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Herbs as antioxidants in oxidation of marine lipids

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Preface

This thesis is submitted in fulfillment of the degree Master of Science in Department of Biotechnology, Faculty of Natural Science and Technology, at Norwegian University of Science and Technology (NTNU).

This project consists of relevant literature study, laboratory experiments and thorough analysis of the results. The idea behind this work is to evaluate the antioxidant activity in some preselected herbs using three different antioxidant capacity assays. Based on the results of these assays, antioxidant capacity was determined in a liposome model system with marine phospholipids, where the rate of oxygen uptake was used to measure rate of lipid oxidation.

Typically, during such projects, some questions will be answered properly, while new hidden problems will reveal themselves, creating thrilling topics for future research.

In order to get valuable directions during any kind of research, the presence of a competent and supportive adviser is necessary. In my case, I've been lucky to have **Turid Rustad** as my supervisor, whom I'm very grateful to. Here, I would like to thank her and my Co-supervisor **Revilija Mozuraityte** for being very helpful, enthusiastic and always having constructive suggestions.

Furthermore, I would like to thank my supportive and understanding family, particularly, my kind parents **Javid** and **Mitra**.

Nonetheless, I should thank **Parsa Rahmanpour**, **Tina Khodadadifar** and **Shabnam Arbab** for being good friends and playing a supportive role as my discussion partners in time of need.

Trondheim, 12 August 2013

Tahereh (Mahsa) Janani

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Abstract

Marine lipids have beneficial health effects due to the high content of long chain polyunsaturated omega-3-fatty acids (LC-PUFA), especially EPA (eicosapentanoic acid) and DHA (docosahexanoic acid) and they are therefore of interest to use in products for human consumption.
Marine phospholipids are very susceptible to lipid oxidation, due to the high amount of n-3 PUFAs, which cause loss of sensory and nutritional value in foods.
In order to prevent the oxidation reactions, it is important to find out more on how different factors and compounds, such as pro- and antioxidants in the food, affect these reactions.
The prooxidant activity of Fe^{3+}, Fe^{2+} and Hemoglobin was tested and Fe^{3+} was selected as a prooxidant in the studied lipid system, which is the most abundant prooxidant in the emulsified system.
The aim of this study was to evaluate the antioxidant activity in 12 selected herbs using three different antioxidant capacity assays: Folin-Ciocalteu (FC), 2,2-diphenyl-1-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Based on the results of these assays, antioxidant capacity of the most prominent antioxidants from the assays was determined in a liposome model system with marine phospholipids, where the rate of oxygen uptake was used to measure rate of lipid oxidation. Propyl gallate, a representative of a synthetic food antioxidant, was used as a reference due to its known high antioxidant capacity. This study also showed inhibitory effects of propyl gallate on iron catalyzed oxidation of marine phospholipids in liposomes.
Antioxidant activity of the 5 selected herbs was measured by means of inhibition percentage of oxygen uptake in the liposome (phospholipid dispersion in buffer). With respect to the obtained results, Sage, Rosemary and Dill exhibited antioxidative effects, while Lemon balm and basil were found to be prooxidants at the tested concentrations.
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

indicates that the AOC of the compounds could be dependent on the oxidation system and the applied prooxidants.

1 Introduction

It has been shown that marine lipids have beneficial health effects, this is due to the high content of long chain polyunsaturated omega-3-fatty acids (LC-PUFA), especially EPA (eicosapentanoic acid) and DHA (docosahexanoic acid). Most people get too little of these fatty acids in their diet and it is therefore of interest to make so- called functional food where these fatty acids are incorporated.

Long chain polyunsaturated fatty acids are however susceptible to oxidation resulting in formation of unwanted flavour and loss of the healthy fatty acids. To retard or prevent the oxidation reactions it is important with more knowledge on how different factors and compounds, including pro- and antioxidants in the food, influence these reactions. Addition of antioxidants is one strategy used to retard lipid oxidation.

1.1 Background

Several synthetic antioxidants are approved for use in food, such as propyl gallate (PG), butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), or ethylenediaminetetraacetic acid (EDTA), in addition nature-identical compounds, such as L-ascorbic, citric, and tartaric acids; natural antioxidants found in rosemary and tocopherol extracts are also commercially available. There is an increasing trend among consumers to prefer foods without synthetic additives, which has resulted in considerable interest in searching for natural antioxidants. Herbs may be a rich source of phenolic compounds with antioxidant activity.

The aim of the work in this thesis was to evaluate the antioxidant activity in 12 selected herbs using three different antioxidant capacity assays: Folin-Ciocalteu (FC), 2,2-diphenyl-1-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Based on the results of these assays, antioxidant capacity was determined in a liposome model system with marine phospholipids, where the rate of oxygen uptake was used to measure rate of lipid oxidation.

1.2 Marine Lipids

Marine lipids possess a high amount of long chain polyunsaturated n-3 fatty acids, which have a documented beneficial effect on human health. Reduced inflammatory (Boissonneault and Chow 2000)and coronary heart diseases (Kris-Etherton PM 2002), reduced susceptibility to mental illness (Peet and Stokes 2005)and cured brain and eye function in infants(Innis 1991)were found as the health benefits of n-3 fatty acids. As far as the health benefits of n-3 fatty acids are concerned, there is a great interest in the food industry to incorporate fats rich in n-3 fatty acids in food products.

Fatty acids have been well known as the major structural components of the phospholipid membranes of tissues throughout the body. They consist of an aliphatic unbranched carbon chain and a carboxylic acid group attached to one end of the chain. In the past 2 decades, views about dietary n-3 fatty acids have introduced n-3 fatty as essential nutrients, which suitably regulate many diseases(Connor 2000). Docosahexaenoic (22:6n-3) is an essential constituent of the phospholipids in all cell membranes. N-3 fatty acids are found in high amounts in the retina, brain, and spermatozoa, where docosahexaenoic (DHA; 22:6 n-3) constitutes less than 34% of total fatty acids ((Connor 2000),(Neuringer, Connor et al. 1986)).

N-3 fatty acids as essential nutrients have some important features. They affect atherosclerosis, coronary heart disease, inflammatory disease and even behavioral disorders. One of the main features of n-3 fatty acids is preventing and modulating the certain diseases that are common in western culture(Connor 2000). The following are some examples of diseases that might be prevented or improved through n-3 fatty acids, in falling down the order of strength of the available evidence as observed by (Connor 2000):

1) Coronary heart disease and stroke
2) Basic fatty acid deficiency in infancy (retina and brain development)
3) Autoimmune disorders (lupus and nephropathy)
4) Crohn disease
5) Breast cancer, Colon cancer and prostate cancer
6) Mild hypertension
7) Rheumatoid arthritis

N-3 fatty acids are important throughout life, and they are especially necessary during pregnancy and infancy(Connor 2000).The studies of lipid

metabolism in infants showed higher DHA absorption from phospholipids than from breast milk (Makrides, Hawkes et al. 2002). During the pregnancy, DHA is transmitted through the placenta to the fetus (Connor 2000). Moreover, DHA is always present in human milk along with other n-3 fatty acids, including ALA.

Triacylglycerols make up the main part of lipid depots in most organisms. Triacylglycerols are built up of a glycerol moiety with fatty acids esterified to the hydroxyl groups. Glycerophospholipids have a glycerol backbone with 2 non-identical fatty acids that might vary in length and the presence or absence of double bonds. The phosphate is always linked to the n-3 position of the glycerol molecule, which is in turn esterified with alcohol. In many phospholipids the hydrophilic group contains quaternary nitrogen atoms. The phosphate group is found negatively charged at physiological pH value, whereas the nitrogen atoms had a positive charge. Figure 1 shows some common phosphoglyceride structures.

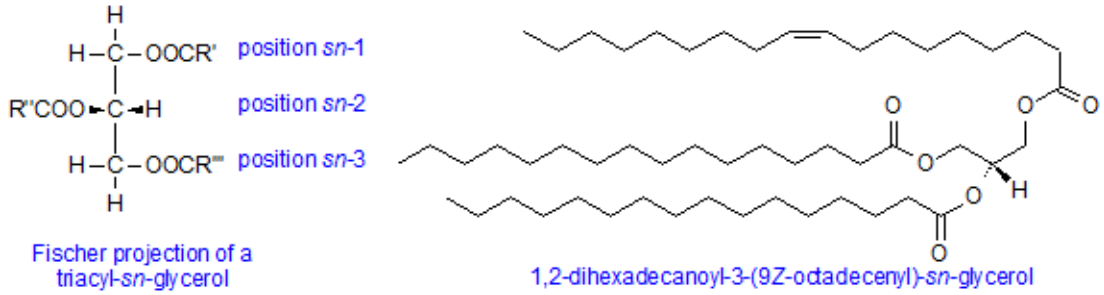


Figure 1: Phosphoglycerides structure (John L. Harwood).

1.3 Marine phospholipids

Many studies have focused on marine phospholipids (MPL) due to their noticeable bioavailability and susceptibility towards oxidation. Marine lipids contain a high amount of polyunsaturated n-3 fatty acids (PUFA), mainly Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)(Lu, Nielsen et al. 2011). Important beneficial health effects have been reported in marine phospholipids as carriers of n-3 PUFA in fish ((Boissonneault and Chow 2000),(Mozuraityte 2007)). N-3 and n-6 polyunsaturated fatty acids (PUFAs)

have documented beneficial effects on human health and therefore they are essential for human diet.

There is a great interest in marine phospholipids as carriers of n-3 fatty acids, because the n-3 fatty acids in phospholipids are more easily accessible for catabolic processes than n-3 fatty acids from triglycerides(Løvaas 2006). Marine phospholipids provide an important source of marine ingredients in functional foods. They are susceptible to lipid oxidation and this may leads to damage in biological and food systems. They also have a large possibility to be used in products for human consumption and as a delivery system in pharmacology (Løvaas 2006).
Phospholipids, as the main components of biological membranes and boundaries, play major roles for metabolic regulation (Berdanier and Chow 2000). Antarctic Krill and fish roe are examples of rich sources of marine phospholipids.
The lipid content in Krill may vary between 12.5 and 0.2% (w/w wet mass) (Pond, Watkins et al. 1995)and the total polar lipid concentration can be up to a maximum of 70% of the total lipids(Phleger, Nelson et al. 2002).Depending on the source, the total lipid content is up to 3-4% of the cod roe weight(Bledsoe, Bledsoe et al. 2003) . Cod roe is a good source for marine phospholipids as the phospholipids make up more than 70% of the total amount of fat (Tocher and Sargent 1984). Natural common emulsifiers, such as egg yolk and soybean lecithin, are rich in phospholipids and often added to food as emulsifiers due to their emulsification properties.

1.4 Phospholipid chemistry

As earlier mentioned, phospholipids, as polar lipids, are the major components of the cellular membrane. All cellular membranes are built up of a lipid bilayer formed by phospholipids. The hydrophilic polar group of phospholipids contains a head, which has a high affinity for water. The polar head is attached to a lipophilic tail group along with a high affinity for oil (Damodaran and Parkin 2008).

Phospholipids have four main constituents: fatty acids as oxidizable substrates, a negatively charged phosphate group, an alcohol and a backbone. Fatty acids have been well known as the major components of lipids, consist of an aliphatic unbranched carbon chain and a carboxylic acid group attached to one end of the chain.

1.5 Liposomes

Liposomes are phospholipids forming bilayer vesicles with amphiphilic structure. Liposomes are found as hollow vesicles surrounded by a fatty envelope with an aqueous core (Arnaud 1995).The principle of the name of liposome comes from the two Greek words (lipo-fat, and soma-structure). Figure 2shows the structure of liposome.

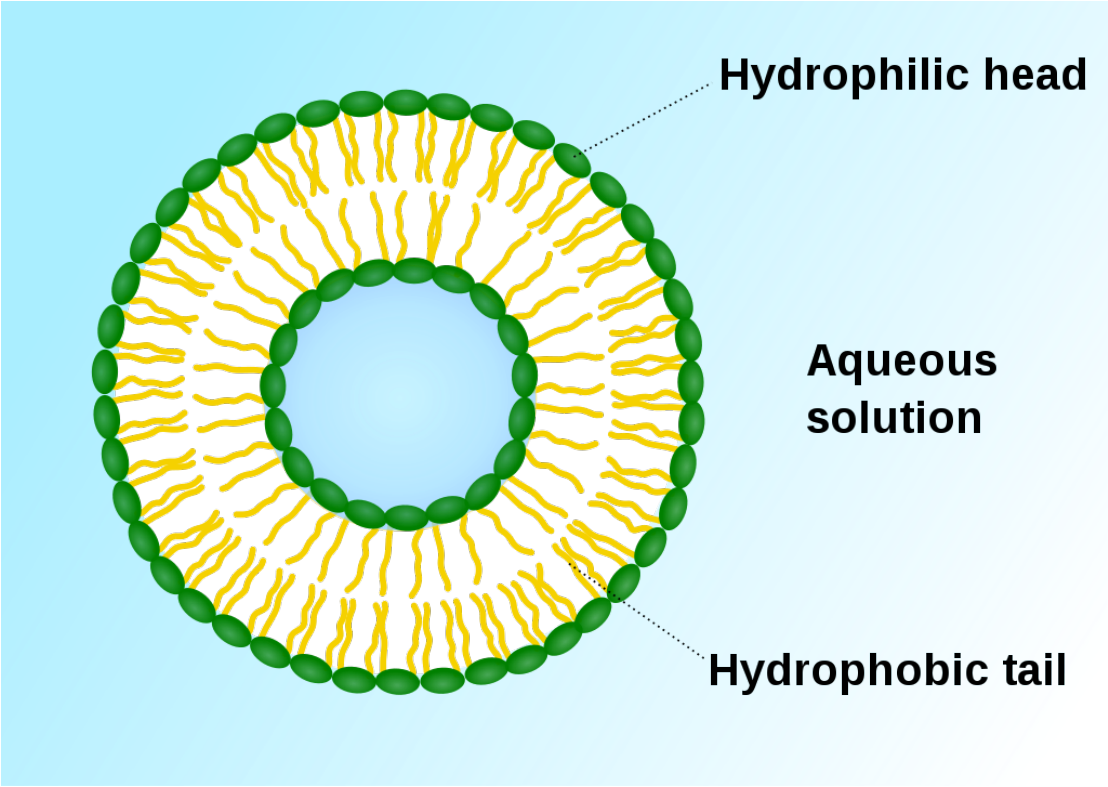


Figure 2: The scheme for the structure of liposome(Boutet 2007).

There is a great interest in using liposomes with different food ingredients (enzymes, flavors, minerals and vitamins) incorporated for human and animal nutrition (Sato and Sunamoto 1992; Arnaud 1995).Due to the observed high bioavailability in liposomes, they might be an interesting material containing

marine phospholipids, which leads to development of liposomes as an oral PUFA supplement (Cansell, Nacka et al. 2003).Liposomes made from marine phospholipids also have a possibility as aα-tocopherol supplement and as a delivery system in pharmacology (Mozuraityte 2007).It should be noted that the absorption of tocopherol increases in the presence of surfactants (Bateman and Uccellini 1984).

1.5.1 Liposomes- model systems for studies of lipid oxidation

Marine phospholipids in liposomes have a high susceptibility to oxidation and can therefore be as a model system for the studies of lipid oxidation. Other model systems include bulk fish oil, emulsions, bilayer structures and fish minces have been used in order to simulate foods or biological samples. When using a complex to study lipid oxidation, different compounds in the system may have an effect on the lipid oxidation(Bateman and Uccellini 1984).In order to identify the components, which affect lipid oxidation, and increase the knowledge on the oxidative pathways in lipid oxidation, the correct model systems and storage conditions should be selected. More complete understanding can be obtained by combining the results from different model systems.

Several advantages have been reported from liposomes as a model system. The liposome system allows manipulation of lipid composition, pH, temperature, and contents of various agents such as salt, antioxidants, prooxidants etc. (Chatterjee and Agarwal 1988). The high oxidative instability of liposomes leads possibility to use this system as a test for various types of oxidative stress and for evaluation of antioxidant efficiency. Thus, liposomes are used as a model system for screening of antioxidant capacity.

Liposomes made from phospholipids have the same structure as cell membranes. Liposome was used with incorporated proteins as a model system in order to study lipid-protein interaction in biological membranes (Re, Pellegrini et al. 1999).

The knowledge gathered from the different studies of lipid oxidation in liposomes can be helpful for choosing liposomes to be used for incorporation of some vitamins, n-3 fatty acids, minerals etc.

1.6 Lipid oxidation

Lipid oxidation is a complicated phenomenon involved in oxidative degradation of lipids. Lipid oxidation occurs through the following reaction pathways:

1) Nonenzymatic chain autoxidation mediated by free radicals;
2) Nonenzymatic and nonradicalphotooxidation; and
3) Enzymatic oxidation.

Based on the type of oxygen involved, autoxidation and photooxidation are responsible for the lipid oxidation during processing and storage of food products (Choe and Min 2005). Triplet oxygen and singlet oxygen are the 2 types of oxygen that react with lipids. Autoxidation is caused by the reaction between atmospheric triplet oxygen and lipid radicals, which is a free radical chain reaction. The presence of light and sensitizers affect the initiation of photosensitized reactions, which produce singlet oxygen from atmospheric triplet oxygen. Then singlet oxygen can react with fatty acids and form lipid hydroperoxides.

When exposed to light of a specific wavelength, a ground singlet state photosynthesizer (Sen^1) changes to an excited singlet state photosynthesizer (Sen^{1*}), which is reversible to ground state through light, internal conversion, or intersystem crossing (ISC). The excited singlet photosynthesizer produces an excited triplet photosynthesizer (Sen^{3*}) (1). The excited triplet photosynthesizermay generate radicals (Type I) (2) by accepting hydrogen from the substrate or donate an electron to the substrate. The energy of the excited triplet sensitizer can be transferred to triplet oxygen in order to produce singlet oxygen or superoxide anion (type II)(3, 4). The excite triplet sensitizer is then reverted back to its ground state ((Laguerre, Lecomte et al. 2007), (Choe and Min 2005)).

$$\text{(1) } Sen^1 \xrightarrow{hv} Sen^{1*} \xrightarrow{ISC} Sen^{3*}$$

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

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

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

Since the singlet oxygen produced by reaction (3) is highly electrophilic, it binds directly to C=C double bonds of fatty acids and causes hydroperoxide formation (LOOH) (5).

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

Nonradicalphotoxidation is believed to be an important mechanism responsible for the onset of lipid autoxidation. The obtained hydroperoxides may break down into the free radicals, which can start the radical oxidation. Autoxidation is an important mechanism in lipid oxidation. It can be described in terms of initiation, propagation and termination(Frankel 2005).

1.6.1 Initiation

Lipid peroxidation is the procedure where free radicals remove an electron from the lipids. During this process, molecular oxygen is incorporated into unsaturated lipids (LH) in order to form a lipid hydroperoxide (LOOH). Initiation is the most important step in terms of the onset of rancidity in food lipids. Since the lipid ground state has an opposite spin direction from that of triplet oxygen, the spin of triplet oxygen prevents direct oxidation of unsaturated fatty acids (LH).
An Initiator (I) helps to easily overcome the spin barrier between lipids and oxygen, and the initiator overcome the separated energy of the allylic bond and therefore cause abstraction of hydrogen atom creating a carbon-centered lipid radical (L·) (6). Afterwards, the reaction between the generated radical and molecular oxygen forms a conjugated diene hydroxyl radical species ((Frankel 2005), (Minotti and Aust 1987)).

(I)

(6) $LH \rightarrow L\cdot + H\cdot$

PUFAs are particularly susceptible to peroxidation because they have a large number of double bonds in a side chain, which causes an easier removal of a

hydrogen atom(Frankel 2005). The chain reaction can start to propagate after formation of lipid radicals.

1.6.2 Propagation

In the propagation phase, the L·radicals is produced and can react with triplet oxygen to generate peroxyl radicals (LOO·) (Min and Boff 2002). Afterwards, the peroxyl radical steals a hydrogen atom from another unsaturated lipid molecule in order to form hydroperoxide (LOOH) (primary oxidation product) and another L· (8), which can react with triplet oxygen in reaction (Laguerre, Lecomte et al. 2007).

(7) L· + O_2 → LOO·

(8) LOO· + LH → LOOH + L·

1.6.3 Termination

Termination is the last stage of autooxidation where the peroxyl radicals interact with each other to form stable non-radical end products (secondary oxidation compounds) and these reactions affect the formation of hydrocarbons, aldehydes, alcohols and volatile ketones. Nonvolatile aldehyde, oxidized triacylglycerols and their polymers are the nonvolatile compounds that are formed by these reactions (Laguerre, Lecomte et al. 2007).

During the oxidation process lipids react with oxygen producing peroxides as primary oxidation products. Afterwards, the peroxides can be broke down to secondary oxidation products such as ketones, aldehydes and alcohols. Volatile oxidation products, which cause off flavor in the products, are formed due to hemolytic β-cleavage of hydroperoxides. The various types of damages such as protein denaturation, polymerization, loss of vitamins, change in texture and color could be due to the reactions between lipid oxidation products and proteins, vitamins etc. Besides, lack of nutritional value in food products is caused by oxidation of essential fatty and amino acids (Frankel 2005).

Lipid molecules within the system and interaction with other molecules (pro-oxidants, antioxidants) have influence on the susceptibility to oxidation. In studies of lipid oxidation, the effects of antioxidants have been more in focus compared to pro-oxidants. Many factors such as light, temperature, enzymes, metals, metalloproteins and microorganisms can affect the oxidation of lipids(Vercellotti, Angelo et al. 1992).

1.7 Prooxidants

Prooxidants are chemicals that encourage oxidation through producing reactive oxygen species or inhibit antioxidant systems. Transition metals with two or more valence states (Fe, Cu, Cr, Ni, V, Zn, Al) can serve as prooxidants and promote lipid oxidation. These metals can cycle between their reduced and oxidized positions transferring electrons (redox cycling), which catalyzes degradation of peroxides (9-11).

1.7.1 Iron

Iron is found as a common constituent of food, and catalyze oxidative changes in lipids. Iron can be added with some food ingredients such as an impurity in salts. A high amount of iron is incorporated in muscle food. In the animal tissue most of the iron is found in hemoproteins, while a number of substances contain nonheme iron (ferritin, transferrin etc.).

The need of iron in oxygen transport, respiration and in activity of some enzymes makes it essential for life (Decker and Hultin 1992). One of the most prevalent nutritional deficiencies is iron deficiency, and this can be alleviated by foods rich in iron, such as red meat, egg yolk, dark, leafy greens (spinach, collards), Dried fruit (prunes, raisins), Iron-enriched cereals and grains (check the labels), Mollusks (oysters, clams, scallops), Turkey or chicken giblets, Beans, lentils, chick peas and soybeans, Liver, and Artichokes (Zelman 2004).It has been suggested that iron fortification of food can be one of the preferred methods to inhibit or reduce iron deficiency (Mehansho 2006).

The food systems and biological membranes usually consist of polyunsaturated fatty acids and liquid rich in oxygen and metal ions (Chatterjee and Agarwal 1988). More knowledge as the effects of different factors on iron induced lipid peroxidation is needed. The studies of iron induced lipid oxidation lead to choice of better storage and processing conditions for food products, and can provide a good knowledge of induced lipid oxidation, especially for those products enriched with iron. This could lead to possibility to produce stable products with high quality, including fortified products.

1.7.1.1 Iron-catalyzed lipid oxidation

Metal activated enzymes and their decomposition products such as heavy metals generally present in food lipids (Ingold 1962), and heavy metals increase the rate of lipid oxidation. The presence or absence of preformed lipid hydroperoxide (LOOH) affects the mechanism of iron-catalyzed lipid peroxidation, which is divided into LOOH-independent (or iron independent) and LOOH-dependent initiation (Girotti 1985).

LOOH-independent oxidation does not need any peroxide to occur, and the initiation mechanism of this is still unknown.
The slow reaction of the breaking down unsaturated lipids (LH) into alkyl radicals (L·) is also directly done by transition metals. Hence, this reaction is not believed to be an important factor in promoting lipid oxidation (Reische, Lillard et al. 1998).
When the oxidation starts either by formation of peroxy radical (H_2O ·) or singlet oxygen (O_2^1), oxygen activation in the presence of transition metals can be one pathway for LOOH-dependent initiation; however, peroxy radical was reported not to play a main role as initiator of peroxidation in liposome systems ((W.W. 1996), (Aruoma, Halliwell et al. 1989)).

$$M^{n+} + O_2 \xrightarrow{-e^-} M^{(n+1)+} + O_2^- \xrightarrow{+H^+} O_2^1, \rightarrow HO_2 \cdot$$

It has been suggested that both Fe^{2+} and $Fe^{3+}$is needed to promote lipid peroxidation in lipid peroxide free lipids(Minotti and Aust 1987); however, (Tadolini and Hakim 1996) believed in no participation of Fe^{3+} in the initiation of peroxide free lipid peroxidation. To start lipid oxidation, forming aFe^{2+}-O_2-Fe^{3+} complex (Tadolini and Hakim 1996) iron-oxygen complexes such as ferrylion and perferrylion have been recommended.

On the other hand, there was an observation that iron complexes have not been isolated (Ohyashiki, Kadoya et al. 2002), and the other observation presented that in order to get through the oxidation procedure, a specific Fe^{2+}- Fe^{3+} complexwould not be needed (Aruoma, Halliwell et al. 1989).

OH· and O_2 ·can be generated in the Fenton reaction (iron-catalyzed degradation of hydrogen peroxide) or by autoxidation of ascorbic acid/Fe^{2+}, and have nothing to do with the process of lipid peroxidation ((Yin, Lingnert et al. 1992), (Ohyashiki, Kadoya et al. 2002), (Fukuzawa, Seko et al. 1993)). On the other hand, some studies reported that $OH^{\bullet}$ is highly reactive and therefore reacts very fast with the first molecule it hits.

Iron is one of the most important prooxidant in many foods, since it catalyzes degradation of peroxides, and peroxides exist in most of lipids (Decker and McClements 2001).It was found that in lipids with no peroxides, iron does not induce lipid peroxidation ((Fukuzawa, Seko et al. 1993), (Tadolini, Cabrini et al. 1997)). Indeed, the main reactions for lipid peroxidation are peroxides dependent oxidation, even with low amount of peroxides.

Metals promote oxidation through 3 following mechanisms:

1) Interaction of iron with unsaturated fatty acids:

(9) Fe^{2+} + LH → Fe^{3+} + H^{+} + L·

2) Interaction of iron with hydroperoxides (called Fenton-type reactions), 2 reaction pathways are possible (Akoh and Min 2002).

(10)Fe^{3+} + LOOH → Fe^{2+} + H^{+} + LOO·

(11) Fe^{2+}+ LOOH → Fe^{3+}+ OH^{-}+ LO·

3) Activation of ground state molecular oxygen to its excited state (Shahidi, Janitha et al. 1992).

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

(13) $Fe^{2+} + O_2^3 \xrightarrow{+H^+} Fe^{3+} + O_2^- \rightarrow HO_2^-$

The interaction of metals with lipid molecules (9) is not considered to be the main mechanism of metal catalysis, since thermodynamic forces, spin barriers and low reaction rate has observed (Damodaran and Parkin 2008).On the other hand, the interaction of metals with lipid hydroperoxides is believed to be the main mechanism of metal catalysis(10,11). It was known that even few amounts of metal-hydroperoxide can increase electron transfer from lipid hydroperoxides, since reaction (10) and (11) can run continuously with regeneration of the lower oxidation state of the metal (Damodaran and Parkin 2008). In order to catalyze hydroperoxide degradation, metals with higher oxidation states have a larger and faster degree than lower oxidation states, and pre-existing lipid hydroperoxides has been reported as a vital condition for these reactions (Mozuraityte, Rustad et al. 2007). Figure 3 shows the mechanism of metal catalyzed lipid peroxidation.

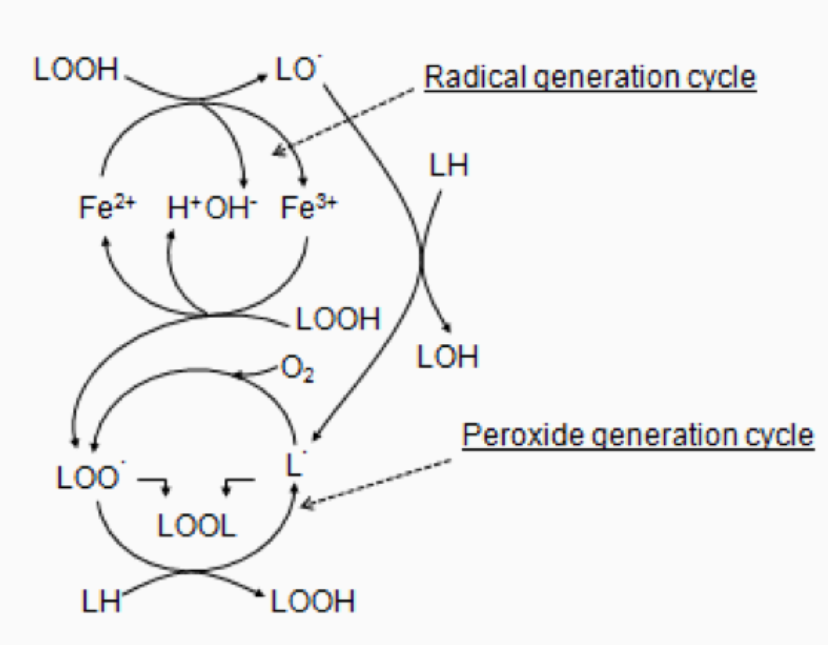


Figure 3: Mechanism of lipid peroxidation promoted by transition metals (Mozuraityte 2007).

1.7.2 Hemoglobin (Hb)

All vertebrates have hemoglobin as an iron-containing oxygen-transport metalloprotein in the red blood cells(Maton Anthea 1993). Hb in mammals has four subunits of the globular protein globin with an embedded heme group. The heme group is responsible for reversible binding of oxygen through ion-induced dipole forces. The heme group consists of a porphyrin ring with a central iron atom ((Damodaran and Parkin 2008), (Belitz 2004)), and it is shown in figure 4.

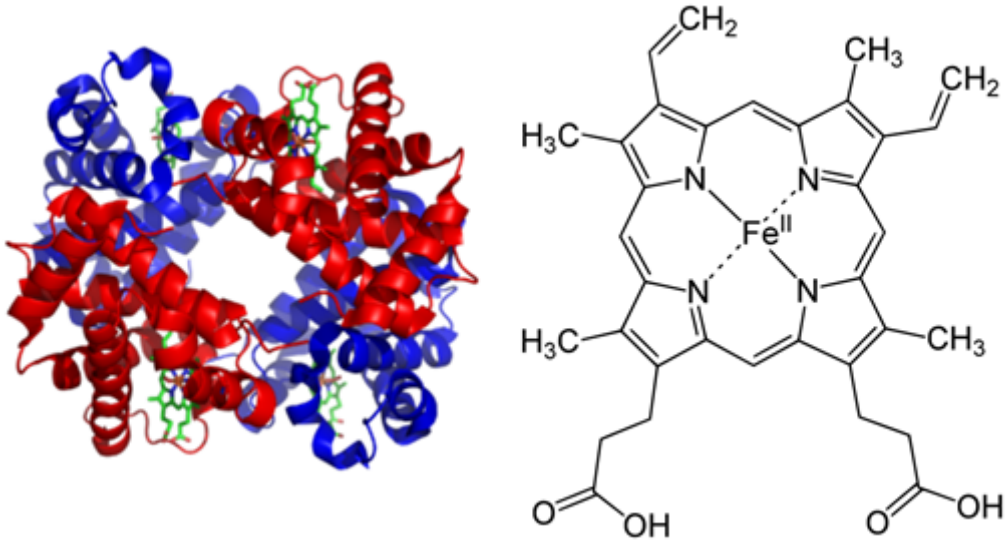


Figure 4: Three-dimensional model of hemoglobin including four globin subunits, each with embedded heme group (left), and a structure of heme group_ a porphyrin ring with iron (right). The proteins α and β subunits are in red and blue, and the iron-containing heme groups in green (Wheeler 2007).

Hemoglobin is found in different forms. O_2 molecule can be bound to the iron (Fe^{2+}) (red oxyhemoglobin)and is stabilized through hydrogen binding by the nearby distal histidine. At low pH or at low oxygen tension the iron can be without oxygen (blue deoxyhemoglobin).

The oxidized state of hemoglobin (Fe^{3+}) is formed through oxidation of iron and is not capabaleof bindingO_2(Kristinsson and Hultin 2004). The strong oxidation agents such as hydrogen peroxide and lipid hydroperoxides affect the oxidation of hemoglobin to ferrylhemoglobin (Fe^{4+}) (Laranjinhas, Vieira et al. 1996). Both Fe^{2+} and Fe^{4+} can act as prooxidants. The relationship between the individual forms of Hb is shown in figure 5.

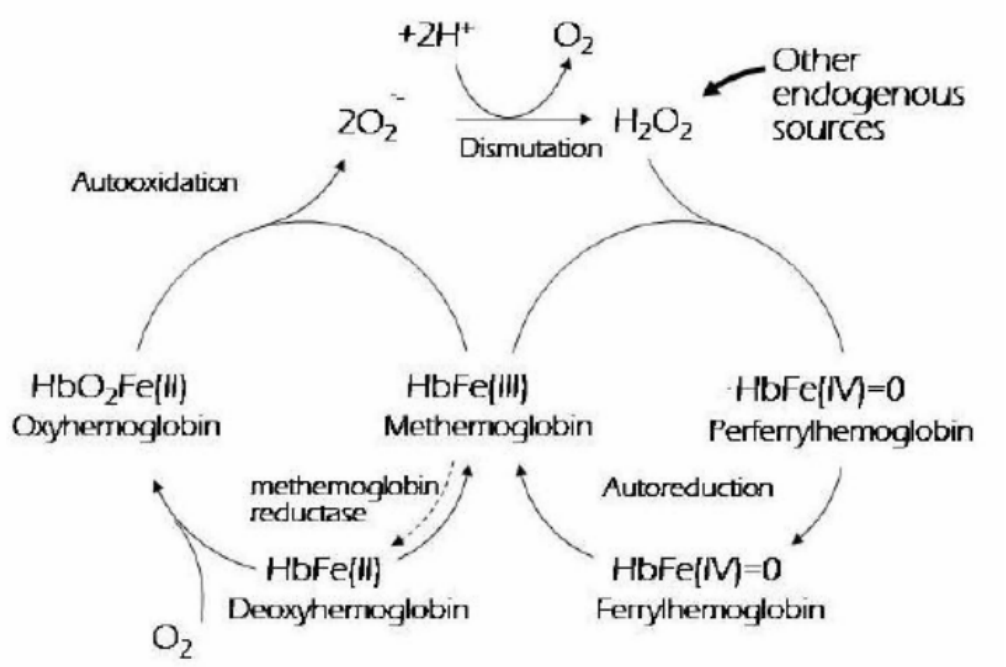


Figure 5: Reaction mechanisms of hemoglobin autoxidation and autoreduction(Kristinová, Mozuraityte et al. 2008).

Oxy-Hb (Fe^{2+}-O_2) can oxidizeto met-Hb(Fe^{3+}) and release oxygen as a superoxide anion radical ($O_2^{\bullet-}$). Later, the generated radical can transform to hydrogen peroxide (H_2O_2), which can activate met-Hb to form a hypervalentferryl-Hb (Fe =O). This form of hemoglobin has a short half-life and is capable of peroxidizing lipids. Moreover, it is assumed to be the major form responsible for Hb-catalyzed oxidation of lipids (Carlsen, Møller et al. 2005).

The ferryl-Hb protein occurs as a radical form, which removes an electron from the lipid substrate resulting in formation of lipid radicals and can cause further oxidation. pH is a factor that affect the prooxidative activity of Hb. The conformation of Hb is less stable at acidic condition. Low pH leads to unfolded Hb structure and more exposed heme group. This results in increasing the prooxidative activity. In contrast, the conformation of hemoglobin is more stable at alkaline pH. Thus, the prooxidative activity of hemoglobin is greatly suppressed in comparison with the activity of native Hb at pH 7 or lower (Kristinsson and Hultin 2004).

Low pH leads to increase in autoxidation, while an alkaline pH reduces autoxidation. The increase in autoxidation at low pH is because of the increased dissociation of the tetramer to dimers for mammalian hemoglobins.

Pre-formed lipid hydroperoxides and other oxidation products may also increase the autoxidation of hemoglobin(Kristinsson and Hultin 2004).Figure 6 shows the mechanism of Hb promoted lipid oxidation.

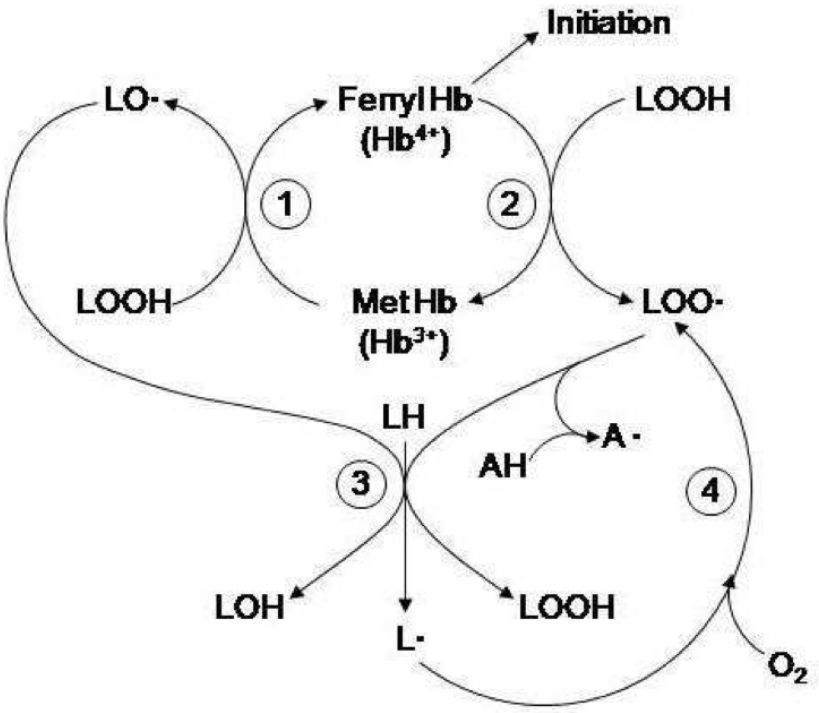


Figure 6: Mechanism of hemoglobin increased lipid oxidation (Kristinová, Mozuraityte et al. 2008).

1.8 Antioxidants

An antioxidant is known as a molecule that inhibits the oxidation of other molecules (proteins, nucleic acids, polyunsaturated lipids, sugars, etc.) through scavenging free radicals or preventing formation of free radicals (Halliwell 1995). Antioxidants works by many different mechanisms, and this can be used to classify the different types of antioxidants. Antioxidants can be categorized as primary and secondary antioxidants. Multiple-function antioxidants are the antioxidants that exhibit more than one mechanism (Akoh and Min 2002).

1.8.1 Primary antioxidants

Primary antioxidants are free radical scavengers (FRS) (Akoh and Min 2002), which are involved in donation of an antioxidant's hydrogen atom to the free radical. The antioxidants then works as free radical scavengers, which inhibits

the initiation phase of lipid peroxidation by scavenging free lipid radicals ($L^{\bullet}$). Free radical scavengers can slow down lipid oxidation through inhibiting the initiation phase of lipid peroxidation (so called preventive primary antioxidants) by scavenging free lipid radicals ($L^{\bullet}$), or by preventing 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).

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

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

FRSs interact with peroxy radicals. It is believed that the low energy of peroxyl radicals causes them to be less reactive and extend their life, and therefore they have a great chance of reacting with FRSs. On the other hand, high-energy free radicals ($OH^{\bullet}$) are so reactive and interact with the molecules nearby to their sites of production. Since antioxidants are found in substrates at low concentrations, they have less possibility to react with the high-energy free radicals (Damodaran and Parkin 2008).

The ability of the compound to donate a hydrogen atom to free radical affects antioxidant efficiency. In order to predict this ability, Standard one-electron reduction potentials ($E°$) could be helpful (Damodaran and Parkin 2008).Antioxidant efficiency is also dependent on the energy of antioxidant radical ($A^{\bullet}$). When energy of an antioxidant radical decreases, the possibility of abstracting one H-atom from an unsaturated fatty acid reduces. FRSs generate low energy radicals due to resonance delocalization of the unpaired electron. They also produce radicals, which do not react hastily with oxygen and form hydroperoxides that could undergo decomposition reactions generating additional free radicals. In order to form nonradical compounds, $A^{\bullet}$ can contribute to termination reactions with other $A^{\bullet}$ or lipid radicals (16 – 18) (Damodaran and Parkin 2008).

(16) $LOO^{\bullet} + A^{\bullet} \rightarrow LOOA$

(17) $LO^{\bullet} + A^{\bullet} \rightarrow LOA$

(18) $A^{\bullet} + A^{\bullet} \rightarrow AA$

Phenolic compounds are known as effective FRSs (Damodaran and Parkin 2008). The mechanism of phenolic antioxidants is discussed further in chapter 1.7.4.1. Carotenoids can act as scavengers of lipid peroxyl radicals in the absence of singlet oxygen or at low oxygen pressure (Akoh and Min 2002). Carotenoids can react with peroxyl radicals to form a resonance-stabilized radicaldue to delocalization of electrons in the their unsaturated structure. These radicals can take part in termination reactions with lipid radicals and are unable to start lipid peroxidation ((Damodaran and Parkin 2008),(Akoh and Min 2002)). β-carotenesas a group of phenolic compounds are mostly active at concentration of 5×10^{-5} mol/L, whereas at higher concentrations the prooxidative effect is predominant(Belitz 2004).
Ascorbic acid (vitamin C) acts as a radical scavenger in aqueous media at high concentrations ($\sim 10^{-3}$). Moreover, prooxidative activity was observed by at low levels (10^{-5} mol/L), especially in the presence of heavy metals (Belitz 2004).

1.8.1.1 Reaction mechanism of hydrogen donation

Antioxidants transfer hydrogen atoms to free radicals by two reaction mechanisms (Wright, Johnson et al. 2001): hydrogen-atom transfer (HAT) and single-electron transfer (SET) or proton-coupled electron transfer (PCET) ((Nikolic 2006), (Wright, Johnson et al. 2001)).
During the HAT mechanism, a hydrogen atom ($H^{\bullet}$) is removed from an antioxidant ($ArOH$) by the free radical using the same sets of orbitals, and the antioxidant becomes a radical (Miliauskas, Venskutonis et al. 2004):
(19) $R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet}$

During the SET mechanism, an antioxidant transfers hydrogen atoms ($H^{\bullet}$) to the free radical using different sets of orbitals. This presents that when the antioxidant turns itself into a radical cation ($ArO^{\bullet+}$), the transferred electron to the free radical turns into an anion (Neuringer, Connor et al. 1986). A quick and reversible deprotonation of the radical cation (Nenadis, Wang et al. 2004) and a neutralization of the anion (Tocher and Sargent 1984)in aqueous phase follow:

(20) $R^{\bullet}$ + $ArOH \rightarrow R^{-}$ + $ArO^{\bullet +}$

(21) $ArO^{\bullet +}$ + $H_2O \Leftrightarrow ArO^{\bullet}$ + H_3O^{+}

(22) R^{-} + $H_3O^{+} \Leftrightarrow RH$ + H_2O

During the HAT mechanism, the bond dissociation enthalpy (BDE) of the O-H bonds is an important parameter for assessing the antioxidant activity. A weak O-H bond makes free radical inactivation easier. On the other hand, the ionization potential (IP) is the most important energetic factor for evaluation of the scavenging ability in SET mechanism. The lower ionization potential makes electron abstraction easier (Wright, Johnson et al. 2001).
It can be difficult to identify electron and hydrogen atom transfer due to the same net result ($R^{\bullet}$ + $ArOH \rightarrow RH$ + $ArO^{\bullet}$) (Huang, Ou et al. 2005).
Both HAT and SET mechanism must always occur in parallel, but at different rates (Wright, Johnson et al. 2001). According to their study about the BDE and IP values for a number of phenolics in the gas phase, they found that the HAT mechanism is predominant for most of the phenolics. Since solution-phase enthalpies of bond dissociation or electron transfer followed the same trends as in the gas phase, they supposed that IP values in solutions have a high correlation with the IP values in gas (Wright, Johnson et al. 2001). The following factors affect the mechanism in solution-phase: 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 (Wright, Johnson et al. 2001).
Hydrogen-bonding characteristics of the solvent (S) is one of the important factors that affect the ratio between the HAT and SET mechanism (Wright, Johnson et al. 2001).
SET mechanism is strongly solvent dependent ((Wright, Johnson et al. 2001),(Foti, Daquino et al. 2004)). Due to solvent stabilization of the antioxidants, the SET mechanism dominates in polar solvents (e.g. alcohols). On the contrary, HAT mechanism is predominant in non-polar solvents (e.g. hexane) and therefore is weakly solvent dependent.
The fact that most of antioxidants are hydrogen-bonded to the solvent (ArOH---S), explains how polar solvents reduce the rate of HAT reactions, though

these species are unable to react by HAT with free radicals. The following parameters are important for the power 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 (Foti, Daquino et al. 2004).

1.8.2 Secondary antioxidants

Secondary antioxidants are oxygen scavengers that decrease the rate of lipid oxidation by several different actions such as chelating and deactivating prooxidant metals, replace hydrogen in primary antioxidants, decompose hydroperoxide to nonradical species, deactivate singlet oxygen, and absorb ultraviolet radiation. Secondary antioxidants promote the antioxidant activity of primary antioxidants and are often referred to as synergists (e.g. ascorbic acid, citric acid, lecithin, etc.) (Foti, Daquino et al. 2004).

1.8.2.1 Metal chelators

Chelators or sequestering agents are the factors that change the prooxidative activity of metals**.** They can inhibit the metal redox cycling by 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) (Damodaran and Parkin 2008). Increase in metal solubility and altering the redox potential of the metal by some metal chelators cause an increase in oxidative reactions. Metal-to-chelator ratio is the factor that influences the tendency of chelators to inhibit or accelerate prooxidative activity. EDTA acts as a prooxidant when EDTA: iron ratio is ≤ 1; whereas it acts as an antioxidant when EDTA: iron ratio is >1 (Damodaran and Parkin 2008).

Foods contain multiple carboxylic acid groups (e.g. EDTA, citric acid) or phosphate groups (e.g. polyphosphates and phytates) were found as metal chelators. Besides, it have been reported that proteins such as transferrin, ferritin, phosvitin, lactoferrin, albumin and casein can control prooxidant metals ((Damodaran and Parkin 2008), (Laguerre, Lecomte et al. 2007)). Under proper conditions, phenolic acids containing catechol and pyrogallol moiety, and flavonoids also show chelating abilities ((Nenadis, Lazaridou et al.

2007), (Laguerre, Lecomte et al. 2007), (Andjelković, Van Camp et al. 2006), (Chvátalová, Slaninova et al. 2008)).

1.8.3 Physical location of antioxidants

It has been reported that the effectiveness of antioxidants does not relate to the factors on the physical nature of the lipid and the polarity of antioxidants (Damodaran and Parkin 2008).They also expressed that lipophilic antioxidants are usually more effective in emulsions than hydrophilic antioxidants; however, hydrophilic antioxidants are more effective in bulk oils than lipophilic antioxidants. This phenomenon has been known as the polar paradox and is shown in Fig 7.

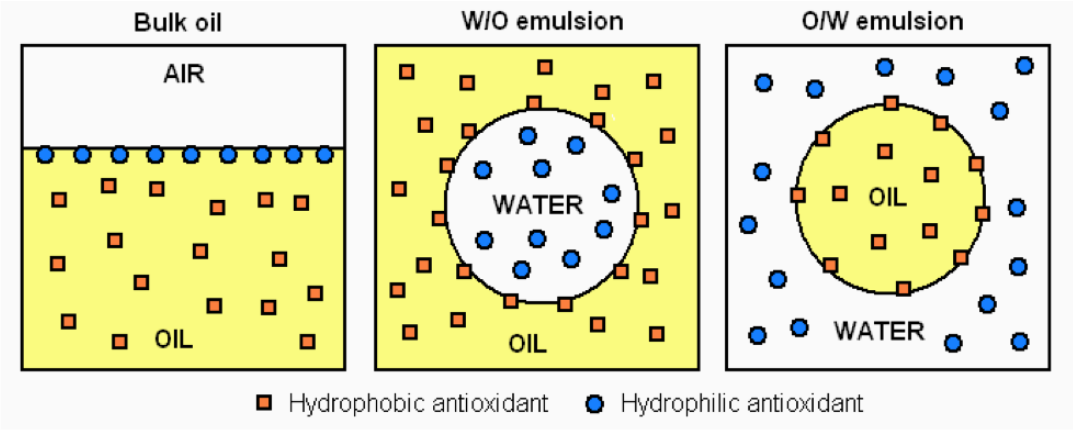


Figure 7: Effects of antioxidant polarity in bulk oil and emulsions (Kristinová, Mozuraityte et al. 2008).

The differentiation in efficiency 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, whereas non-polar antioxidants are more effective in emulsions. Polar antioxidants can accumulate at the air-oil interface or in reverse micelles within the oil, the location where lipid oxidation reaction would be high owing to high concentrations of oxygen and prooxidants; however, non-polar antioxidants can accumulate at the oil-water interface (created by emulsifiers, e.g. lecithin), the location where hydroperoxides at the droplet surface interacts with prooxidants in the aqueous phase. In the other words, polar antioxidants are less able to protect the lipid in emulsions, due to their partition in the aqueous phase (Damodaran and Parkin 2008).

1.8.4 Natural antioxidants

Natural antioxidants are mainly plant phenolics (flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols, etc.), also carotenoids or vitamin C, and may exist in different parts of the plant. The present study deals with one of the phenolic antioxidants, propyl gallate. The following overview of antioxidants is focused mainly on these substances.

1.8.4.1 Phenolic compounds

Phenolic compounds (phenolics) found in a group of approximately 8000 natural compounds, which have a phenol (an aromatic ring bearing at least one hydroxyl group) as a common structural feature (Shahidi, Janitha et al. 1992). Phenolic compounds are divided into three major groups according to the number of phenol subunits in the molecule:

1) Simple phenols – phenolics containing one phenol unit
2) Flavonoids – phenolics containing two phenol subunits
3) Tannins – phenolics consisting of at least three phenol subunits

Flavonoids and tannins are classified aspolyphenols category (PP).
The main groups of flavonoid antioxidants are shown in figure 8.

Phenolic antioxidantsas secondary plant metabolites are developed all over the plants, and they are conjugated to various molecules (quinic acid, sugars) (Clifford 1999).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

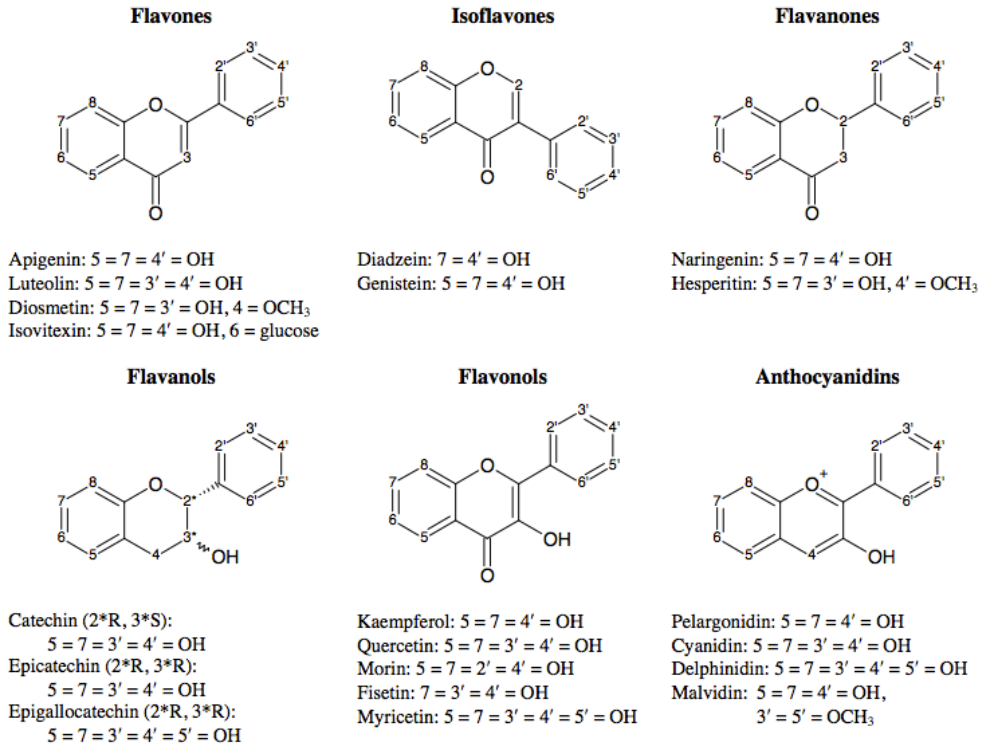


Figure 8: Main flavonoid antioxidants found in plants (Laguerre, Lecomte et al. 2007).

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.) are examples of plants rich in phenolics ((Akoh and Min 2002), (Clifford 1999), (Yanishlieva, Marinova et al. 2006)). It was reported that high antioxidant activity in beverages made of these plants such as red wine, juices, tea and coffee (Yanishlieva, Marinova et al. 2006).

1.8.4.2 Phenolic acids

Phenolic acids refer tophenols that contain one carboxylic acid group; however, in the description of plant metabolites they refer to a distinct group of organic acids. Phenolic acids have two different carbon frameworks: hydroxycinnamic, andhydroxybenzoic structures. The number and position of the hydroxyl groups on the aromatic ring lead to a variety of compounds, whereas the basic skeleton stays the same(Figure 8). In some cases,

aldehyde analogues are also within the mentioned grouped, and are referred to as, phenolic acids (e.g. vanillin).

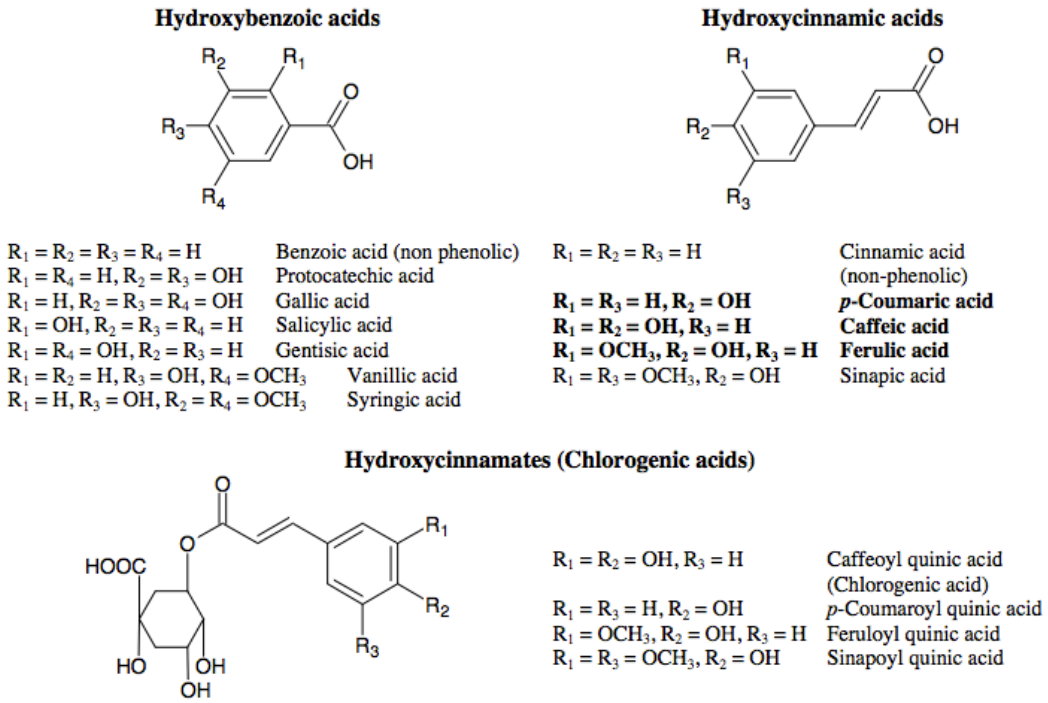


Figure 9: Exhibition of main phenolic acids and esters that found in the plant family (Laguerre, Lecomte et al. 2007).

Hydroxyl groups in phenolic compounds are involved in donation of hydrogen. The generated radical ($ArO^{\bullet}$) has low energy due to a delocalization of the unpaired electron throughout the phenolic ring structure (Fig 10). Replacement groups on the phenolic ring cause an increase in the efficiency of phenolics. These replacements raise the hydrogen donating ability of $ArOH$ in order to donate hydrogen and further increase the stability of the $ArO^{\bullet}$.

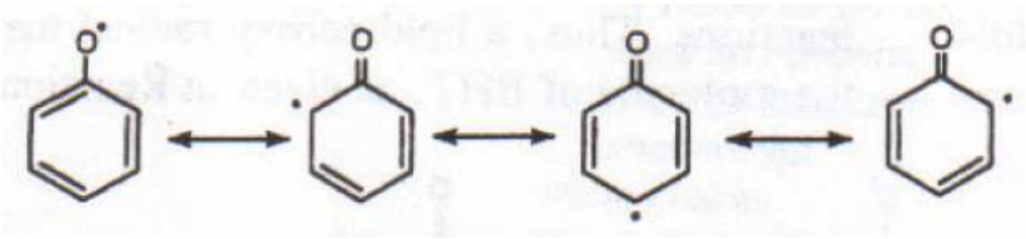


Figure 10: Delocalization of unpaired electrons around the aromatic ring of a phenoxy radical (Shahidi, Janitha et al. 1992).

Phenol is an inactive antioxidant. The electron density of the OH moiety is

increased by replacement 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) and by inductive effect and therefore its reactivity is improved toward lipid radicals. Bulky groups increase the steric barrier in the region of the radicals. Hence, the stability of the phenoxy radical is increased by these substituents at the ortho-position. Subsequently, they decrease the rate of possible propagation reactions mediated by $ArO^{\bullet}$ (Shahidi, Janitha et al. 1992).

A second hydroxy group at the ortho- or para-position of the hydroxy group of a phenol leads to an increase in antioxidant activity. Consistency of the phenoxy radical affects the efficiency of a 1,2- dihydroxybenzene derivative (catechol, e.g. caffeic acid) through an intermolecular hydrogen bond (Figure 11).

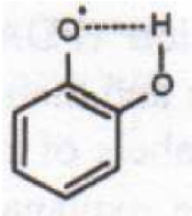


Figure 11: Stabilization of the phenoxy radical through an intramolecular hydrogen bond in 1,2-dihydroxybenzene derivatives (catechols)(Kristinová, Mozuraityte et al. 2008).

The produced semi-quinoid radical can react with another lipid radical or another $ArO^{\bullet}$ and can oxidize to a quinone, and thus this can increase antioxidant activity of dihydroxybenzene derivatives (Figure 12).

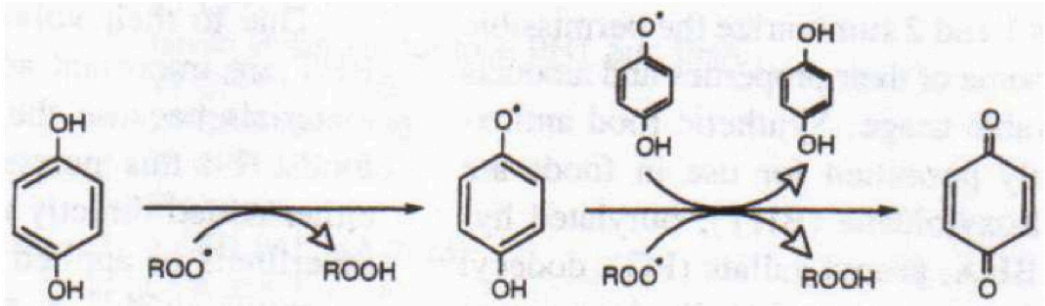


Figure 12: Oxidation of dihydroxybenzene derivative forms a quinone (Kristinová, Mozuraityte et al. 2008).

When antioxidants are added to lipids with a low degree of oxidation, they become more effective; however, they are not so effective in delaying

oxidation in highly oxidized lipids (Shahidi, Janitha et al. 1992).

1.8.5 Synthetic antioxidants

Synthetic antioxidants are efficient inhibitors of lipid oxidation found in a wide range of food products. Antioxidant interact with lipid radicals andthe bulky substituents (such as tert-butyl or methoxy groups) in ortho- and para-positions relevant to the OH group on the benzene ring leads to generation of a very low energy resonance-stabilized phenolic radical. This produced radical does not catalyze the oxidation of unsaturated fatty acids rapidly due to its low energy. Synthetic antioxidants tend to react in radical-radical termination reactions. In order to form unstable antioxidant hydroperoxides, synthetic antioxidants may decompose into high-energy free radicals that could promote oxidation (Damodaran and Parkin 2008).
Synthetic antioxidants are important to use, since they can extend the shelf life of foods. They can also reduce wastes and nutritional losses through inhibiting and delaying oxidation (Shahidi, Janitha et al. 1992).
Each country has different restrictions and regulations for the use of the synthetic antioxidants. The potential toxicity of synthetic antioxidants is the major factor limiting the acceptability of these compounds, which has been widely studied and the effect on health is still not clear (Shahidi, Janitha et al. 1992).

1.8.5.1 Propyl gallate

Propyl gallate, an n-propyl ester of 3,4,5-trihydroxybenzoic acids, has been known as a synthetic phenolic compound. Propyl gallate was used as an antioxidant in food (Halliwell 1995) and cosmetic industry to protect fats and oils towards oxidation. Figure 11 shows the structure of propyl gallate.

Propyl gallate as a food additive is used and has the number E 310 (Kristinová, Mozuraityte et al. 2008). Among synthetic antioxidants,propyl gallate was found with the lowest polarity (Damodaran and Parkin 2008) because of the esterification of the acid group with an aliphatic chain.

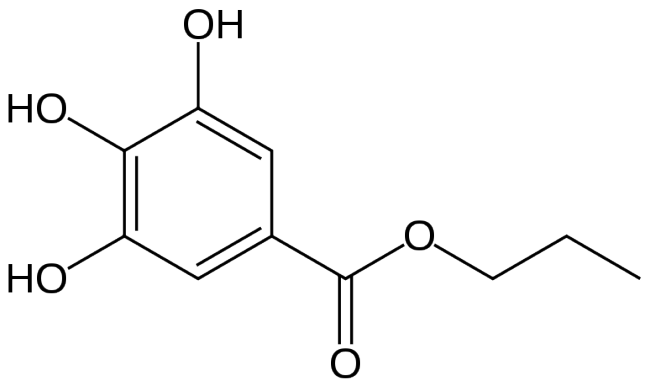


Figure 13: Structure of Propyl gallate(Kristinová, Mozuraityte et al. 2008).

It has been observed that propyl gallate is a white crystalline powder in which can be soluble in water, fats, oils, glycerylmonooleate, alcohols, glycerol and propylene glycol ((Shahidi, Janitha et al. 1992), (Akoh and Min 2002)). Moreover, (Shahidi, Janitha et al. 1992)argued that propyl gallate can chelate iron ions forming a blue-black complex.

1.9 Evaluation of antioxidant activity

During the past 2 decades, evaluation of AOC of various media has received great attention. Many studies reported an opposite relationship between the intake of natural antioxidants and the occurrence of oxidative stress related diseases, such as inflammation, cardiovascular disease, cancer and aging-related disorders ((Huang, Ou et al. 2005),(Connor 2000)).There is a great interest of natural origin antioxidants and some specific plant materials (grape pomace, rosemary or berry extracts)for food industry. They have been used as food additives to protect against oxidative deterioration and therefore evaluation of their antioxidant capacity has been as an important issue. Many published methods have been used to measure total antioxidant capacity in vitro so far. However, lack of a certified assay that could reliably measure the antioxidant capacity assay (AOC) evaluate the AOC is still a large problem; some reviews have been previously done on this topic ((Huang, Ou et al. 2005), (Prior, Wu et al. 2005), (Roginsky and Lissi 2005),(Frankel and Meyer 2000)).

1.9.1 Methods for antioxidant capacity assay (AOC) evaluation

A great interest in knowledge about antioxidant potentials of phenolic rich media has influence the development of assays for determination of antioxidant capacity. There are two main approaches in order to evaluate AOC:

1) Direct methods
2) Indirect methods (Roginsky and Lissi 2005).

1.9.1.1 Direct methods

Direct methods are established on studying the effects of antioxidants on the oxidative degradation of a system for biological relevance (individual lipids, lipid mixtures – oils, lipid membranes, low density lipoprotein, DNA, blood, plasma, etc.) (Roginsky and Lissi 2005).
Direct approach of evaluation that applies various lipid model systems rather than the indirect approach was preferred, where the antioxidant activity is assessed artificially by means of so called one-dimensional AOC assays ((Frankel and Meyer 2000), (Laguerre, Lecomte et al. 2007)). The kind of oxidative substrate in the model systems and the conditions of system play an important role for choosing the methods.
Direct methods are often time consuming and they do not achieve the demand for quick and easy assessments important mainly for food industry.
It have been suggested that the use of different analytical methods for evaluation of antioxidant activity could lead to get more knowledge about antioxidant potentials of various compounds ((Laguerre, Lecomte et al. 2007), (Frankel and Meyer 2000), (Huang, Hopia et al. 1996)).

1.9.1.2 Indirect methods

The majority of the indirect methods are based on spectrophotometric measurements. These measurements are rapid and easy to perform. They also supply reproducible data.
It has been discussed that the methodology, chemistry, biological relevance, ways of quantification and interpretation of results of spectrophotometric methods provide knowledge of the antioxidant capacity for tested materials

((Huang, Ou et al. 2005), (Prior, Wu et al. 2005), (Roginsky and Lissi 2005), (Nenadis, Lazaridou et al. 2007), (Frankel and Meyer 2000), (Connor 2000)). The biological relevance of these methods has been argued, since the results of these in vitro assays often conclude with potential in vivo activity. The spectrophotometric assays are based on chemical reactions in vitro and they are not related to biological systems. Moreover, it has been reported that the mentioned assays do not measure bioavailability, in vivo stability, and preservation of antioxidants by tissues and reactivity in situ (Huang, Ou et al. 2005).It has been suggested that AOC assays may underestimate the real physiological antioxidant capacity in food extracts(Serrano, Goñi et al. 2007).

1.9.2 Antioxidant capacity assays

Several reviews have thoroughly discussed the difficulties and drawback of the antioxidant capacity (AOC) assays ((Huang, Ou et al. 2005), (Prior, Wu et al. 2005), (Roginsky and Lissi 2005), (Frankel and Meyer 2000), (Connor 2000)). Based on the mechanisms of chemical reactions, AOC assays can be classified to two categories: hydrogen atom transfer (HAT) and single electron transfer (SET).

Generally, the SET-based assays are responsible for measuring an antioxidant's reducing capacity, while hydrogen atom donating capacity is measured by the HAT-based assays (Huang, Ou et al. 2005).

1.9.2.1 HAT-based assays

HAT-based assays measure the capability of an antioxidant to suppress free radicals by hydrogen atom donation. HAT-based assays are made up of a synthetic free- radical generator, an oxidizable probe, and an antioxidant. There is a challenge between generated peroxyl radicals ($ROO^{\bullet}$) of antioxidants and probes, and explanation is originated from the kinetic curves after monitoring the competitive reaction kinetics (Sun and Tanumihardjo 2007).

The following assays have been known as the most biologically relevant HAT-based assays:

1. Oxygen radical absorbance capacity (ORAC)
2. Total radical-trapping antioxidant parameter (TRAP)

3. Inhibition of autoxidation of induced low-density lipoprotein (LDL) oxidation (Prior, Wu et al. 2005).

Since hydrogen atom transfer is an important reaction mechanism in the radical chain reactions, HAT-based in vitro assays has been supposed to more close reflect in vivo action ((Prior, Wu et al. 2005), (Huang, Ou et al. 2005)).

Since ORAC assay uses a controllable source of peroxyl radicals and can detect both hydrophobic and hydrophilic antioxidant, it can be used as a standard method for quality control and measurement of food AOC ((Prior, Wu et al. 2005), (Sun and Tanumihardjo 2007)).

1.9.2.2 SET-based assays

SET-based assays evaluate the ability of an antioxidant to transfer single electron in order to reduce oxidants such as radicals, metals and carbonyls. The oxidant acts as a probe for monitoring the reaction and also servesas an indicator of the reaction endpoint (Sun and Tanumihardjo 2007).

SET-based assays are similar to redox reactions in usual chemical analysis and can be explained by the following electron-transfer reaction:

Oxidant (probe) + e (from antioxidant) → reduced probe + oxidized antioxidant

The oxidant (probe) has the ability to absorb light in the visible spectrum (VIS) with a specific wavelength. It also has a specific colour, which normally changes when an electron is abstracted from the antioxidant. The endpoint is reached as soon as the colour change stops.

Normally, absorbance is sketched against the antioxidant concentration to give a linear curve. The slope of the curve presents the antioxidant's reduction capacity and is expressed as equivalents of a selected standard compound (Trolox, propyl gallate, gallic acid, etc.).

SET-based assays do not have any oxygen radical and competitive reaction involved. The following methods have been known as the most popular SET-based assays:

1) 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

2) 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay
3) Ferric reducing antioxidant power (FRAP) assay
4) Folin-Ciocalteu (FC) assay
5) Cupric reducing antioxidant capacity (CUPRAC) (Prior, Wu et al. 2005).

1.9.2.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was introduced as a suitable free radical method in order to evaluate antioxidant capacity of pure compounds (Brand-Williams, Cuvelier et al. 1995). DPPH is a simple and common assay for AOC assessments of a wide array of food matrices. The DPPH radical ($DPPH^{\bullet}$) is a stable organic nitrogen-radical that has a strong purple colour with UV-VIS absorption maximum at 515 nm. Figure 14 shows the structure of DPPH radical.

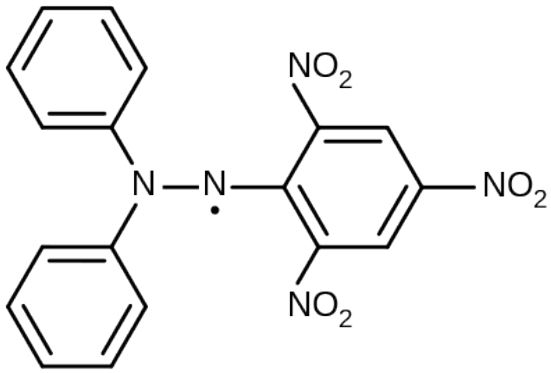


Figure 14: Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

$DPPH^{\bullet}$ loses its colour after having reacted with an antioxidant and the loss of colour can be measured by the DPPH assay. On the other hand, the radical can be deactivated by both HAT and SET reaction mechanisms. The SET reaction mechanisms are considered to play a major role in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. Spectrophotometer is used to observe the reaction progress ((Huang, Ou et al. 2005), (Prior, Wu et al. 2005), (Connor 2000)).

The following versions of DPPH assay can be used:

1) Dynamic
2) Static

The rate of $DPPH^{\bullet}$ decolorization is measured after the addition of a phenolic- containing sample; however, the amount of $DPPH^{\bullet}$, which scavenged by a defined amount of sample, is measured in the static process (Roginsky and Lissi 2005). According to published studies, different methods have been used to analyze the results and this led to diversity in expressed results. Analyzing the percentage of the initial amount of $DPPH^{\bullet}$ scavenged by a defined amount of sample/antioxidant and EC_{50} value (the amount of antioxidant necessary (efficient concentration) for reducing the initial DPPH·concentration by 50 %) are the most common expression in the static version. The Kinetic curve is used to find the necessary time to reach the steady state withEC_{50}(Huang, Ou et al. 2005).

Although the $DPPH^{\bullet}$ assay has been known as a good method, some disadvantages have been found which narrows the application of the assay and makes it less reliable for AOC measurements ((Huang, Ou et al. 2005), 110).

Since $DPPH^{\bullet}$ acts as radical probe and oxidant, it is not a competitive assay. SET and HAT reactions can decolorize $DPPH^{\bullet}$ as well as some irrelevant reactions. Steric approach is a major cause of the reaction (Prior, Wu et al. 2005). Hence, the small molecules close to the radical site perform higher antioxidant capacity. They also mentioned $DPPH^{\bullet}$ as stable nitrogen radical that is not similar to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Steric unavailability may either lead to slow reaction between some antioxidants with peroxyl radicals or even may cause no reaction with ($DPPH^{\bullet}$) (Prior, Wu et al. 2005).

1.9.2.4 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) assay

The ABTS assay measures the capability of antioxidants to neutralize the pre-formed absorbing turquoise ABTS radical monocation ($ABTS^{\bullet+}$) (Figure 15). This assay is also known as TEAC (Trolox Equivalent Antioxidant Capacity) assay.

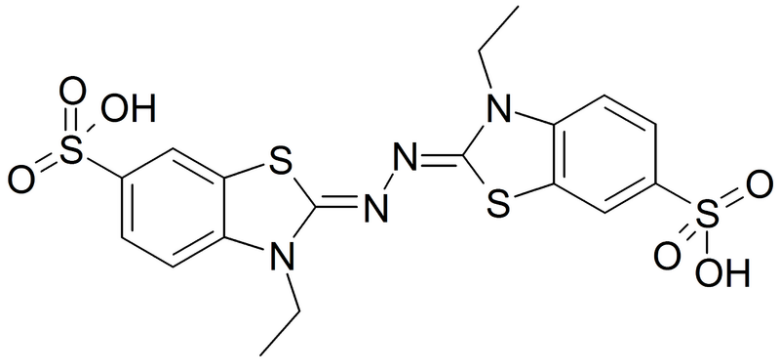


Figure 15: Chemical structure of ABTS.

The electron/hydrogen donors, which have redox potential lower than 0.68 V (redox potential of ABTS) can react with radical and produce a colourless product. Oxidation reaction of ABTS with potassium persulfate produces the radical. The process of generating radical is pH-independent and is not influenced by ionic force. Antioxidant concentration has a linear correlation with decreasing of the $ABTS^{\bullet+}$((Prior, Wu et al. 2005), 62, (Connor 2000)). The $ABTS^{\bullet+}$is soluble in both aqueous and organic solvents and thus it can be proper to use for determination of both hydrophilic and lipophilic antioxidants in various matrices (food extracts, body fluids, etc.). One of the most important drawbacks of this assay is the variable mechanism of $ABTS^{\bullet+}$ deactivation (HAT or SET). The mechanism may change because of having a slow reaction with antioxidants, and may be affected by pH (Prior, Wu et al. 2005). Another limitation of the ABTS assay is the poor selectivity of $ABTS^{\bullet+}$to H-atom donors. It has been found that $ABTS^{\bullet+}$reacts with OH-groups of hydroxylated aromatics, which do not contribute to the antioxidation (Roginsky and Lissi 2005).

A short incubation time, usually 4 – 6 minute, may not provide long enough periods for the reaction to be completed and this has been the most arguable criticized aspect in methodology of the assay ((Prior, Wu et al. 2005),(Roginsky and Lissi 2005)).

1.9.2.5 The total phenol assay by Folin-Ciocalteu reagent

Folin-Ciocalteu assay (FC) is the oldest assay for measuring the reducing

capacity of a sample and total phenolic assay in food research laboratories. The oxidation-reduction reaction between the Folin-Ciocalteu reagent (FCR) and a phenolic compound is the basis of FC assay (Foti, Daquino et al. 2004).Separation of a phenolic proton leads to formation of a phenolate anion and reduces FCR. The proper conditions required for the proton dissociation is pH~ 10 and this is achieved by the use of a sodium carbonate solution ((Huang, Ou et al. 2005), (Prior, Wu et al. 2005)), and the yellow colour of FCR changes to blue after accepting an electron.

Since 1927 the method has been developed and changed many times. Primarily it was developed in order to determine proteins and taking advantage of the reagent's activity toward tyrosine. Further, some changes in composition of the FCR were done in order to develop the assay to the analysis of total phenols in wine (Singleton, Orthofer et al. 1999).The improved method meet mandatory steps and conditions in order to obtain reliable data. Since then, total phenolics in natural products (e.g. teas, juices) have been measured.

The total phenols assay by FCR can be measured in aqueous phase, since FCR is soluble in water. Hence, this assay is not applicable for lipophilic antioxidants. FCR is nonspecific to phenolic compounds and also many nonphenolic compounds such as vitamin C, Fe^{2+} and Cu^{+} can reduce this reagent ((Huang, Ou et al. 2005), (Prior, Wu et al. 2005)). This fact presents a significant disadvantage of the FC method.

2 Materials

2 kg fresh Cod roe was bought from fish market in Trondheim, Norway and was extracted the same day.
Hydrochloric acid (HCl), Methanol, Chloroform and Acetonewere purchased from Merck KGaA (Darmstadt, Germany).
Ethanol 96% was purchased from VWR chememicals.
The following chemicals were purchased at Sigma-Aldrich ChemieGmbh (Steinheim, Germany):
2.0 M Folin-Ciocalteu'sphenol reagent
MES (2-(N-Morpholino) ethanesulfonic acid), minimum 99.5 % titration
2,2-Diphenyl-1-picrylhydrazyl (DPPH)
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) ~ 98%
Sodium dithionite $(NaO_2S)_2$, 85+%
Potassium persulfate ($K_2S_2O_8$), 99+%
Iron (III) chloride anhydrous ($FeCl_3$) was purchased from at Sigma-Aldrich
Propyl Gallate, 97%
NAOH and Iron (II) chloride tetrahydrate ($FeCl_2.4\ H_2O$) were purchased at FlukaChemie (Buchs, Germany).
N_2gas (99.999%) was purchased at AGA AS, Oslo-Norway.

The dried herbs were bought in a food store (those marked Hindu), some dried herbs were supplied by Mills and some plant materials were bought fresh in a food store. Fresh plant samples were cleaned, freeze-dried and ground into a fine powder by laboratory mortar. An overview of the different herbs is given in Table 2.

Table 1: Herbs and their information of the used herbs in the present study

Norwegian	English	Latin	Comment
Dill (Mills)	Dill	*Anethum graveolens*	Dried, supplied by Mills
Dill	Dill		Hindu, Olaf Ellingsen AS Best before 18th apr. 2015
Basilikum	Basil	*Ocimumbasilicum*	Fresh herbs freeze dried in the lab.
Sitronpepper	Lemon pepper	Salt (40 %), black pepper (28 %), (citric acid), sugar, potato starch, flavor enhancer (E621), onion, lemon juice concentrate (0,3 %).	Santa Maria AB, Sweden Best before 5th des. 2009
Sitronmelisse	Lemonbalm	*Melissa officinalis*	Fresh herbs freeze dried in the lab.
Estragon	Tarragon	*Artemisiadracunculus*	From Hindu, Olav Ellingsen AS, Best before 24th aug. 2015
Gressløk	Chive	*Alliumschoenoprasum*	Dried, supplied by Mills
Timian	Thyme	*Thymusvulgaris*	From Hindu, Olav Ellingsen AS, Best before 3th des. 2015
Persille	Parsley	*Petroselinum*	Santa Maria AB, Sweden Best before 11th aug. 2015
Karve	Caraway	*Carumcarvi*	From Hindu, Olav Ellingsen AS (webpage hindu.no, down at the moment but might have some more information) Best before 15th nov. 2015
rosmarin	rosemary	*Rosmarinusofficinalis*	Fresh herbs, freeze dried in the lab
salvie	sage	*Salviaofficinalis*	Fresh herbs, freeze dried in the lab

3 Methods

3.1 Preparation of extracts from dried herbs

The (Hossain, Barry-Ryan et al. 2010) method was used for the preparation of extracts. The dried herbs were powderedin a mortar. 0.5 g of powdered herbs were mixed with 25 ml of 80% methanol. The sample suspension was shaken overnight with avortex at 500 rpm at room temperature. The sample suspension was then centrifuged for 15 min at 5000g and then filtered through blackband filter paper. Extracts were kept at $-40℃$till used.

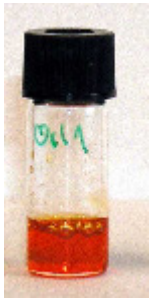
3.2 Phospholipid isolation

The marine phospholipids used to prepare liposomes were isolated from cod (*Gadusmorhua*) roe. The (Bligh and Dyer 1959) method was used for the extraction of lipids from cod roe. 100 g of fish roe was homogenized using an ultra Turrax homogenizer in 100 ml distilled water, 400 ml methanol and 200 ml chloroform for 2 min. 200 ml chloroform was added afterwards and kept to homogenize for 1 min. Then 200 ml distilled water was added and the suspension was homogenized for 1 min. The mixture was centrifuged for 15 min at 4920 g and the chloroform (bottom) phase was transferred by glass pipettes. During the whole procedure the chloroform was kept on ice in order to make pipetting easier and minimize evaporation. A Rotary evaporator was used to evaporate the chloroform, but all the chloroform was not removed. The concentrated oil was kept at$-20\ ℃$ and used for isolation of phospholipids.

The phospholipids were isolated from the total lipids by the acetone precipitation method as explained by (Kates 1991)and modified by (Mozuraityte and Rustad 2006).The principle of the method is the insolubility of phospholipids in cold acetone. 15 ml aliquot of concentrated oil in chloroform (approximately 250mg/1 ml), was mixed with 200 ml of acetone and stored at $-20℃$ overnight. The acetone phase was decanted fast and the precipitated phospholipid layer was dissolved in chloroform and gathered afterwards. Therefor PL1 fraction was reached. In order to find the amount of made precipitate, the total volume of oil/chloroform was measured after the first phospholipids precipitation. 2 ml of chloroform was placed in a

preweighed tube. The chloroform was evaporated under a stream of N_2(70℃) and cooled to room temperature. The weight of tube was measured to find out the total amount of precipitate. To get more precipitation from oil/chloroform, a second isolation was performed in the same way to get the PL2 fraction. The total amount of phospholipid was measured as described above. The final phospholipids were stored at −20℃ until use. During the extraction and isolation, 5 products were obtained, total lipids, OIL1, PL1, OIL2, final phospholipids (PL2).

100% oil



acetone soluble phase **72%**

precipitate **28%**

acetone soluble phase **32%**

precipitate **68%**

3.3 Preparation of liposomes

1.5 ml of phospholipid in chloroform was transferred into a tube with known weight. Nitrogen gas was used to remove the chloroform from the liposome solution. Next, the phospholipid was kept in a covered vacuum exsiccator for 2 hours to evaporate the left solvent. The weight of tube was measured to calculate the amount of needed MES buffer to add to make 3% phospholipid solution. The dried volume of phospholipid was dissolved in 5 mM MES buffer (pH 5.6).MES buffer was used because has very low solubility in non-polar solvents and does not bind to iron (Kates 1991).The solution was sonicated afterwards with 50 % amplitude and 6 seconds pulse for total of 3 minutes with a Vibra Cell sonicator.Phospholipids were kept in an ice bath during and after the sonication process to prevent the rise of temperature.

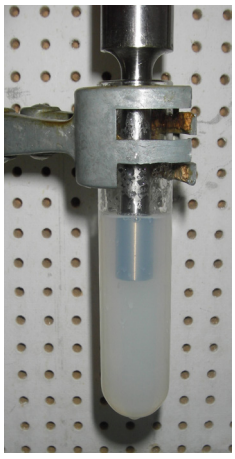


Figure 16: Sonication of phospholipids dispersed in MES buffer in order to prepare liposome solution (Kristinová, Mozuraityte et al. 2008).

3.4 Antioxidant capacity assays

For the antioxidant capacity assays, a 10 mM propyl gallate in 80%methanol was made as a stock solution, whichwas usedto make standard solutions for the all assays. This solution was stored in dark at 4 ℃. The spectrophotometer (Ultrospec 2000) was used for measuring the absorbance.

3.4.1 Free radical-scavenging ability by the use of Folin-Ciocalteu assay

The total phenolic content was determined by Folin-Ciocalteu Assay which was described by(Singleton, Orthofer et al. 1999; Nenadis, Lazaridou et al.

2007).A dilution series of the propyl gallate stock solution (0.5 mM, 1mM, 1.5 mM and 2mM) was made and used as standard.A mixture of H_2O(5 ml), Folin-Ciocalteu phenol reagent (0.5 ml), and (0.5 ml)herbextracts or standard solutions was made. 1 ml 20% Na_2CO_3 was added after exactly 3 min, and the volume was brought up to 10 ml by adding 3 ml H_2O. The samples were covered and incubated for 1 hour at room temperature. The absorbance was recorded at 725 nmwith water as reference. For each compound and concentration, suitable blank solutions were used for measurement.

3.4.2 Free radical-scavenging ability by the use of $DPPH^{\bullet}$ assay

The radical scavenging activity was measured by DPPH assay (2,2-diphenlhydrazyl). The method was done as described by (Nenadis, Lazaridou et al. 2007; Thiansilakul, Benjakul et al. 2007):
A series of dilutions from the propyl gallate stock solution (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) was preparedand the volume was reached to 10 ml, made standard solutions. An aliquot (1.5 ml) diluted extract/standard and 0.15 mMDPPH in 96% ethanol (1.5 ml) were added and mixed. The samples werestored in the dark place at room temperature for 30 min. Then the absorbance was recorded at 517 nm with 96% ethanol as reference. The working solution of the stable radical $DPPH^{\circ}$ was made with magnetic stirring 1 day before the analysis and was kept overnight at 4 ℃.

3.4.3 Free radical-scavenging ability by the use of$ABTS^{\bullet+}$ assay

The radical scavenging activity was also measured with ABTS assay. The method was done as described by ((Nenadis, Lazaridou et al. 2007), (Re, Pellegrini et al. 1999), (Nenadis, Wang et al. 2004)).
The $ABTS^{\circ+}$ solution was prepared by reaction of 25 ml of a 7 mM aqueous ABTS solution and 440 μl of a 7 mM potassium persulfate$K_2S_2O_8$. The solution was kept overnight in the dark and at room temperature.
A series of dilutions from the propyl gallate stock solution (10 μM, 20 μM, 30 μM, 40 μM and 50 μM) was made and the volume was brought up to 10 ml by methanol 80% to make propyl gallate standard solutions. ABTS (2 ml) was

mixed with 200 μl of extracts/standard solutions and left to incubate for 6 min at room temperature. Theradical cation solution was diluted in methanol until the absorbance value of 0.75 $\pm$0.05 at 734 nm was reached. Water was used as a reference.

3.5 Oxygen uptake measurements

This method is based on the methodology described by Mozuraityte et al. who studied free iron-catalyzed oxidation of cod roe phospholipids using the oxygen uptake method ((Mozuraityte 2007), (Mozuraityte, Rustad et al. 2006)), and found working conditions for this system and method.
The initial stage of lipid oxidation can proceedrapidly by adding a promoter of lipid oxidation into a lipid system. In addition, lipid oxidation can be easily observed by recording oxygen consumption. Meanwhile, the effect of an antioxidant on the system can be monitoredas fast as the oxygen consumption is altered (inhibited or accelerated or unchanged).Thus,the consumption of dissolved oxygen by liposomes was used to measure lipid oxidation.
A polarographic oxygen electrode, which is a pivotal part of the Oxygraph system (Hansatech Instruments Ltd., Norfolk, UK), was used to continously measure the concentration of dissolved oxygen in the reaction mixture.
The Oxygraph system (Figure 16) consists of an electrode unit fixedon a control unit. The electrode disk is mounted as the surface of the reaction cell, and liposomes or other reaction mixtures can be added to it. A magnet can help to mix the reaction mixture to maintain equally distributed oxygen all over the volume.A plunger inhibits oxygen diffusion from the atmosphere and its capillary hole allows the injection of reactants during measurements.The reaction cell is water-jacketed and the holes of a water jacket arelinked to a circulating water bath. The circulating bath allows running experiments at different temperatures.

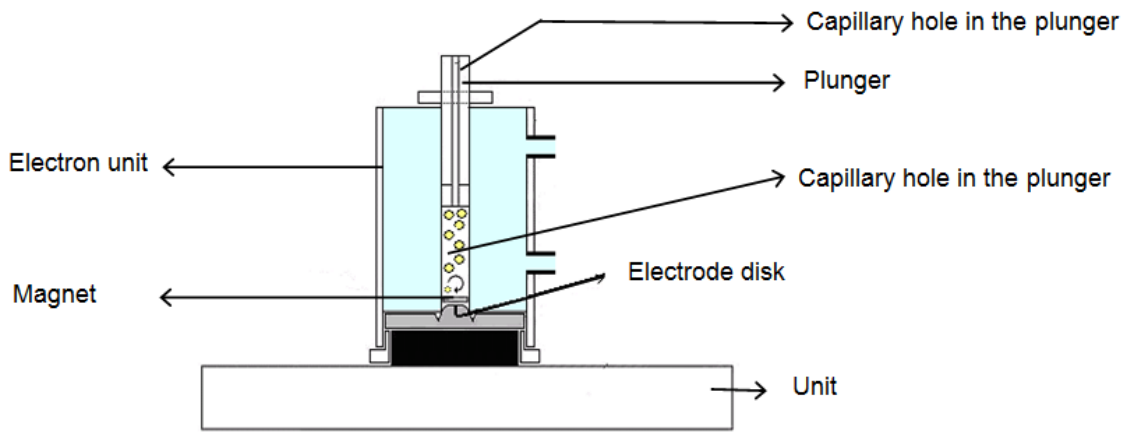


Figure 17: Schematic presentation of the oxygragh (Mozuraityte 2007).

The oxygen electrode (Figure 18) contains a platinum cathode and a silver anode. An electrolyte link between the electrodes is established by using a small amount of electrolyte solution (3 M KCl) on the dome area of the electrode disk, which is kept by a paper spacer and a polytetrafluorethylene membrane. Application of a stable polarizing voltage between the electrodes from the electrode control box causes 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 is proportional to the concentration of oxygen in the surrounding media (solution in the reaction cell) (Mozuraityte 2007).

Figure 18: Electrode disc (Mozuraityte, Rustad et al. 2006).

The following conditions areused for the reaction cells throughout this work:

1) Concentration of phospholipids: 3 % (w/v)
2) Volume of reaction mixture (liposomes dispersed in a 2 ml MES buffer)
3) pH 5.6 (this pH was selected because a maximum OUR for Fe-induced oxidation was observed at this pH).
4) Temperature: 30 ℃.

Stock solutions of propyl gallate (PG) were prepared in 80%methanol and stored at 4 ℃. Working solutions of propyl gallatewere prepared daily by diluting an appropriate aliquot of the stock solution with 80% methanol.

Stock solution of 30 mMFe^{2+} ($FeSO_4.7H_2O$)and30 mMFe^{3+} ($FeCl_3$)in 0.5 MHCLwere 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 (0.1 g/ml) was prepared daily by dissolving an appropriate amount of *Hb* in 5mM MES buffer (pH 5.6)(the working solution of 20 μl of Fe^{2+}, Fe^{3+} and *Hb* were added). The concentrations of prooxidants used in all this experiments were (calculated as a final concentration in the reaction mixture):

C (Fe^{2+}) = 20 μM
C (Fe^{3+}) = 20 μM
C (HB) = 3μM

3.5.1 Oxygen uptake rate (OUR)

When measuring concentration of dissolved oxygen, a background oxygen uptake rate (OUR) was observed for 4 – 6 minutes before addition of an antioxidant or MES buffer (blank) into the system.After injection of an antioxidant (or MES), a background OUR was observed again until it became steady for at least 2 minutes.A prooxidant (Fe^{2+}, Fe^{3+} and Hb) was added to the system as soon as a constant backgroundOUR (r_2) was reached. Afterwards, concentration of dissolved oxygen rapidly decreased.The OUR of total oxidation (r_2)was assayed after addition of a prooxidant.
The rate of oxidation (r) was obtained by reducing the background OUR from the total OUR (Pulido, Bravo et al. 2000). In the other words, rate calculation function calculates the rate between a pair of user-define Start and End points. An example of an OUR measurement is shown in Figure 19. The period of experiments for oxygen uptake ranged 20 – 40 min.

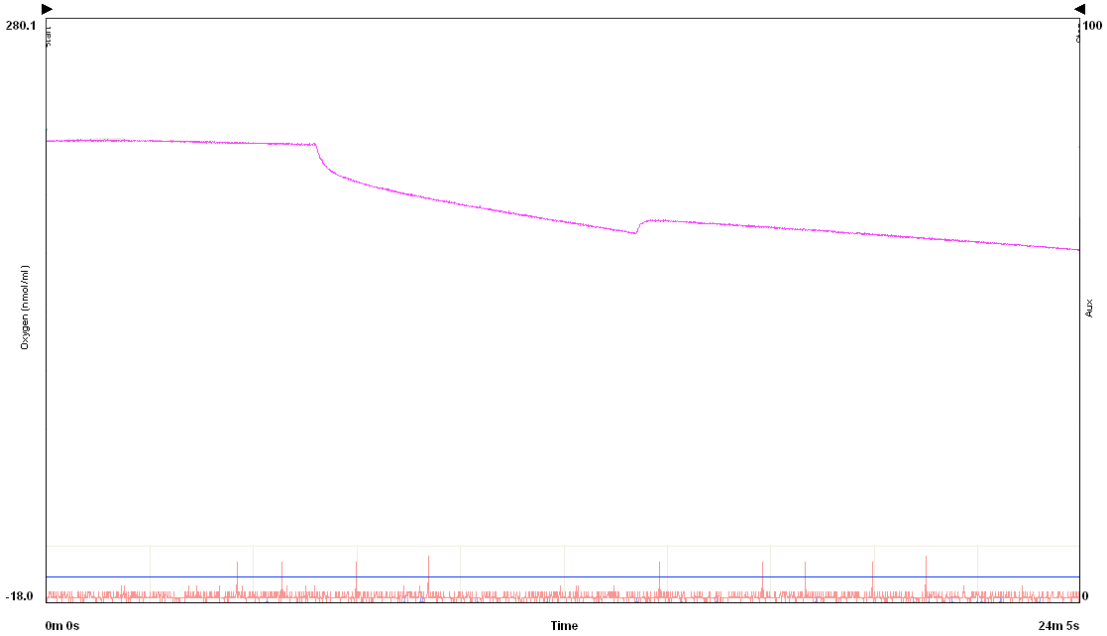


Figure 19: Oxygen uptake rate measurement by polarorganic oxygen electrode.

Oxygraph software oxyg32” was used to measure the OURs.In order to measure the antioxidant effect, the rate of inhibited oxidation was compared to the rate of non-inhibited oxidation (appropriate blank).Three or four parallel measurements were run for each concentration of each antioxidant.

4 Results and Discussion

4.1 Antioxidant capacity assays

In this study, three spectroscopic antioxidant capacity (AOC) assays were used to measure the antioxidant capacity of propyl gallate and 12 different herbs (Lemon balm, Tarragon, Chive, Thyme, Parsley, Sage, Rosemary, Caraway, Dill, Lemon pepper, Dill (Mills) and Basil).

1) Folin-Ciocalteu Assay (FC assay)
2) 2,2-Diphenyl1-1-picrylhydrazyl Radical Scavenging Assay (DPPH assay)
3) 2,2-Azinobis-3-ethylbenzotiazoline-6-sulfonic acid Assay (ABTS assay)

Propyl gallate (PG) was used as a reference due to its high efficiency in all the assessments above. A standard curve was made using PG for the three assays.

4.1.1 Folin-Ciocalteu Assay

Since phenols are responsible for the majority of the antioxidant activity in most plant- derived products (Singleton, Orthofer et al. 1999), the antioxidant activity can be found bymeasuring the total amount of phenols. The Folin-Ciocalteuassay was therefore used to determine the total amount of phenols. The Folin-Ciocalteu (FC) assay is also known as the Gallic acid Equivalence method (GAE), which is a mixture of phosphomolybdate and phosphotungstate (Miliauskas, Venskutonis et al. 2004). The assay is used for the colorimetric in vitro measurement of phenolic and polyphenolic antioxidants (Miliauskas, Venskutonis et al. 2004)The reagent will react with all the reducing substances in the samples (Prior, Wu et al. 2005). The slope value of a linear curve shows the reduction capacity. In other words, it explains the dependency of absorbance as a function of phenol concentration. Propyl gallate was used as a reference due to its high reactivity with the FC reagent. The standard curve was also used to determine the total phenolic content of the herbs.

The herb extracts were diluted in both 1:10 and 1:100 and the absorbance values were measured based on the FC method. Since most of the absorbance values for dilution 1:100 were too low, their phenolic content was

not measured. Figure 1 shows the absorbance valuesfor the PG standard curve based on the FC measurement.

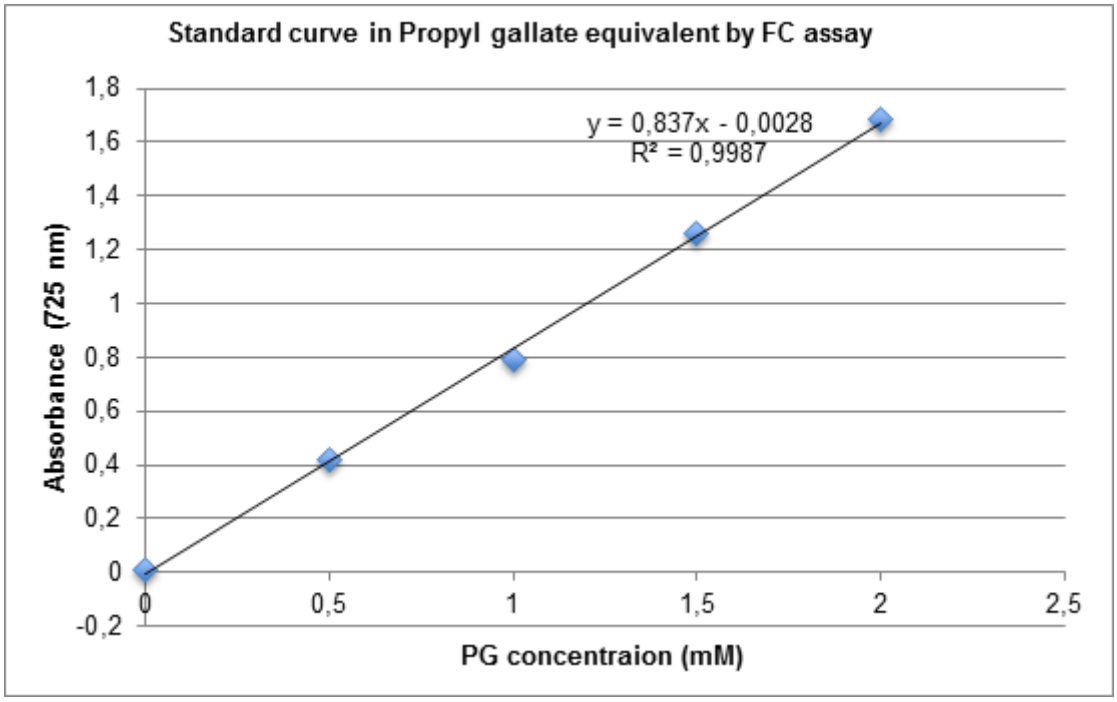


Figure 20: Exhibition of absorbance values by different concentrations of propyl gallate (PG) based on the Folin-Ciocalteu (FC) Assay. Methanol 80 % was used as blank. The reducing capacity is expressed as the slope value (0.837) of the linear curve.

According to the trend above, increasing the concentration of PG solution leads to an enhancement in absorbance. Thus, the obtained FC results show a direct relationship between absorbance and the different concentrations of PG. The standard curve was linear between the concentration of 0.5mM and 2 mM, where absorbance ranging from 0.012 to 1.68.
80% methanol was used as blank.The herb extracts with an absorbance that is within the range of the standard curve can be used for determination of total phenolic contents.
The reducing capacity is expressed as the slope value (0.837) of the linear curve and shows how much the absorbance increases with regard to the concentration. Total phenolic compound, determined using FC method and antioxidant activity in propyl gallate, showed a good correlation (R^2= 0.9987).

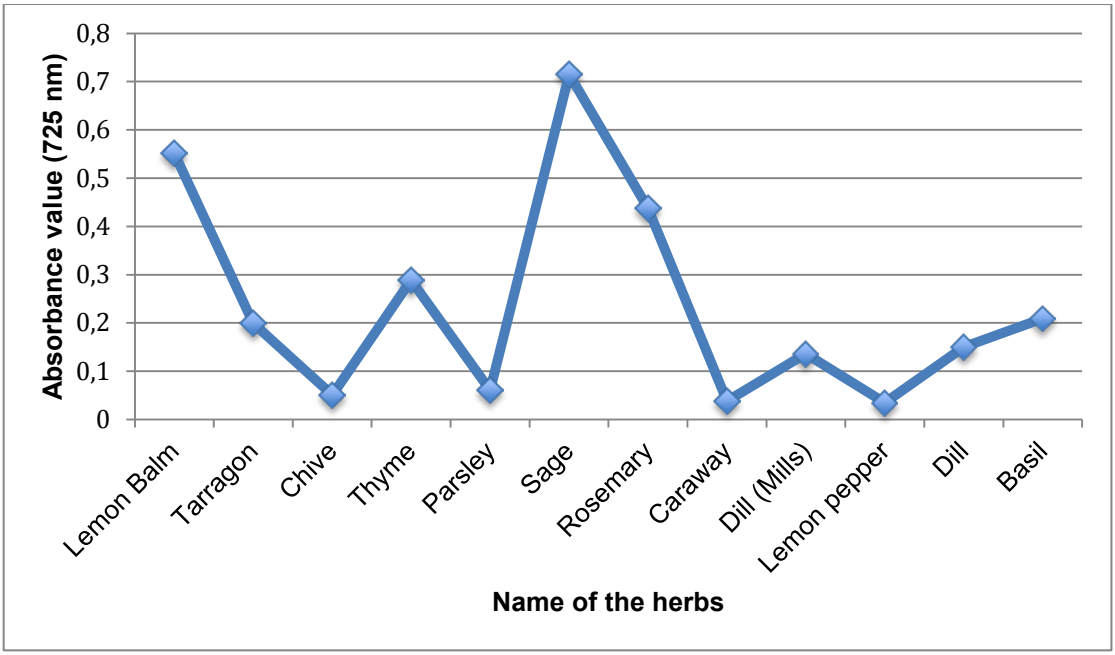


Figure 21: Absorbance values for 12 herbs (with dilution 1:10) based on the Folin-Ciocalteu (FC) Assay. Distilled water was used as reference.

Figure 2 shows the absorbance values for the herb extracts, which where diluted 1:10. The absorbance value measured by FC assay method,varied widely between the herbs and ranged from0.03to 0.71. The highest level of absorbance was found in sage (0.71), while the lowest level of absorbance was seen in Lemon pepper (0.03).Theabsorbancevalues of all the herbswere within the range of the standard curve values. Lemon balm (0.55), Rosemary (0.43), Thyme (0.28) and Basil (0.20) also had high levels of absorbance. Tarragon (0.19), Dill (Mills) (0.13) and Dill (0.15) had relatively low values of absorbance, whereas Caraway (0.03), Chive (0.05), Parsley (0.06) the absorbance values were below the lowest standard.Based on(Kim and Lee 2004; Shan, Cai et al. 2005), the differences between the herbscould probablybe due to variations in genotypic and environmental properties (climate, location, temperature, fertility and pest exposure). Among the studied herbs, Lemon balm, Thyme,Rosemary and Basil showed good absorbance.

Based on the FC assay, the total phenolic content for the 1:10 diluted extracts measured in PG equivalents, and is displayed in table 2.

Table 2: Overview of the results obtained by the FC assay for 1:10 diluted extracts. According to the correlation formula, the PG concentration was calculated: (Absorbance +0.0028) /slope (0.837). The absorbance values were used to measure the phenolic content i PG equivalents per gram dried herb.

Name of the herbs	PG equivalentsper gram dried herbs
Lemon Balm	331.42
Tarragon	120.55
Chive	32.14
Thyme	174.31
Parsley	38.11
Sage	428.79
Rosemary	263.32
Caraway	24.37
Dill (Mills)	82.92
Lemon pepper	22.58
Dill	91.28
Basil	126.52

As mentioned in the methods, 0.5 g of herb extracts were diluted in 25 ml methanol 80%. So there would be 0.02 g extract in 1 ml methanol. To calculate the phenolic content, PG amount was measured in the extracts through the following formula: PG concentration= (absorbance+0.0028) / 0.837.

Among the obtained values for total amount of phenols in PG equivalents, Lemon Balm (331.42), Sage (428.79), Rosemary (263.32) and Thyme (174.31) had the highest concentration of propyl gallate antioxidant, whereas Chive (32.14), Parsley (38.11), Caraway (24.37) and lemon Pepper (22.58) had the lowestvalues.

(Singleton, Orthofer et al. 1999) suggested Gallic acid as a suitable reference substance in their modified FC method. This study presentsthat propyl gallate has a good reactivity with the FC reagent and relatively low absorbance values. In some other studies Gallic acid was replaced with the other reagents such as catechin, tannic acid, chlorogenic acid, vanillic acid or ferulic acid (Prior, Wu et al. 2005). Since the reference substance will have an effect on

the value for the total phenolic content, the reference compound should be selected carefully. The reducing capacity of the samples might look to be too high if the reference compound provides low absorbance values.

In the structure of antioxidants, a high number of available hydroxyl groups in the aromatic ring increase reduction capacity. Moreover, the presence of other substituents such as methoxy group could have an effect on reduction ability (Rice-Evans, Miller et al. 1996). Indeed, the methoxy-substituted benzene in PG could further present high reduction ability, and this could be the explanation for high reaction of PG with the FC reagent. On the basis of the FC results, PG shows a good protection because of the redox reactions in its mechanism. The ability of reduction activity cause probable redox reactions with transition metals (Fe, Cu). PG can chelate metal ions and promote redox reactions, which is one of the indirect antioxidant mechanisms (Damodaran and Parkin 2008) .

The procedures was based on the improved FC assay and described by (Singleton, Orthofer et al. 1999) (Nenadis, Lazaridou et al. 2007).In this experiment timing of additions and length of incubation was changed and 20% Na_2CO_3; however, (Miliauskas, Venskutonis et al. 2004)used 25% Na_2CO_3 in their project.

Precipitation and dispersed particles were made by saturated solution, which made the measurement of the absorbance values difficult. Since 1-hour reaction time has been announced to be appropriate for the completion of reactions, the incubation time was changed to 1 hour. Based on (Stratil, Klejdus et al. 2006), (Singleton, Orthofer et al. 1999) longer reaction time might leads to the unstable reaction products.

Although several studies of bioactive compounds and their total content have been done so far, the structure-function relationship of phenolic compounds in the studied herbs is not entirely explained.

There have been some studies that have used the FC assay to measure the phenolic compounds in some selected herbs. Phenolic acids and flavonoids are known asthe main typical phenolics that have antioxidant activity. Particularly, phenolic acids are a major class of phenolic compoundsfound in fruits and vegetables.(Wojdyło, Oszmiański et al. 2007)used HPLC analysis to evaluate the phenolic acids and flavonoids as the major phenolic

compoundsin 32 herbs. Their research group foundCaffeic acid (CA), Neochlorogenic acid (NCA), p-coumaric acid (p-CA) and Ferric acid (FA) as phenolic acids in some herbs.
They quantified the absorbance with respect to the standard curve of Gallic acid and they expressed the results as Gallic acid equivalents. Based on the FC assay, they foundphenolic acids in Sage, Rosemary, Caraway and Thyme; however, flavonoidswere found just in Sage, Rosemary and Caraway.Of the five herbs (Lemon balm, Rosemary, Thyme, Caraway, Sage) that were studied both in their research and this project, they found the highest phenolic content in Lemon balm, while the lowest was found in Caraway.

Furthermore, some flavonoidsaglycones such as quercetin (QUE), kaempferol (KAEM), luteolin (LUT), apigenin (API), myricetin (MYR) and isorhamnetin (IZORHA) were found as the phenolic contents in some of the herbs (Wojdyło, Oszmiański et al. 2007). They also found high amount of flavonoids (LUT) in Rosemary.
Volatile oils, phenolic acids and flavonoids (kaempferol), coumarins are found as the major types of phenolic compounds in Caraway (Shan, Cai et al. 2005).
They also reported phenolic acids (rosmarinic acid,caffeoyl derivatives),phenolic diterpenes,volatile compounds(carvacrol), flavonoids(catechin) in Basil. Additionally, phenolic acids (caffeic acid,rosmarinic acid,caffeoyl derivatives),phenolic diterpenes(carnosic acid, carnosol,epirosmanol),volatile compounds(carvacrol) and flavonoids were found in Rosemary. Phenolic acids (rosmarinic acid),phenolic diterpenes(carnosic acid), volatile compounds and flavonoids were found as major compounds in Sage. Moreover, phenolic acids(gallic acid, caffeic acid,rosmarinic acid),volatile compounds(thymol), phenolicditerpenes and flavonoids were found in Thyme.
It was found that Rosemary has high antioxidant activity, this was also found in this work (Wojdyło, Oszmiański et al. 2007).
The results from (Wojdyło, Oszmiański et al. 2007) present antioxidant properties in Rosemary, Sage and Lemon bam, which belong to Labiatae

family. According to their results based on the FC assay, it was expected to find phenolic acid and flavonoids such as flavones and flavonols in the herbs. The obtained absorbance ranges represent a range of phenolic content in the herbs.

A high concentration of antioxidant was found in Lemon balm and Rosemary on the basis of FC assay (Wojdyło, Oszmiański et al. 2007), and this result was in agreement with our results.Basil also had a high antioxidant concentration in this study and could be a choice for oxygen uptake along with Lemon balm, Rosemary, Sage and Thyme.

It has been reported that Sage did not have a high phenolic content (Wojdyło, Oszmiański et al. 2007) and this data was not in agreement with the data from the present study. In this study, the phenolic content of Thyme was found to be the 4th highest of the herbs studied (about the half value of Lemon balm) and had a high phenolic content measured by FC assay, estimated by PG equivalents, and the result was in agreement with the data reported by (Wojdyło, Oszmiański et al. 2007).

Different results from the (Wojdyło, Oszmiański et al. 2007) observations in some herbs can be explained bythe fact that the phenolic compounds in the same herbs can be influenced by several internal and external factors such as climate, location, temperature, ways of preparation and stage of growth.

Folin-Ciocalteu assay was used to determine the total content of phenolic compounds in vegetables (Stratil, Klejdus et al. 2006). They selected Gallic acid equivalent as the standard and compared their results with published results of(Vinson, Dabbagh et al. 1995; Kähkönen, Hopia et al. 1999). They all reported a low phenolic content of Parsley, which is in agreement with the results of the present study.

Totally on the basis of the FC assay, the selected three herbs (Lemon balm, Rosemary and Sage) performeda high phenolic content. Based on the results for determination of total phenolic contents, the studied herbs can work as antioxidants.

4.1.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging Assay

The DPPH·is one of the most commonly used methods to determine antioxidant activity. Free radical-scavenging activity was determined using the DPPH method.

The herb extracts were diluted both 1:10 and 1:100 and the absorbance values were measured based on the DPPH method. Since some of the absorbance values for dilution 1:10 were too low and some were not within the range of the standard curve, the phenolic content was not measured. Figure 3 shows the absorbance valuesfor the PG standard curve based on the DPPH measurement.

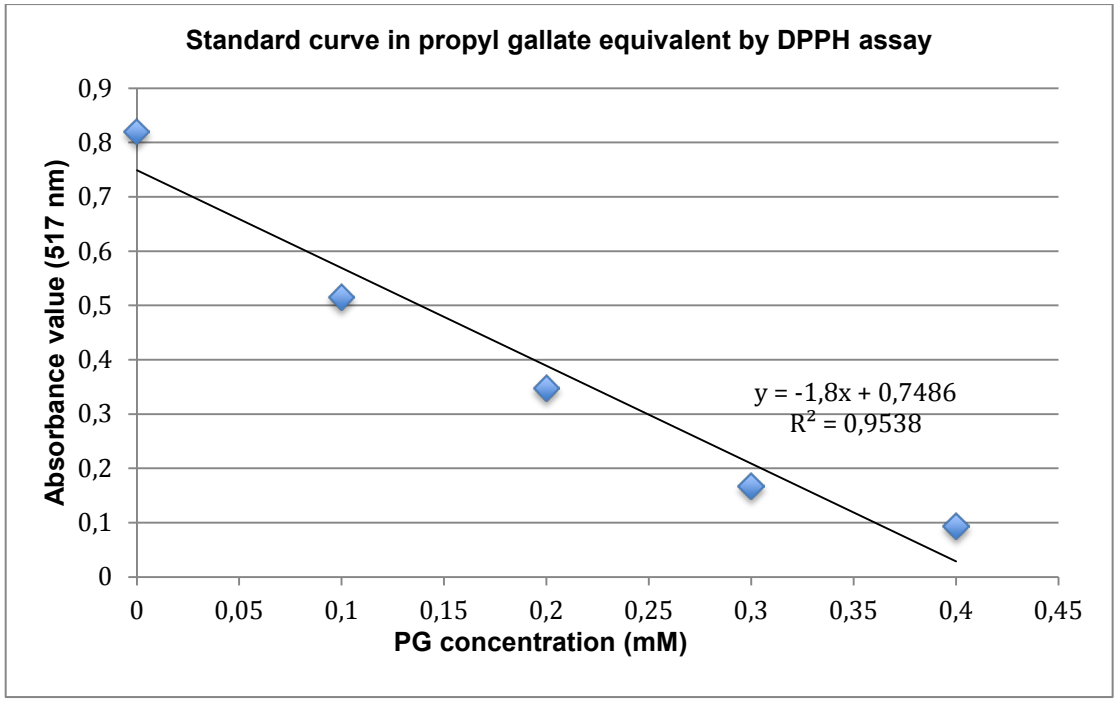


Figure 22: Exhibition of absorbance values by different concentrations of propyl gallate (PG) based on the DPPH Assay. Methanol 80 % was used as blank.

The figure above presents thatincreasing the PG concentration causes a decrease in absorbance on the basis of DPPH method. The obtained DPPH results show a direct relationship between absorbance and the different concentrations of PG. The total antioxidant activity, measured by the DPPH method, ranged from 0.094 to 0.820.

The reducing capacity is expressed as the slope value (-1.8) of the linear curve and shows how much the absorbance decrease with regard to the concentration.

DPPH method was used to measure the radical scavenging activity in Gallic acid equivalent by (Stratil, Klejdus et al. 2006).

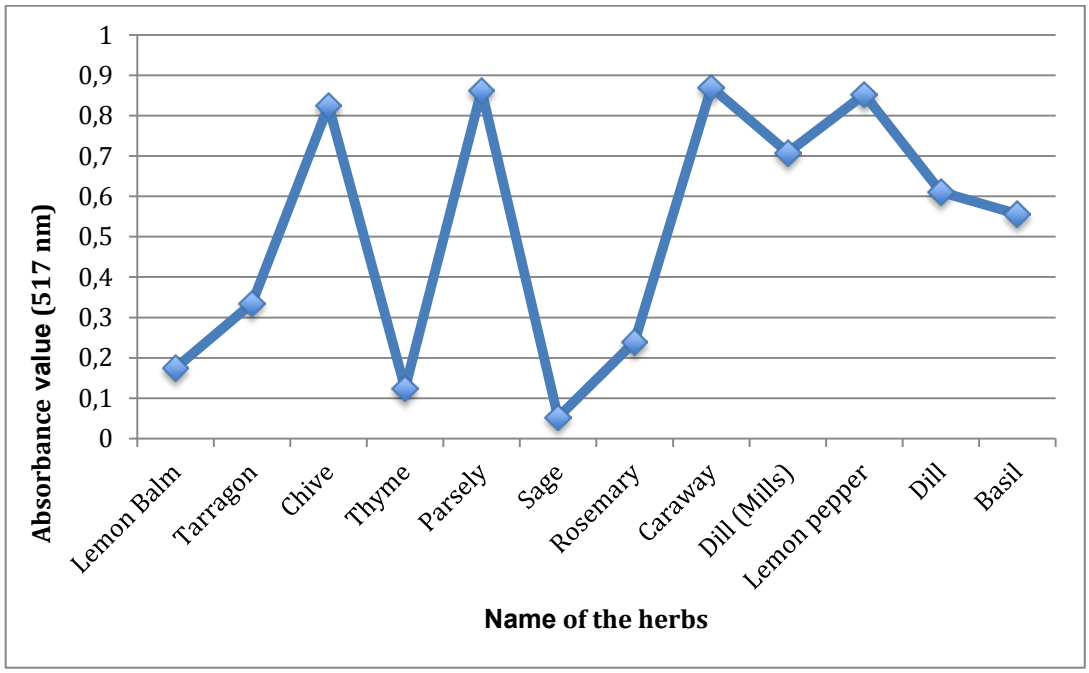


Figure 23: Absorbance values for 12 herbs (with dilution 1:100) based on the DPPH assay. Ethanol 96% was used as reference.

In the present study allthe substances used for the DPPH method were dissolved in 80% methanol as a polar solvent; however, the DPPH was dissolved in 96% methanol. Figure 4 shows the absorbance values for the herb extracts, which where diluted 1:100 by the DPPH method. The absorbance value measured by DPPH method, varied widely between the herbs and ranged from 0.05 to 0.87. The high absorbance values were found in Chive, Parsley, Caraway, and Lemon pepper, and were outside the range of the standard curve. The highest level of absorbance with respect to the standard was presented by Dill (Mills) (0.70), while the lowest was seen in Sage (0.05)andthis value was below the lowest standard. Among the 12 herbs tested on the DPPH basis, Rosemary (0.24), Tarragon (0.33), Dill (0.61) and Basil (0.55) also had high levels of absorbance. Lemon balm (0.17) and Thyme (0.12) had relatively low values of absorbance. Low absorbance values present a high antioxidant capacity/scavenging activity.As explained by (Kim and Lee 2004)(Shan, Cai et al. 2005), variations in genotypic and environmental properties such as climate, location, temperature and pest

exposure could lead to the different results amongst the herbs. Among the studied herbs, Rosemary, Tarragon, Dill (Mills), Dill and Basil showed low absorbance values.

Based on the DPPH assay, radical scavenging activity for both 1:10 and 1:100 diluted extracts were measured in PG equivalent, and is presented in table 3.

Table 3: Overview of the results obtained by DPPH assay for both 1:10 and 1:100 diluted extracts. According to the correlation formula, the PG concentration was calculated: (Absorbance - 0.7486) /slope (-1.8). The absorbance values were used to measure the radical scavenging activity in PG equivalents per gram dried herb.

Name of the herbs	PG equivalentsper gram dried herbs
Lemon Balm	1593.33
Tarragon	1148.89
Chive	-49.28
Thyme	1735.00
Parsley	-30.39
Sage	1932.22
Rosemary	1412.78
Caraway	30.72
Dill (Mills)	184.61
Lemon pepper	37.94
Dill	382.22
Basil	535.00

Dilution 1:100 was used for the following herb extracts: Lemon balm, Sage, Rosemary, Thyme, Tarragon, Dill and Basil. Dilution 1:10 was also used for the rest of the herbs (Chive, Caraway, Parsley, Dill (Mills) Lemon pepper).

To calculate thescavenging activity, PG value was measured in different extracts through the following formula:

PG concentration= (absorbance−0.7486) / -1.8.

According to table 3, the absorbance values for Chive and Parsleywere so high that they reached negative PG equivalent values based on the DPPH method.This means that nothing has happened to the DPPH radical. PG equivalent value for Parsley was close to zero in both dilutions, neither dilution

1:10 nor 1:100 could be used. Undiluted extracts could have been analysed, but as the aim of the AOC assays were to pick good candidates for the measurements using oxygen uptake rate, this was not done.

The DPPH method is based on the reaction with electron-donating or hydrogen radicals (H·) producing compounds/antioxidants regarding to the following reaction (Stratil, Klejdus et al. 2006):

$R\cdot + Aox^{-} H \rightarrow RH + Aox\cdot$

The purple colour of radical is lost during the reaction and the loss of absorbance was measured by a spectrophotometer. Basis on the obtained data, the PG presented a strong reaction with DPPH·. According to the former studies, DPPH· does not react with flavonoids without any OH-group in the B-ring or with monophenols containing only one OH-group attached to the benzene ring.

There have been many studies to prove both HAT and SET mechanisms are involved in the reaction of hydrogen-atom concept by the DPPH·, although one of the mechanisms is usually minor while the other dominates (Foti, Daquino et al. 2004). The hydrogen-binding characteristics of the sample solvent probably are a factor affecting on the ratio between the HAT and SET mechanisms. Thekinetic analysis of the reactions between phenolics (ArOH) and DPPH· in alcohol was done by (Foti, Daquino et al. 2004). It was proposed that the presence of phenoxide anions and a cascade of SET reactions affect the reaction of phenolicswith DPPH· (Foti, Daquino et al. 2004).The solvents with strong hydrogen bond slowly receive the hydrogen-atom abstracted from neutral ArOH by DPPH· and this becomes a marginal reaction. The researchers also determined the effect of acids or bases on the ionization equilibrium of the phenols. This leads to a reduction or enhancement in reaction rate.

ArOH$\Leftrightarrow$ Ar$O^{-} + H^{+}$ (Singleton, Orthofer et al. 1999)

ArO^{-} + DPPH·$\Longrightarrow$ Ar$O^{-} + DPPH^{-}$ (Huang and Frankel 1997)

$DPPH^{-} + H^{+} \Longrightarrow$H-DPPH (Foti, Piattelli et al. 1996)

Some other compounds in the extracts could react with DPPH· and cause the additional quenching (Prior, Wu et al. 2005). Since phenols react with highly reactive and transient radicals, they are usually involved in lipid peroxidation throughHAT mechanism (Halliwell 1995). This has beenone of the main criticisms in several reviews about DPPH assay ((Prior, Wu et al. 2005), (Roginsky and Lissi 2005)).

It is difficult to compare the data from other studies directly, since the results are obtained from different assays with varying conditions. It is even difficult to compare the same assays due to the several ways of expressing the results, which could be expressed in different equivalents (different standards) or different units.

The DPPH free radical (DPPH·) is known as a simple method that does not need any special preparation (Arnao 2000). It has been said that DPPH can only be dissolved in organic media such as ethanol, which is an important limitation, due to the hydrophilic and lipophilic nature of the compounds in samples (Wojdyło, Oszmiański et al. 2007).

Since the structure-activity of phenolic compounds requires extensive investigations, the phenolic correlation data figured out by previous studies are not sufficient enough.

(Wojdyło, Oszmiański et al. 2007) determined the DPPH radical scavenging activity by following (Yen and Chen 1995). The incubation time used for their prepared mixture was 10 min, while for the present study the incubation time was 30 min. They expressed the results in Gallic acid equivalents and quantified the absorbance with respect to the standard curve of Gallic acid. According to their report, the radical scavenging activity for Rosemary and Thyme was very high, whilst it was low for Sage, Lemon balm and Caraway. The results from (Wojdyło, Oszmiański et al. 2007) present the antioxidant properties in Rosemary, and Thyme.

There are some weaknesses in the DPPH method, which can affect the values (Prior, Wu et al. 2005). Many substances react slowly with DPPH· (Fukumoto and Mazza 2000), therefore low valued and underestimated radical scavenging activity could be obtained due to a short incubation period.

The influence of solvent to dissolve the sample on antioxidant activity was examined by (Pérez-Jiménez and Saura-Calixto 2006), and they observed that the effect of solvent in the DPPH assay was relatively low compared to the ABTS assay.

4.1.3 ABTS assay (2,2'-azino-bis), (3-ethylbenzothiazoline-6-sulphonic acid)

$ABTS^{+}$ is a popular antioxidant method, which measures the capability of an antioxidant to neutralize a radical cation formed by a single-electron to absorb $ABTS^{\cdot+}$ radical (Pérez-Jiménez and Saura-Calixto 2006).$ABTS^{\cdot+}$can be dissolved in aqueous and organic media where the antioxidant activity can be measured (Re, Pellegrini et al. 1999; Wojdyło, Oszmiański et al. 2007) used the decolorization to define the free radical scavenging activity.

In the present study allthe substances used for ABTS method were dissolved in 80% methanol as a polar solvent; however, $ABTS^{\cdot+}$ solution was dissolved with $K_2S_2\,O_8$.
In this study 6 min was used for incubation time and 80% methanol was used as a solvent, which were the same as (Wojdyło, Oszmiański et al. 2007).
Based on the ABTS method, the absorbance values were used to measure the radical scavenging activity in PG equivalent in the selected herbs. Figure 5 shows the absorbance valuesfor the PG standard curve based on the ABTS measurement.

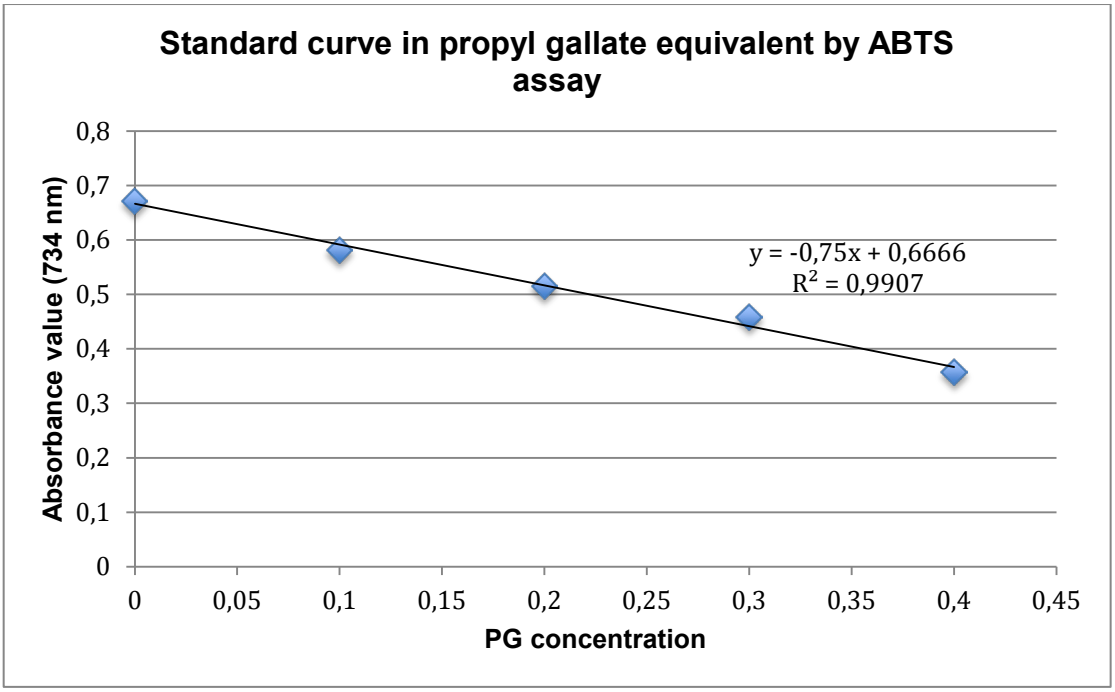


Figure 24: Exhibition of absorbance values by different concentrations of propyl gallate (PG) based on the ABTS Assay. Methanol 80 % was used as blank.

The figureshows that increaseinPG concentration leads to decrease in absorbance. The obtained ABTS results display a direct relationship between absorbance and the different concentrations of PG.

The absorbance values, measured by the ABTS method, ranged from 0.330 to 0.671.

The slope value (-0.75) of the linear curve shows how much the absorbance decrease with respect to the concentration. Antioxidant activity measured in propyl gallate equivalent, showed a good correlation (R= 0.99532).

(Stratil, Klejdus et al. 2006) observed a linear relationship between the decrease of $ABTS^{\cdot+}$ and the antioxidant concentration, and the antioxidant activity (ABTS value) was expressed as the slope value andcalculated by linear regression. Their observation is in agreement with our results.

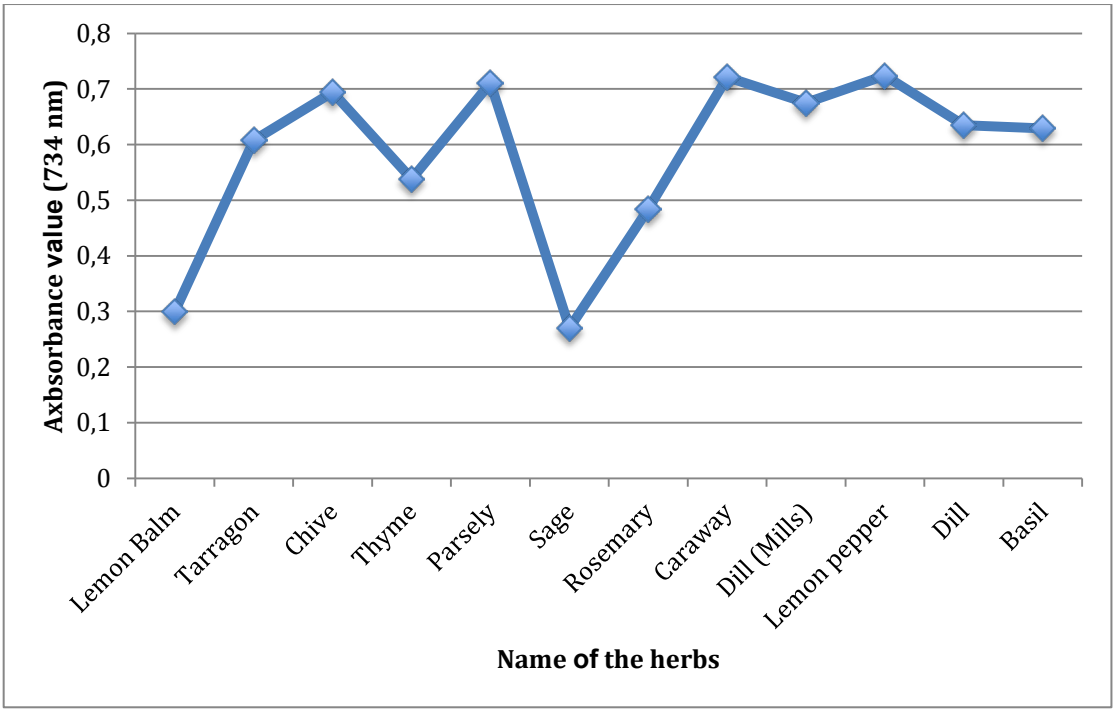


Figure 25: Absorbance values for12 herbs (with dilution 1:100) based on the ABTS assay. Distilled water was used as reference.

Figure6 shows the absorbance values of the herb extracts, which where diluted 1:100 by the ABTS method.

The absorbance value measured by ABTS assay, varied between the herbs and ranged from 0.033 to 0.671. The high absorbance values were found in Chive, Parsley, Caraway, Dill (Mills) and lemon pepper, which were not within the range of the standard curve. The highest level of absorbance with respect to the standard curve was presented by Dill (0.63), while the lowest was found in Sage (0.27). Based on the ABTS method,Tarragon (0.60) and Basil (0.62) also had high levels of absorbance among the selected herbs. In contrast, the lowest value of absorbancewas found in Sage (0.27).

On the basis of the ABTS assay, the recorded results following the standard curve indicate thatLemon balm, Sage and Rosemary exhibited good (low) absorbance values among the studied herbs.

Based on the ABTS assay, the radical scavenging activities for the 1:100 diluted extracts measured in PG equivalent, and are presented in table 4.

Table 4: Overview of the results obtained by the ABTS assay for 1:100 diluted extracts. According to the correlation formula, the PG concentration was calculated: (Absorbance - 0.6666) /slope (-0.75). The absorbance values were used to measure radical scavenging activity in PG equivalents per gram dried herb.

Name of the herbs	PG equivalentsper gram dried herbs
Lemon Balm	4604.67
Tarragon	2551.33
Chive	1978.00
Thyme	3018.00
Parsley	1864.67
Sage	4804.67
Rosemary	3378.00
Caraway	1798.00
Dill (Mills)	2104.67
Lemon pepper	1778.00
Dill	2371.33
Basil	2411.33

To calculate the radical scavenging activity PG value was measured in the extracts through the following formula:

PG concentration= (absorbance−0.6666) / -0.75.

Some of the values are well within the range of the standard curve; however, some herbs have absorbance values that are out of the range of the standard curve. On the basis of the ABTS assay, the recorded results following the standard indicate that Sage had the highest radical scavenging activity, while the lowest scavenging activity was found in Caraway.It should be noted that Sage should have been more diluted.

There have been other studies that have used the ABTS assay to measure the scavenging activities in some selected herbs.

Originally, the HAT reaction mechanism; atom donation by antioxidants was said to be the only mechanism responsible for quenching $ABTS^{\cdot+}$; however, advanced studies has reported that the reduction mechanism is involved in quenching $ABTS^{\cdot+}$ (Re, Pellegrini et al. 1999). It has been explained that the difference of the ratio between the HAT and SET mechanism could be due to the hydrogen binding of the solvent (Kristinová, Mozuraityte et al. 2009). For

instance, polar solvents like methanol promote the SET mechanism. The mechanisms probably change with respect to pH; for example, acid pH is a favour forelectron transfer (Prior, Wu et al. 2005).It was believed that HAT mechanism makes the phenols involved in lipid peroxidation (Halliwell 1995). Therefore, $ABTS^{\cdot+}$ as a scavenger of lipid radicals does not directlyreflect the antioxidant activity. It was observed that Propyl gallate with three OH group was the most active compound among the other antioxidants such as Ferulic acid (one OH group), Caffeic acid (two OH groups) and ρ-Coumaric (one OH group) (Kristinová, Mozuraityte et al. 2009). Indeed, more OH group makes PG more active than the other selected antioxidants.

One of the most controversial issues referring to ABTS limitations is that the ABTS values describe the ability of the tested substance to react with $ABTS^{\cdot+}$ in comparison with inhibiting the oxidative process (Roginsky and Lissi 2005), and this feature is common for DPPH assay.

It was found that Caffeic has a slow reaction with $ABTS^{\cdot+}$(Roginsky and Lissi 2005). Therefore, the time of incubation and the ratio of sample quantity affect the $ABTS^{\cdot+}$ concentration. In this study a 6 min incubation time was used. For substances that have a slow reaction with $ABTS^{\cdot+}$ this period might not be sufficient and may lead to miscalculated values.

Another limitation that has been reported by (Roginsky and Lissi 2005) was that the ABTS method has a poor selectivity in the reaction with H-atom donors. They declared that the correlation between $ABTS^{\cdot+}$ and hydroxylated aromatics did not depend on their real antioxidative potential. In their review, the ABTS assay was reduced in order to titrate the aromatic OH group, and those OH groups did not contribute to the antioxidative action.

(Pulido, Bravo et al. 2000) showed that the used solvents had a strong association with the ABTS values. It was said that the $ABTS^{\cdot}$ reagent was very unstable, and it was slowly degraded at the given experimental conditions (Stratil, Klejdus et al. 2006). They also observed a constant decrease of the initial absorbance of the $ABTS^{\cdot+}$ solution.

(Wojdyło, Oszmiański et al. 2007) determined the $ABTS^{\cdot+}$radical scavenging activity by followingthe method of (Re, Pellegrini et al. 1999). The incubation

time used for their prepared mixture was 6 min, and was exactly the same as used in our study. They expressed the results in Gallic acid equivalents and quantified the absorbance with respect to the standard curve of Gallic acid. Regarding to their report, the majority of their selected herbs had medium amounts of antioxidants.Theradical scavenging activity for Rosemary and Thyme wasrelatively high, whilst it was low for Sage and Lemon balm. The results from (Wojdyło, Oszmiański et al. 2007) present the antioxidant properties in Rosemary, and Thyme.

As mentioned earlier, (Pérez-Jiménez and Saura-Calixto 2006) examined the influence of solvent used to dissolve the sample on antioxidant activity, and observed a higher effect in the ABTS assay than the DPPH method.

The herb extracts were also diluted 1:10 and the absorbance values were measured based on the ABTS method. Most of the absorbance values for dilution 1:10 were too low and some were below the standard and therefore their scavenging activities were too high.

In this method, the antioxidant activities ofRosemary, Sage, Lemon balm and Thyme were high .The selected herbs might act as antioxidants or prooxidants, which have influence on the oxidation process.

As formerly noticed, it would be difficult to compare the data from other studies directly, since the results obtained from different assay versions and conditions. It is also impossible to compare the same versions because of several ways of expressing the results.

4.1.4 Comparison of the antioxidant capacity results

The three spectroscopic methods ($DPPH$, $ABTS$ and FC) were used to determine the antioxidant activity of 12 selected herbs with regard to phenolic contentand radical-scavenging activities.

Comparison of PG equivalents shows differences in the degree of the capacities for the same compound in the different assays (figure 26).

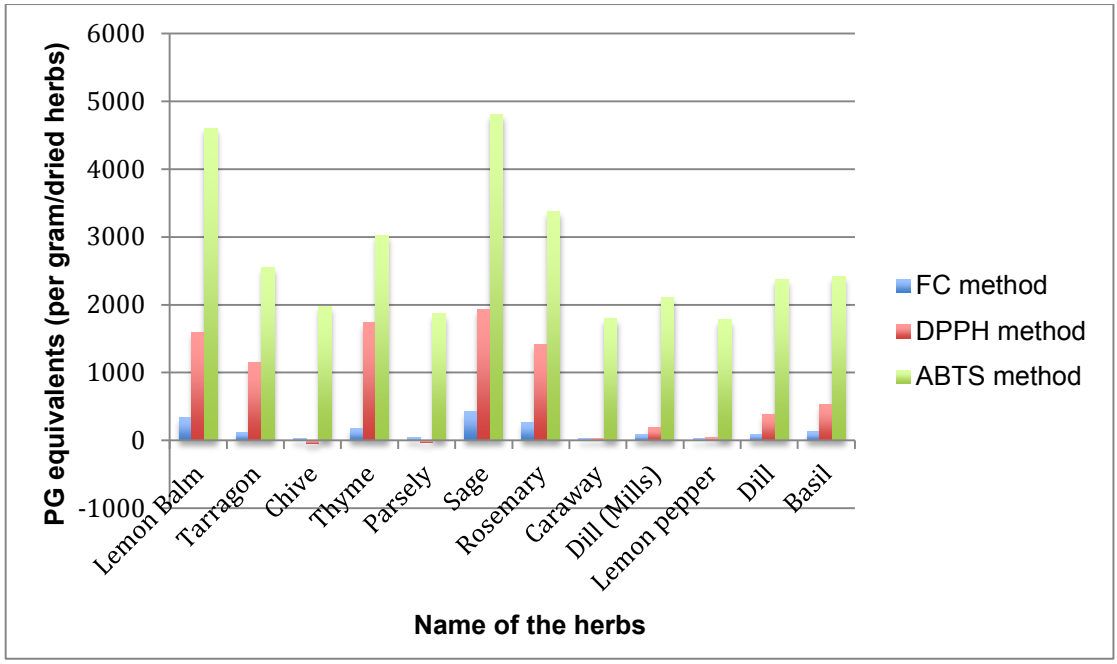


Figure 26: Comparison of the antioxidant capacities of the tested herbs determined by the different AOC assays. The values are expressed in propyl gallate (PG) equivalents (per gram/dried herb).

According to figure 26, among 12 herbs analyzed, Sage exhibited the highest scavenging of $ABTS^{\cdot+}$ and $DPPH^{+}$. Sage had also the highest content of phenolics measured by the FC assay, and high content of phenolics estimated in PG equivalents.Rosemary and Lemon balm had also high content of phenolics evaluated by the FC assay. Besides, they both exhibited a high scavenging of $ABTS^{\cdot+}$ and $DPPH^{+}$. On the contrary, Chive had the lowest scavenging of$DPPH^{+}$ in PG equivalents.

The relationship between the three AOC assays is shownin different figures.

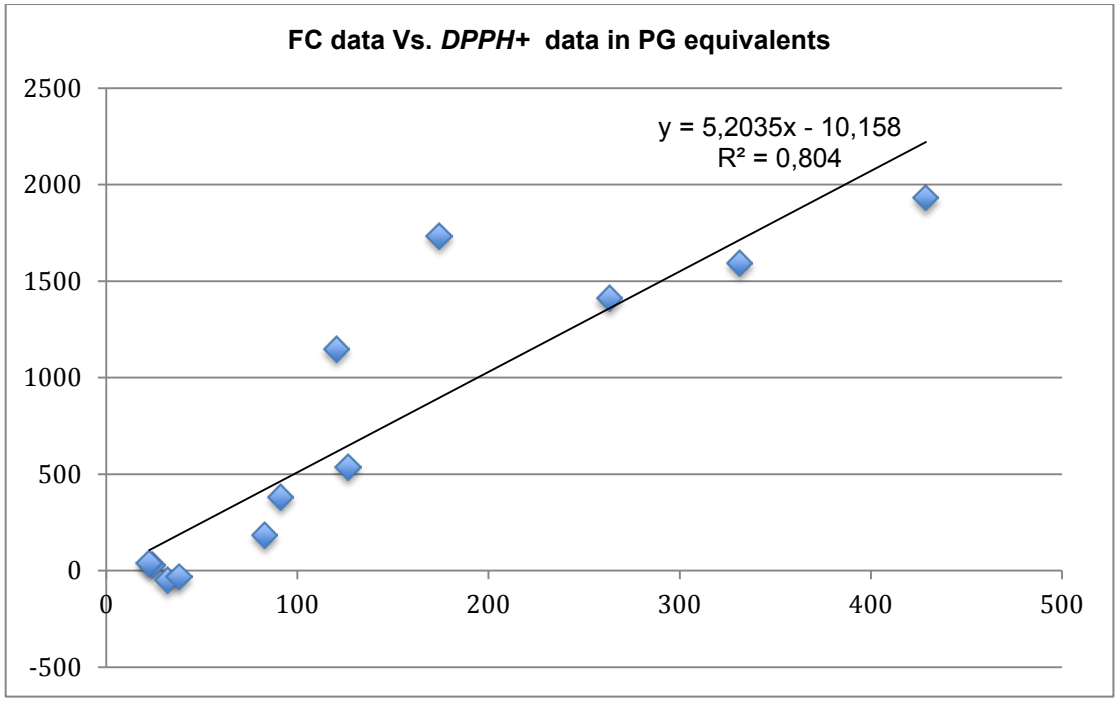


Figure 27: Comparison of the antioxidant capacities of the tested herbs determined by the FC and $DPPH^+$ methods expressed in PG equivalents per gram dried herbs.

Antioxidant activity, determined using FC and $DPPH^+$ in the 12 different plants showed a kind of correlation. The correlations with the selected herbs were: R^2 = 0.80 between $DPPH^+$ and phenolic content.

The recorded values showed some variation in antioxidant activity. These variations might be due to different reaction mechanisms and some specific interactions that may occur between the assay reagents and the studied herbs including some unrelated reactions, such as dimerization of the antioxidants, in the reaction mixture.The pH values also affect the reducing capacity of antioxidants (Huang, Ou et al. 2005).

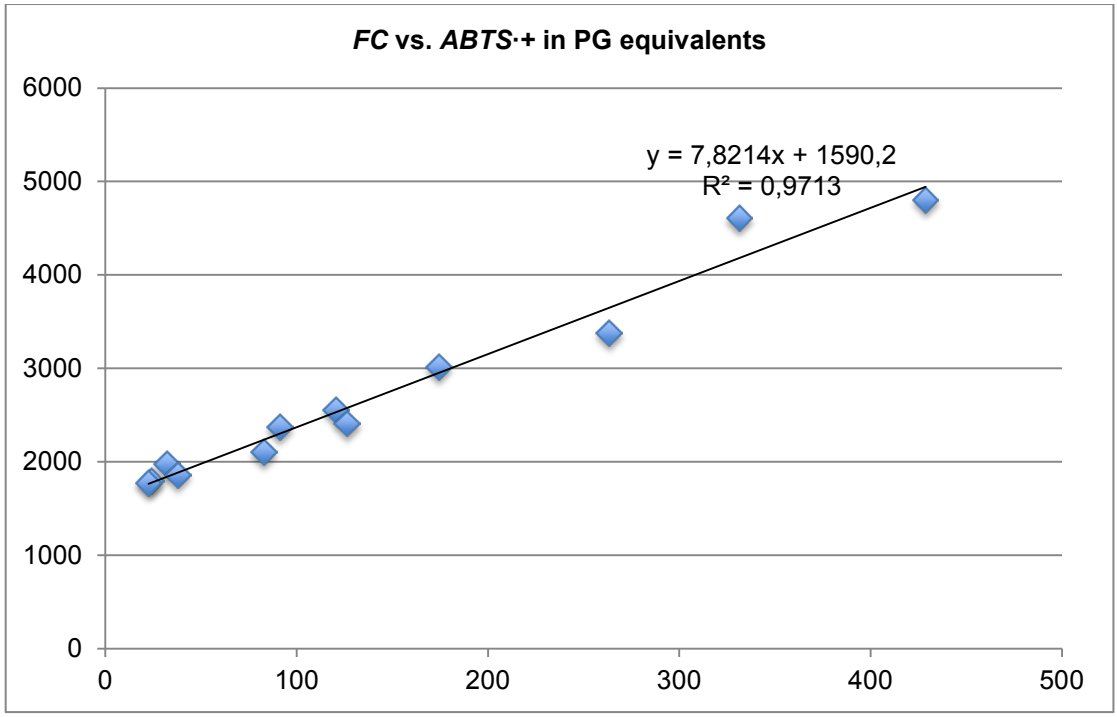


Figure 28: Comparison of the antioxidant capacities of the tested herbs determined by the FC and $ABTS^{\cdot+}$ methods expressing in PG equivalents per gram dried herbs.

Antioxidant activity, determined using FC and $ABTS^{\cdot+}$ in the 12 different plants showed a good correlation. The correlations with the selected herbs were: R^2 = 0.971 between $ABTS^{\cdot+}$ and phenolic content.

Totally, a significant linear relationship was found between the antioxidant activity, especially with FC and $ABTS^{\cdot+}$, while radical scavengers were major contributors to antioxidant activity.

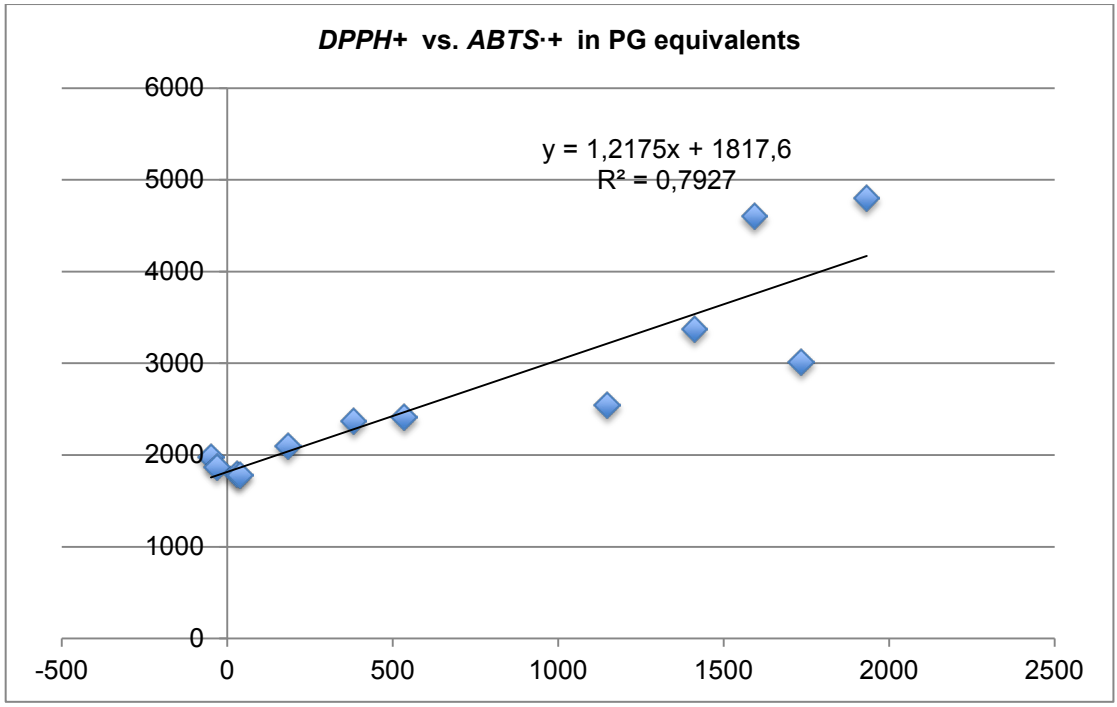


Figure 29: Comparison of the antioxidant capacities of the tested herbs determined by the $DPPH^+$ and $ABTS^{\cdot+}$ methods expressing in PG equivalents (per gram/dried herbs).

Antioxidant activity, determined using $DPPH^+$and $ABTS^{\cdot+}$ in the 12 different plants showed a kind of good correlation. The correlations with the selected herbs were: R^2 = 0.79 between $ABTS^{\cdot+}$ and $DPPH^+$.

The variation presented by figure 29 could be generated of reactions between some reducing compounds and FC reagent.

Antioxidant activity, determined using $DPPH^+$, $ABTS^{\cdot+}$ and FC in the 12 different plants showed the best correlation between $ABTS^{\cdot+}$ and FC (R^2 =0.97).

The results of this study proved the importance of radical scavengers in the antioxidant behaviour of the herb extracts and also they contribute significantly to the antioxidant capacity. Moreover, our results showed that Rosemary, Sage, Lemon balm and Thyme were rich in phenolic constituents and demonstrated good antioxidant activity measured by different methods. These plants, rich in flavonoids and phenolic acids could be a good source of natural antioxidants.

A positive and significant correlation existed between antioxidant activity and radical scavenging, measured by $ABTS^{\cdot+}$in most of the selected herbs,

revealing that free radical scavengers were the dominant antioxidant components.

It have been reported that the total phenolic content measured by the Folin–Ciocalteu procedure does not give a full picture of the quality or quantity of the phenolic constituents in the extracts ((Katsube, Tabata et al. 2004), (Wu, Beecher et al. 2004)). It has been found that caffeicand neochlorogenicacidare the main hydroxycynnamic acids in Lemon balm and Thyme (Wojdyło, Oszmiański et al. 2007). Though, ferulic acid occurred in small quantities in these plants. They found Caffeicacid with higher activity, comparable to quercetin (QUE).

Ferulic acid showed an inhibition effect on the photo-peroxidation of linoleic acid at high concentrations (Carvajal). Rosmarinic acid is the main antioxidant constituentalso containing hydrocaffeic and caffeic acids(Kim and Lee 2004). Hydrocaffeic acid and caffeic acid originates from enzymatic degradation of rosmarinic acid.A trace amount of flavonoids and phenolic acids in Rosemary was found by (Wojdyło, Oszmiański et al. 2007), but nohydrocaffeic or caffeic acid was found. Theyrevealed that rosmarinic acid holds higher antioxidant activity thanCaffeic acid.

Generally, antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups. 3',4'-orthodihydroxy configuration in ring B and 4-carbonyl group in ring C is the essential necessity for effective radical scavenging.The presence of 3-OH group or 3- and 5-OH groups, giving a catechol-like structure in ring C also affect the antioxidant activity of flavonoids. The presence of the C2–C3 double bond configured with a 4-keto arrangement is known to be responsible for electron delocalization from ring B and leads to an increase in the radical-scavenging activity.A catechol structure in ring A cancompensate for flavonoid antioxidant activity in the absence of the o-dihydroxy structure in ring B.

The relationship between the chemical structure of flavonoids and their radical-scavenging activities was studied by (Bors, Heller et al. 1990). They found that Quercetin has a catechol structure in ring B, as well as a 2,3-double bond in conjunction with a 4-carbonyl group in ring C,admitting for delocalization of the phenoxyl radical electron to the flavonoid nucleus.The

presence of a 3-hydroxy group with a 2,3-double bond increases the resonance stabilization for electron delocalization and therefore it has a higher antioxidant value. Flavonols (quercetin, myricetin, kaempferol and isorhamnetin) have a hydroxyl group at position 3 (Kim and Lee 2004), which proposes an important role of the 3-OH group of the chroman ring leading to an enhancement of antioxidant activity.

High antioxidant capacities in Thyme, Rosemary and Sage (Wojdyło, Oszmiański et al. 2007) and they also isolated methylated flavones and essential oil from these plants. Additionally, many studies revealed that essential oils (thymol, thyme, rosmanol) were main components that showed high antioxidant and antimicrobial activity ((Shan, Cai et al. 2005),(Wang 2002)).

Through our systematic comparative study of 12 selected herbs, some herbs were excellent free radical-scavengers and potent natural phenolic antioxidants for oxygen uptake measurement. 5 herbs were selected to be used for oxygen uptake measurement, including Sage, Rosemary, Lemon balm, Dill and Basil. Sage, Rosemary and Lemon balm was chosen due to their high antioxidant activity, determined by three AOC methods. Dill and Basil were selected due to their high antioxidant activity, determined by $ABTS^{\cdot+}$method.

4.2 Oxidation of liposomes

Transition metals and some metalloproteins, such as hemoglobin (Hb) and myoglobin, are known as potent promoters of lipid oxidation even in trace amounts (Damodaran and Parkin 2008). In this study, the effects of Fe^{2+}, Fe^{3+} and bovine hemoglobin (Hb), as initiators (prooxidants) of lipid oxidation was observedin the liposome system.

Figure 30, 31 and 32 show oxygen consumption by liposomesbefore and after adding prooxidants.

Figure 30: The kinetics of oxidation of 0.6% liposomes induced by Fe^{2+} (50 µM). 5 *m*MMES buffer was used as solvent.

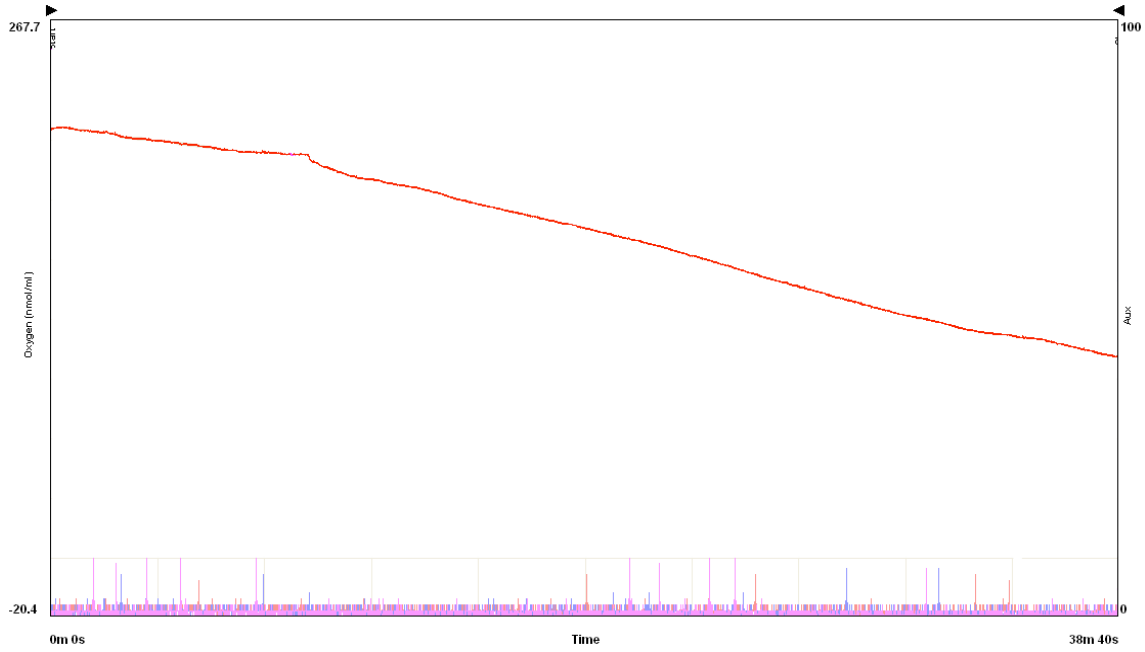


Figure 31: The kinetics of oxidation of 0.6% liposomes induced by Fe^{3+} (µM). 5*m*MMES buffer was used as solvent.

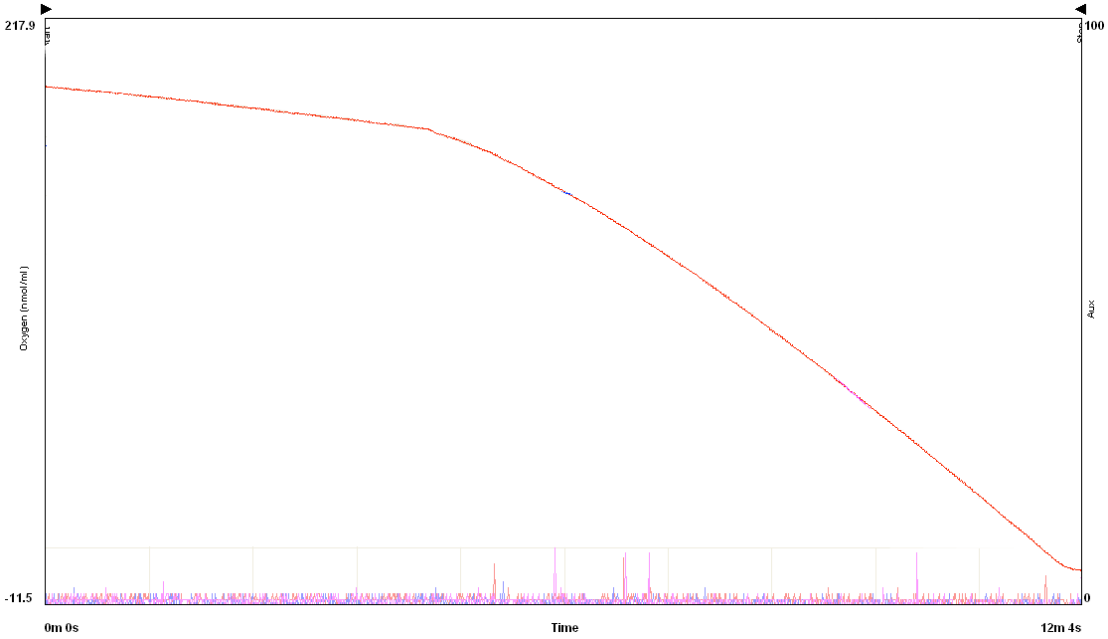


Figure 32: The kinetics of oxidation of 0.6% liposomes induced by *Hb* (60 µM).5 *m*MMES buffer was used as solvent.

There is a slow and linear decrease in concentration of dissolved oxygen before adding the prooxidants. This consumption of dissolved oxygen by liposomes is referred to liposome initial activity. The oxygen uptake rate (OUR) of pure liposomes was measured as background OUR.

The presence of pre-formed (endogenous) peroxides and the presence of endogenous transition metals (Pt, Fe, Cr) could be responsible for the liposome initial activity. These metals can be released into the liposome solution during sonication of phospholipidsor can be found as contaminants in chemicals.

When Fe^{3+} was added to the liposome, an initial drop in concentration of dissolved was observed which was followed by the slower linear decrease in concentration of dissolved oxygen. The initial drop is caused by oxidation of Fe^{2+} to Fe^{3+}. After the initial fast oxygen uptake, equilibrium between Fe^{2+}andFe^{3+} is achieved and a constant rate of oxygen consumption is observed (Mozuraityte 2007).

As mentioned earlier, the mechanism of Hb-induced oxidation is more complicated compared to the free iron induced oxidation and this complexity might lead to variable consumption of dissolved oxygen after the addition of Hb. After the addition of Hb, more or less Constant OUR was immediately

observed, and the oxygen consumption slowed down non-linearly (Figure 32). The average OUR ofFe^{2+}, $Fe^{3+}$and Hb-catalyzed oxidation ofliposome solution (0.6%) are presented in Table 5.

Table 5: Average oxygen uptake rates (OUR) of oxidation catalyzed by Fe^{2+}, Fe^{3+} and Hemoglobin in the liposome solution. N- number of experiments, R3 oxygen uptake rate.

Prooxidant	R3	SD	N
***Fe2+* (50 μM)**	4.17	0.2	4
***Fe2+* (100 μM)**	3.8	0.77	5
***Fe3+* (30 μM)**	6.14	0.81	5
***Fe3+* (60 μM)**	5.17	1.07	8
***Fe3+* (100 μM)**	6.3	1.75	13
***Fe3+* (160 μM)**	5.46	1.65	6
***Fe3+* (240 μM)**	7.15	3.08	6
***Hb* (50 μM)**	18.69	4	8
***Hb* (100 μM)**	30.69	4	10
***Hb* (150 μM)**	34.25	4.2	9
***Hb* (200 μM)**	35.1	7.22	8

N- number of experiments**, R3** oxygen uptake rate.

According to the three studied prooxidants, hemoglobin presented the higher antioxidative effect. Many factors may affect consumption of oxygen by liposomes, such as preparation of liposome and prooxidant working solutions, pH and freshness of the solution. Oxidation of electrodes could also lead to high deviations, whichdecreases electrode sensitivity. Among the 3 studied prooxidants (Fe^{2+}, Fe^{3+} and HB),Fe^{3+}as the most abundant prooxidant of emulsified foods was used as a prooxidant in the oxidation of fatty acids and to study the antioxidaitve effect of PG and the chosen herb extracts.

4.3 Influence of solvents on OUR

The solubility of phenolics in water depends on the polarity of each compound. The solubility and stability of phenolics in aqueous solutions is limited. The stability of phenolicshigher in organic solvents (methanol, ethanol) compared to aqueous solutions. In order to dissolvecompounds

properly and maintain their stability, stock solutions of all the phenolics were prepared in 80% methanol. Keeping the solutions at low temperatures (< 4°C)increases stability of compounds in organic solvents.
The influence of methanol 80% on the consumption of oxygen by liposomes is shown in figure 33.

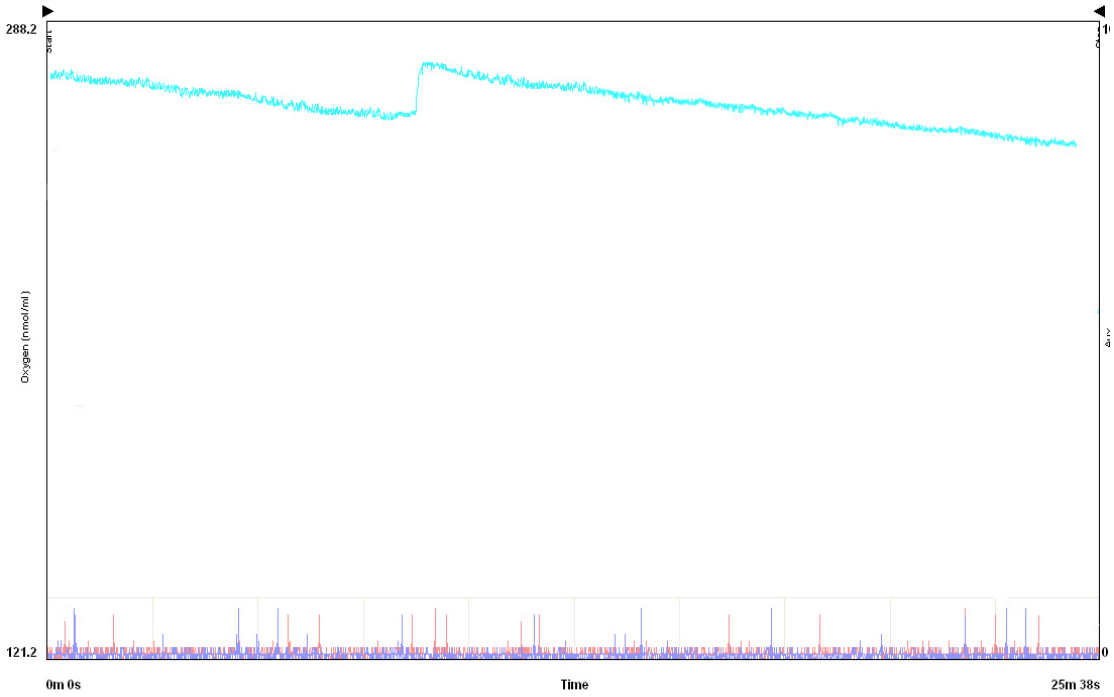


Figure 33: Influence of methanol 80% on the initial activity of liposomes.

Figure 33 shows liposome initial activity before and after the addition of 20 μl of methanol 80% into the system. After injection of methanol 80%, a rapid increase in dissolved oxygen was observed. After the increase, the OUR almost remained the same and was constant as before the increase.
This indicates that addition of methanol 80% does not affect the liposomes in a way that would enhance their initial activity.Higher amounts of methanol 80% (or any other organic solvents) might lead to destabilization of the liposomes.

After addition of methanol 80%, no significant deviations between the OUR was observed. As reported by (Asakura, Adachi et al. 1978),low concentrations of solvents(up to 5 % (v/v)), such as alcohols and ketones, stabilize the tertiary and quaternary structure of proteins; however, the same

solvents lead to denaturation of proteins at high temperature.

4.4 Propyl gallate (PG)

As mentioned earlier, propyl gallate (PG) as a synthetic antioxidant was used as standard in this study. Propyl Gallate is on the FDA's (U.S. Food and Drug administration) list of ingredients that are Generally Recognized As Safe (GRAS) for use, but it is limited to a maximum concentration of 0.02% (200 ppm) of the fat or oil content of the food (FDA).

In this study, PG was observed to have antioxidant effects and prooxidant effects in different concentrations. The antioxidative activity of PG was calculated in different concentrations, using oxygen uptake rates and the percent inhibition calculation.

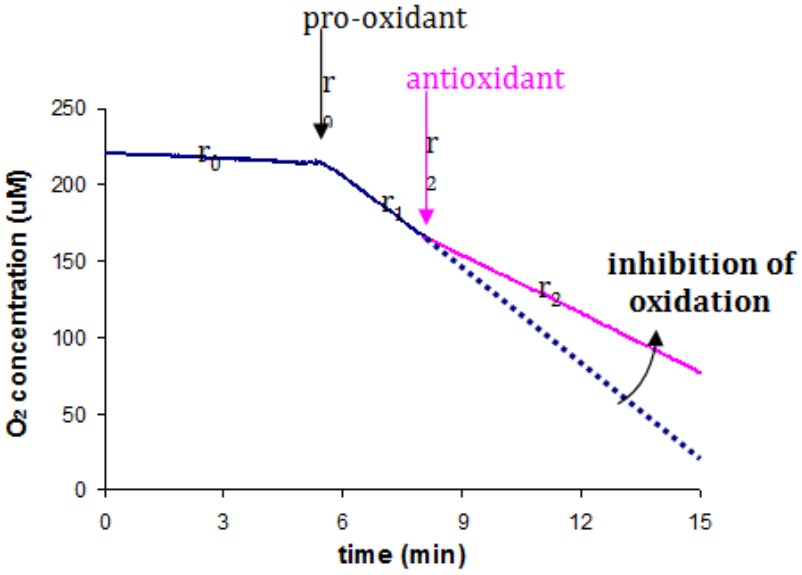


Figure 34: Oxygen uptake rate measurement

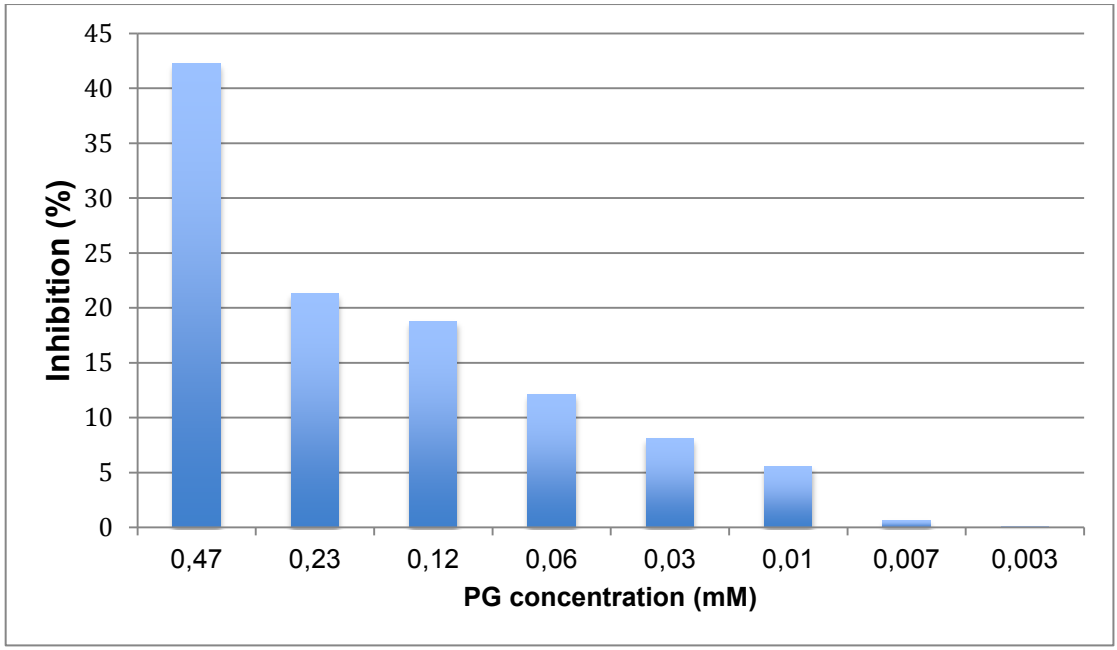


Figure 35: Inhibition (%) of oxygen uptake rate of Fe^{3+}(20 μl) by different concentrations of PG (0.47 mM, 0.23 mM, 0.12 mM, 0.06 mM, 0.03 mM, 0.01 mM, 0.007 mM, 0.003 mM). Percent inhibition =(OUR of PG – OUR of background/OUR of prooxidant (Fe^{3+})) ×100

The tested concentration range was 0.8 – 100 ppm (0.47 – 0.003 mM). In the liposome system PG turned out to be a good inhibitor of oxidation. The type of the initiator and the prooxidant-to-PG ratio can limit antioxidant activity.
In oxidation promoted by Fe^{3+}, the highest inhibitory effect was observed at 0.47 mM concentration and inhibited the OUR by 0.68%, while the lowest inhibition effect was seen at 0.007 mM and inhibited the OUR by 0.68%. Last concentration (0.003mM) has reached almost zero, which means that the oxidation of the liposome was stopped. According to the given results, PG presented a high antioxidant activity by Fe^{3+}- induced oxidation of liposome. It was reported that PG was efficient in inhibition of iron-ascorbate and Hb-promoted oxidation of fish microsomes (Pazos, Lois et al. 2006). It was also found that PG strongly inhibits rancidity of bulk oil, and itdoes not have any strong prooxidative activity in various emulsions(Schwarz, Huang et al. 2000).(Gal, Lichtenberg et al. 2007) observed prooxidative effect of PG at 0.1 - 5 μM by Cu-promoted (5 μM) oxidation of palmitoyllinoleoylphosphatidylcholine liposomes.
The location of antioxidants in multi-phase systems has been found as an important factor that influences the antioxidant activity ((Huang and Frankel

1997), (Schwarz, Huang et al. 2000), (Pekkarinen, Stöckmann et al. 1999), (Chatterjee and Agarwal 1988), (Frankel, Huang et al. 1994)).

The affinity of antioxidants towards the phospholipid bilayers is a main factor in phospholipid bilayers systems (Pazos, Lois et al. 2006). (Nakayama, Ono et al. 1998) have found that liposome membranes have 10% PG. The affinity of PG was measured for incorporation into microsomal phospholipid membranes (Pazos, Lois et al. 2006) and they have reported 52,1 % for PG amount.

The polarity of a moleculeis the other factor that affects the antioxidant activity.I has been reported that separation of PG in the oil phase of emulsions is relatively high (Jacobsen, Schwarz et al. 1999). For instance, they reported that PG was used as emulsifierin the oil phase of mayonnaise with the egg lecithin,and its partitioning was 44,9 % and 7 % in the interface (represented by phospholipids).It was also found high partitioning of PG in biphasic systems, and the partition coefficient of PG was reported to be 0,895 for an oil-water (1:10) system (Schwarz, Huang et al. 2000).

According to the examples given above, a substantial part of PG could be located within the phospholipid bilayer, while a smaller part could be held in the water phase.

Another pathway by which antioxidants can inhibit lipid oxidation was reported on the chelating properties of phenolic compounds ((Nenadis, Lazaridou et al. 2007), (Pazos, Lois et al. 2006), (Andjelković, Van Camp et al. 2006), (Chvátalová, Slaninova et al. 2008)). They found that phenolics bearing catechol or pyrogallol moiety are capable of metal chelation and PG belongs to this compounds. (Pazos, Lois et al. 2006)found that 10 μM PG could chelate 56,6% of 20 $\mu M Fe^{2+}$. Apparently, only the fraction of PG present in the aqueous phase would interfere in chelation. Additionally, PG-to-iron ratio and pH of solutionwere found to be important affecting the antioxidant activity (Damodaran and Parkin 2008).

((Schwarz, Huang et al. 2000), (Kikuzaki, Hisamoto et al. 2002)) used gallic acid as a precursor of PG, and they found that gallic acid, as a polar compound, does not separate in the oil phase and even remains in the aqueous phase of emulsions. They tested 100 μM of gallic acid

in MES buffer (5.5 *mM*, PH 5.5) using Fe^{2+}induced oxidation (GA: Fe = 10:1), and did not observe any significant decrease in OUR. This indicates that either chelation does not occur, oronly occur to a very small degree. ((Andjelković, Van Camp et al. 2006), (Chvátalová, Slaninova et al. 2008))reported low chelating abilities of gallic acid at physiological pH (7.4)by using more metals. (Andjelković, Van Camp et al. 2006) also found a relatively low gallic acid-Fe binding constant (4.78 M^{-1}) at physiological PH. Regarding to their experiment, iron chelating capability of PG remaining in the aqueous phase
likely does not contribute to the overall inhibition effect of PG, and only scavenging of free radicals takes place. Therefore, free radical scavenging activity is probably the main mechanism.
Once amount of iron is huge, the capacity of PG to scavenge free radicals seems to be insufficient. It was also reported thatPG contains a strong metal reducing power (Pazos, Lois et al. 2006). The amount of PG activated as a free radical scavenger, may be quickly depleted and simultaneously, the proportion remaining in the aqueous phase may reduce ferric iron, which may lead to an overall acceleration of lipid oxidation.

4.5 Oxygen uptake measurement of the 5 selected herb extracts

As mentioned formerly, Lemon balm, Sage, Rosemary, Dill and Basil were chosen to measure their oxygen uptake rates. Inhibition (%) of oxygen uptake rate of the herb extracts was calculated by different concentrations (Figure 36).

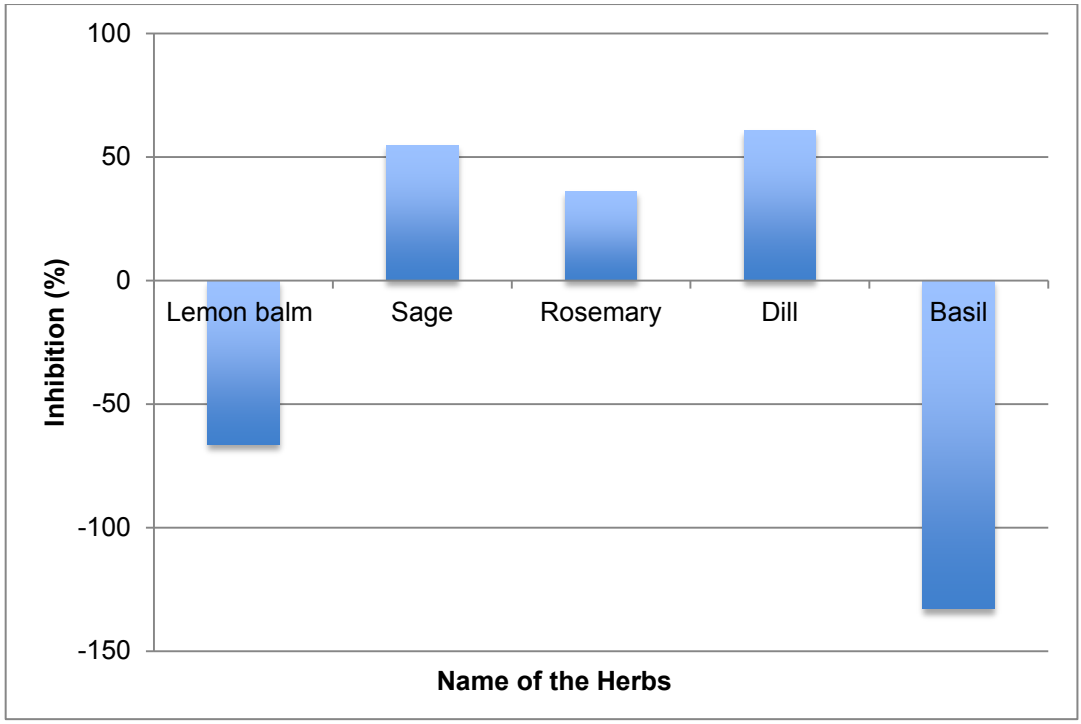


Figure 36: Inhibition (%) of oxygen uptake rate of the 5 herb extracts (20 μl). Percent inhibition = (OUR of herb extract– OUR of background /OUR of prooxidant (Fe^{3+})) × 100.

The figure above presents that Lemon balm and Basil have prooxidant effects, since they showed negative inhibition. Sage, Rosemary and Dill showed antioxidant activity, due to shownpositive inhibition.
(Wojdyło, Oszmiański et al. 2007) determined the relationship between antioxidant activity and phenolic compounds of Sage, Rosemary, Lemon balm, Thyme and Caraway to confirm that phenolic constituents are responsible for antioxidant activity of the plants.
They found a significant linear relationship between the antioxidant activities, especially with$ABTS^{\cdot+}$ and $FRAP$; however, phenolic compounds were major contributors to antioxidant activity.They found the importance of phenolic compounds in the antioxidant behavior of herb extracts. They also concluded that phenolic compounds contribute to the total antioxidant capacity.
A linear relationship between the content of total phenolic compounds and their antioxidant capacity was found by ((Cai, Luo et al. 2004), (Djeridane, Yousfi et al. 2006), (Katalinic, Milos et al. 2006), (Katsube, Tabata et al. 2004)), while ((Capecka, Mareczek et al. 2005),(Wong, Li et al. 2006))

demonstrated poor linear relationship between the content of total phenolic compounds and their antioxidant capacity.

According to results given by (Wojdyło, Oszmiański et al. 2007), Rosemary and Sage exhibit antioxidant properties, which is in agreement with our results.Our results are also in agreement with ((Lamaison, Petitjean-Freytet et al. 1990), (Shan, Cai et al. 2005), (Zheng and Wang 2001)); who presented that Sage and Rosemary had a very strong antioxidant capacity.

The inhibition (%) of the 5 selected herbs was compared using the three spectroscopic antioxidant capacity (AOC) assays.

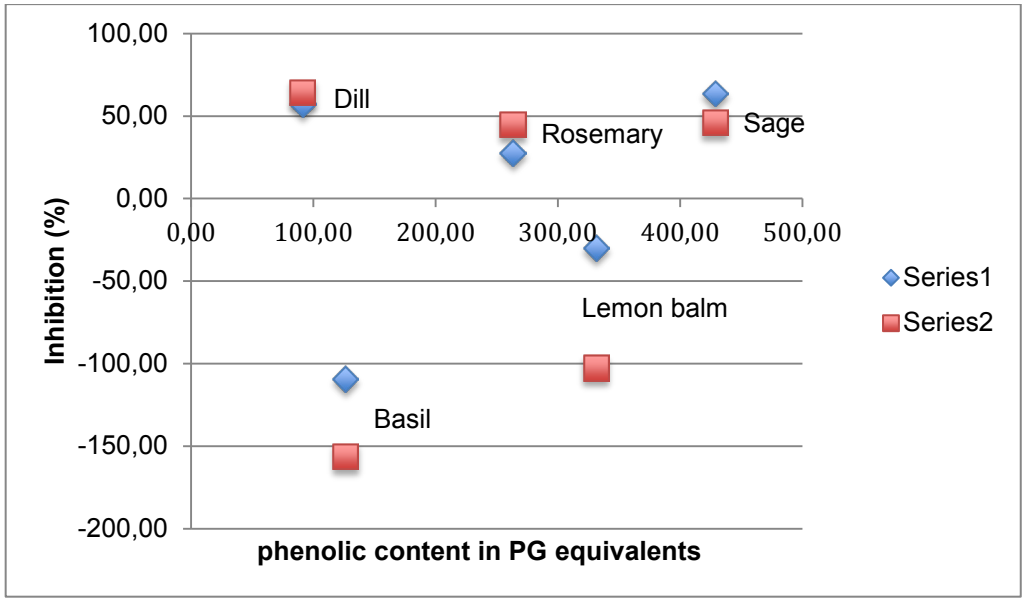


Figure 37: Relationship between inhibition (%) and phenolic content in PG equivalents in 5 selected herbs using the *FC* assay. OUR measurement of the herbs was done 2 times, which was followed by series1 and series 2.

Based on the *FC* assay, Lemon balm and Basil showed no antioxidant activity due to their negative percent of inhibition, while Dill, Sage and Rosemary presented antioxidant activities due to their positive inhibition (%). The correlation between phenolic content and inhibition was found low (R^2 = 0.028). Based on the *FC* assay and among the selected herbs, no direct relationship was found between phenolic content and antioxidative effect of the selected herb extracts.

Sage and Lemon balm both presented high phenolic content, though an

antioxidant effect was only found in Sage, and Lemon balm was found as a prooxidantprooxidant on iron induced oxidation at the studied lipid oxidation conditions.

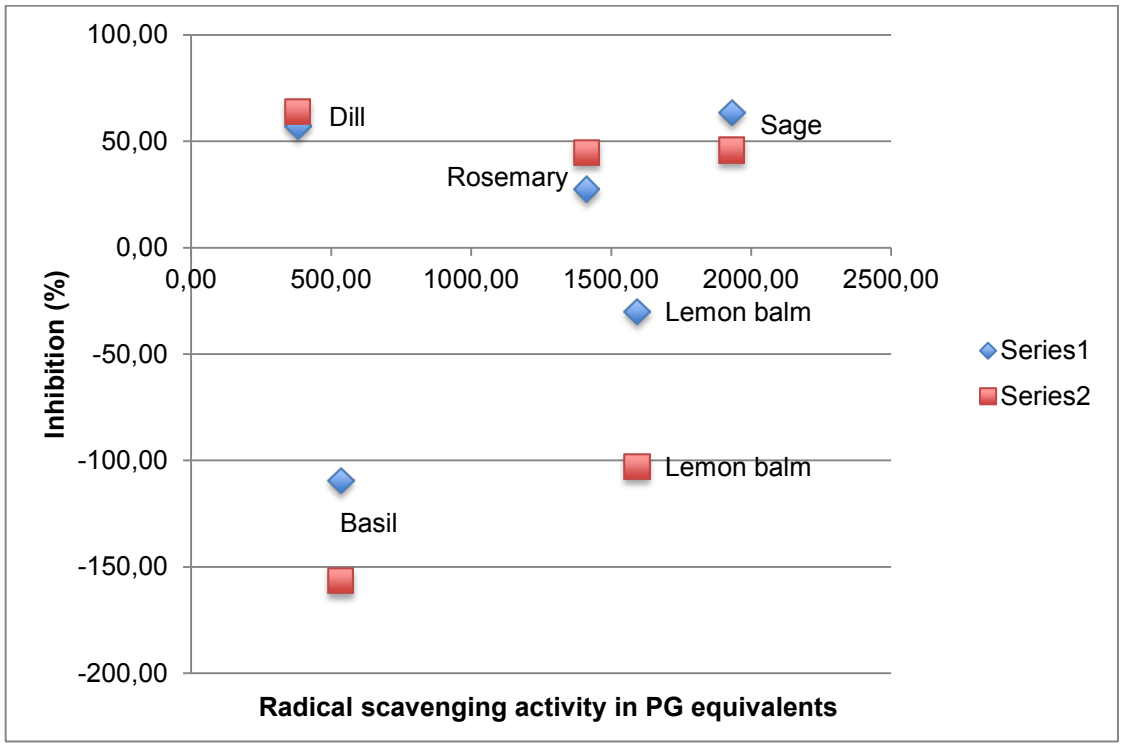


Figure 38: Relationship between inhibition (%) and radical scavenging activities in PG equivalents in 5 selected herbs using the *DPPH* assay. OUR measurement of the herbs was done 2 times, which was followed by series1 and series 2.

The correlation between phenolic content and inhibition was found low (R^2 = 0.035). No direct relationship was found betweenradical scavenging activity and antioxidativeeffect among the selected herb extracts and on the basis of *DPPH* assay.

Rosemary and Lemon balm both presented high scavenging activities; Rosemary presented an antioxidant effect, while Lemon balm showed a prooxidative effect.

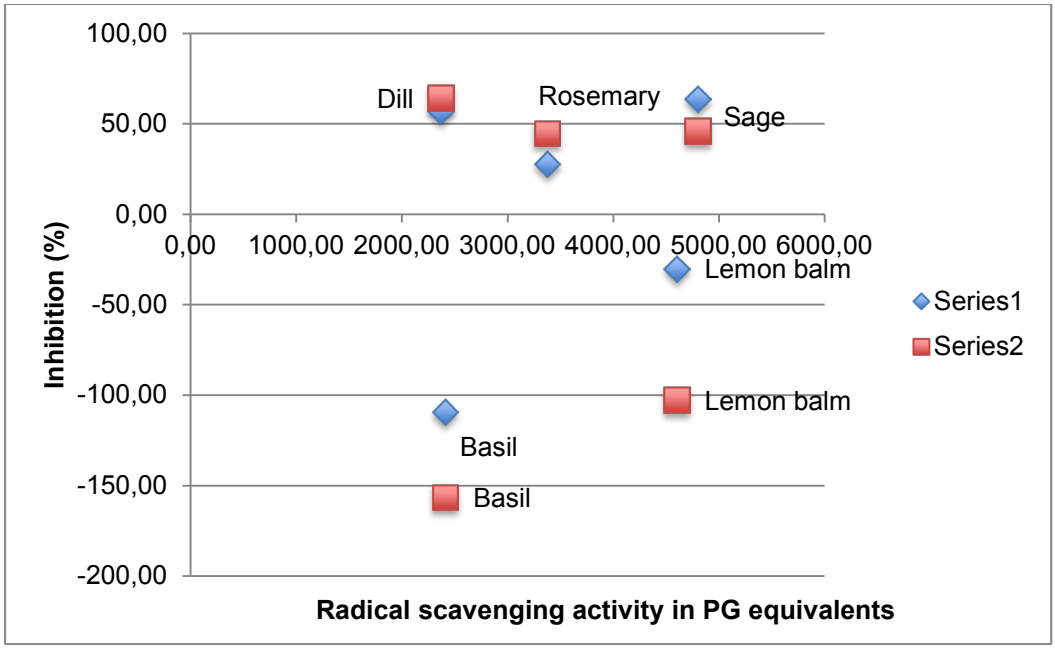


Figure 39: Relationship between inhibition (%) and radical scavenging activities in PG equivalents in 5 selected herbs using the *ABTS* assay. OUR measurement of the herbs was done 2 times, which was followed by series1 and series 2.

According to the results and based on the *ABTS* method, no direct relationship was found between radical scavenging activity and antioxidative effect among the selected herbs. The correlation between phenolic content and inhibition was found low (R^2 = 0.09).

Sage and Rosemary presented high scavenging activities and acted as good antioxidants, while Lemon balm showed high scavenging activities but appeared as a prooxidant.

(Shan, Cai et al. 2005) estimated total phenolic contentin 26 spice extracts using the Folin-Ciocalteucolorimetric method and using TEAC (trolox equivalent antioxidant capacity). Their results were expressed as g of gallic acid/ 100 r DW (dried weight).

They observed highly positive linearrelationship (R^2= 0.95) between TEAC values and total phenolic content, and they concluded that phenolic compounds in the tested spices contributed significantly to their antioxidant capacity. They foundphenolic acids, phenolic diterpenes, flavonoids, and volatile oils (e.g., aromatic compounds) as the major types of phenolic

constituents in the spice extracts. They reported rosmarinic acid as themain phenolic compound in Sage, Rosemary and Lemon balm.

(Shan, Cai et al. 2005) determined the antioxidant capacity of Rosemary, Sage, Basil, Thymeand Caraway measuring phenolic content. The first three herbs had high phenolic content, though a relatively low phenolic content was observed in Caraway.
The importance of phenolic compounds in the antioxidant behaviour of spice extracts was reported by (Wojdyło, Oszmiański et al. 2007). They also said that phenolic compounds contribute significantly to the total antioxidant capacity. They believed that qualitative and quantitative analysis of major individual phenolic in Sage, Rosemary, Dill, Basil and Lemon balm could be helpful for explaining the relationships between total antioxidant capacity and total phenolic contents in the herb extracts. They found a positive and significant correlation existed between antioxidant activity and total phenolics, measured by HPLC analysis in some selected family herbs, indicating that phenolic compounds were the major antioxidant components.

According to our results, Sage and Rosemary were excellent free radical-scavengers and a potent natural phenolic antioxidant for commercial exploration.The three spectroscopic antioxidant capacity (AOC) assays can be used to detect oxidation products of phospholipids. However (Kristinová, Mozuraityte et al. 2008) observed that antioxidative activity of phenolic compounds can be affected by prooxidants in the lipid oxidation system, such as caffeic acid asprooxidant in iron-catalized oxidation and antioxidant in haemoglobin-induced oxidation. pH can also affect antioxidative effect. Therefore in order to get better view of possible effect of herbal extracts on lipid oxidation, different affects of prooxidants and pH buffers could probably be investigated.

5 Conclusion

In this study, both direct and indirect methods were used to measure antioxidantive activity of the 12 different herb extracts were. Three antioxidant capacity assays (*FC*, *ABTS*, *DPPH*) have been used to evaluate antioxidant activity of propyl gallate and the herb extracts. Propyl gallate was used as a reference due to its high antioxidant capacity, which led to obtaining good results in all the assays.

Among the tested herbs, Lemon balm, Rosemary, Basil and Thyme had high phenolic content using *FC* method. Base on the *DPPH* assay, Rosemary, Dill, Dill (Mills), Tarragon and Basil showed high radical scavenging activities, while Lemon balm, Sage and Rosemary presented high radical scavenging activities on the basis of *ABTS* assay.

The different results of the radical scavenging activities of the selected herbs, using *ABTS* and *DPPH* methods, indicate that more than one AOC assay is required in order to correctly evaluate antioxidant capacity.

Although the antioxidant capacities of all the given herbs were high, Lemon balm, Sage, Rosemary, Dill and Basil were finally selected for oxygen uptake measurement. Oxygen uptake method uses a direct approach to determine the antioxidant ability in order to reduce the lipid oxidation. In this study, the inhibitory effects of phenolics and radical scavengers were not correlated with their reduction potentials.

The prooxidant activity of Fe^{3+}, Fe^{2+} and *Hb* was tested and Fe^{3+} was selected as a prooxidant in the studied lipid system as it is the most common prooxidant in the emulsified system. *Hb* was observed to be a strong prooxidant, but due to time limit it was left for further investigation in future.

Antioxidant activity of the 5 selected herbs was measured by means of inhibition percentage of oxygen uptake in the liposome (phospholipid dispersion in buffer). Following the obtained results, Sage, Rosemary and Dill exhibited antioxidative effects, while Lemon balm and basil were found to be prooxidants at the tested concentrations.

The contribution of radical scavenging could be significant only for Rosemary, Sage and Basil. 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 indicates that the AOC of the compounds was not the only factor affected the liposome model system. Other factors, such as reducing abilities determined the effectiveness of the studied compounds in the liposome system. The results show that antioxidant activity could be dependent on the oxidation system and the applied prooxidants.

Propyl gallate, a representative of a synthetic food antioxidant showed inhibitory effects on catalyzed oxidation of marine phospholipids in liposomes. The results of this work gave better prospective of some basic pro- and antioxidant mechanisms and factors affecting lipid oxidation.

More investigation of antioxidants effects is still needed to clarify the mechanisms and factors determining the antioxidant efficacy. Food-related conditions should be focused ahead.

Reference

Akoh, C. and D. Min (2002). "Structured lipids." Food lipids: chemistry, nutrition, and biotechnology(Ed. 2): 877-908.

Andjelković, M., J. Van Camp, et al. (2006). "Iron-chelation properties of phenolic acids bearing catechol and galloyl groups." Food chemistry**98**(1): 23-31.

Arnao, M. B. (2000). "Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case." Trends in Food Science & Technology**11**(11): 419-421.

Arnaud, J. (1995). "Liposomes in the agro food-industry." Agro Food Industry Hi-Tech**6**(5): 30-36.

Aruoma, O., B. Halliwell, et al. (1989). "The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron (II)-iron (III) complex." Biochem. j**258**: 617-620.

Asakura, T., K. Adachi, et al. (1978). "Stabilizing effect of various organic solvents on protein." Journal of Biological Chemistry**253**(18): 6423-6425.

Bateman, N. and D. Uccellini (1984). "Effect of formulation on the bioavailability of retinol, d - α - tocopherol and riboflavine." Journal of pharmacy and pharmacology**36**(7): 461-464.

Belitz, H.-D., Grosch, W.,Schieberle, P (2004). Food Chemistry. r. ed. Berlin, Springer.

Berdanier, C. and C. Chow (2000). "Fatty acids and membrane function." Fatty acids in foods and their health implications.(Ed. 2): 569-584.

Bledsoe, G., C. Bledsoe, et al. (2003). "Caviars and fish roe products."

Bligh, E. and W. J. Dyer (1959). "A rapid method of total lipid extraction and purification." Canadian journal of biochemistry and physiology**37**(8): 911-917.

Boissonneault, G. and C. Chow (2000). "Dietary fat, immunity and inflammatory disease." Fatty acids in foods and their health implications.(Ed. 2): 777-807.

Bors, W., W. Heller, et al. (1990). Radical chemistry of flavonoid antioxidants. Antioxidants in therapy and preventive medicine, Springer**:** 165-170.

Boutet, E. (2007). "Liposome." Retrieved 01.08.2013, 2013, from http://en.wikipedia.org/wiki/File:Liposome_scheme-en.svg.

Brand-Williams, W., M. Cuvelier, et al. (1995). "Use of a free radical method to evaluate antioxidant activity." LWT-Food Science and Technology**28**(1): 25-30.

Cai, Y., Q. Luo, et al. (2004). "Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer." Life sciences**74**(17): 2157-2184.

Cansell, M., F. Nacka, et al. (2003). "Marine lipid-based liposomes increase in vivo FA bioavailability." Lipids**38**(5): 551-559.

Capecka, E., A. Mareczek, et al. (2005). "Antioxidant activity of fresh and dry herbs of some< i> Lamiaceae</i> species." Food chemistry**93**(2): 223-226.

Carlsen, C. U., J. K. Møller, et al. (2005). "Heme-iron in lipid oxidation." Coordination Chemistry Reviews**249**(3): 485-498.

Carvajal, A. C. Lipidoksidasjon i liposomer, Master Thesis, 2007. Trondheim: NTNU, Faculty of Natural Science and Technology, Department of Biotechnology, 2007. Supervisor: Turid Rustad.

Chatterjee, S. and S. Agarwal (1988). "Liposomes as membrane model for study of lipid peroxidation." Free Radical Biology and Medicine**4**(1): 51-72.

Choe, E. and D. B. Min (2005). "Chemistry and reactions of reactive oxygen species in foods." Journal of Food Science**70**(9): R142-R159.

Chvátalová, K., I. Slaninova, et al. (2008). "Influence of dietary phenolic acids on redox status of iron: Ferrous iron autoxidation and ferric iron reduction." Food chemistry**106**(2): 650-660.

Clifford, M. N. (1999). "Chlorogenic acids and other cinnamates–nature, occurrence and dietary burden." Journal of the Science of Food and Agriculture**79**(3): 362-372.

Connor, W. E. (2000). "Importance of n− 3 fatty acids in health and disease." The American journal of clinical nutrition**71**(1): 171S-175S.

Damodaran, S. and K. L. Parkin (2008). Fennema's food chemistry, CRC press Boca Raton, FL.

Decker, E. A. and H. O. Hultin (1992). "Lipid oxidation in muscle foods via redox iron." Lipid oxidation in food**500**: 33-54.

Decker, E. A. and D. J. McClements (2001). Transition Metal and Hydroperoxide Interactions: An Important

Determinant in the Oxidative Stability of Lipid Dispersions. Inform. **12:** 4.

Djeridane, A., M. Yousfi, et al. (2006). "Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds." Food chemistry**97**(4): 654-660.

FDA, U. S. (21.03.2013). "Propyl Gallate." from http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm091048.htm.

Foti, M., M. Piattelli, et al. (1996). "Flavonoids, coumarins, and cinnamic acids as antioxidants in a micellar system. Structure-activity relationship." Journal of agricultural and food chemistry**44**(2): 497-501.

Foti, M. C., C. Daquino, et al. (2004). "Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions." The Journal of organic chemistry**69**(7): 2309-2314.

Frankel, E. N. (2005). Lipid oxidation, The Oily Press.

Frankel, E. N., S.-W. Huang, et al. (1994). "Interfacial phenomena in the evaluation of antioxidants: bulk oils vs emulsions." Journal of agricultural and food chemistry**42**(5): 1054-1059.

Frankel, E. N. and A. S. Meyer (2000). "The problems of using one‐dimensional methods to evaluate multifunctional food and biological antioxidants." Journal of the Science of Food and Agriculture**80**(13): 1925-1941.

Fukumoto, L. and G. Mazza (2000). "Assessing antioxidant and prooxidant activities of phenolic compounds." Journal of agricultural and food chemistry**48**(8): 3597-3604.

Fukuzawa, K., T. Seko, et al. (1993). "Dynamics of iron-ascorbate-induced lipid peroxidation in charged and uncharged phospholipid vesicles." Lipids**28**(6): 497-503.

Gal, S., D. Lichtenberg, et al. (2007). "Copper-induced peroxidation of phosphatidylserine-containing liposomes is inhibited by nanomolar concentrations of specific antioxidants." Chemistry and physics of lipids**150**(2): 186-203.

Girotti, A. W. (1985). "Mechanisms of lipid peroxidation." Journal of free radicals in biology & medicine**1**(2): 87-95.

Halliwell, B. (1995). "Antioxidant characterization: methodology and mechanism." Biochemical Pharmacology**49**(10): 1341-1348.

Hossain, M., C. Barry-Ryan, et al. (2010). "Effect of drying method on the antioxidant capacity of six Lamiaceae herbs." Food chemistry**123**(1): 85-91.

Huang, D., B. Ou, et al. (2005). "The chemistry behind antioxidant capacity assays." Journal of agricultural and food chemistry**53**(6): 1841-1856.

Huang, S.-W. and E. N. Frankel (1997). "Antioxidant activity of tea catechins in different lipid systems." Journal of agricultural and food chemistry**45**(8): 3033-3038.

Huang, S.-W., A. Hopia, et al. (1996). "Antioxidant activity of α-tocopherol and trolox in different lipid substrates: bulk oils vs oil-in-water emulsions." Journal of agricultural and food chemistry**44**(2): 444-452.

Ingold, K. U. (1962). Lipids and Their Oxidation. Symposium on Foods. H. W. Schultz, Day, E.A., and Sinnhuber, R.O., AVI Publishing Company. **5:** 93–121.

Innis, S. M. (1991). "Essential fatty acids in growth and development." Progress in lipid research**30**(1): 39-103.

Jacobsen, C., K. Schwarz, et al. (1999). "Partitioning of selected antioxidants in mayonnaise." Journal of agricultural and food chemistry**47**(9): 3601-3610.

John L. Harwood, R. J. W. (15.08.2012). "Phosphoglycerides structure." from http://lipidlibrary.aocs.org/lipids/tag1/index.htm.

Katalinic, V., M. Milos, et al. (2006). "Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols." Food chemistry**94**(4): 550-557.

Kates, M. (1991). Separation of lipid mixtures. In Techniques of Lipidology: Isolation, analysis, and identification of lipids. Amsterdam, The Netherlands, Elsevier Science Publishers.

Katsube, T., H. Tabata, et al. (2004). "Screening for antioxidant activity in edible plant products: comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay." Journal of agricultural and food chemistry**52**(8): 2391-2396.

Kikuzaki, H., M. Hisamoto, et al. (2002). "Antioxidant properties of ferulic acid and its related compounds." Journal of agricultural and food chemistry**50**(7): 2161-2168.

Kim, D.-O. and C. Y. Lee (2004). "Comprehensive study on vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship." Critical reviews in food science and nutrition**44**(4): 253-273.

Kris-Etherton PM, H. W., Appel LJ (2002). "Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease." **106** (Circulation).

Kristinová, V. r., R. Mozuraityte, et al. (2008). Evaluation of antioxidant effect using different analytical methods. Faculty of chemistry, institute of food science and biotechnology, Brno university of technology.

Kristinová, V. r., R. Mozuraityte, et al. (2009). "Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants." Journal of agricultural and food chemistry**57**(21): 10377-10385.

Kristinsson, H. G. and H. O. Hultin (2004). "The effect of acid and alkali unfolding and subsequent refolding on the pro-oxidative activity of trout hemoglobin." Journal of agricultural and food chemistry**52**(17): 5482-5490.

Kähkönen, M. P., A. I. Hopia, et al. (1999). "Antioxidant activity of plant extracts containing phenolic compounds." Journal of agricultural and food chemistry**47**(10): 3954-3962.

Laguerre, M., J. Lecomte, et al. (2007). "Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges." Progress in lipid research**46**(5): 244-282.

Lamaison, J., C. Petitjean-Freytet, et al. (1990). "[Medicinal Lamiaceae with antioxidant properties, a potential source of rosmarinic acid]." Pharmaceutica Acta Helvetiae**66**(7): 185-188.

Laranjinhas, J., O. Vieira, et al. (1996). "Inhibition of metmyoglobin/H< sub> 2</sub> o< sub> 2</sub>-dependent low density lipoprotein lipid peroxidation by naturally occurring phenolic acids." Biochemical Pharmacology**51**(4): 395-402.

Lu, F. S. H., N. S. Nielsen, et al. (2011). "Oxidative Stability of Marine Phospholipids in the Liposomal Form and Their Applications." Lipids**46**(1): 3-23.

Løvaas, E. (2006). "Marine phospholipids (MPL): resources, applications and markets." Seafood research from fish to dish, 1st ed. edn. Wageningen Academic Publishers, The Netherlands: 17-28.

Makrides, M., J. S. Hawkes, et al. (2002). "Nutritional effect of including egg yolk in the weaning diet of breast-fed and formula-fed infants: a randomized controlled trial." The American journal of clinical nutrition**75**(6): 1084-1092.

Maton Anthea, J. H., Charles William McLaughlin, Susan Johnson, Maryanna Quon Warner, David LaHart, Jill D. Wright (1993). Human Biology and Health. Englewood Cliffs, New Jersey, USA, Prentice Hall**:** 52-59.

Mehansho, H. (2006). "Iron fortification technology development: new approaches." The Journal of nutrition**136**(4): 1059-1063.

Miliauskas, G., P. Venskutonis, et al. (2004). "Screening of radical scavenging activity of some medicinal and aromatic plant extracts." Food chemistry**85**(2): 231-237.

Min, D. B. and J. M. Boff (2002). "Lipid oxidation of edible oil." FOOD SCIENCE AND TECHNOLOGY-NEW YORK-MARCEL DEKKER-: 335-364.

Minotti, G. and S. D. Aust (1987). "The role of iron in the initiation of lipid peroxidation." Chemistry and physics of lipids**44**(2): 191-208.

Mozuraityte, R. (2007). Oxidation of marine phospholipids in liposomes, Norwegian University of Science and Technology.

Mozuraityte, R. and T. Rustad (2006). "Oxidation of cod phospholipids in liposomes: Effects of salts, pH and zeta potential." European Journal of Lipid Science and Technology**108**(11): 944-950.

Mozuraityte, R., T. Rustad, et al. (2006). "Pro-oxidant activity of Fe2+ in oxidation of cod phospholipids in liposomes." European Journal of Lipid Science and Technology**108**(3): 218-226.

Mozuraityte, R., T. Rustad, et al. (2007). "The role of iron in peroxidation of polyunsaturated fatty acids in liposomes." Journal of agricultural and food chemistry**56**(2): 537-543.

Nakayama, T., K. Ono, et al. (1998). "Affinity of antioxidative polyphenols for lipid bilayers evaluated with a liposome system." Bioscience, biotechnology, and biochemistry**62**(5): 1005-1007.

Nenadis, N., O. Lazaridou, et al. (2007). "Use of reference compounds in antioxidant activity assessment." Journal of agricultural and food chemistry**55**(14): 5452-5460.

Nenadis, N., L.-F. Wang, et al. (2004). "Estimation of scavenging activity of phenolic compounds using the ABTS+ assay." Journal of agricultural and food chemistry**52**(15): 4669-4674.

Neuringer, M., W. E. Connor, et al. (1986). "Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys." Proceedings of the National Academy of Sciences**83**(11): 4021-4025.

Nikolic, K. M. (2006). "Theoretical study of phenolic antioxidants properties in reaction with oxygen-centered radicals." Journal of Molecular Structure: THEOCHEM**774**(1): 95-105.

Ohyashiki, T., A. Kadoya, et al. (2002). "The role of Fe 3+ on Fe 2+-dependent lipid peroxidation in phospholipid liposomes." Chemical and pharmaceutical bulletin**50**(2): 203-207.

Pazos, M., S. Lois, et al. (2006). "Inhibition of hemoglobin-and iron-promoted oxidation in fish microsomes by natural phenolics." Journal of agricultural and food chemistry**54**(12): 4417-4423.

Peet, M. and C. Stokes (2005). "Omega-3 fatty acids in the treatment of psychiatric disorders." Drugs**65**(8): 1051-1059.

Pekkarinen, S. S., H. Stöckmann, et al. (1999). "Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate." Journal of agricultural and food chemistry**47**(8): 3036-3043.

Pérez-Jiménez, J. and F. Saura-Calixto (2006). "Effect of solvent and certain food constituents on different antioxidant capacity assays." Food Research International**39**(7): 791-800.

Phleger, C. F., M. M. Nelson, et al. (2002). "Interannual and between species comparison of the lipids, fatty acids and sterols of Antarctic krill from the US AMLR Elephant Island survey area." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**131**(4): 733-747.

Pond, D., J. Watkins, et al. (1995). "Variation in the lipid content and composition of Antarctic krill Euphausia superba at South Georgia." Marine ecology progress series. Oldendorf**117**(1): 49-57.

Prior, R. L., X. Wu, et al. (2005). "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements." Journal of agricultural and food chemistry**53**(10): 4290-4302.

Pulido, R., L. Bravo, et al. (2000). "Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay." Journal of agricultural and food chemistry**48**(8): 3396-3402.

Re, R., N. Pellegrini, et al. (1999). "Antioxidant activity applying an improved ABTS radical cation decolorization assay." Free Radical Biology and Medicine**26**(9): 1231-1237.

Reische, D., D. Lillard, et al. (1998). "Antioxidants." Food lipids: chemistry, nutrition, and biotechnology.: 423-448.

Rice-Evans, C. A., N. J. Miller, et al. (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Radical Biology and Medicine**20**(7): 933-956.

Roginsky, V. and E. A. Lissi (2005). "Review of methods to determine chain-breaking antioxidant activity in food." Food chemistry**92**(2): 235-254.

Sato, T. and J. Sunamoto (1992). "Recent aspects in the use of liposomes in biotechnology and medicine." Progress in lipid research**31**(4): 345-372.

Schwarz, K., S.-W. Huang, et al. (2000). "Activities of antioxidants are affected by colloidal properties of oil-in-water and water-in-oil emulsions and bulk oils." Journal of agricultural and food chemistry**48**(10): 4874-4882.

Serrano, J., I. Goñi, et al. (2007). "Food antioxidant capacity determined by chemical methods may underestimate the physiological antioxidant capacity." Food Research International**40**(1): 15-21.

Shahidi, F., P. Janitha, et al. (1992). "Phenolic antioxidants." Critical Reviews in Food Science & Nutrition**32**(1): 67-103.

Shan, B., Y. Z. Cai, et al. (2005). "Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents." Journal of agricultural and food chemistry**53**(20): 7749-7759.

Singleton, V. L., R. Orthofer, et al. (1999). "[14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent." Methods in enzymology**299**: 152-178.

Stratil, P., B. Klejdus, et al. (2006). "Determination of total content of phenolic compounds and their antioxidant activity in vegetables evaluation of spectrophotometric methods." Journal of agricultural and food chemistry**54**(3): 607-616.

Sun, T. and S. Tanumihardjo (2007). "An integrated approach to evaluate food antioxidant capacity." Journal of Food Science**72**(9): R159-R165.

Tadolini, B., L. Cabrini, et al. (1997). "Iron (III) stimulation of lipid hydroperoxide-dependent lipid peroxidation." Free radical research**27**(6): 563-576.

Tadolini, B. and G. Hakim (1996). "The mechanism of iron (III) stimulation of lipid peroxidation." Free radical research**25**(3): 221-227.

Thiansilakul, Y., S. Benjakul, et al. (2007). "Antioxidative activity of protein hydrolysate from round scad muscle using alcalase and flavourzyme." Journal of Food Biochemistry**31**(2): 266-287.

Tocher, D. R. and J. R. Sargent (1984). "Analyses of lipids and fatty acids in ripe roes of some northwest European marine fish." Lipids**19**(7): 492-499.

Vercellotti, J., A. J. S. Angelo, et al. (1992). "Lipid oxidation in foods." Lipid oxidation in food**500**: 1-11.

Vinson, J. A., Y. A. Dabbagh, et al. (1995). "Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease." Journal of agricultural and food chemistry**43**(11): 2800-2802.

W.W., N. (1996). Lipids. Food chemistry. F. O.R. New York, Marcel Dekker**:** 225-319.

Wang, S. Y. (2002). Antioxidant capacity of berry crops, culinary herbs and medicinal herbs. XXVI International Horticultural Congress: Asian Plants with Unique Horticultural Potential: Genetic Resources, Cultural 620.

Wheeler, R. (2007). "Hemoglobin." 2013, from http://en.wikipedia.org/wiki/File:1GZX_Haemoglobin.png.

Wojdyło, A., J. Oszmiański, et al. (2007). "Antioxidant activity and phenolic compounds in 32 selected herbs." Food chemistry**105**(3): 940-949.

Wong, C.-C., H.-B. Li, et al. (2006). "A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay." Food chemistry**97**(4): 705-711.

Wright, J. S., E. R. Johnson, et al. (2001). "Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants." Journal of the American Chemical Society**123**(6): 1173-1183.

Wu, X., G. R. Beecher, et al. (2004). "Lipophilic and hydrophilic antioxidant capacities of common foods in the United States." Journal of agricultural and food chemistry**52**(12): 4026-4037.

Yanishlieva, N. V., E. Marinova, et al. (2006). "Natural antioxidants from herbs and spices." European Journal of Lipid Science and Technology**108**(9): 776-793.

Yen, G.-C. and H.-Y. Chen (1995). "Antioxidant activity of various tea extracts in relation to their antimutagenicity." Journal of agricultural and food chemistry**43**(1): 27-32.

Yin, D., H. Lingnert, et al. (1992). "Fenton reagents may not initiate lipid peroxidation in an emulsified linoleic acid model system." Free Radical Biology and Medicine**13**(5): 543-556.

Zelman, K. M. (2004). "Top 10 Iron-Rich Foods." from http://www.webmd.com/diet/features/top-10-iron-rich-foods.

Zheng, W. and S. Y. Wang (2001). "Antioxidant activity and phenolic compounds in selected herbs." Journal of agricultural and food chemistry**49**(11): 5165-5170.

6 Appendix

A. Experimental data for the antioxidant capacity assays.

A. 1 FC assay values for PG

Concentration (mM)	Absorbance (725 nm, 60 min)
0	0.012
0.5	0.421
1	0.792
1.5	1.262
2	1.684

A. 2 Absorbance values of the herbs using FC assay

Herbs	Absorbance	PG equivalents
Lemon Balm	0.552	331.42
Tarragon	0.199	120.55
Chive	0.051	32.14
Thyme	0.289	174.31
Parsley	0.061	38.11
Sage	0.715	428.79
Rosemary	0.438	263.32
Caraway	0.038	24.37
Dill (Mill))	0.136	82.92
Lemon pepper	0.035	22.58
Dill	0.15	91.28
Basil	0.209	126.52

A.3 DPPH assay values for PG

Concentration (mM)	Absorbance (517 nm, 30 min)
0	0.82
0.1	0.515
0.2	0.347
0.3	0.167
0.4	0.094

A. 4 Absorbance values of the herbs using DPPH assay

Dilution 1:10	Herbs	Absorbance	PG equivalents
	Lemon Balm	0.175	1593.33
	Tarragon	0.335	1148.89
	Thyme	0.124	1735
	Sage	0.053	1932
	Rosemary	0.24	1412.78
	Dill	0.611	1932.22
	Basil	0.556	1414.78
Dilution 1:10	Chive	0.926	-49.28
	Parsley	0.858	30.39
	Caraway	0.638	30.72
	Dill (Mills)	0.084	382
	Lemon pepper	0.612	535

A. 5 ABTS assay values for PG

Concentration (mM)	Absorbance (734 nm, 6 min)
0	0.671
0.1	0.581
0.2	0.515
0.3	0.459
0.4	0.357

A. 6 Absorbance values of the herbs using DPPH assay

Herbs	Absorbance (734 nm, 6 min)	PG equivalents
Lemon Balm	0.3	4604.67
Tarragon	0.608	2551.33
Chive	0.694	1978.00
Thyme	0.538	3018.00
Parsley	0.711	1864.67
Sage	0.27	4804.67
Rosemary	0.484	3378.00
Caraway	0.721	1798.00
Dill	0.675	2104.67
Lemon pepper	0.724	1778.00
Dill	0.635	2371.33
Basil	0.629	2411.33

A. 7 Comparison of AOC results (in PG equivalents per gram dried herbs)

	FC	DPPH	ABTS
Lemon Balm	331.42	1593.33	4604.67
Tarragon	120.55	1148.89	2551.33
Chive	32.14	-212.22	1978.00
Thyme	174.31	1735.00	3018.00
Parsley	38.11	-317.78	1864.67
Sage	428.79	1932.22	4804.67
Rosemary	263.32	1412.78	3378.00
Caraway	24.37	30.72	1798.00
Dill	82.92	112.78	2104.67
Lemon pepper	22.58	37.94	1778.00
Dill	91.28	382.22	2371.33
Basil	126.52	535.00	2411.33

B. Oxygen Uptake Rate (OUR) measurements

B.1 OUR for Fe2+

Prooxidant	**Background**	**Adding Fe2+**				
Fe2+ conc	r1	r2	r3 = r2-r1	avg r3	pH	SD
50 mM	2.8	7	4.2	4.175	5.6	0.206155281
50 mM	2.1	6.3	4.2		5.6	
50 mM	1.9	5.8	3.9		5.6	
50 mM	1.7	6.1	4.4		5.6	
100 mM	1.2	4	2.8	3.78	5.6	
100 mM	1.7	5.5	3.8		5.6	0.77588659
100 mM	1.3	6.2	4.9		5.6	
100 mM	2.4	5.8	3.4		5.6	
100 mM	2.1	6.1	4		5.6	

B.2 OUR for HB

Prooxidant	**Background**	**Adding Hb**				
Hb conc	R1	R2	R3 = r2-r1	Avg of r3	pH	SD
50 mM	2	24.4	22.4	18.6875	5.6	4.00657941
50mM	2.5	17.2	14.7		5.5	
50mM	2.9	19.3	16.4		5.5	
50 mM	2.3	17.3	15		5.5	
50 mM	2.8	23.1	20.3		5.5	
50 mM	2.5	21.3	18.8		5.5	
50 mM	2	17.9	15.9		5.5	
50 mM	2	28	26		5.5	

Hb	**Background**	**Adding Fe3+**	**R3 = r2-r1**	**Avg of r3**	**pH**	**SD**
100 mM	1.8	28.2	26.4	30.69	5.5	3.962729138
100 mM	2	33	31		5.5	
100 mM	2.1	37	34.9		5.5	
100 mM	2.4	38	35.6		5.5	
100 mM	2.2	37.6	35.4		5.5	
100 mM	2.4	34	31.6		5.5	
100 mM	1.5	28.7	27.2		5.5	
100 mM	2.4	35	32.6		5.5	
100 mM	2.2	28.4	26.2		5.5	
100 mM	1.7	27.7	26		5.5	

Hb	Background	Adding	r3 = r2-r1	Avg of r3	pH	SD
150 mM	3.7	31.7	28	34.25555556	5.5	4.203008182
150 mM	3.1	36.7	33.6		5.5	
150 mM	3.1	39.7	36.6		5.5	
150 mM	2.4	36.5	34.1		5.5	
150 mM	2.1	38	35.9		5.5	
150 mM	2.1	37.3	35.2		5.5	
150 mM	2	43.7	41.7		5.5	
150 mM	2.1	30.3	28.2		5.5	
150 mM	2.6	37.6	35		5.5	

Hb	Background	Adding Fe3+	R3 = r2-r1	Avg of r3	pH	SD
200 mM	3.1	33.8	30.7	35.1	5.5	7.225154472
200 mM	3.6	38.3	34.7		5.5	
200 mM	3.7	24	20.3		5.5	
200 mM	3.3	48	44.7		5.5	
200 mM	3.6	41.3	37.7		5.5	
200 mM	2.6	38	35.4		5.5	
200 mM	2.7	40.2	37.5		5.5	
200 mM	2.4	42.2	39.8		5.5	

B.3 OUR for Fe3+

Prooxidant	Background	Adding Fe3+				
Fe3+ conc	R1	R2	R3 = r2-r1	Avg of r3	pH	SD
30 mM	3.7	8.9	5.2	6.14	5.5	0.814248119
30 mM	2	7.6	5.6		5.5	
30 mM	1.7	8.2	6.5		5.5	
30 mM	2.7	8.8	6.1		5.5	
30 mM	2.4	9.7	7.3		5.5	

Fe3+	Background	Adding Fe3+	R3 = r2-r1	Avg of r3	pH	SD
60 mM	2.5	7	4.5	5.1675	5.5	1.072190681
60 mM	2.8	6.84	4.04		5.5	
60 mM	3.4	9.2	5.8		5.6	
60 mM	2.1	8.6	6.5		5.6	
60 mM	1.8	7.8	6		5.6	
60 mM	1.2	6.9	5.7		5.6	
60 mM	1.9	5.3	3.4		5.6	
60 mM	1.6	7	5.4		5.6	

Fe3+	Background	Adding Fe3+	R3 = r2-r1	Avg of r3	pH	SD
100 mM	1.6	7	5.4	6.307692308	5.5	1.175770342
100 mM	1.2	7.3	6.1		5.5	
100 mM	1.1	8.4	7.3		5.5	
100 mM	3.1	9.7	6.6		5.5	
100 mM	3.8	8.5	4.7		5.5	
100 mM	3.7	8	4.3		5.5	
100 mM	2.3	8	5.7		5.6	
100 mM	2.2	8.5	6.3		5.6	
100 mM	1.7	9.8	8.1		5.6	
100 mM	1.2	9	7.8		5.6	
100 mM	2.5	9.2	6.7		5.6	
100 mM	2.8	8.3	5.5		5.6	
100 mM	2.2	9.7	7.5		5.6	

Fe3+	Background	Adding Fe3+	R3 = r2-r1	Avg of r3	pH	SD
160 mM	2.7	6.7	4	5.466666667	5.6	1.647624553
160 mM	2	6.1	4.1		5.6	
160 mM	1.3	7.5	6.2		5.6	
160 mM	3	7.5	4.5		5.6	
160 mM	2.3	8	5.7		5.6	
160 mM	1.7	10	8.3		5.6	

Fe3+	Background	Adding Fe3+	r3 = r2-r1	Avg of r3	pH	SD
240 mM	3.6	13.4	9.8	7.15	5.5	3.076849038
240 mM	3.3	13.2	9.9		5.5	
240 mM	3.8	13.5	9.7		5.5	
240 mM	3.2	6	2.8		5.6	
240 mM	2.8	8.8	6		5.6	
240 mM	2.3	7	4.7		5.6	

B.4 OUR for PG

	Background	Adding Fe3+	80% met		
Control PG	R1	R2	R3= r2-r1	R4	R5=r4-r1
Fe3+ + 80% Met (100 ppm)	1.65	3.5	1.85	3.4	1.75

B.5 OUR for different concentrations of PG

	Background	Adding Fe3+		Adding PG		
PG	R1	R2	R3= r2-r1	R4	r5=r4-r1	inhibition
PG (0.47 mM)	1.7	8.3	6.6	3.4	1.7	20.48
PG (0.47 mM)	1.2	3.3	2.1	3.8	2.6	78.79
PG (0.47 mM)	2.4	6	3.6	4.4	2	33.33
PG (0.47 mM)	2.1	5.5	3.4	4.1	2	36.36
PG (0.23 mM)	1.7	9.5	7.8	3.8	2.1	22.11
PG (0.23 mM)	2.4	8.3	5.9	4.1	1.7	20.48
PG (0.12 mM)	2	7	5	3.5	1.5	21.43
PG (0.12 mM)	1.2	6.8	5.6	2.3	1.1	16.18
PG (0.06 mM)	1.9	7.1	5.2	2.8	0.9	12.68
PG (0.06 mM)	2.2	8.6	6.4	3.2	1	11.63
PG (0.03 mM)	1.7	8.1	6.4	2.1	0.4	4.94
PG (0.03 mM)	1.2	8.9	7.7	2.2	1	11.24
PG (0.01 mM)	1.5	6.7	5.2	1.8	0.3	4.48
PG (0.01 mM)	1.2	6	4.8	1.6	0.4	6.67
PG (0.007 mM)	2.8	12.1	9.3	1.2	1	8.26
PG (0.007 mM)	1.8	12.5	10.7	1.3	-0.5	-4.00
PG (0.007 mM)	1.8	18	16.2	1.4	-0.4	-2.22
PG (0.003 mM)	2.8	12	9.2	1.1	-1.7	-14.17
PG (0.003 mM)	1.9	12.6	10.7	1.1	1.8	14.29

B.6 Inhibition of the herbs applying ABTS, DPPH and FC assay

				1	2
Herbs	FC	DPPH	ABTS	Inhibition	Inhibition
Lemon Balm	331.42	1593.33	4604.67	130.00	202.78
Sage	428.79	1932.22	4804.67	36.36	54.10
Rosemary	263.32	1412.78	3378.00	72.41	55.36
Dill	91.28	382.22	2371.33	42.86	35.90
Basil	126.52	535.00	2411.33	209.30	256.52

B.7 Inhibition of the herbs applying ABTS, DPPH and FC assay

Herbs	**Series 1**	**Series 2**
Lemon balm	-30.00	-102.78
Sage	63.64	45.90
Rosemary	27.59	44.64
Dill	57.14	64.10
Basil	-109.30	-156.52