 AU.*

antioxidant	slope[1]	PG equivalents	EC_{50} (μM)[2]	effective concentration range (μM)
Caffeic acid	6,4 ± 0,1	0,32	7,86	0 – 10
Ascorbic acid	4,8 ± 0,1	0,24	10,43	0 – 10
Propyl gallate	20,0 ± 0,4	1,00	2,51	0 – 5
Ferulic acid	8,5 ± 0,2	0,43	5,87	0 – 10
p-Coumaric acid	8,7 ± 0,4	0,44	5,74	0 – 10

[1] Each value is a mean of duplicate determinations ± standard deviation (SD)

[2] The efficient antioxidant concentration for scavenging 50 % of $ABTS^{\bullet+}$ (100 μM)

Originally, only hydrogen atom donation (HAT reaction mechanism) by antioxidants was thought to be responsible for quenching $ABTS^{\bullet+}$. [20] Subsequent studies reported also electron donation (reduction) to be involved. At present, a combination of both mechanisms is believed to be the overall reaction leading to scavenging $ABTS^{\bullet+}$. As explained for the DPPH assay (section 4.2.3), hydrogen-bond characteristics of the solvent play an important role in influencing the ratio between the HAT and SET mechanisms; briefly, polar solvents, such as methanol, favor the SET mechanism. The mechanisms may also switch with pH; for instance, electron transfer is preferential at acidic pH. [12] Phenols usually react with radicals involved

in lipid peroxidation by the HAT mechanism [1]. From this point of view, the ability of a compound to scavenge $ABTS^{\bullet+}$ does not clearly reflect the compound's antioxidant activity as a scavenger of lipid radicals.

The achieved order does not correlate with SAR principles. Propyl gallate bearing three OH-groups was the most active compound, but *p*-coumaric acid and ferulic acid with only one OH-group each were more active than caffeic acid with two OH-groups. The results of several studies have shown that TEAC values of phenolics often do not correlate with SAR. [13, 21] Our results support these reports. For example, compounds, such as coumaric acids or isoferulic acid were even more reactive with $ABTS^{\bullet+}$ than certain diphenolic counterparts, e.g. rosmarinic acid. [21] Since monophenols are known to be less active as radical scavengers than polyphenols, such findings were rather confusing. The phenomenon has been attributed to the reaction mechanisms of antioxidants with $ABTS^{\bullet+}$, which are still unclear and seem to be different for each group of phenolics. [21]

Up to date, a large amount of information on TEAC values for individual phenolics has been collected. Often, the reported data differ substantially; in some cases they even provide conflicting information. Thus there have been many studies where the limitations of the ABTS method have been evaluated.

One of the most criticized limitations is that the ABTS (or TEAC) values actually characterize the capability of the tested substance to react with $ABTS^{\bullet+}$ than to inhibit the oxidative process. [13] This feature is common with the DPPH assay. $ABTS^{\bullet+}$ is nonphysiological radical that has nothing in common with the highly reactive peroxyl radicals involved in lipid oxidation.

It has been reported that with many phenolics the reaction occurs rather slowly. Caffeic acid belongs to such compounds. The results are therefore dependent on the time of incubation as well as on the ratio of sample quantity to $ABTS^{\bullet+}$ concentration. In the present study a 4 min incubation time was used. This period may not be efficient for substances that react with $ABTS^{\bullet+}$ slowly, which may lead to underestimated final values.

Another limitation is the poor selectivity of $ABTS^{\bullet+}$ in the reaction with H-atom donors. It has been reported that $ABTS^{\bullet+}$ reacts with any hydroxylated aromatics independent of their real antioxidative potential. In this view, the ABTS assay is reduced to titration of aromatic OH-groups including OH-groups which do not contribute to the antioxidative action. [13]

The study of Pulido et al. has shown that the ABTS values are strongly dependent also on the solvent used. [28] Instability of the $ABTS^{\bullet+}$ solution has been reported by Stratil et al. The $ABTS^{\bullet+}$ was slowly spontaneously degraded and continuous decrease of the initial absorbance of pure $ABTS^{\bullet+}$ solution was observed during time. [24] This may affect the absorbance values of samples and lead to overestimated results.

The ABTS assay undoubtedly has some limitations; some seem to be random and uncontrollable, while others could be difficult to reduce or control. The order of antioxidant activity obtained by this assay in this study differs substantially differs from the orders obtained by the other assays, especially from the AOC order obtained by the DPPH assay which is conceptually similar to the ABTS assay. The assumption therefore could be that the antioxidant capacity is not fairly measured by the ABTS assay and the use of this assay for the purposes of AOC assessment should be reappraised.

Table 4.10 *AOC of the tested compounds as analyzed with the ABTS assay in different studies*

ABTS values	TEAC (mM) [1]	Molar TAC (µmol/µmol) [2]	slope [3]	$TEAC_{but}$ [4]	slope [5]
propyl gallate	×	×	×	×	×
caffeic acid	0,98 ± 0,06	1,18	-0,0179	1,15 ± 0,09	2,26 ± 0,06
ferulic acid	1,90 ± 0,05	3,51	-0,0624	1,97 ± 0,02	×
p-coumaric acid	2,00 ± 0,07	×	×	2,39 ± 0,09	×
ascorbic acid	1,05 ± 0,02	×	-0,0172	×	2,00 ± 0,05

[1] Re et al. (1999) [20]; TEAC – Trolox Equivalent Antioxidant Capacity values
[2] Nilsson et al. (2005) [23]; TAC – Total Antioxidant Capacity values
[3] Stratil et al. (2006) [24]; slope value of a calibration equation A = a × C [µmol/L] + b (A – absorbance, C – antioxidant concentration)
[4] Nenadis et al. (2004) [21]; $TEAC_{but}$ – Trolox Equivalent Antioxidant Capacity values, butanol was used as a sample solvent
[5] Nenadis et al. (2007) [15]; slope value of a linear calibration curve A = *f*(C)

4.2.5 Comparison of the AOC results

The orders of AOC obtained by the FC, FRAP and DPPH assay have a very similar trend (PG > CaA > AsA > FeA > CoA). However, comparison of PG equivalents reveals differences in the degree of the capacities for the same compound in the different assays (Figure 4.3). For instance, while in the FC assay CaA shows activity almost equal to that of PG, in the DPPH assay, the activity of CaA is ca. one half of the activity determined by the FC or the DPPH assays.

Different reaction mechanisms and some specific interactions that may occur between the assay reagents and the studied compounds including some unrelated reactions, such as dimerization of the antioxidants, in the reaction mixture might most likely be the reason for these inconsistencies. Another factor determining the extent of the capacity is the chosen end-point for the reactions. It should be noted that each assay is carried out at different pH (FRAP – acidic, FCR – basic, ABTS, DPPH – neural). The pH values also have influence on the reducing capacity of antioxidants. [11] The mechanisms, reactions and important limiting factors are more closely described and discussed in the respective sections to each assay (section 2.3.8 and subsections, section 4.2 and subsections).

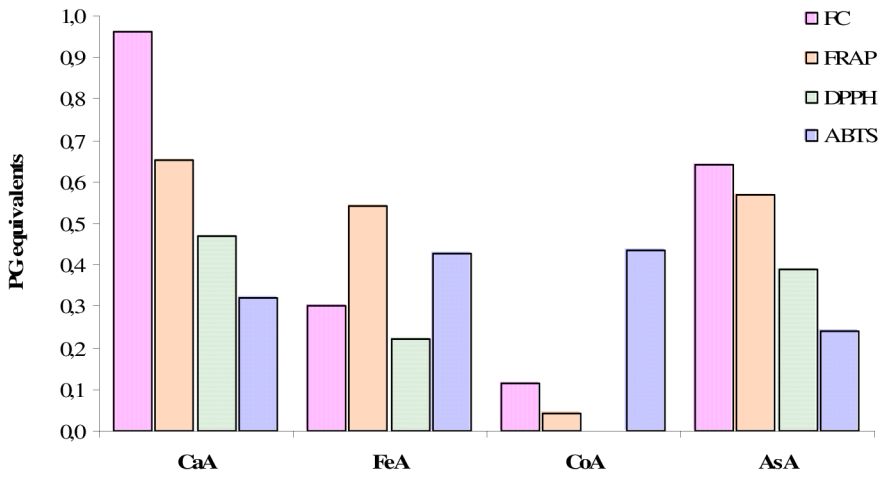


Figure 4–3 *Comparison of the antioxidant capacities of the tested compounds determined by the different AOC assays. The values are expressed as propyl gallate (PG) equivalents (PG having a value equal to 1).*

4.3 Study of antioxidant effects in a liposome system

To obtain more reliable information on the antioxidant potentials of various compounds, it is recommended to use more than one analytical method, and also to use different model systems. A direct approach of evaluation that utilizes lipid model systems has been suggested as being superior to the indirect approach utilizing artificial AOC assays. [7, 18, 30]

The reasons for choosing a liposomal model system containing high amounts of PUFAs as a lipid model system, and the use of iron and hemoglobin as lipid oxidation catalysts are briefly given in the Introduction to the thesis.

It was observed that many studies that assess the antioxidant activity of phenolics in various systems have focused on one or two fixed concentrations of the antioxidant in the system [30, 33, 34, 35, 69]. In our study, various concentrations were tested in order to see whether different proportions of antioxidants in relation to the given amount of lipids have any influence on lipid oxidation catalyzed by a fixed amount of prooxidant.

4.3.1 Oxidation of liposomes

Transition metals and some metalloproteins, such as hemoglobin or myoglobin, are known as potent promoters of lipid oxidation even in trace amounts. [5] Iron ions (Fe^{2+}, Fe^{3+}) and bovine hemoglobin (Hb) were used as initiators (prooxidants) of lipid oxidation in the liposome system.

Oxygen consumption by liposomes before and after addition of prooxidants is shown in Figure 4–4. Before the addition of prooxidants, a slow and linear decrease in concentration of dissolved oxygen was observed. This consumption of dissolved oxygen by liposomes themselves is further referred to as *liposome initial activity* and the oxygen uptake rate (OUR) measured in pure liposomes is referred to as *background OUR*. The liposome activity could be attributed to the presence of pre-formed (endogenous) peroxides and/or to the presence of endogenous transition metals (Pt, Fe, Cr)). Traces of these metals can be released into the liposome solution for example during sonication of phospholipids, or can be found as contaminants in chemicals. The phospholipids had a low content of endogenous peroxides (see determination of peroxide values, Table 4.2).

When Fe^{2+} was added to the liposomes an initial drop in concentration of dissolved oxygen was observed, which was followed by a slower linear decrease in concentration of dissolved oxygen. When Fe^{3+} was added, only the slow linear decrease in concentration of dissolved oxygen was observed. According to Mozuraityte et al. [4], this fast drop is due to oxidation of Fe^{2+} to Fe^{3+}. This process is accompanied by generation of alkoxyl radicals that trigger a chain of branching reactions leading to production of several peroxides, which results in rapid oxygen consumption. When equilibrium between Fe^{2+} and Fe^{3+} is achieved after the initial fast oxygen uptake, a constant rate of oxygen consumption is observed.

When Hb was added to the liposomes, no initial drop was observed. This could be attributed to the met-hemoglobin form of Hb that binds ferric (Fe^{3+}) iron, and which is predominant in aqueous solutions. The mechanism of Hb-induced oxidation is more complex compared to the free iron induced oxidation (see section 2.1.3). Probably due to this, the consumption of dissolved oxygen after the addition of Hb was not constant. The fastest and more or less constant OUR was observed immediately after the addition of Hb; after that the oxygen consumption slowed down non-linearly. The constant initial OUR was measured and used as a reference rate for further experiments with antioxidants (Figure 4–8).

Hb gave a higher OUR value than iron even though the amount of iron donated *via* hemoglobin was ca. one eight of the amount of free iron used. This clearly demonstrates very strong prooxidative properties of Hb.

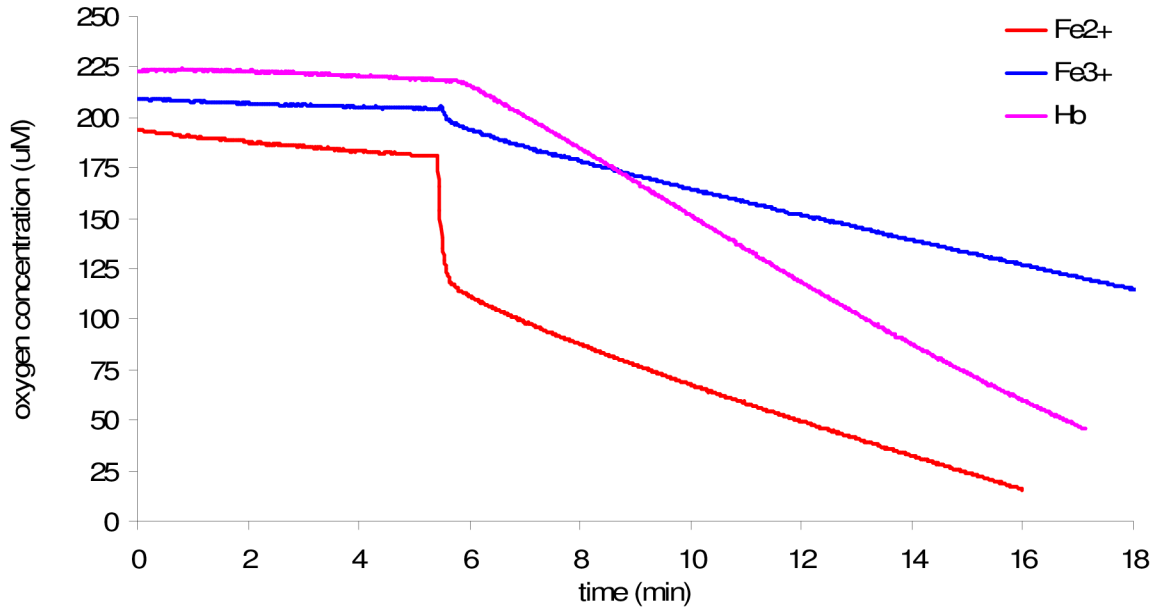


Figure 4–4 *The kinetics of oxidation of 1.5 % liposomes induced by Fe^{2+} (10 μM), Fe^{3+} (10 μM) and bovine hemoglobin (Hb) (1,24 μM).*

4.3.2 Influence of solvents on OUR

The solubility of phenolics in water is limited and depends on the polarity of each compound. The stability of phenolics in aqueous solutions is also limited; the stability of phenolics is much higher in organic solvents, such as methanol or ethanol, than in aqueous solutions. Thus, stock solutions of all the phenolics were prepared in 96 % ethanol to ensure both that the compounds were properly dissolved and mainly to maintain their stability; the stability in organic solvents is also strongly enhanced by keeping the solutions at low temperatures (< 4°C).

The influence of ethanol, as a solvent of the phenolics, on the consumption of oxygen by liposomes themselves (liposome initial activity) as well as on the OUR after addition of prooxidants was therefore first investigated.

Figure 4–5 shows liposome activity before and after the addition of 5, 10 and 20 μL of 96 % ethanol into the system (0.5, 1.0 and 2.0 % of ethanol in a total reaction volume, respectively). After addition of ethanol, a rapid increase in dissolved oxygen was observed. This increase was proportional to the amount of added ethanol. The OUR after the increase remained approximately the same and was constant as before the increase, which indicates that ethanol added in small amounts does not affect the liposomes in a way that would enhance their initial activity. Higher amounts of ethanol (or any other organic solvents) might lead to destabilization of the liposomes.

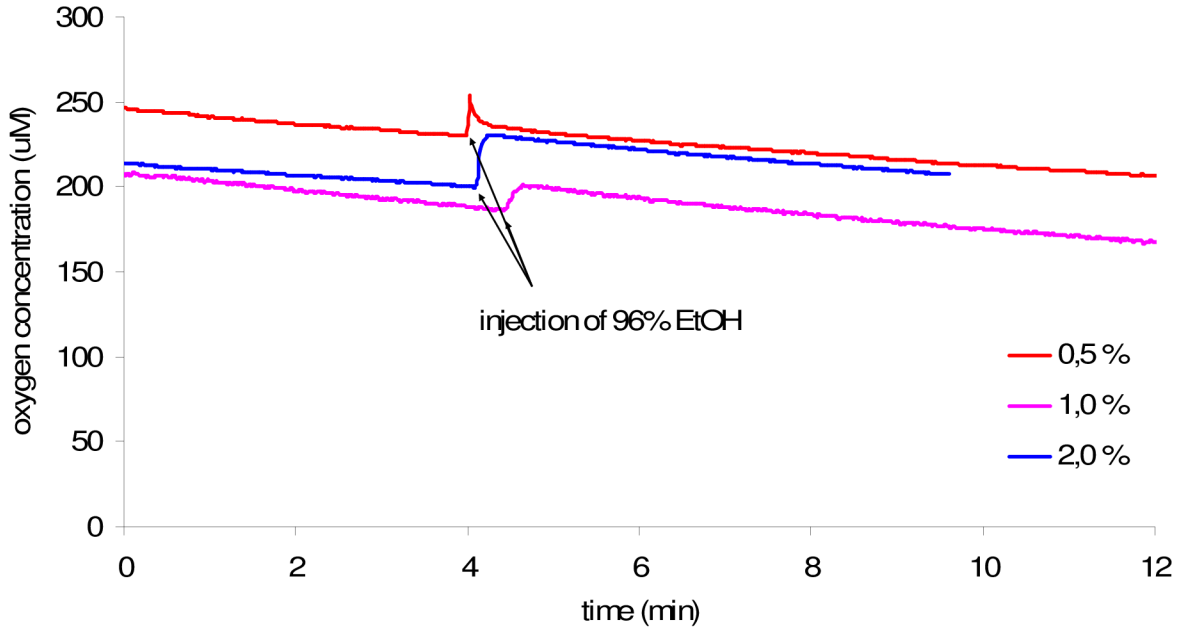


Figure 4–5 *Influence of ethanol (0.5, 1 and 2% in the reaction mixture) on the initial activity of liposomes*

Investigation of the influence of ethanol (2 %) on the OUR of Fe^{2+} and Fe^{3+} induced oxidation is shown in Figure 4–6 and Figure 4–7, respectively. No significant deviations between the OUR in liposomes without ethanol (red lines) and with added ethanol (blue lines) were observed.

Ethanol added into the liposome solution (2 %) slightly inhibited the OUR of Hb-promoted oxidation in trial measurements. Assakura et al. (1977) reported that solvents, such as alcohols and ketones, at low concentrations (up to 5 % (v/v)) stabilize the tertiary and quaternary structure of proteins while high concentrations of the same solvents lead to denaturation of proteins. [61] A stabilized (less unfolded) hemoglobin exhibits lower prooxidative effects, since the heme group containing iron is less exposed to the oxidative substrate. [74] This might explain the inhibition of OUR that was observed in several of our experiments. In order not to overestimate the antioxidant effects of the phenolic compounds, a blank with added ethanol was always used as a reference in Hb-induced oxidation for measurements with compounds dissolved in ethanol.

The average OUR ± standard deviation of Fe- and Hb-catalyzed oxidation of a pure liposome solution and a solution containing 2 % of ethanol (further referred to as *blank samples*) are presented in Table 4.11. The standard deviation encompasses many factors that may influence consumption of oxygen by liposomes, such as preparation of liposome and prooxidant working solutions, pH and freshness of the solutions; high deviations can be also caused by the state of electrodes, oxidation of which decreases their sensitivity.

Table 4.11 *Average oxygen uptake rates of oxidation catalyzed by iron ions and hemoglobin in the absence and presence of ethanol (2 %) in the liposome solution (OURs of blanks samples)*

Prooxidant	**r** (μM/min)	**SD**	**n**
Fe^{2+} (10 μM)	6,98	2,94	17
Fe^{2+} (10 μM) + EtOH	7,20	1,22	8
Fe^{3+} (10 μM)	6,05	2,24	25
Fe^{3+} (10 μM) + EtOH	6,97	1,81	9
Hb (1,24 μM)	20,77	5,65	21
Hb (1,24 μM) + EtOH	19,40	3,62	22

r – oxygen uptake rate, SD – standard deviation, n – number of experiments

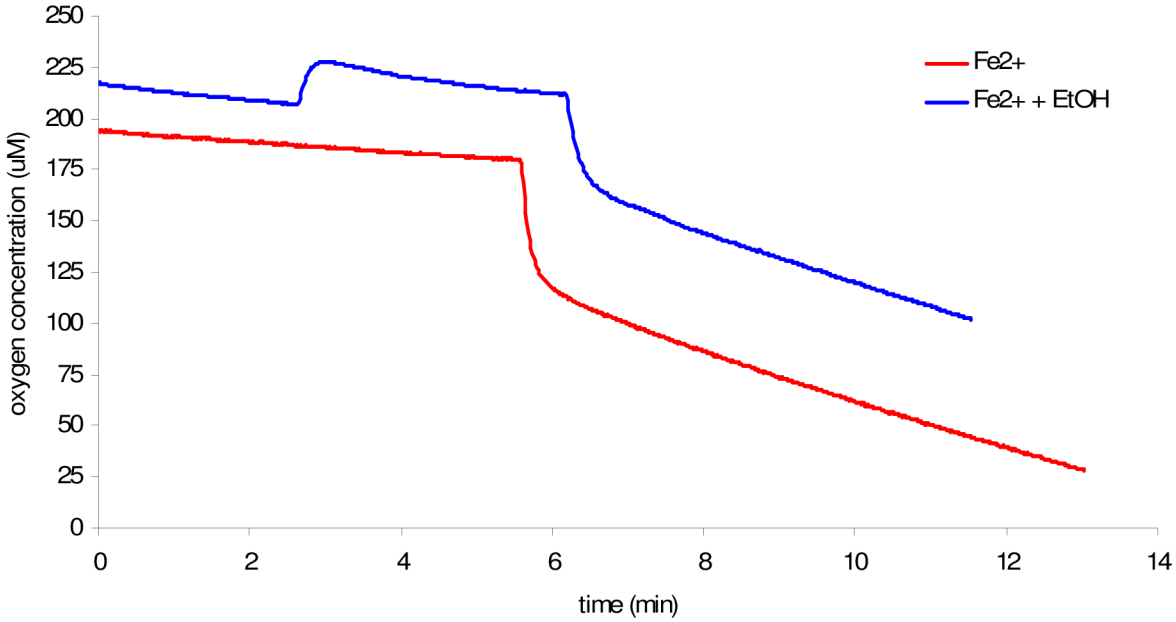


Figure 4–6 *Influence of ethanol (2 % in the reaction mixture) on OAR when Fe^{2+} (10 μM) was added as an initiator of liposome oxidation*

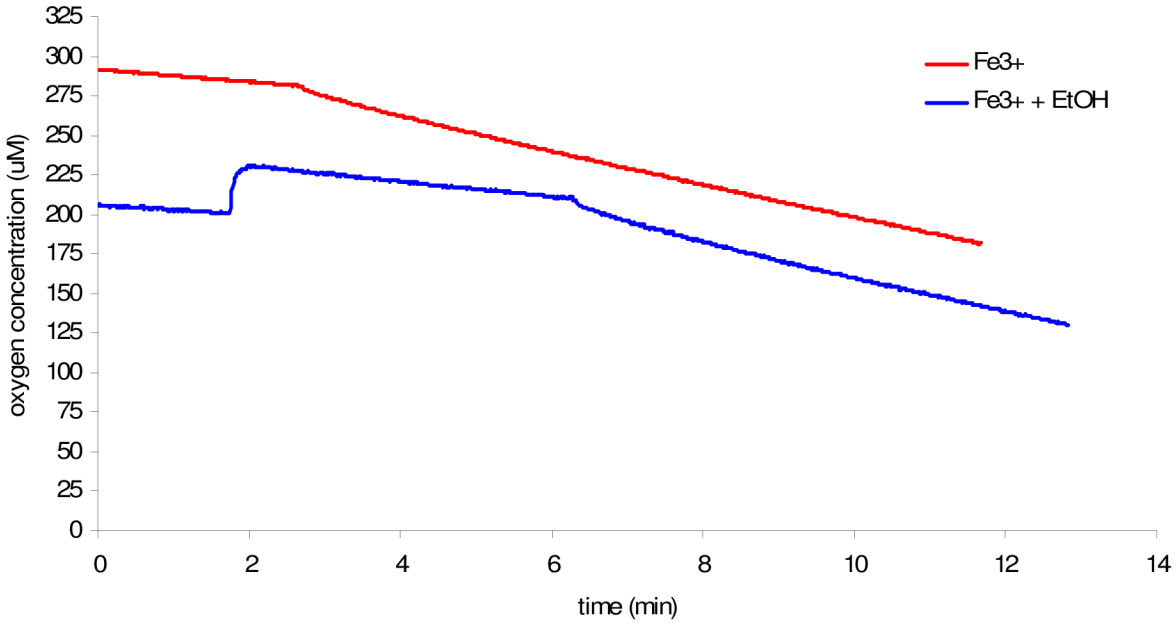


Figure 4–7 *Influence of ethanol (2 % in the reaction mixture) on OUR when Fe^{3+} (10 μM) was added as an initiator of liposome oxidation*

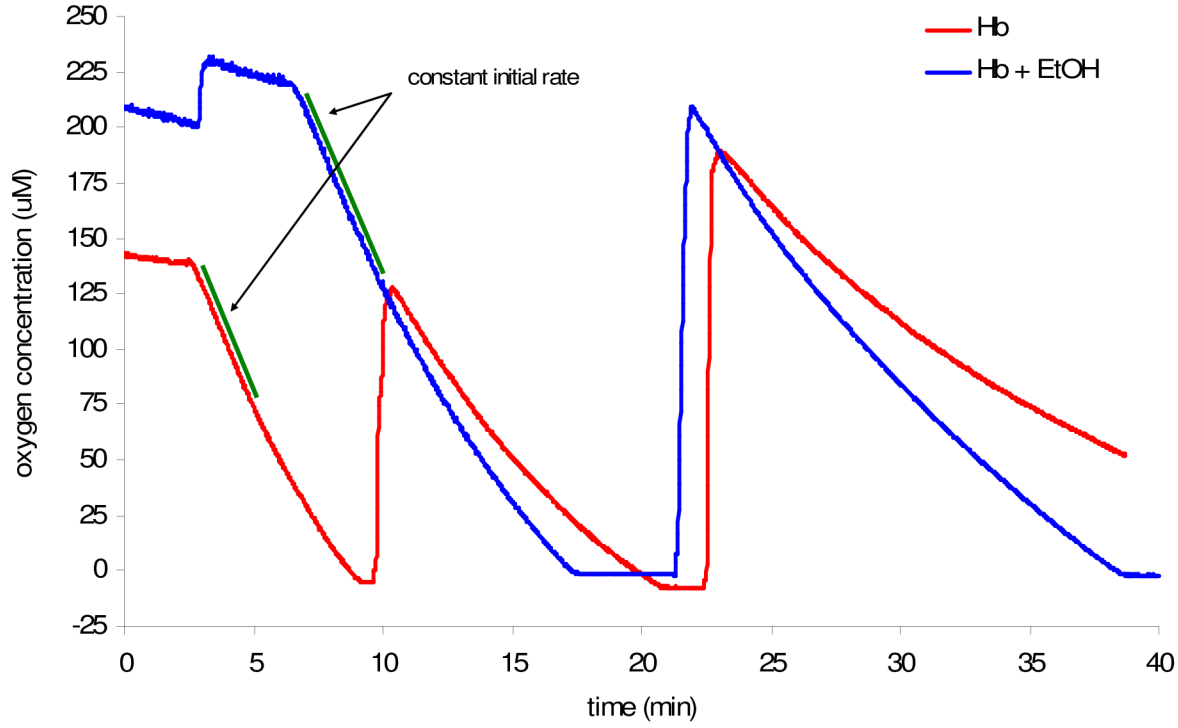


Figure 4–8 *Kinetics of oxygen consumption during the oxidation of liposomes (1,5 %, pH 5.5) initiated by Hb (1,24 μM) in the absence (red lines) and presence (blue lines) of ethanol (2 %). The green marks depict the constant initial oxygen uptake rate observed immediately after the addition of Hb into the system.*

4.3.3 Propyl gallate

Propyl gallate (PG) was the only synthetic antioxidant evaluated in this study. The tested concentration range was 1 – 200 μM. In the liposome system PG turned out to be a very effective inhibitor of oxidation, but its antioxidant activity seems to be limited by the prooxidant-to-PG ratio and also by the type of the initiator.

In oxidation promoted by hemoglobin (1,24 μM), all the concentrations of PG were efficient in inhibiting oxidation. The inhibitory effect increased with increasing concentration and showed a linear dependence above a concentration of 50 μM. The highest tested concentration (200 μM) inhibited the initial OUR by 77 %. Discussion on inhibition of Hb-induced oxidation by phenolic antioxidants is given in section 4.3.7.

In oxidation promoted by ferrous and ferric iron (10 μM), only the concentrations above 10 μM (ratio PG/Fe ≥ 1) were efficient in inhibiting oxidation. The concentration of 200 μM inhibited lipid peroxidation initiated by free iron completely (Figure 4–10). Interestingly, when 1 μM PG concentration was tested (ratio PG/Fe < 1) an increase in oxygen consumption was observed (ca. 20 %). All the obtained results are shown in Figure 4–9.

Different antioxidant properties of PG are reported in the literature: PG was efficient in inhibition of iron-ascorbate and Hb-promoted oxidation of fish microsomes [35], it was able to strongly inhibit rancidity of bulk oil, but had either no activity or strong prooxidative activity in various emulsions [34], a prooxidative effect of PG at 0,1 – 5 μM was observed in Cu-promoted (5 μM) oxidation of palmitoyllinoleoylphosphatidylcholine liposomes by Gal et al. [65]

The location of antioxidants in multi-phase systems has been found to be an important factor that influences the antioxidant activity. [30, 34, 36, 62, 88] In systems consisting of phospholipid bilayers the affinity of antioxidants toward the phospholipid bilayers is a key parameter. [35]. Nakayama et al. has found the amount of PG incorporated into liposome membranes to be 10 %. [72] A different number was reported by Pazos et al. who measured the affinity of PG for incorporation into microsomal phospholipid membranes; the incorporation percentage was found to be 52,1 %. [35]

The polarity of a molecule determines its lipophilicity or hydrophilicity. [5] Among the tested antioxidants, PG is the least polar compound. Partitioning of PG in the oil phase of emulsions is reported to be relatively high; for example, Jacobsen et al. reported that partitioning of PG in the oil phase of mayonnaise with the egg lecithin used as emulsifier was 44,9 % and 7 % in the interface (represented by phospholipids). [38]. Partitioning of PG in biphasic systems is reported to be very high: the partition coefficient of PG was found to be 0,895 for an oil-water (1:10) system [34] and 0,95 for an octanol/PBS (1:1) system. [69]

From the examples given above it could be assumed that a substantial part of PG would be located within the phospholipid bilayer while a smaller part would be retained in the water phase. When PG was added into the liposomes, a total inhibition of the initial liposome activity was observed, which can also serve as an indicator that PG was active in the phospholipid bilayer.

A number of studies reported on the chelating properties of phenolic compounds [15, 35, 46, 47] as another pathway by which antioxidants can contribute to inhibition of lipid oxidation. According to these studies, only phenolics bearing catechol or pyrogallol moiety are capable of metal chelation. PG belongs to this type of compounds. Pazos et al. found that 10 μM PG was able to chelate 56,6 % of 20 μM Fe^{2+} iron. [35] Presumably, only the fraction

of PG present in the aqueous phase would participate in the chelation. PG-to-iron ratio and pH of solution are important factors. [5]

However, to distinguish between the two mechanisms – radical scavenging and chelation – is often problematic. Therefore, an experiment with gallic acid, a precursor of PG, was performed. Gallic acid, as a polar compound, does not partition in the oil phase and remains predominantly in the aqueous phase of emulsions [34, 69]. When 100 µM of gallic acid in MES buffer (5,5 mM, pH 5.5) was tested in Fe^{2+} induced oxidation (GA : Fe = 10 : 1) no significant decrease in OUR was observed, which indicates that chelation does not occur, or only to a very small degree. Low chelating abilities of gallic acid at physiological pH (7.4) was reported when metal was used in excess. [46, 47] A relatively low gallic acid-Fe binding constant (4.78 M^{-1}) was determined at physiological pH. [46]

Due to this experiment, iron chelating ability of PG remaining in the aqueous phase probably does not contribute to the overall inhibition effect of PG, and only scavenging of free radicals takes place.

A prooxidative effect was observed at PG concentration of 1 µM (PG : Fe = 1 : 10). As described above, scavenging free radicals is presumably the main reaction mechanism. The capacity of PG to scavenge free radicals seems to be insufficient when iron is in abundance. Moreover, PG possess a strong metal reducing power as previously verified by the FRAP assay in this study and by other researchers [35]. The proportion of PG that is active as a free radical scavenger may be rapidly depleted and at the same time, the proportion remaining in the aqueous phase may reduce ferric iron which may results in an overall acceleration of lipid oxidation.

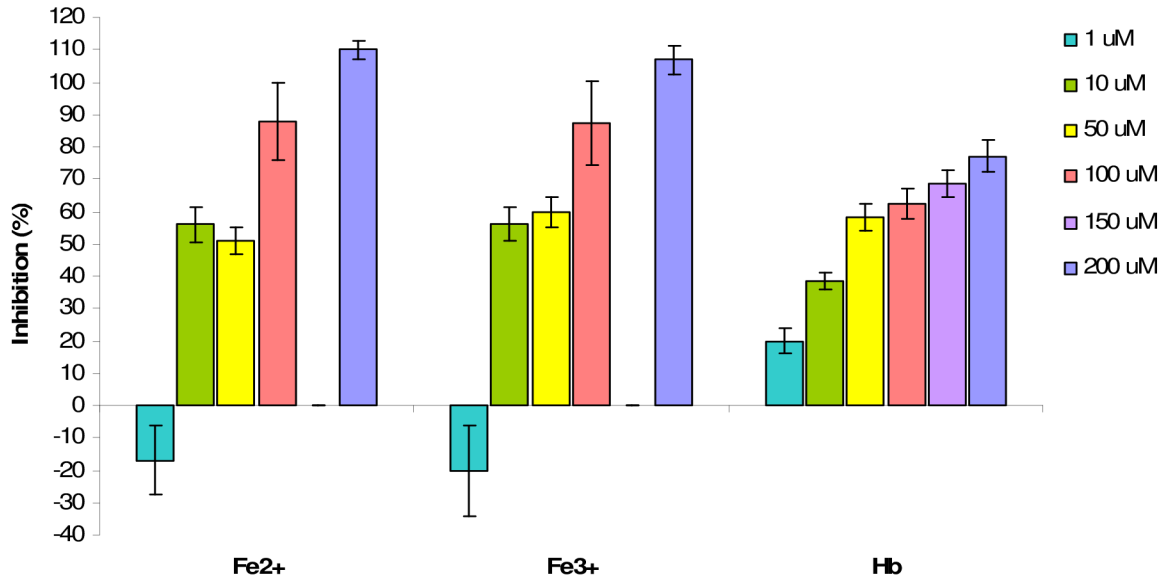


Figure 4–9 *Inhibition (%) of oxygen uptake rate of Fe^{2+} (10 µM), Fe^{3+} (10 µM) and Hb (1,24 µM) initiated oxidation of liposomes (1,5 %, pH 5.5) by different concentrations of propyl gallate. The negative inhibition values represent an increase of the oxygen uptake rate, thus a prooxidative effect.*

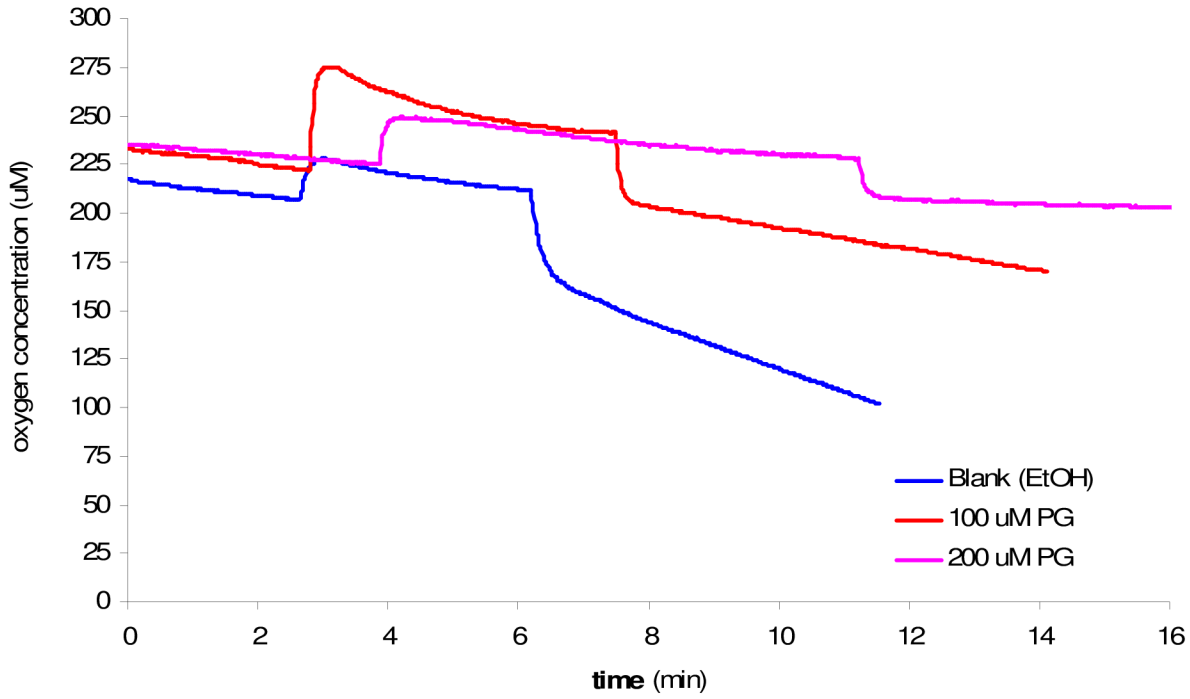


Figure 4–10 *Behaviour of propyl gallate (100 µM and 200 µM) in Fe^{2+}-induced oxidation of liposomes (1,5 %, pH 5.5). 200 µM concentration of propyl gallate totally suppressed the oxidative process.*

4.3.4 Caffeic acid

The effectivity of CaA in the liposome system was not the same when different prooxidants were applied (Fe and Hb) and also varied with the CaA-to-prooxidant ratio. The tested concentration range was 0,1 – 1000 µM. CaA was efficient in inhibiting Hb-induced oxidation at all levels of addition except for 0,1 µM, while in the free iron induced oxidation, a prooxidative behaviour was observed.

In the latter, concentrations above 50 µM promoted the rate of oxidation more strongly (concentration of 50 µM increased the OUR more than 10 times) than concentration of 1 µM; 0,1 µM CaA did not have any significant effect on the rate of oxidation. This phenomenon is discussed later. The quantification (measuring OURs) of these "rapid" reactions was rather problematic due to fast oxygen consumption as shown in Figure 4–14. Especially in Fe^{2+}-induced oxidation the initial drop was often difficult to distinguish from the slower OUR that follows after the drop.

It should be noted that such strong prooxidative behaviour was not observed with any of the other tested phenolic compounds. The results of Fe- and Hb-induced oxidation are shown in Figure 4–11 and Figure 4–15, respectively.

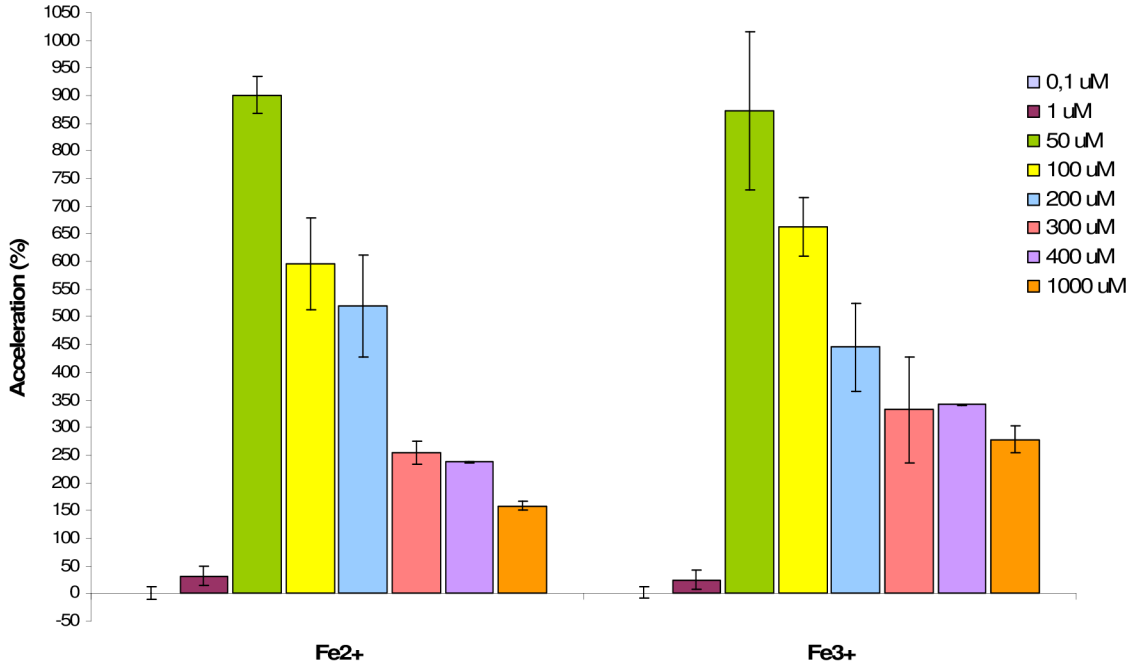


Figure 4–11 *Prooxidant effects of caffeic acid – acceleration (%) of oxygen uptake rate of Fe^{2+} (10 μM)- and Fe^{3+}(10 μM)-initiated oxidation of liposomes (1,5 %, pH 5.5) by different concentrations of caffeic acid. The values are means ± SD of two to seven parallel experiments.*

The ability of CaA to retard lipid oxidation has been examined in a number of studies. In these studies, various lipid model systems and methods for screening the degree of lipid oxidation were used and the behaviour of CaA varied from a potent antioxidant to a strong prooxidant. For example, CaA strongly inhibited the formation of hydroperoxides in bulk methyl linoleate after 7 day oxidation, but slightly promoted or did not show any inhibiting effect in methyl linoleate emulsions after 4 days of oxidation; in both cases, the effectiveness of CaA was system- and concentration-dependent. [36] CaA showed a very low activity as a protector of linoleic acid in aqueous micelles of sodium dodecyl sulfate (SDS) [31], but on the other hand delayed the induction period (autoxidation) of lard as well as of LDL oxidized with copper ion. [37]. In another study, CaA acted as an anti- or a prooxidant, depending on a level of addition, in copper induced oxidation of phosphatidylcholine liposomes and acted as a prooxidant in a 10 % O/W emulsion [16]. The examples given above show that the type of system is an important parameter that determines the effectiveness of CaA to act as an antioxidant, and the "polar paradox" (see section 2.2.3 for explanation) seems to be fully applicable in the case of CaA. Other important parameters determining the action of CaA that can be deduced from the examples are the presence of metals as prooxidants, and the amount of CaA in the system.

To explain the prooxidative behaviour of CaA in our system, several factors must be considered. First, the distribution of CaA between the water phase and the phospholipid bilayer (interface) of liposomes. CaA, as a polar compound, is expected to be present predominantly in the aqueous phase and when considering biphasic systems, partitioning of CaA in the oil phase was reported to be generally very poor [31, 36]; for example, its partitioning in W/O emulsion (9 : 1) was only 1,2 % in the oil phase. [36] Several studies also attempted to estimate the proportion of antioxidants distributed in the different phases of

emulsified systems. [38, 39] In the study by Jacobsen et al. [38], CaA partitioned in mayonnaise fortified with egg lecithin by 19,0 % in the oil phase and by 5,7 % in the interface (the data were achieved by application of various phase separation techniques). [38] Pekarinnen et al. [36] found a high proportion of CaA (48,8 %) dissolved in micelles of surfactant Tween 20. It should be noted that Tween 20 is, compared to phospholipids, a non-charged and differently structured emulsifier, thus similarity with our system could be questioned.

The assumption therefore is that the proportion of CaA retained in the oil phase and more importantly in the interface of emulsions, as the phase closely resembling a phospholipid bilayers in liposomes, would be low.

It should be mentioned here that all the studied antioxidants were added to the liposome solution after preparation of liposomes. This may lead to even lower distribution of the compounds within the phospholipid bilayer. A different distribution profile could be achieved by addition of the compounds to phospholipids prior to formation of liposomes by sonication.

The charge of the liposome surface is another important factor that must be taken into account. Phospholipids are polar compounds [5]. According to Mozuraityte et al. the zeta potential of cod roe phospholipid liposomes at pH 5.5 is negative (~ –20 mV). [56] Although the zeta potential is not a direct measure of surface charge, it can provide tentative information on the net charge of the particles. [56] At pH 5.5 CaA acid is present largely as anion (pK_{a1} = 4.36 [50]) owing to the ionized acidic group. Due to this, the accessibility of negatively charged molecules of CaA to the inside of the liposome bilayer may be lowered since they might be repulsed into the aqueous phase by the negatively charged outer surface of liposomes.

CaA consists of a catechol moiety – two OH groups attached to the benzene ring in *ortho* position (see Figure 2–11). Such substances are under the right conditions (pH, CaA/Fe ratio, type of buffer in the solution) capable of forming complexes with transition metals [43, 45, 46, 47, 48], which inactivates the redox cycling of prooxidant metals. [5] We observed a strong prooxidative behaviour of CaA at the ratios CaA/Fe > 5 (excess of CaA) and significantly lower prooxidative effect at the ratio CaA/Fe ≤ 0,1 (excess of Fe). This indicates that chelating (forming stable metal complexes) probably does not occur. A weak complex formation between Fe^{2+} and CaA at acidic pH and excess of CaA in aqueous solutions has been reported. [45, 48]

Yet, the strong prooxidative effect observed may be attributed to the presence of catechol moiety [43, 49] when reducing properties of caffeic acid are taken into consideration. The ability of CaA to reduce Fe^{3+} was previously verified by the FRAP assay (see section 4.2.2) and is also well documented in the literature. [15, 28, 32] The oxidative potential of CaA was reported to be relatively low, 0,212 V (*vs* Ag/AgCl). [43]

As shown in the work of Zheng et al. [49], CaA is capable of reducing cupric ions (Cu^{2+}) into cuprous ions (Cu^{+}). The so called *intramolecular electron transfer* (IET) was proposed to be the mechanism leading to the reduction of copper. The same mechanism was reported to be predominantly involved also in reduction of ferrous ions (Fe^{3+}) by CaA as examined by Hynes et al. [45] The process is based on formation of temporary monodentate complexes between CaA and Fe^{3+} (32), which subsequently decays by means of intramolecular electron transfer releasing Fe^{2+}.

$$Fe^{3+} + H_2L \Leftrightarrow Fe(LH)^{2+} + H^{+} \qquad (32)$$

It was reported that fully protonated catecholate ligands are the reactant species involved. [45] The pK_{a2} value of CaA is 8,48 [60]. Despite this, in the presence of metal ions, the proton (H^+) is dissociated at much lower pH values, e.g. 2.0 – 8.0. [45, 49] Interactions of protonated phenolic ligands can also be viewed as metal catalyzed deprotonation reactions. [45]

Therefore, CaA can dissociate to form an *ortho*-hydroxyphenoxide anion capable of binding Fe^{3+} into a temporary monodentate complex. This initial complex can rapidly undergo intramolecular electron transfer to form *ortho*-hydroxyphenoxyl radical. The acidity dissociation constant of this radical is much lower (pK_{a3} = 4.1) than that of CaA (pK_{a3} = 11.17 [50]). Thus, *ortho*-hydroxyphenoxyl radical dissociates to form *ortho*-semiquinone anion binding Fe^{2+}. The *ortho*-semiquinone radical anion is further oxidized by another Fe^{3+} to yield the final products, *ortho*-quinone or possibly some products of dimerization reactions [43] releasing reduced iron [49] that can further circle and enhance lipid oxidation. [5] The process is depicted in Figure 4–12.

Hotta et al. reported that polymerization of *ortho*-semiquinone radicals releases additional electrons and in some polymeric products oxidizable catechol moieties are reproduced (Figure 4–13). [50] The released electrons may contribute to reduction of ferrous iron as well as the newly restored catechol moieties by further polymerization.

Moreover, superoxide anion ($O_2^{\bullet -}$) is generated from triplet oxygen during the process that can reduce ferric iron ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$) or form hydrogen peroxide that can participate in Fenton type reactions. [9]

The oxygen consumption after the addition of iron ions into the liposomes with added CaA was not constant – the fastest consumption of oxygen was observed immediately after the addition of prooxidants, thereafter it slowed down non-linearly. This observation indicates that during the prooxidative process the concentration of CaA in the reaction mixture changes – it is depleted by conversion of CaA into different products as described above. [43, 49] To quantify the prooxidative effect, the OUR of the first 2 minutes after the addition of prooxidants was measured.

The rapid formation and subsequent decomposition of CaA-Fe monodentate complexes due to the intramolecular electron transfer, which facilitates the reduction of ferric iron, is characteristic for systems where CaA is present in a large excess relative to iron. [45] In the systems with a ratio CaA/Fe ≤ 1 (excess of iron), the reaction pathways of CaA-Fe complex formation and decomposition may differ [45]. A total breakdown of CaA rather than formation of quinones and subsequent dimers was reported to follow after the monodendate complex formation when iron was used in great abundance. [45] This could explain the markedly lower prooxidative activity at 1 μM CaA concentration and possibly no effect at 0,1 μM CaA concentration.

Interestingly, the acceleration of Fe-induced oxidation was significantly lowered also at 1000 μM CaA concentration. This could be attributed to the radical scavenging abilities of the proportion of CaA that was located in the interface. A switch between a pro- and antioxidant behaviour is possible when the antioxidant is added in a huge excess (1000 times) relative to prooxidant; ascorbic acid is a typical example of such behaviour. [6]

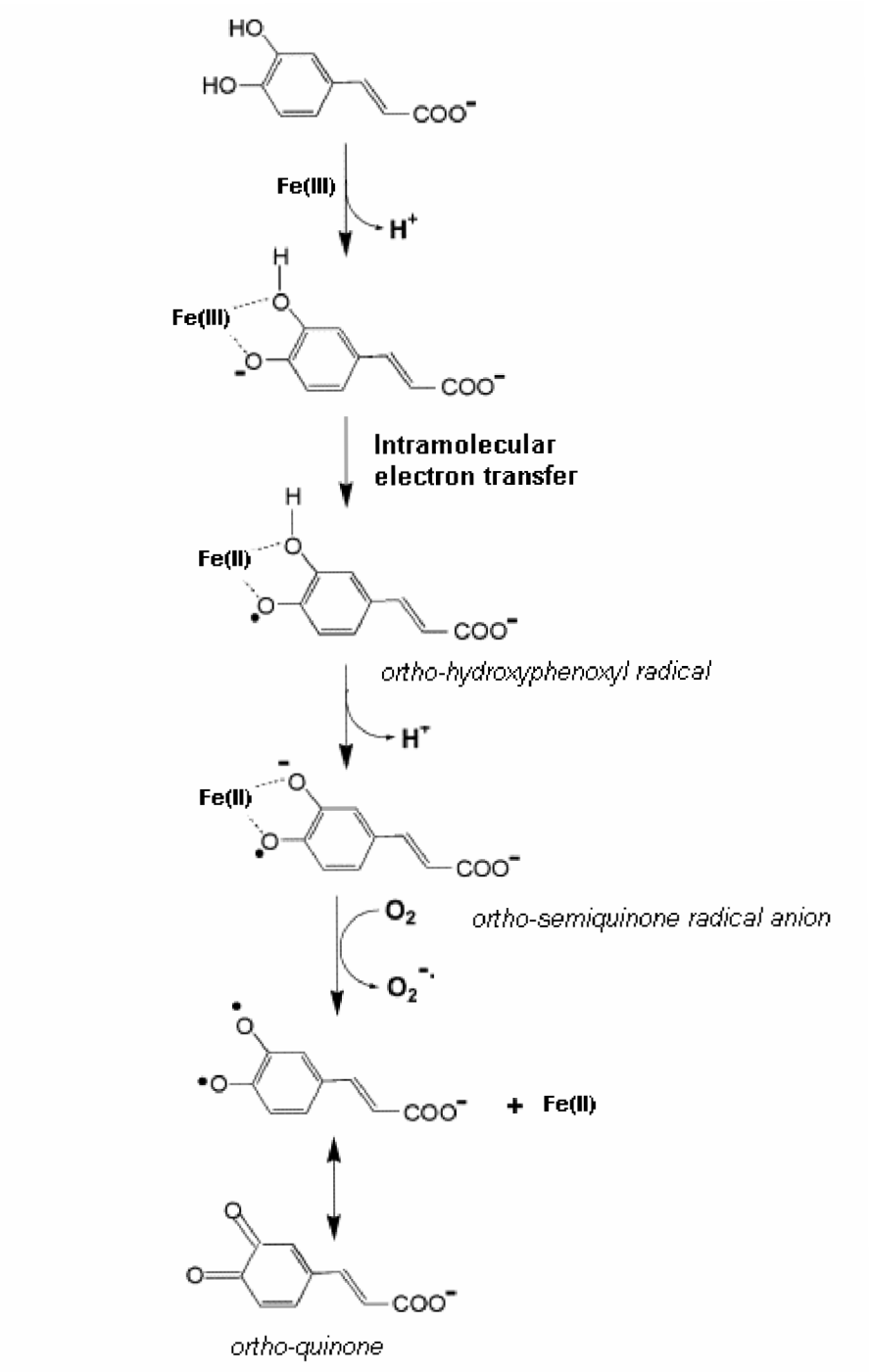


Figure 4–12 *Prooxidative mechanism of caffeic acid (adapted from* [49]*)*

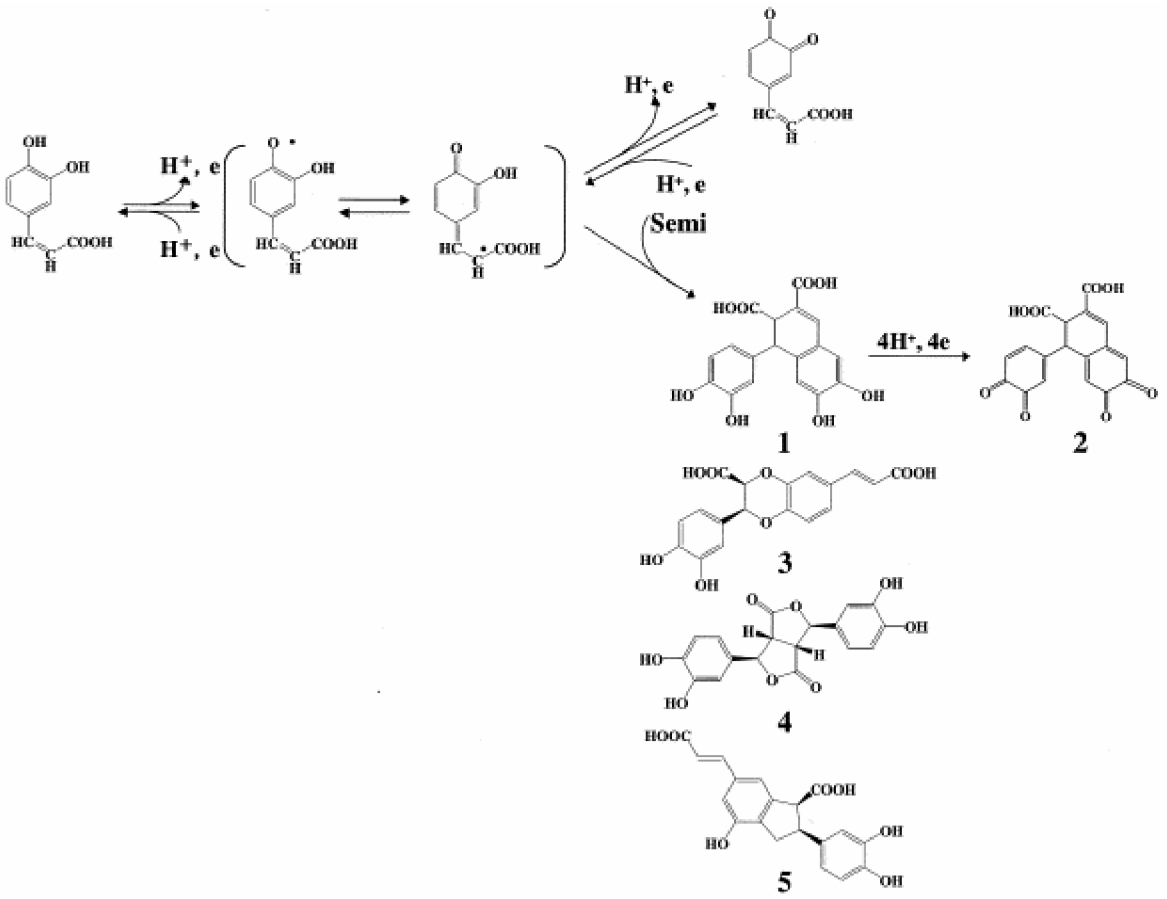


Figure 4–13 *Possible mechanisms and products of the oxidative dimerization of caffeic acid* [50]

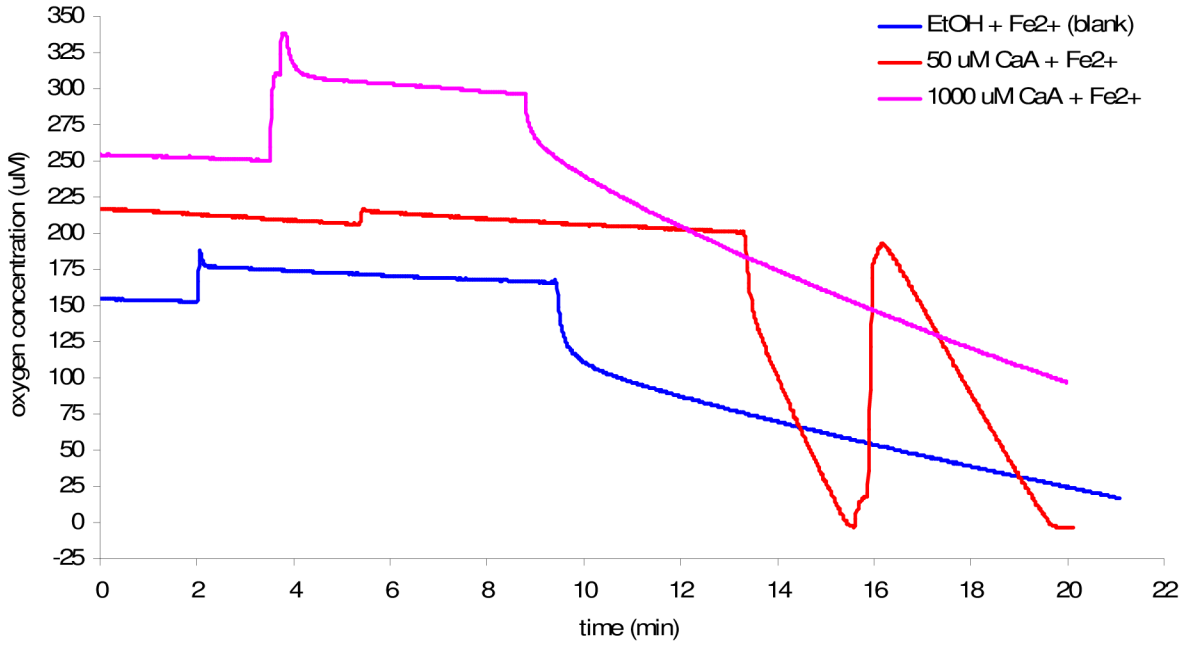


Figure 4–14 *Prooxidative effect of caffeic acid (50 and 1000 μM) in Fe^{2+}-induced oxidation – rapid acceleration of OUR after addition of Fe^{2+}(10 μM).*

Contrary to Fe-induced oxidation of liposomes, in Hb-induced oxidation CaA inhibited the rate of oxidation at concentrations ranging from 1 to 1000 µM. The inhibition ranged from 30 to 57 % at different concentrations as shown in Figure 4–15. No distinct dependence was observed between the degree of inhibition and the level of addition. However, higher concentrations of CaA exhibited better antioxidant effects than the lower concentrations.

Inhibition of Hb-induced oxidation by phenolic compounds is discussed in section 4.3.7. The kinetics of oxygen consumption in Hb-promoted oxidation in the presence of CaA (50 µM) is shown in Figure 4–16. Exponentially decreasing concentration of dissolved oxygen after addition of Hb was observed and verified by performing linearization of the kinetic curve ($\ln(C_{O2}) = f(t)$) (Figure 4–16). Kinetic characterization of catalyzed oxidation inhibited by antioxidants is a matter that still needs to be elucidated.

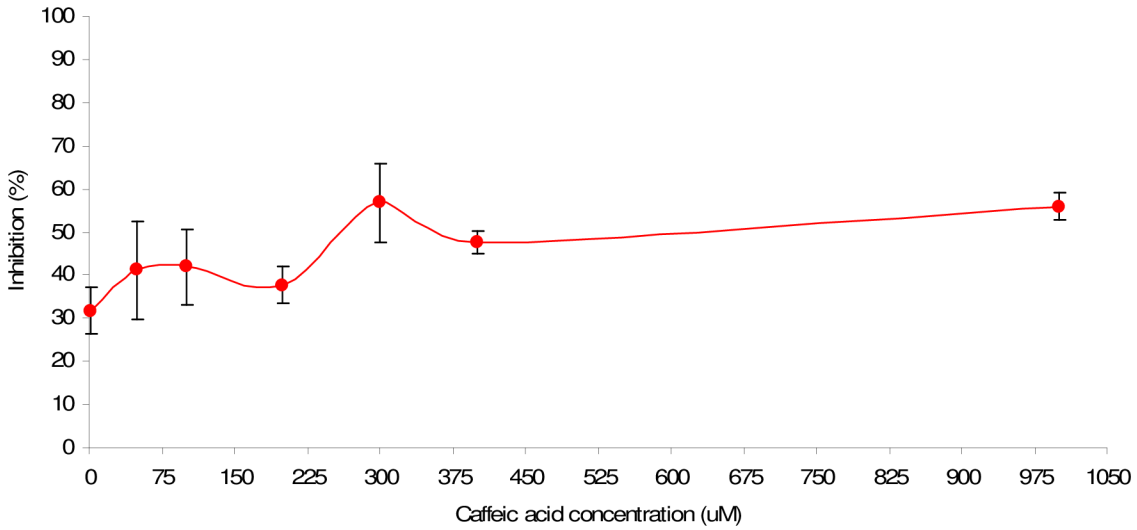


Figure 4–15 *Inhibition (%) of Hb-initiated oxidation of liposomes (1,5 %, pH 5.5) by different concentrations of caffeic acid. The values are means ± SD of two to five parallel experiments.*

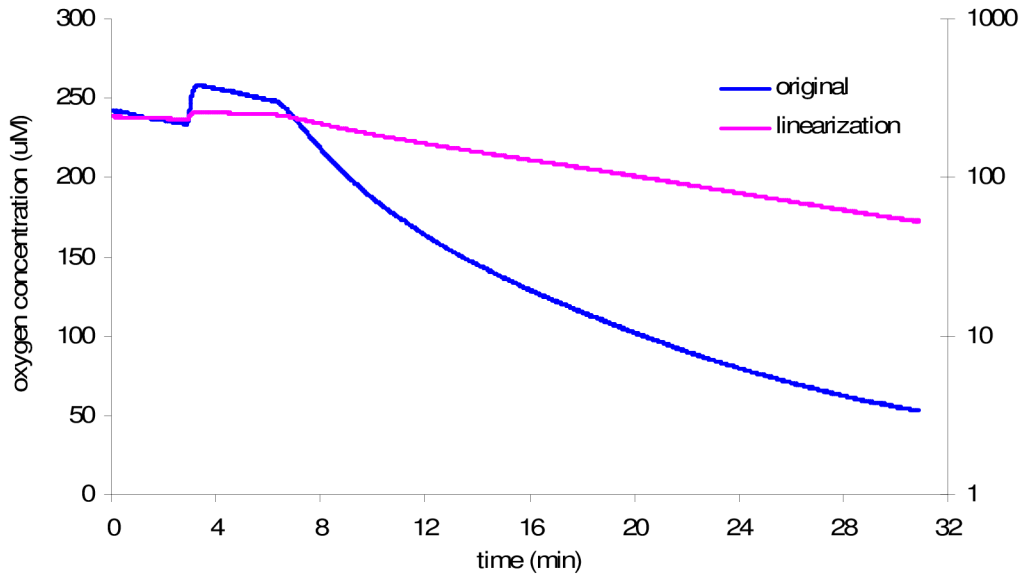


Figure 4–16 *Kinetics of oxygen consumption of Hb (1,24 µM)-induced oxidation of liposomes in the presence of caffeic acid (50 µM) (blue line; y-axis on the left side of the graph) and linearization of the kinetic curve* ($\ln C_{O2} = f(\text{time})$) *(pink line; y-axis on the right side of the graph).*

4.3.5 Ferulic acid

Ferulic acid (FeA) was tested in the concentration range 50 – 400 µM, thus only in abundance relative to promoters of oxidation. At these levels of addition, FeA was capable of inhibiting both free iron and Hb promoted oxidation of liposomes. The results are shown in Figure 4–17. However, in free iron induced oxidation, the degree of inhibition did not markedly increase with increasing concentration as was observed previously with propyl gallate (section 4.3.3), but on the other hand, no prooxidative behaviour was observed as in the case of caffeic acid (section 4.3.4) and ascorbic acid (section 4.3.8). Also in Hb-induced oxidation the degree of inhibition did not markedly increase with increasing concentration and the inhibitory effect was approximately equal to that of caffeic acid. FeA turned out to be a better antioxidant than *p*-coumaric acid (section 4.3.6). Among the tested compounds, FeA cold be characterized as a less potent antioxidant.

At the highest level of addition (400 µM), the degree of inhibition of oxidation promoted by Hb (1,24 µM) reached 47,0 ± 2,9 %, and of oxidation promoted by ferrous and ferric iron (10 µM) 35,7 ± 2,9 % and 32,7 ± 4,6 %, respectively.

Similarly to caffeic acid, the OUR was not constant after addition of free iron to the liposomes containing FeA. The initial (fastest) OUR was therefore measured and used to quantify the inhibitory effect of FeA. When the kinetic curve of oxygen consumption after adding iron ($C_{O2} = f(t)$) was linearized by calculating the dependence $\ln(C_{O2}) = f(t)$, a linear dependence was obtained. This revealed an exponential decrease in oxygen consumption in the presence of FeA (Figure 4–18).

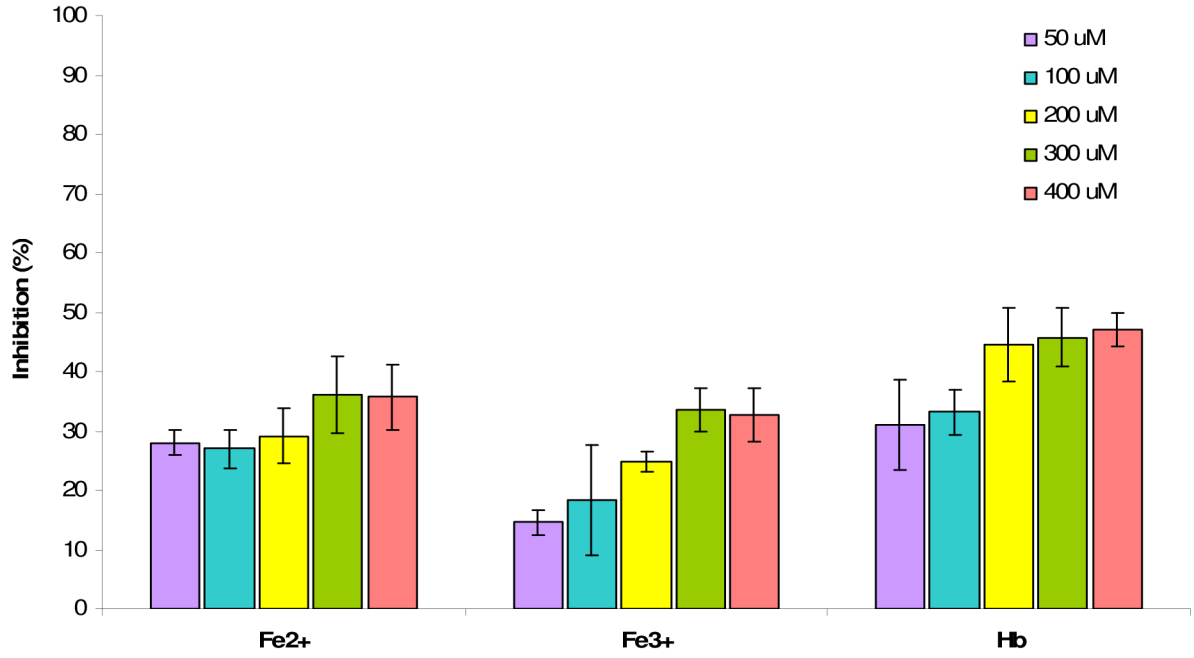


Figure 4–17 *Inhibition (%) of oxygen uptake rate of Fe^{2+} (10 µM), Fe^{3+} (10 µM) and Hb (1,24 µM) initiated oxidation of liposomes (1,5 %, pH 5.5) by different concentrations of ferulic acid. The values are the means ± SD of two to seven parallel experiments.*

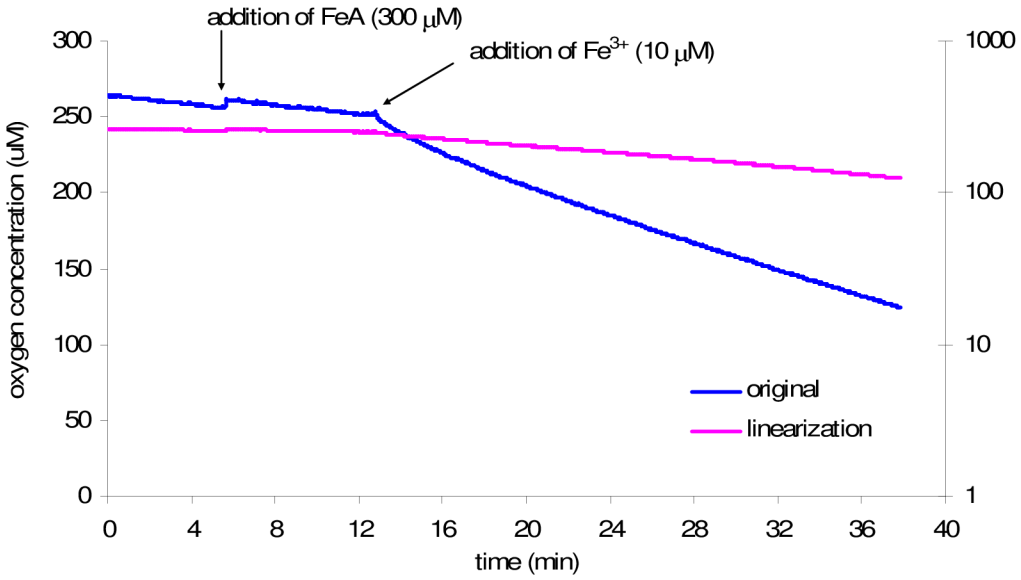


Figure 4–18 *Kinetics of oxygen consumption during the oxidation of liposomes (1,5 %, pH 5.5) induced by Fe^{3+} (10 μM) in the presence of ferulic acid (300 μM) (blue line; y-axis on the left side of the graph) and linearization of the kinetic curve (pink line; y-axis on the right side of the graph).*

FeA is well soluble in organic solvents, such as methanol or ethanol, and insoluble in aqueous solutions; needle-like crystals formed in the aqueous solution consisting of FeA (10 mM) and MES buffer (5 mM, pH 5.5) after 2 days of storage in dark and at low temperature.

A small number of studies on the antioxidant activity of FeA in different lipid systems has been published. For example, a low protective action of FeA toward linoleic acid in a SDS micellar system was reported [31]; on the contrary, FeA strongly inhibited lipid peroxidation in microsomal membranes and intact cells (fibroplasts) induced by *tert*-BOOH and AAPH. [68] FeA was also an effective antioxidant in bulk methyl linoleate, ethanol-buffered solution of linoleic acid and liposomes with oxidation induced by AAPH. [69]

The overall lower antioxidant effect than the one observed with propyl gallate could be attributed to several factors: Firstly, to the location of FeA in the liposome system. Partitioning of polar compounds in the oil phase of both biphasic systems and emulsions has been reported to be generally low. [31, 36, 38] Trombino et al. measured the affinity of FeA for incorporation into microsomal membranes and reported that the percentage of FeA associated with the membranes was about 5 %, while the remaining percentage was found in the aqueous phase. [68]

Similarly to CaA, at pH 5.5 FeA bears a negative charge due to the ionized acidic group (pKa ~ 4). The accessibility of FeA molecules to the phospholipid bilayer might be more difficult due to repulsion of FeA molecules by negatively charged outer surface of liposomes. Thus, a larger proportion of FeA is likely to be located outside the phospholipid bilayer whereas a minor part is likely to be found inside the phospholipid interface, which decreases its activity as a radical scavenger.

It is commonly accepted that the antioxidant activity of phenolic acids and their esters depends on the number of hydroxy groups in the molecule and other electron donating or withdrawing substituents. [3] FeA possesses only one hydroxy group and one methoxy group (see Figure 2–11), which makes FeA a weaker antioxidant compared to more substituted

molecules, such as CaA or PG, but a stronger antioxidant than *p*-coumaric acid bearing only a single OH group.

The capability of compounds bearing 3-methoxy-4-hydroxy groups on the benzene ring to interact with transition metal ions – either to reduce/oxidize them or chelate – was reported in several studies. [45, 46, 47, 49] All these studies agreed on very weak or no chelating abilities of such compounds. A relatively high redox potential of FeA (0,430 V *vs* Ag/AgCl) [43] does not favor redox reactions with free metals.

So called interfacial phenomena (or polar paradox) is most often attributed to low activities of polar antioxidants in emulsified systems. [33, 34] Another theory explaining the low activity of phenolics in emulsions was proposed by Pekkarinen et al. According to this study, some specific interactions, such as hydrogen bonding, of antioxidant with emulsifiers can have a marked effect on the activity of individual phenolics in emulsions. Hydrogen bonds between the ether oxygen of an emulsifier and OH groups of an antioxidant may enhance the solubility of the antioxidant in the interface, but at the same time these relatively strong hydrogen bonds may weaken the ability of the antioxidant to donate hydrogen. The donation of hydrogen depends among other factors on the strength of the hydrogen bonds between the antioxidant and the solvent. The latter is represented by the phospholipids in our case. Phenolic acids in W/O emulsion fortified with emulsifier Tween 20 showed a high proportion in the emulsifier interface (micelles of Tween 20) but a low antioxidant activity in the emulsion. It has therefore been concluded that the proportion of the antioxidant solubilized in the lipid phase and in the interface does not necessarily mirror the antioxidant activity of the compound. [36] Also some other works do not exclude such interactions. [30] However, it should be noted that Tween 20 has much more ether groups compared to phospholipids and does not have a charged head that could influence the antioxidants by repulsing or attracting them. Thus, in our case, this aspect might not be so relevant.

4.3.6 *p*-Coumaric acid

In the liposome system, CoA turned out to be the least active compound of all the tested compounds. Over the tested concentration range (50 – 300 μM) CoA did not exhibit any significant anti- nor prooxidative effect in both Fe- and Hb-induced oxidation of liposomes (Figure 4–19).

However, this finding is not surprising when properties, such as molecule structure, polarity, and reduction potential of CoA are considered. Analogical features concerning polarity and location of CoA in the liposome system can be made as in the case of caffeic and ferulic acids (see section 4.3.4 and section 4.3.5, respectively). As was the case of ferulic acid, CoA (10 mM) was insoluble in 5 mM MES buffer – flake-like clumps of precipitated particles appeared in the aqueous solution.

Absence of substituents, such as methoxy group, on the phenolic ring ranks CoA among the less potent radical scavengers. [3] Chelating properties of CoA acid are fully excluded since the compound does not possess any site that could bind metals.

The use of CoA as a protector of different lipid systems has been rather scarce. For example, CoA did not exhibit any activity as a protector of linoleic acid in aqueous micelles of SDS. [31]

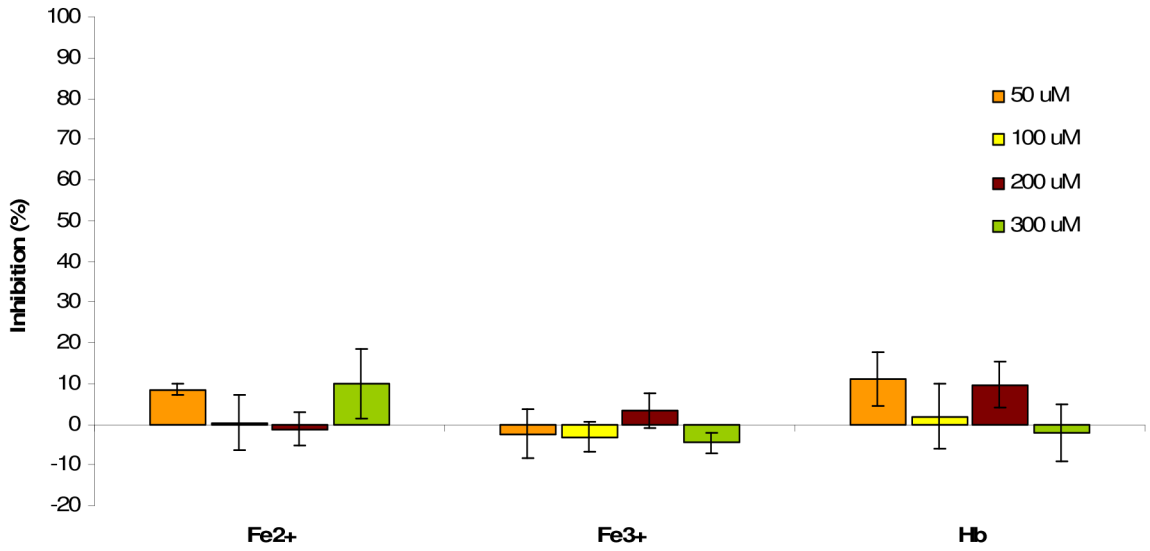


Figure 4–19 *Inhibition (%) of Fe^{2+} (10 μM), Fe^{3+} (10 μM) and Hb (1,24 μM) induced oxidation of liposomes (1,5 %, pH 5.5) by different concentrations of p-coumaric acid. The values are the means ± SD of two to five parallel experiments.*

4.3.7 Hb-induced oxidation inhibited by phenolic antioxidants

Except for *p*-coumaric acid, all the tested phenolics were efficient in inhibiting Hb-induced oxidation of liposomes. The relative order of antioxidative activity of the phenolics tested at the same molar concentration (200 μM) was:

Propyl gallate > Caffeic acid ~ Ferulic acid >> *p*-Coumaric acid

The inhibitory effect of phenolic compounds on lipid oxidation promoted by Hb has not so far been fully clarified and different explanations can be found in literature. It is presumed that the inhibitory effect of phenolics is a consequence of scavenging lipid free radicals and interactions of phenolics with Hb. [36, 51]

As shown in Figure 2–6, Hb-promoted oxidation is a complex process involving at least two different mechanisms and several different states of Hb [63]. Hemoproteins (Hb, Mb) containing oxoferyll complex (Fe^{4+}=O) are considered to be the main driving force of heme-iron-induced oxidation [63]. Moreover, heme pigments are capable of generating singlet oxygen that reacts directly with the double bonds of fatty acids causing induction of lipid oxidation. [7, 9]

Phenolic compounds are capable of noncovalent binding to various proteins, including Hb. [66] Carlsen et al. reported that binding of chlorogenate (a derivative of chlorogenic acid) to the highly reactive ferrylmyoglobin (Mb^{4+}=O) lead to the reduction of the oxoferryl moiety to ferric iron (Fe^{3+}), thus to the less reactive metmyoglobin [67]. Laranjinha et al. studied metmyoglobin/H_2O_2 dependent oxidation of LDL and also reported reduction of ferrylmyoglobin to metmyoglobin by phenolic acids. In the same study, the efficiency of phenolics was dependent on the chemical nature of substituents on the phenolic ring – the most effective compounds were catechol derivatives of cinnamic acid, namely chlorogenic, caffeic and protocatechuic acids – and the efficiency of these compounds also increased with increasing concentration. [51] Similarly, binding of phenolics to ferrylhemoglobin (Hb^{4+}=O)

and subsequent reduction of the oxoferryl moiety could explain the inhibition of Hb-induced oxidation observed in the liposome system. It should be said that compared to myoglobin the chemical structure of hemoglobin is more complicated (4 subunits) and also the interactions of phenolics with Hb can slightly differ.

Pazos et al. reported that the mechanism of phenolics for inhibiting lipid oxidation promoted by Hb does not seem to be related to a direct effect of phenolics on Hb autoxidation (Hb-Fe^{2+} ⇔ Hb-Fe^{3+}). However, they did not strictly exclude this possibility if the experiments were performed under different reaction conditions. Unfortunately, alternative conditions were not suggested. [35]

The relative effectivity of phenolic compounds in our study correlates with the proposed theory of inhibiting Hb-induced oxidation by reduction of ferrylhemoglobin. Phenolics with a higher reduction potential (which is ruled by composition of substituents on a benzene ring – by an electron donating and withdrawing groups) inhibited the rate of oxidation more strongly that those with a lower reduction potential. The interactions of phenolics with Hb itself seem to be more important in our liposome system than the radical scavenging abilities for, apart from propyl gallate, all the studied compounds are likely to be predominantly located in aqueous phase of emulsions, which is also the location site of Hb.

Propyl gallate turned out to be the most effective compound in protection against Hb-promoted lipid oxidation. Low polarity of PG and relatively high affinity for incorporation into phospholipid membranes [35] leads to its distribution very near or inside the phospholipid bilayer of liposomes. Here, it probably functions both as an efficient scavenger of free lipid radicals and as a powerful reductant of ferryl-Hb, which results in the overall strong inhibiting effect. This may also explain the increasing inhibitory effect observed with increasing concentration of PG. Chelating as one of the antioxidant protective mechanisms was excluded in free iron induced oxidation and it is unlikely also in Hb induced oxidation because the iron is bound to the hem structure of Hb.

As explained in section 4.3.4, the ability of caffeic acid to donate an electron to ferric iron (Fe^{3+}) and reduce it to the more prooxidative ferric (Fe^{2+}) state, caused acceleration of liposome oxidation. In Hb-induced oxidation the same reducing abilities probably brought out exactly the opposite effect, inhibition of liposome oxidation.

This shows that when evaluating antioxidant effects of different compounds the presence and the type of a promoter (e.g. metals, metal-containing proteins, or synthetic stable free radicals) of lipid oxidation must also be taken into account as factors influencing the action of antioxidants. Scavenging free radicals by CaA may also contribute to the overall inhibitory effect. However, low partitioning of CaA in the phospholipid interface is expected (see section 4.3.4 for explanation), thus such contribution may be low.

The inhibitory effect on Hb promoted oxidation of ferulic acid was very similar to that of caffeic acid although reduction potential of FeA has been reported to be one half of that of caffeic acid. [43] This implies that the ability of phenolics to reduce ferryl iron of heme is not the only factor that rules the inhibitory effect of phenolics. The accessibility of antioxidant molecules to the heme group or strength of noncovalent bonds between proteins and phenolics may also play an important role. However, a high dependence between the structure of phenolic acids (the nature of substituents on aromatic ring) and the ability to reduce oxoferryl moiety to the ferric form in myoglobin has been reported. [52]

p-Coumaric was completely inactive towards Hb-induced oxidation at all tested concentrations as well as being inactive towards free iron induced oxidation. Standard reduction potential of CoA has been reported to be relatively high, 0,583 V (*vs* Ag/AgCl). [43] Weak reducing abilities toward iron were also previously verified by the FRAP assay (section 4.2.2). When this feature is considered along with the low capability of CoA to scavenge free radicals (section 4.2.3, section 4.3.6), the inability of CoA to reduce the oxoferryl moiety and thus to inhibit Hb-induced oxidation is not surprising.

4.3.8 Ascorbic acid

L(+)-Ascorbic acid (AsA), Vitamin C, was the only non-phenolic and totally water soluble compound [6] tested in our study. AsA belongs to important components of living systems and is a natural component of a wide array of foods and food products including fish meat, so it was attractive to test its properties also in the liposome model system.

Unlike the phenolic compounds, AsA itself promoted the oxidation of liposomes. When AsA was added to the liposomes, an increase in background OUR was observed. This increase was proportional to the amount of AsA added, except for a concentration of 1 μM. At this concentration, no significant change in the background OUR was observed. Up to the concentration of 100 μM, the increase in the background OUR was constant (linear), at higher concentrations non-linear increase in consumption of dissolved oxygen was observed. Kinetic curves of oxygen consumption by liposomes after addition of different concentrations of AsA can be seen in Figure 4–20, Figure 4–21 and Figure 4–23.

It is likely that the oxidation of liposomes promoted by AsA continues, at least partially, also after the addition of prooxidants (Fe or Hb) into the liposomes. Moreover, AsA also interacts with the added prooxidants. The overall rate of oxidation therefore characterizes the sum of all reactions simultaneously occurring in the reaction mixture. When evaluating the effect of AsA on free iron- or Hb-promoted oxidation of liposomes, the prooxidative effect of AsA itself cannot be anyhow excluded and is included in the overall effect. Thus, the rate of oxidation was found by subtracting the overall OUR from the background OUR that was measured before addition of AsA, not after the addition of AsA, as it was done with all the phenolic compounds.

The concentrations of AsA tested in free iron induced oxidation were 30 μM and 50 μM (molar ratio AsA/Fe = 3:1 and 5:1, respectively). At these concentrations AsA behaved as a strong prooxidant after the addition of iron ions. Quantification (measuring the OUR) of Fe^{2+}- and Fe^{3+}-induced oxidation was rather problematic. Consumption of dissolved oxygen by liposomes was very rapid immediately after the addition of iron and then decreased non-linearly as amount of AsA was depleted due to its oxidation. Moreover, the initial drop in dissolved oxygen that is characteristic for Fe^{2+}-induced oxidation was difficult to distinguish from the slower oxygen consumption rate that usually follows after the trop. Thus, to demonstrate the prooxidative effect of AsA, direct comparison of the kitetic curves with AsA with the kinetics curves of the respective blanks was done, see Figure 4–20 for Fe^{2+}- and Figure 4–21 for Fe^{3+}-induced oxidation.

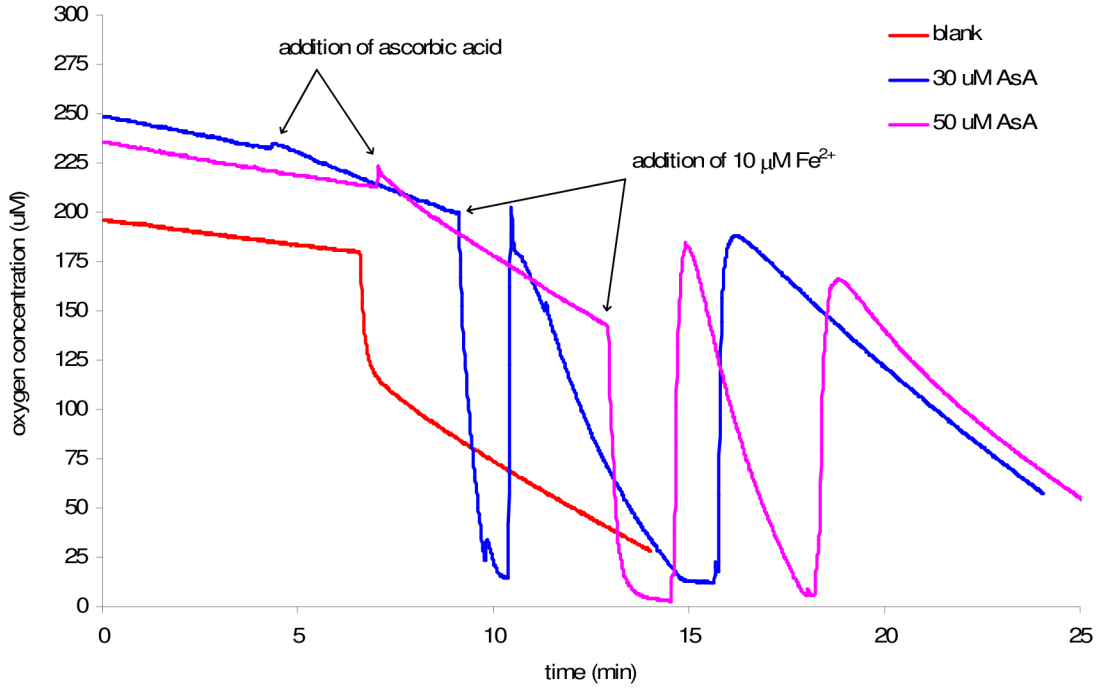


Figure 4–20 *Kinetics of oxygen consumption during the oxidation of liposomes (1,5 %, pH 5.5) initiated by Fe^{2+} (10 μM) in the presence of 30 μM and 50 μM ascorbic acid. An increase in background OUR was observed after addition of ascorbic acid.*

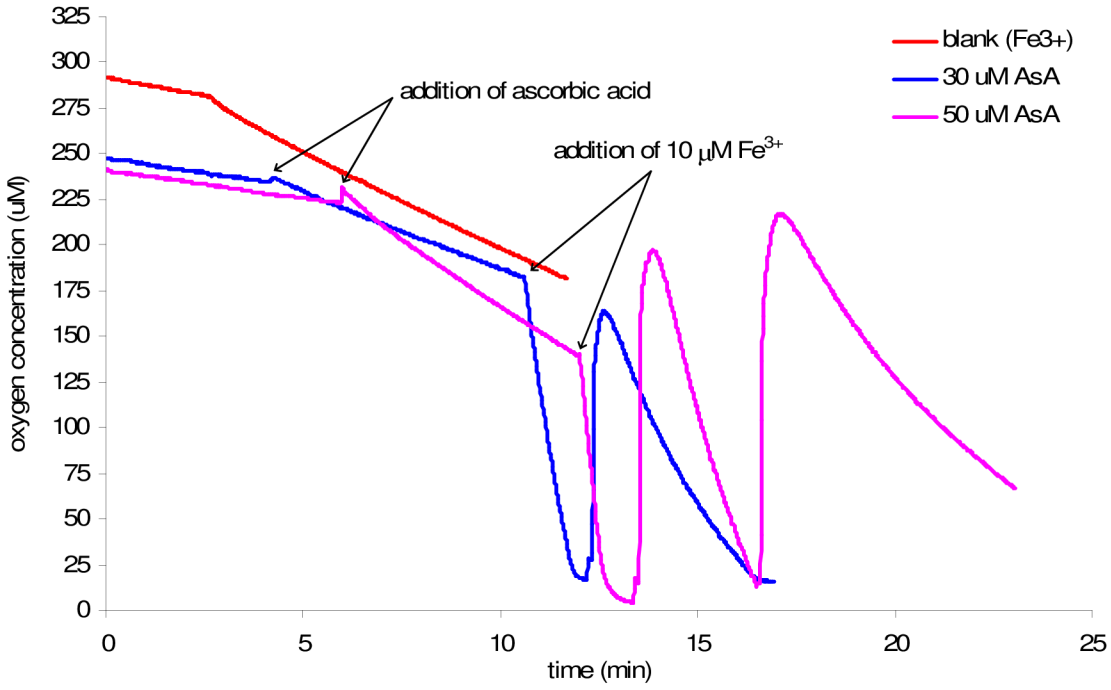


Figure 4–21 *Kinetics of oxygen consumption during the oxidation of liposomes (1,5 %, pH 5.5) initiated by Fe^{3+} (10 μM) in the presence of 30 μM and 50 μM ascorbic acid. Increase in background OUR was observed after addition of ascorbic acid.*

In Fe-induced oxidation the effect of AsA was strongly prooxidative at the tested concentrations. In Hb-induced oxidation the effect of AsA at the same concentrations could not be so plainly categorized. The tested concentration range was 1 – 300 µM. At concentration of 1 µM, AsA did not exhibit any significant effects, at concentration of 30 µM AsA slightly inhibited the rate of oxidation, and above the concentration of 30 µM AsA accelerated the rate of oxidation (Figure 4–22).

It should be pointed out again that the resulting effect consists possibly of simultaneous interactions of AsA with both phospholipids and Hb. Thus, the direct effect of AsA on Hb itself, which is likely to occur because both substances are in close contact in the aqueous phase, is not clear from the measured data. Investigation of interactions of AsA with Hb itself is not the objection of this study. However, elucidation of such reactions might be useful to better understand the effects of AsA in Hb-induced oxidation of liposomes.

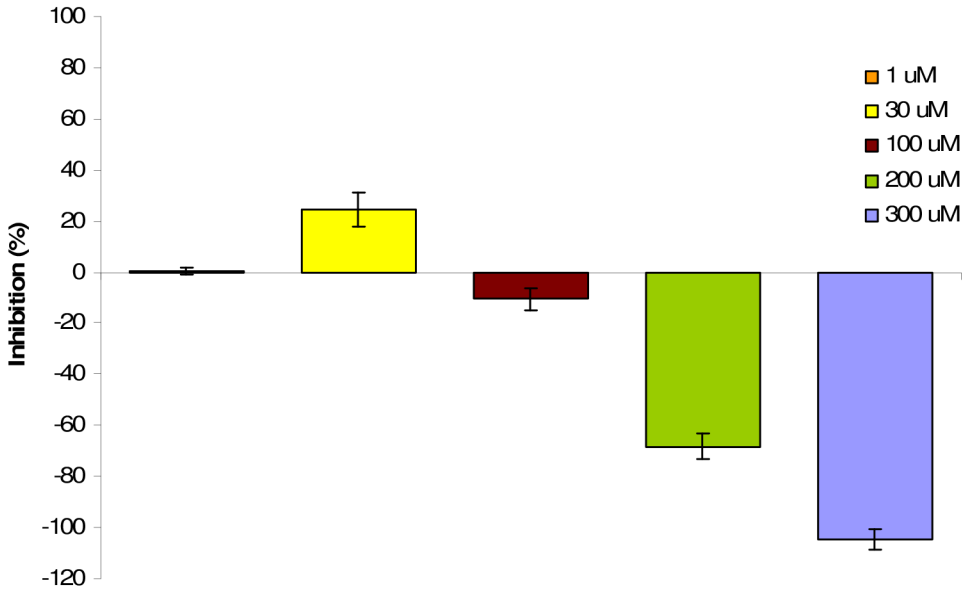


Figure 4–22 *Inhibition (%) of Hb-induced oxidation of liposomes by different concentrations of ascorbic acid. The values are the means ± SD of two to three parallel experiments.*

Numerous investigations have been carried out on the combination of AsA and metal ions (Fe, Cu). AsA was found to act either as a prooxidant or an antioxidant, mostly depending on the experimental conditions (pH, nature of lipid substrate, incubation time) and most importantly on the molar ratio between AsA and a metal. [53, 57, 70, 71] A switch between pro- and antioxidant actions of AsA was observed at certain critical concentrations of AsA. [70] The prooxidative behaviour arises from the ability of AsA to reduce ferric ions to ferrous ions and so maintaining the “iron redox cycle”. This usually occurs at low AsA/Fe ratios and the process proceeds as long as AsA is available for the reduction reaction. Above the critical concentration (at higher AsA/Fe ratios) AsA acts predominantly as a radical scavenger and so the antioxidative effect is observed.

Apart from reduction of metal ions, prooxidative effects of AsA are also dependent on the level of pre-formed lipid hydroperoxides, which AsA can break down. [70, 71] *In vitro* induction of lipid oxidation by ascorbate-iron systems is a standard test for inducing oxidative stress and testing antioxidant activity of other antioxidants. [54]

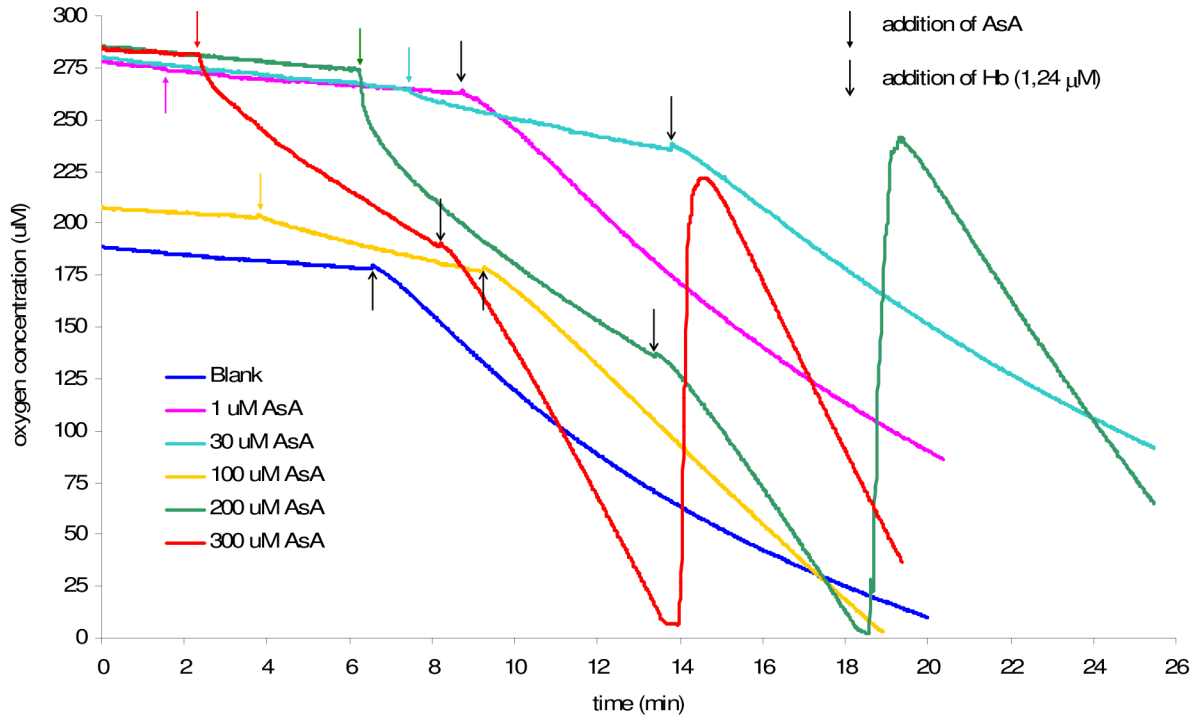


Figure 4–23 *Kinetic curves of oxygen consumption during the oxidation of liposomes (1,5 %, pH 5.5) initiated by Hb (1,24 μM) in the presence of different concentrations of ascorbic acid (AsA).*

A strong prooxidative effect of AsA in free iron induced oxidation that was observed in our study seems to meet the observations reported by Fukuzawa et al.

According to Fukuzawa et al., a low level of endogenous (pre-formed) lipid hydroperoxides (L-OOH) is necessary for initiating lipid oxidation by AsA/Fe^{2+}. Addition of AsA/Fe^{2+} did not induce lipid peroxidation in liposomes with chemically removed L-OOH. Moreover, AsA was oxidized only slightly by Fe^{2+} in such liposomes. [53] The same study also reported that the cleavage of L-OOH by Fenton-like reactions was catalyzed by the weakly charged Fe^{2+}-AsA complex. The formation of a Fe-AsA complex was found to be essential for the initiation of lipid oxidation also by other studies. [57, 71] However, some studies bring evidence that AsA-Fe^{2+} complex can initiate lipid peroxidation irrespective of the existence of pre-formed lipid peroxides. [71] The study of Jacobsen et al. on oxidation of fish oil enriched mayonnaise by ascorbic acid in a presence of iron also support the importance of pre-formed lipid hydroperoxides. The PV decreased upon addition of AsA, which was accompanied by an increase in total volatiles. [57]

A site-specific mechanism of lipid peroxidation in liposomes induced by the addition of AsA and Fe^{2+} was proposed (see Figure 4–24). [53] The OOH-group of L-OOH may be cleaved near the membrane surface by the Fe^{2+}-AsA complex (reaction ①), and the resulting alkoxyl radical (L-$O^{\bullet}$) may penetrate into the hydrophobic region of the membranes (reaction ②) and react with the unsaturated fatty acids (reaction ③) resulting in a chain reaction (reaction ④). Due to many double bonds in n-3 PUFAs, the relatively polar OOH-group of a new L-OOH then moves towards the surface (reaction ⑤) and reacts with the Fe^{2+}-AsA complex (reaction ①). The resulting Fe^{3+}-AsA complex is then reduced by a new molecule of AsA to regenerate Fe^{2+} complex (reaction ⑦) resulting in further degradation of AsA. AsA may also scavenge L-$O^{\bullet}$ (reaction ⑥). [53]

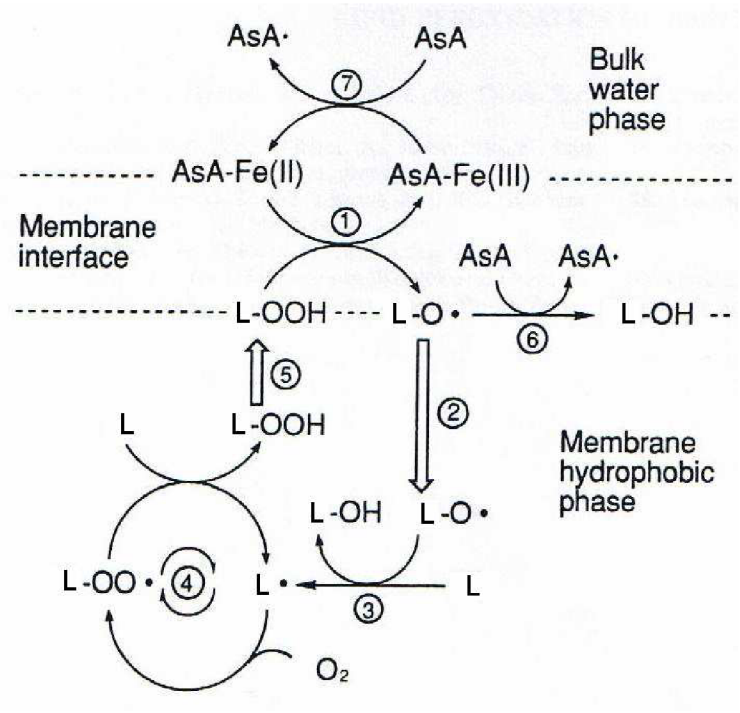


Figure 4–24 *Proposed mechanism of lipid peroxidation induced by ascorbic acid (AsA)/Fe^{2+} in liposomal membranes. Adapted from [53].*

AsA is a di-acid (pK_{a1} = 4,1; pK_{a2} = 11,8). Under the conditions in our study (pH 5.5) AsA is present as monoanion (ascorbate). The dissociated form of AsA has a stronger electron donating ability than the undissociated form. [9] During the process described above AsA (ascorbate) is oxidized. The resulting product of the reaction is dehydroascorbic acid (DHA). Dehydroascorbic acid is unstable and may break down rapidly to produce oxalic acid and L-threonic acid [9] (Figure 4–25) or convert into simple carbohydrates (L-xylose). [6]

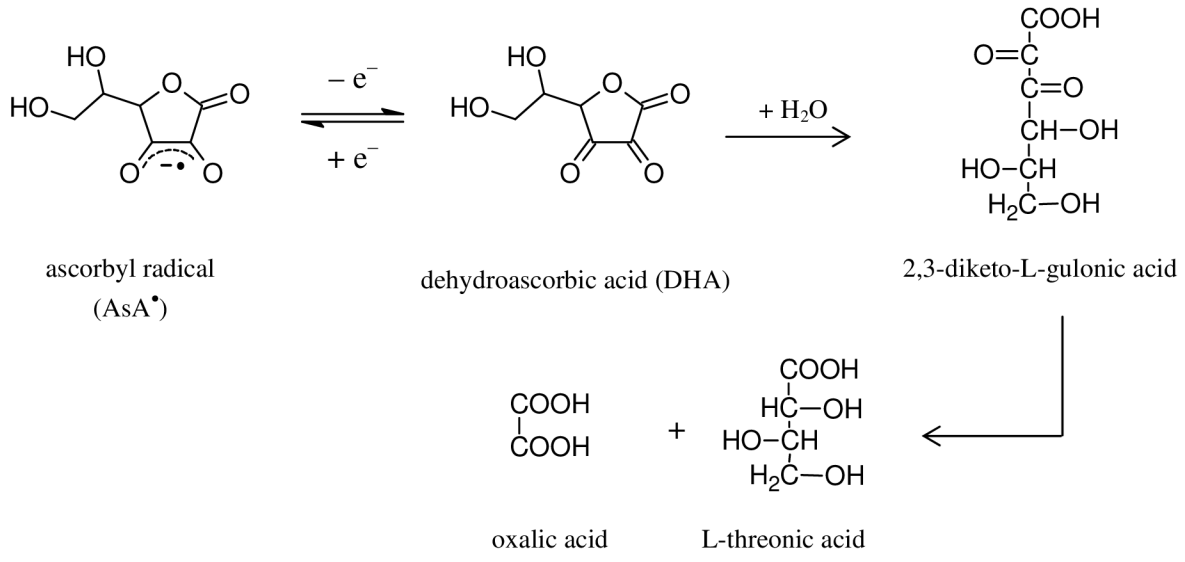


Figure 4–25 *Degradation of ascorbic acid after the ascorbyl radical formation*

Different behaviour of AsA has been reported also in systems with Hb-promoted lipid oxidation. Antioxidant action of AsA is attributed to the ability of AsA to reduce ferrylhemoglobin by one-electron transfer into less prooxidative methemoglobin and scavenging free radicals, while the prooxidative effect include reduction of ferrylhemoglobin by two-electron transfer to more prooxidative ferrous hemoglobin and breaking down of lipid peroxides. [58] Also here, the action of AsA is concentration dependent. More research on interactions of AsA with Hb is needed to elucidate which action is predominant in our study. However, an undoubtedly prooxidative effect was observed at the highest tested concentration of AsA (300 μM).

5 CONCLUSION

Four conventional AOC assays have been performed to evaluate antioxidant capacity of five different compounds that could be of interest for marine food industry as antioxidants for fatty products. The orders of AOC of the tested compounds obtained by the FC, FRAP and DPPH assays had a similar trend: PG > CaA > AsA > FeA > CoA. However, the degree of the antioxidant activity differed for the same compound in the different assays. The AOC order obtained by the ABTS assay differed substantially from the other orders: PG > CoA ~ FeA > CaA > AsA. The inconsistencies in the orders and degrees could be most likely attributed to specific reactions between the different assay reagents and the antioxidants, and to some unrelated reactions, such as polymerization, that probably occur in the reaction mixtures. The ABTS assay seems to be the least reliable AOC assay. Not only due to the dissimilar AOC order, but also due to variable chemistry of the assay that have been reported in a number of papers. Propyl gallate gave the best results in all the assays and its high antioxidant capacity is therefore beyond question. However, on the basis of this comparative study, the use and reliability of these commonly used AOC assays could be questioned.

All the assays used in this study measured mainly the reducing abilities (ability to donate en electron) of the compounds. This property is important for some water soluble antioxidants, such as ascorbic acid, or metal chelators. However, the assays did not provide any sufficient information on the ability of the compounds to retard lipid oxidation mediated by either lipid radicals or prooxidant agents.

Many drawbacks and limitations in chemistry and methodology of the AOC assays that have been reported in the literature suggest that the AOC assessment by means of these assays and the interpretation of the results must be done with care taking into account these drawbacks and limitations; the nature of the tested samples must be considered as well. On the example of the ABTS assay, this study demonstrated that the use of a single AOC assay to evaluate antioxidant capacity may give misleading information.

It could be concluded that AOC determined by these assays should serve as a tentative or preliminary estimation of antioxidant capacity. Any unequivocal conclusions on protection of *in vitro* lipid systems (foods) by the studied antioxidants based on the results of the four AOC assays would be unreasonable. This suggestion is supported by the comparison of the results obtained by the assays and by the study of the antioxidant effects in the liposome model system with catalyzed oxidation. The AOC of the compounds was reflected only partially in the liposome model system. Other factors than reducing abilities determined the effectiveness of the studied compounds in the liposome system.

The type of oxidation promoter (prooxidant), free iron *vs* metalloprotein Hb, and the antioxidant-to-prooxidant ratio were found to be factors of great importance. In Fe-promoted oxidation, scavenging lipid free-radicals by the tested phenolics seems to be predominant antioxidant mechanism unless the compounds interact directly with iron as was observed in the case of CaA, while in Hb-mediated oxidation reduction of ferryl-Hb by phenolics appears to be the most decisive antioxidant mechanism.

In the latter, the inhibitory effect of phenolics correlated with their reduction potentials. PG gave the best results; moreover, the efficiency increased with increasing concentration. CaA and FeA were found to be equally potent and CoA did not exhibit any protective actions at the tested concentrations. The contribution of radical scavenging could be significant only for PG, as the least polar of the studied compounds, that partition in the phospholipid bilayer.

The effectivity of the phenolics in Fe-mediated oxidation was found to be dependent on the occurrence of direct interactions of the tested phenolics with iron; red-ox reactions appear to be more significant than iron chelation which is feasible for PG and CaA. A strong ability of PG to inhibit Fe-mediated oxidation was observed at molar prooxidant-to-antioxidant ratios ≥ 1. PG showed the tendency to promote oxidation when the ratio was 0,1 (excess of Fe). CaA, on contrary, was found to be a potent prooxidant at the ratios ≥ 0,1. When the ratio vas 0,01 (relatively great excess of Fe), CaA did not have any effect on oxidation. Reduction of Fe^{3+} to Fe^{2+} by CaA *via* so called intra-molecular electron transfer is the mechanism responsible for acceleration of oxidation. FeA inhibited Fe-mediated oxidation when tested at the ratios ≥ 5; however, the effectivity was significantly lower than that of PG. CoA was found to be completely inactive at all tested ratios, which were ≥ 5. The study showed that a turning point between antioxidative and prooxidative behaviour of phenolics that are capable of ferric iron reduction exist at a molar ratio close to the value 1.

Ascorbic acid itself promoted oxidation of liposomes, presumably *via* breaking down pre-formed lipid hydroperoxides and reduction of endogenous transition metals. After addition of Fe the prooxidative effect was further intensified owing to reduction of Fe^{3+} to Fe^{2+} facilitated by AsA due to its strong reducing abilities. The effects of AsA on Hb-induced oxidation varied in a concentration range 1 – 100 μM. Above a concentration of 100 μM a prooxidative effect was observed and the effect increased with increasing concentration.

With the exception of CaA, the polar phenolics (FeA, CoA) showed lower relative protective effects on Fe-catalyzed oxidation than less polar PG. The effectivity decreased with decreasing number of OH groups on aromatic ring. The conclusion therefore is that the structure of the compounds and their location in the system are other important factors determining their antioxidant effectivity.

The raised interest in natural substances as food antioxidant additives calls for studies on antioxidant properties of these compounds in lipid systems. Phenolic acids and ascorbic acid, compounds of natural origin that were used in this study, did not perform any exceptional inhibitory effects on catalyzed oxidation of marine phospholipids in liposomes, at least not under the conditions of the performed experiments. On contrary, propyl gallate, a representative of a synthetic food antioxidant, still provided the best results. However, the outcomes of this work contributed to better understanding some basic pro- and antioxidant mechanisms and factors influencing the phenomena called lipid oxidation that could be applicable on cell membranes, liposome solutions, or lipid oil-in-water emulsions.

Evaluation of antioxidants effects is a very complex field, even when simplified model systems are used, and deeper investigations are still needed to clarify the mechanisms and factors determining the antioxidant efficacy. Focus on food-related conditions should be put forefront.

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7 LIST OF ABBREVIATIONS

$A^{\bullet}$, $ArO^{\bullet}$	antioxidant radical
AAPH	2,2'-azobis(2-amidinopropan)
ABTS	2,2’-Azinobis-3-ethylbenzotiazoline-6-sulfonic acid
AsA	Ascorbic Acid
BDE	Bond Dissociation Energy
CaA	Caffeic Acid
CoA	*p*-Coumaric Acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediamine tetraacetic acid
FeA	Ferulic Acid
FC	Folin-Ciocaltau
FCR	Folin-Ciocaltau Reagent
FRAP	Ferric Reducing/Antioxidant Power
FRS	Free Radical Scavenger
HAT	Hydrogen Atom Transfer
Hb	Hemoglobin
IET	Intramolecular Electron Transfer
IP	Ionization Potential
$L^{\bullet}$	lipid radical
LDL	Low Density Lipoprotein
$LO^{\bullet}$	lipid alkoxyl radical
$LOO^{\bullet}$	lipid peroxyl radical
LOOH	lipid hydroperoxide
MES	2-(N-Morpholino)ethanesulfonic acid
NMR	Nuclear Magnetic Resonance
O/W	oil-in-water (type of emulsion)
OUR	Oxygen Uptake Rate
PG	Propyl Gallate
PP	Polyphenol
PUFA	Poly Unsaturated Fatty Acid
PV	Peroxide Value
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SET	Single Electron Transfer
TBARS	Thiobarbituric Acid Reactive Substances
tert-BOOH	*tert*-butyl hydroperoxide
TLC-FID	Thin Layer Chromatography – Flame Ionization Detection
W/O	water-in-oil (type of emulsion)