ISOLATION, IDENTIFICATION AND EVALUATION OF NATURAL ANTIOXIDANTS FROM AROMATIC HERBS CULTIVATED IN LITHUANIA

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ISOLATION, IDENTIFICATION AND EVALUATION OF NATURAL ANTIOXIDANTS FROM AROMATIC HERBS CULTIVATED IN LITHUANIA

(with a summary in English)
(met een samenvatting in het Nederlands)
(su santrauka lietuviškai)
(com resumo em Português)

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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op dinsdag 29 januari 2002
des namiddags te twee uur in de Aula

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To my children Ieva and Simonas

Due to its specific aroma and the volatility of thymol and carvacrol, the essential oil from thyme has a limited use as a food antioxidant.

This thesis.

Due to the complexity of food products and the variety of possible oxidation mechanisms, it is not possible to find a single antioxidant that can control all factors involved in food oxidation.

This thesis.

Six, P. 1994. Current research in natural food antioxidants. Inform, 5, 679–687.

Decker, E.A. 1998. Strategies for manipulating the prooxidative/antioxidative balance of foods to maximize oxidative stability. Trends Food Sci. Technol., 9, 241–248.

It is perfectly possible for an antioxidant to protect lipids against oxidative damage whilst accelerating damage to other biomolecules.

Aruoma, O.I., Spencer, J.P.E., Warren, D., Jenner, P., Butler, J. and Halliwell, B. 1997. Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. Food Chem., 60, 149–156.

"Natural ≠ safe".

Reische, D.W., Lillard, D.A. and Eitenmiller, R.R. 1998. Antioxidants. p. 423–448. In: C.C. Akoh and D.B. Min (eds). Food Lipids. Marcel Dekker, New York.

Pokorny, J. 1991. Natural antioxidants for food use. Trends Food Sci. Technol., 2, 223–227.

When reporting analytical results on commercial phytopharmaceuticals or dietary supplements, researchers should always report brand names, type, dosage and batch numbers so that others may repeat their investigations.

Petty, H.R., Fernando, M., Kindzelskii, A.L., Zarewych, B.N., Ksebati, M.B., Hryhorczuk, L.M. and Mobashery, S. 2001. Identification of colchicine in placental blood from patients using herbal medicines. Chem. Res. Toxicol., 14, 1254–1258.

Economical considerations are just one – and not always the most important – reason for the brain-drain from Eastern European countries.

Life is a constant battle to avoid becoming rancid.

Gary G. Duthie

Stellingen behorend bij het proefschrift: Isolation, Identification and Evaluation of Natural Antioxidants from Aromatic Herbs Cultivated in Lithuania

Airidas Dapkevicius, 29 januari 2002

GENERAL INTRODUCTION

1.1 Lipid oxidation in general

Oxidative spoilage of lipids has become increasingly important to both the food producer and the consumer, due to several reasons. Firstly, polyunsaturated lipids are an obligatory part of healthy modern diets, secondly, usage of food antioxidants is still unappealing to consumers and, finally, the practice of enriching some food products with minerals like iron renders them, in some cases, susceptible to oxidation[1]. Certain additives (e.g., colourants, flavourings) used in the food industry may promote oxidation of lipids as well. While a certain level of oxidative changes that occur in lipid containing foods during various technological processes may be welcome (e.g., cured ham, fermented sausage, several types of cheese), the undesirable oxidation of oils and edible fats may often induce product discolouration, polymerisation and nutritional loss[2]. Chemical and physical changes which occur in stored food products due to lipid oxidation are usually followed by deterioration of organoleptic properties, which is defined as oxidative rancidity.

Rancid lipids in foods not only decrease their organoleptic value, but also constitute health hazards. It has been reported that primary and secondary lipid oxidation products may affect vital cell functions by damaging proteins, membranes and biological components[3]. Over-consumption of oxidised fat may result in poor growth rate, cardiomyopathy and a generalised myopathy, hepatomegaly, hemolytic anemia as well as proliferation of the lymphatic system[4]. In general, the toxicity of lipid oxidation products leads to increased degenerative processes in the human body and accelerates ageing[3,5,6]. Some authors have indicated that primary products of lipid oxidation (lipid hydroperoxides and oxidised cholesterol) can promote tumor formation, while malonaldehyde, a secondary lipid oxidation product, can act as a catalyst resulting in substances (e.g. *N*-nitrosamines) with a high risk of mutagenesis[3]. More detailed definitions of primary and secondary products of lipid oxidation will be given in the succeeding sections of this chapter.

The need to protect food lipids from oxidation in order to supply the consumer with wholesome, safe and palatable food products is of utmost concern for food chemists and technologists. The usage of antioxidants in modern food manufacturing, therefore, should be perceived as a requirement, not just as a necessary evil[7].

1.2 Food lipid oxidation mechanisms

1.2.1 Free radical autoxidation

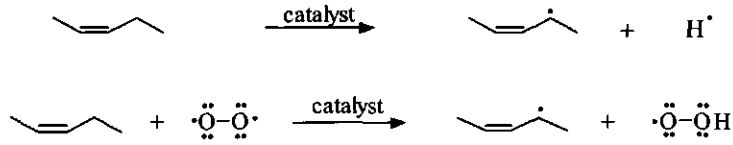
Autoxidation is generally defined as a reaction with molecular oxygen via a self-catalytic mechanism[8]. Autoxidation that occurs in foods and bulk oils leads to formation of off-flavours. Living organisms have their own protective mechanisms (e.g. flavonoids, antioxidant vitamins E and C, and the enzymes superoxide dismutase, and glutathione peroxidase) to counteract reactive free radicals, which are key intermediates in autoxidation processes. This is the reason why living matter does not become rancid[4].

Free radicals are atoms or groups of atoms that possess one or more unpaired valence electrons and are capable of mostly short independent existence. This wide definition also covers transition metals, the hydrogen atom and the oxygen molecule[9]. The most important pathway of rancidification of food lipids follows the so-called classical free radical autoxidation mechanism.

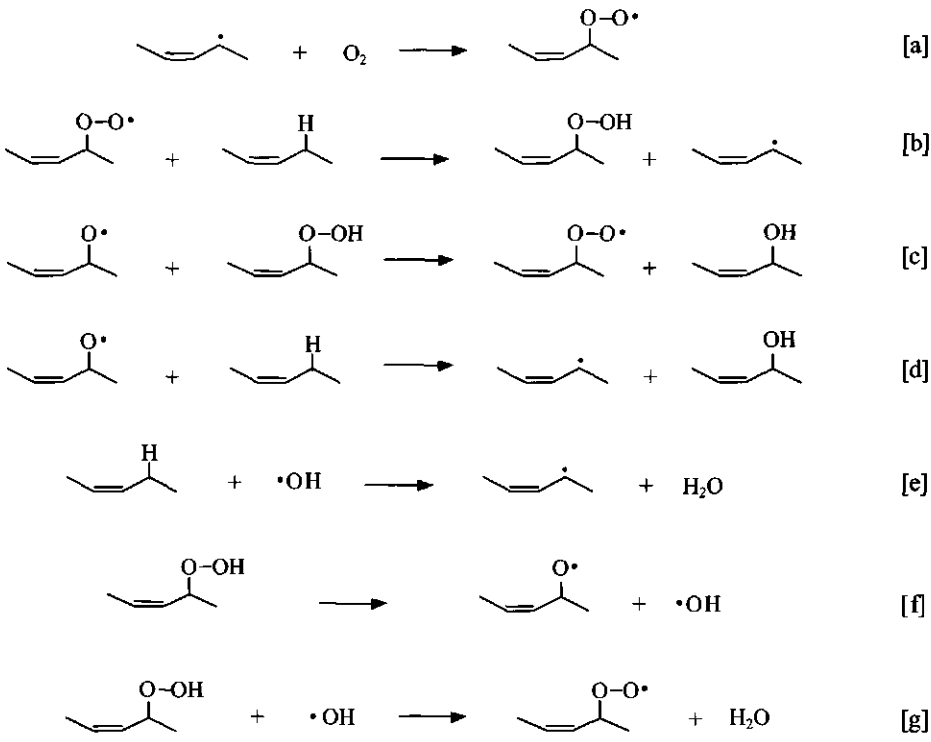
Direct oxidation of unsaturated fats has a high activation energy (~ 35 to 65 kcal/mol) and is thus, under normal storage conditions, thermodynamically improbable[10]. Unsaturated lipids (RH), however, may undergo oxidation that starts by formation of resonance stabilised allylic free radicals through the action of trace metals, metalloproteins, enzymes, microorganisms, high temperatures, light or other types of radiation[5,11]. The chain reaction of lipid oxidation may be divided into three phases: initiation, propagation and termination. The *initiation* step (Scheme 1.1) starts at an allylic methylene group of an unsaturated lipid molecule and may be catalysed by one or several of the phenomena mentioned above[12]. The exact mechanism of the generation of the initial free radicals is still controversial[9,12].

The free radicals generated during the *initiation* step react further with a molecule of ground state triplet oxygen (a diradical) via a radical-radical coupling mechanism[13] producing a peroxy radical $ROO^{\bullet}$ (Scheme 1.2. [a]). This peroxy

radical reacts further with a lipid molecule to form another free allylic radical and a hydroperoxide ROOH, the initial product of lipid oxidation (Scheme 1.2. [b]).



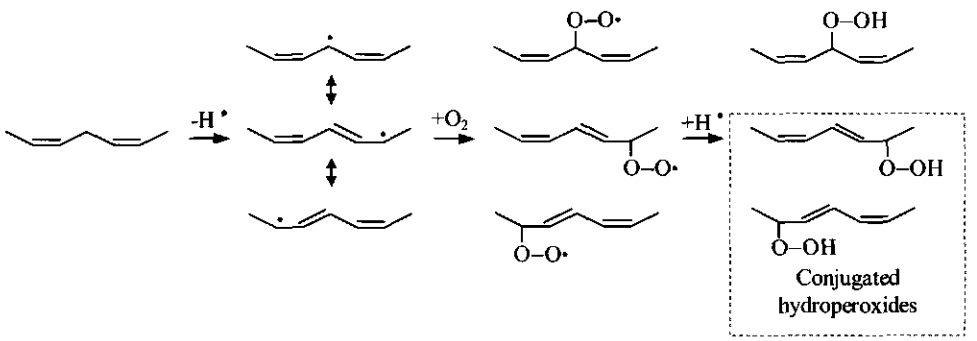
Scheme 1.1 *Formation of free radicals during the initiation step of lipid autoxidation.*



Scheme 1.2 *The propagation step of lipid autoxidation.*

The formed hydroperoxides are subject to fragmentation, which can be accelerated by heat, metal ions, or UV light[14] and may follow a monomolecular (Scheme 1.2. [f]) or bimolecular [f]+[g] route depending on the amount of energy absorbed and on the concentration of ROOH.

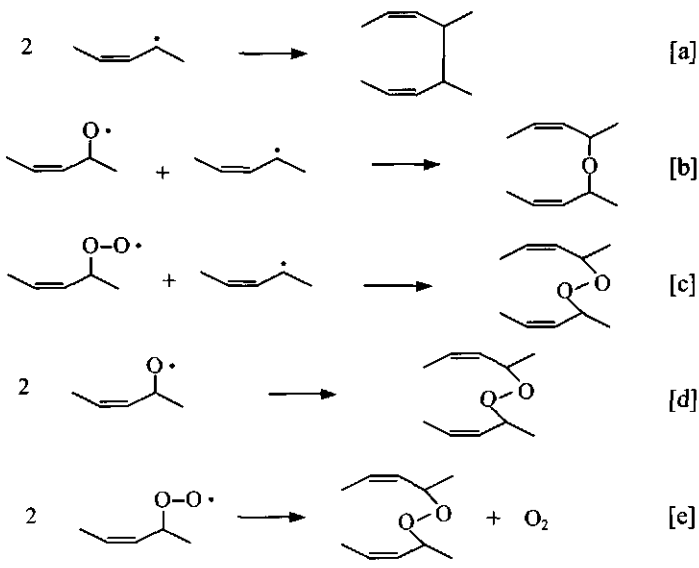
The free radical formed during the reactions [b], [d] and [e] of the *propagation* step (Scheme 1.2.) re-enters lipid oxidation, making this reaction to a self-propagating chain process. The reaction of a free radical with oxygen [a] requires almost no activation energy[11]. The abstraction of a hydrogen atom from an unsaturated lipid molecule [b] is the rate-determining step[15], which highly depends on bond dissociation energies. The degree of unsaturation and the length of the fatty acid influence the C–H bond strength at methylene groups next to double bonds, which are the most susceptible parts of triglycerides. The C–H bond at a double allylic methylene group of linoleic acid has a 1.5 times weaker bond strength than a methylene C–H bond at the position α of the double bond of oleic acid[11]. Relative rates of oxidation for arachidonic, linolenic, linoleic and oleic acid are about 40:20:10:1, respectively[8]. High amounts of free fatty acids also accelerate oxidation, since fatty acids in a free form react faster than when they are esterified to glycerol[16]. During the propagation step, linoleic acid or more highly unsaturated fatty acids may form conjugated hydroperoxides (Scheme 1.3).



Scheme 1.3 *Formation of conjugated hydroperoxides during oxidation of polyunsaturated fatty acids*[15].

The *termination* step occurs when free radicals react among themselves yielding non-radical products (Scheme 1.4).

At low oxygen conditions, the termination mostly follows the reactions [a], [b] and [c][11]. In an oxygen saturated atmosphere, the predominant termination pathway is the reaction among two peroxyl radicals [e] due to rapid coupling of allylic radicals with molecular oxygen[10,17]. Hydroperoxides formed during radical chain-reactions as well as peroxides that derive from other mechanisms of lipid oxidation (e.g. photooxidation, enzymatic oxidation) may further undergo various changes, which will be discussed later in this chapter.



Scheme 1.4 *Radical reactions that constitute the termination step of lipid oxidation.*

1.2.2 Photooxidation

Formation of singlet oxygen ($^1O_2^*$) through a photochemical reaction in the presence of a sensitiser initiates a rancidification process in edible oils, which is called photosensitised oxidation. Together with photolytic autoxidation (primary formation of free radicals from lipids exposed to light), photosensitised oxidation plays a significant role in spoilage of fat containing foods that are stored in light[11].

Some synthetic dyes as well as natural pigments (e.g. chlorophyll, pheophytin, hematoporphyrins, riboflavin and myoglobin), when being photochemically excited, can donate energy to triplet oxygen and eventually yield singlet oxygen[10,11,14]. Singlet oxygen, being a very electrophilic compound, easily reacts with electron-rich unsaturated fatty acids and forms primary free radicals that trigger the chain reaction of lipid oxidation.

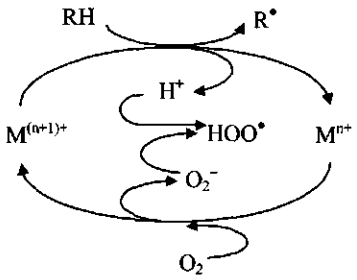
1.2.3 Enzyme-initiated lipid oxidation

Crushing or otherwise physically processing animal or vegetable tissue releases lipases that start off lipolysis of glycerides, glycolipids and phospholipids. The free fatty acids produced, in the first place unsaturated ones, may further react with lipoxygenases, which are broadly available in plant and animal tissues[9,18]. Lipoxygenase isoenzymes that are able to react with triglycerides directly are less frequently encountered. The lipoxygenase enzymatic reaction leads to specific hydroperoxides, which may enter reaction with hydroperoxide lyase or may break down and enter the chain of free radical oxidation reactions. Hydroperoxide lyase is able to convert the hydroperoxide to aldehydes, ketols, divinyl ether fatty acids, epoxy hydroxy fatty acids and trihydroxy fatty acids[9].

1.2.4 Meta-catalysed lipid oxidation

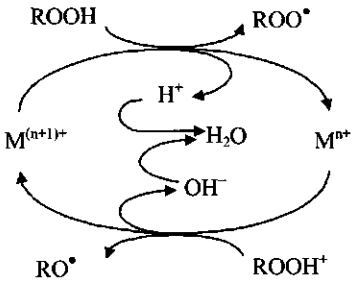
Metals naturally occur in trace amounts[15] (10^{-3} to 500 mg/kg) in all food materials of plant and animal origin. Under natural conditions, the presence of water and other constituents of biological systems seem to reduce the accelerating effect of metals on lipid oxidation. However, pure fats and oils are very susceptible to oxidation by trace amounts of metals. Grinding, deboning, and heating meat as well as products containing vegetable oils release active metals from inactive bound complexes and mobilise them for the catalysis of lipid oxidation[19]. Metal-catalysed lipid oxidation normally involves a one electron transfer between the metal with two or more valency states (Fe, Cu, Ni, Co, and Mn) and a lipid molecule. The mechanisms of such reactions are rather complicated and are grossly dependent on the amount and form of the trace metals available[20]. There are two steps of lipid oxidation in which metal ions play the most significant role.

Firstly, the activation energy of the initiation step in presence of trace metals is reduced to 15–25 kcal/mol[10]. Thus, transition metals may generate free radicals directly from unsaturated fatty acid via a single-electron transfer and proton abstraction[8,21,22]. Dependently on the available form of metal and oxygen, metal ions can interact with triplet oxygen to generate superoxide radicals (Scheme 1.5).



Scheme 1.5 *Generation of superoxide radicals by metal ions.*

Secondly, transition metals increase the rate of decomposition of hydroperoxides and promote the branching steps of lipid autoxidation. Depending on the metal ion, either a alkoxy or a peroxyl radical may be generated[8,14,23] (Scheme 1.6).



Scheme 1.6 *Decomposition of hydroperoxides catalysed by transition metals.*

1.2.5 Formation of secondary products of lipid oxidation

Most of the hydroperoxides, the primary products of food lipid autoxidation, are predominantly derived from unsaturated fatty acids, such as oleic, linoleic, linolenic and arachidonic acid. These hydroperoxides can break down further and react among themselves as well as with other food components following various mechanisms. Formation of the secondary products of lipid peroxidation starts up soon after the first lipid hydroperoxides are generated. The plentitude of volatile products such as saturated and unsaturated aldehydes (butanal, hexanal, heptanal, (*Z*)-3-hexenal, (*Z*)-4-heptenal, (*E*)-6-nonenal, (*E,Z*)-2,4-heptadienal, (*E,Z*)-2,6-

nonadienal, (*E,Z,Z*)-2,4,7-decatrienal), acids (acetic, butyric, hexanoic, 2-octenoic), alcohols (1-heptanol, 2-heptanol, 2-nonanol, (*E*)-2-octen-1-ol, 1-octen-3-ol), esters (ethyl butanoate, ethyl pentanoate), and ketones (2-hexanone, 2-heptanone, 2-octanone, oct-1-ene-3-one, 1-penten-3-one, (*Z*)-1-5-octadien-3-one), which is formed during the breakdown of hydroperoxides, contributes to the rancid off-flavour formation[10,16,18]. Due to the low threshold of the volatiles formed (Table 1.1), their off-flavour can be detected in oil long before formation of peroxides reaches its maximum. The outcome of advanced oxidation in foods may be alteration in colour, loss of solubility and other technological properties, decrease of nutritive value and reduced shelf life (Figure 1.1).

Table 1.1 *Threshold values of volatiles formed during oil oxidation*[24]

Compounds	Threshold (ppm)
Hydrocarbons	90-2150
Substituted furans	2-27
Vinyl alcohols	0.5-3
1-Alkenes	0.02-9
2-Alkenals	0.04-2.5
Alkanals	0.04-1.0
(*E,E*)-2,4-Alkadienals	0.04-0.3
Alkadienals with isolated double bonds	0.002-0.3
(*Z*)-Alkenals with isolated double bonds	0.0003-0.1
(*E,Z*)-Alkadienals	0.002-0.006
Vinyl ketones	0.00002-0.007

Decomposition of monohydroperoxides occurs by homolytic and heterolytic cleavage mechanisms[25]. As a result of homolytic cleavage, alkoxy radicals are rapidly produced from their precursors by loss of hydroxyl radical (Scheme 1.7). The alkoxy radicals can further undergo homolytic β-scission of a C–C bond to yield oxo-compounds and an alkyl or alkenyl radical. Alkyl and alkenyl radicals as well as the aldehyde produced may undergo further oxidation yielding various saturated and unsaturated hydrocarbons, alcohols, aldehydes and secondary hydroperoxides[11,21]. These secondary hydroperoxides may form an aldehyde or they can undergo further C–C cleavage producing new shorter length

alkyl radicals. It has been reported, for example, that homolytic cleavage of 13-hydroperoxy methyl linoleate, affords pentane, methyl-13-oxo-9,11-tridecadienoate and hexanal, while methyl octanoate, 2,4-decadienal and methyl-9-oxononanoate are produced during homolytic cleavage of 9-hydroperoxy methyl linoleate[23].

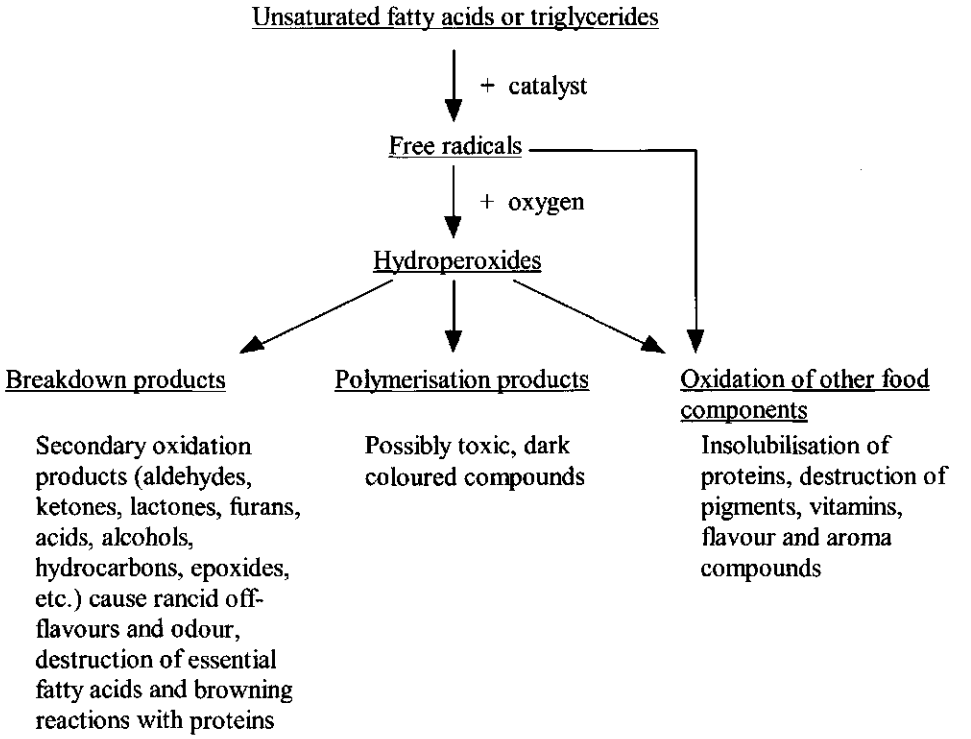
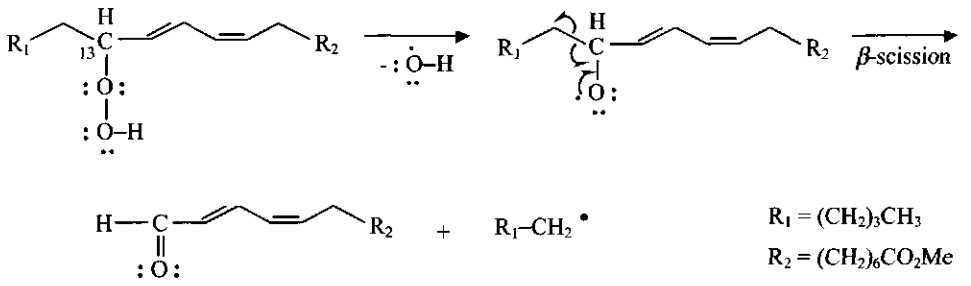
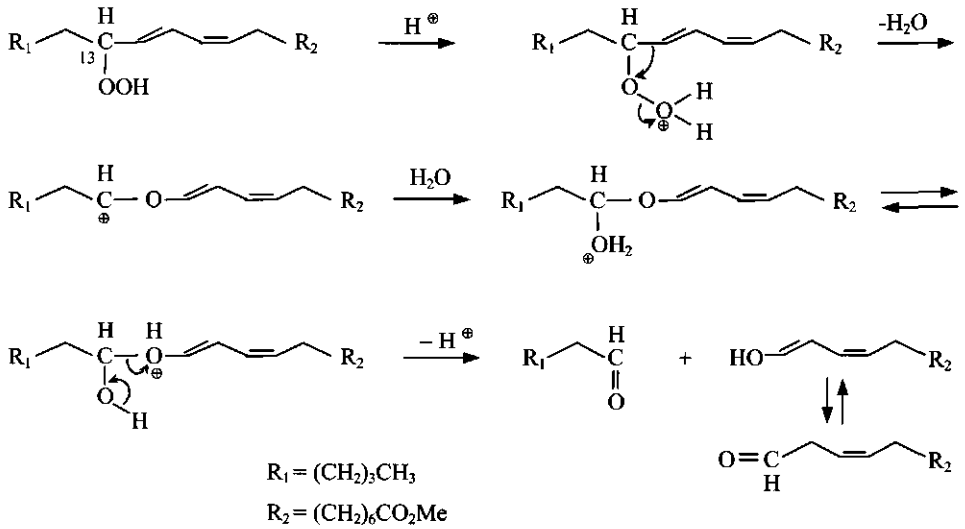


Figure 1.1 *Overall mechanisms involved in lipid oxidation[10,16].*



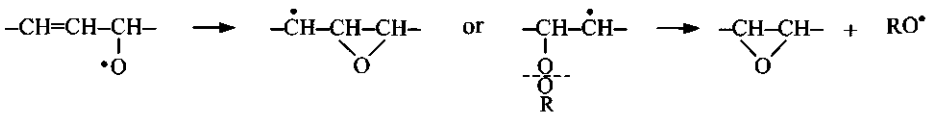
Scheme 1.7 *Homolytic cleavage of 13-OOH methyl linoleate hydroperoxide[17].*

Under acidic conditions, heterolytic cleavage between the hydroperoxide group and the allylic double bond occurs (Scheme 1.8). This results in carbocation intermediates from which a broad variety of characteristic carbonyl compounds are derived. For example, heterolytically cleavaged methyl linoleate hydroperoxides selectively form hexanal, methyl-9-oxononanoate, nonanal, and methyl-12-oxododecanoate[8,23].



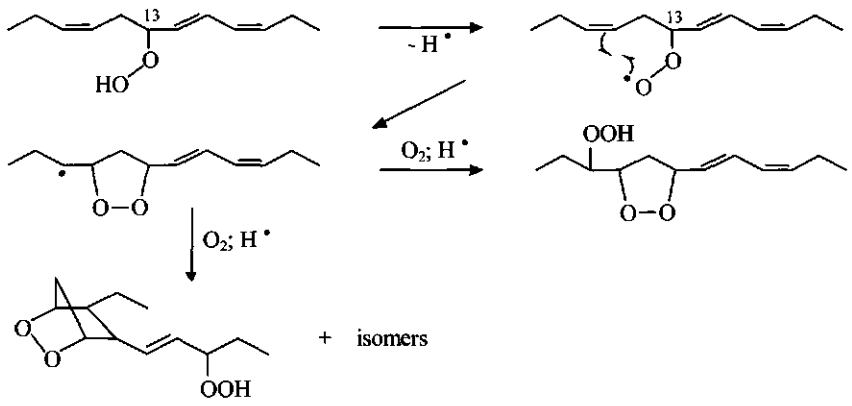
Scheme 1.8 *Heterolytic cleavage of 13-OOH methyl linoleate hydroperoxide.*

Various epoxides can be formed from unsaturated alkoxy and peroxy radicals (Scheme 1.9).



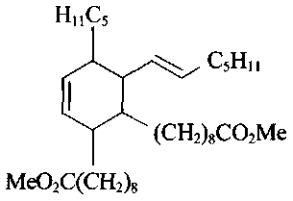
Scheme 1.9 *Formation of epoxides from alkoxy and peroxy radicals*[8].

Primary hydroperoxides of unsaturated fatty acids may form cyclic peroxides (Scheme 1.10). The amount of cyclic peroxides detected in products of methyl linolenate oxidation is of the same order of magnitude as that of the monohydroperoxides.



Scheme 1.10 *Mechanism of 1,3-cyclisation of 13-hydroperoxide of linolenate.*

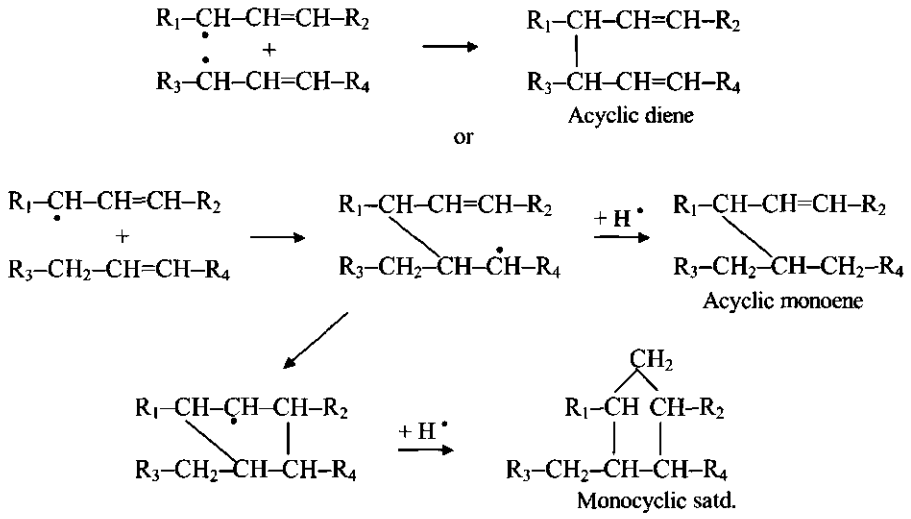
Hydroperoxides formed during primary and secondary steps of oxidation can also condense to cyclic and acyclic dimers and polymers which may, in their turn, further oxidise and breakdown forming a multitude of volatile products[3]. A conjugated double-bond system formed during linoleate oxidation can react with linoleate (or with oleate) and yield a tetrasubstituted cyclohexene (Scheme 1.11).



Scheme 1.11 *A cyclic dimer formed during oxidation of linoleate*[8].

An acyclic dimer can be generated when two oleate allyl radicals react. The reaction between oleate and its allyl radical may form cyclic saturated hydrocarbons (Scheme 1.12).

The formation mechanisms and presence of substituted furans, epoxy aldehydes, ketones, lactones, alkynes and aromatic compounds among hydroperoxide decomposition products are still not fully explained[5].



Scheme 1.12 *Reactions of oleate allyl radicals*[9].

1.3 Prevention of lipid oxidation by antioxidants

One of the natural mechanisms used by all aerobic organisms, including humans, to counteract autoxidation is the utilisation of a variety of antioxidant defence systems, which can be non-enzymatic or enzymatic. A spacious definition that covers antioxidative compounds acting *in vivo* was proposed by Halliwell and co-workers[6]. According to these authors, "an antioxidant is any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate". In food science, antioxidants are usually defined as "naturally present or added substances that retard the onset or slow down the rate of oxidation of oil or food lipids"[26].

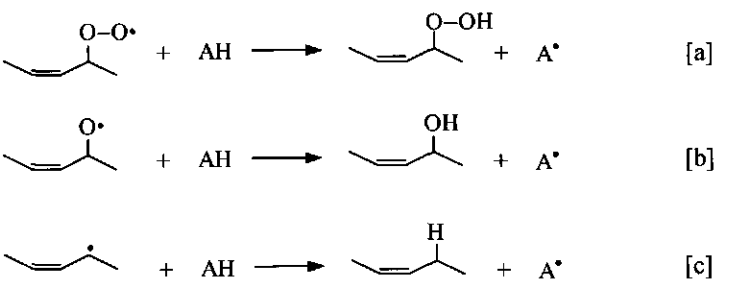
Since 1947, antioxidants and their combinations are used in U.S.A. food industry to protect oils, fats and fat containing foods from oxidative deterioration[27]. These compounds cannot reverse advanced oxidation of food lipids, or improve the quality of the product. The function of food antioxidants is to maintain the quality and extend the shelf life of oils, fats and fat containing foods by preventing oxidation of labile lipid components and consequently delaying negative oxidative changes in foods (e.g. loss of nutritive value, decrease in solubility, browning, discolouration, etc.).

Almost half a century of research on food antioxidants has led to several different classification systems. The mechanism of antioxidative action, the occurrence in nature, the enzymatic/non-enzymatic character of inhibition are just some of them. A certain confusion when defining secondary antioxidants and synergists of antioxidation can be noticed in the literature[1,10,12,27].

1.3.1 Primary and secondary antioxidants

By far the most common way to classify antioxidants is to divide them into two mechanistically distinct groups: *primary* and *secondary* antioxidants. According to their mode of action, they can also be named as *chain-breaking* and *preventive* antioxidants.

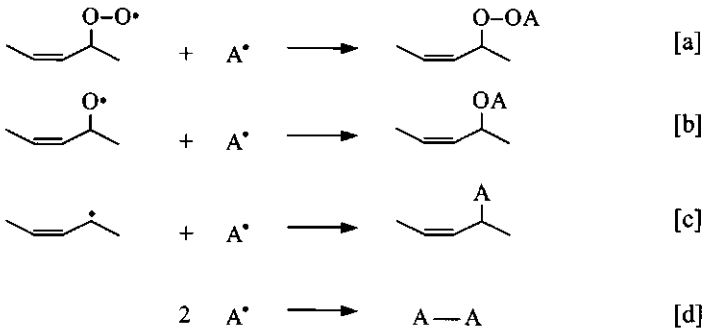
Antioxidants belonging to the class of *primary* antioxidants delay or inhibit the initiation step and interrupt the propagation step of the radical chain reaction. Primary antioxidants (AH) function by a radical chain-breaking mechanism that involves hydrogen donation to peroxy or oxy free radicals (Scheme 1.13 [a] and [b]). Antioxidants may also react directly with lipid radicals [c]. Such reactions result in lipid derivatives and a more stable antioxidant radical ($A^{\bullet}$)[18,21,26].



Scheme 1.13 *Free radical scavenging by a primary antioxidant.*

The propagation is a relatively slow step in lipid oxidation, therefore the peroxy radical which is formed during this step (Scheme 1.2 [a], [c]) is the major lipid radical at normal oxygen pressures. Moreover, peroxy radicals have lower energies than other radicals in the system (e.g. alkoxy)[28]. Thus, the reaction [a] in Scheme 1.13 influences the overall inhibition rate constant most strongly and is more important than reactions [b] and [c][15,22].

The antioxidant radical A$^{\bullet}$ may enter termination reactions with peroxy, oxy and other types or radicals yielding stable non-radical products (Scheme 1.14).



Scheme 1.14 *Termination reactions in which the antioxidant radical A$^{\bullet}$ takes part.*

The majority of antioxidants presently used are primary antioxidants which are mono- or polyhydroxy phenols that have various ring substitutions: synthetic phenolics (BHT, BHA, TBHQ, PG); tocopherols; plant phenolics (simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives and flavonoids). Hydrophilic primary antioxidants such as ascorbic acid, glutathione and certain amino acids (e.g. histidine, tyrosine, tryptophan, cysteine, proline and lysine) are reported to be able to scavenge free radicals present in the water phase of foods[28]. Molecular structures of some of the primary antioxidants are shown in Figure 1.2.

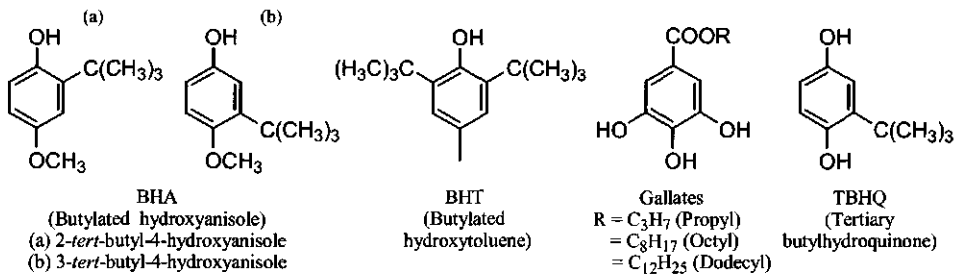


Figure 1.2 a *Chemical structures of some primary synthetic antioxidants.*

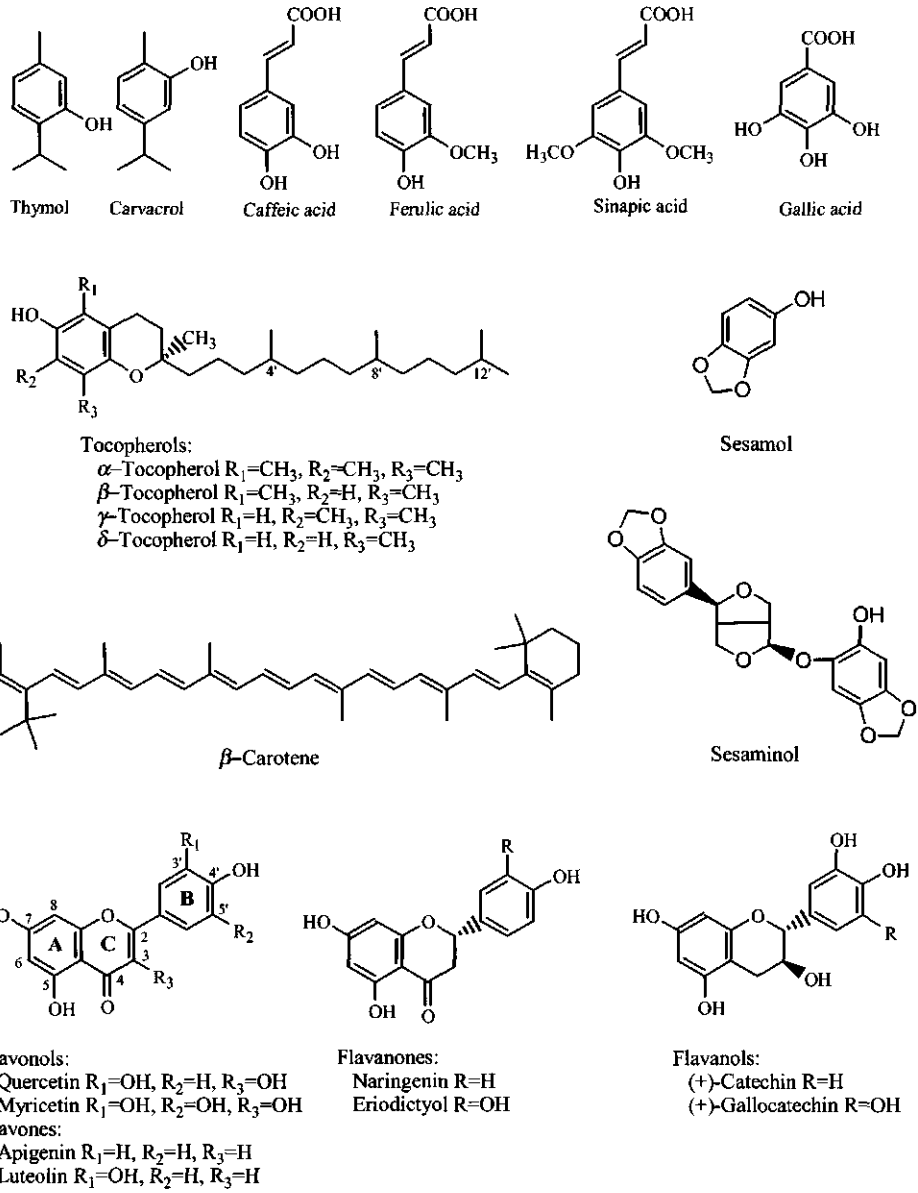
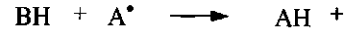


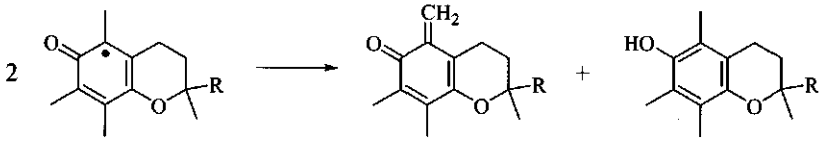
Figure 1.2 b *Chemical structures of some primary natural antioxidants.*

It has been demonstrated that some combinations of primary antioxidants (e.g. BHA with PG, BHT with BHA) exhibit synergistic effects. This occurs when another free-radical scavenging compound BH regenerates the antioxidant AH (Scheme 1.15).



Scheme 1.15 *Regeneration of the antioxidant (AH).*

Such a reaction is possible when the dissociation energy of B–H is smaller than that of A–H and when the reaction of BH with peroxy radicals is slow due to steric hindrance[29]. In some cases, a partial auto-regeneration of primary antioxidants (e.g. α-tocopherol) can take place as well (Scheme 1.16).



α-Tocopheryl radical Methyl tocopherylquinone α-Tocopherol

Scheme 1.16 *Partial auto-regeneration of α-tocopherol (R = $C_{16}H_{33}$).*

Another class of antioxidants is called *secondary antioxidants* or *preventive antioxidants*. These compounds reduce the rate of free radical chain reactions by processes other than conversion of free radicals to more stable species:

- *Metal chelation agents* affect metal-catalysed initiation of the chain reaction by raising its activation energy. Such compounds can form σ-bonds with a metal, reduce their redox potential and in this way stabilise the oxidised form of the metal ion. Besides preventing metal redox cycling, metal chelators can form insoluble metal complexes or occupy metal coordination sites. Steric hindrance of interactions between metals and lipids is thought to be another action mechanism[28]. It is known that antioxidant activity or even manifestation of pro-oxidant properties of some metal chelators (e.g. ethylenediamine tetraacetate, citric acid and ascorbate) depends on their concentrations, the moisture content and pH[10,28].
- *Oxygen scavengers* are another group of the secondary antioxidants that are capable to eliminate oxygen *via* hydrogen atom transfer[27]. Such compounds as ascorbyl palmitate, ascorbic acid, erythorbic acid, sodium erythorbate and sulphites are used to lower the concentration of dissolved or headspace oxygen from finished products[26].

- Secondary antioxidants, called *singlet oxygen quenchers*, can deactivate singlet oxygen physically or chemically. The chemical pathway consists of preferential oxidation of an antioxidant rather than lipids. Trapping a molecule of singlet oxygen by an antioxidant (e.g. β-carotene, lycopene and lutein) and dissipation of $^1O_2$ excess energy in the form of heat is the basis of physical inactivation[22,26].
- The non-enzymatic mechanism of peroxide decomposition involves secondary antioxidants called *peroxide decomposers*. Such compounds as thiodipropionic acid and dilauryl thiodipropionate[3] as well as ascorbic acid[22] may react with lipid hydroperoxides yielding stable non-radical products. Due to their low efficiency, peroxide decomposers are used more in stabilising polyolefin resins than foods[3,28].
- Antioxidants called *UV light deactivators* (e.g. carbon black, phenyl salicylate, and hydroxybenzophenone) are also rarely used in food industry.

Some antioxidants such as ascorbic acid or carotenoids can affect the course of lipid oxidation by several mechanisms, therefore, they are sometimes called *multifunctional antioxidants*. Ascorbic acid, for instance, may act as a quencher of singlet oxygen, hydroxyl radicals or superoxide radical anion, as a reducer of free radicals and radicals of primary antioxidants, as a molecular oxygen scavenger, as well as a decomposer of lipid peroxides[26,30]. Some carotenoids may function as singlet oxygen inhibitors, synergists of primary antioxidants or free radical inactivators, which at low oxygen pressures act by physical and chemical radical trapping mechanisms[22,28]. Antioxidative phospholipids are able to chelate metal ions, decompose hydroperoxides and regenerate primary antioxidants, whereas antioxidative compounds of the Maillard reaction are reported to have chain-breaking, metal chelating and hydrogen peroxide reducing abilities[22]. The abundance of types and groups of antioxidants that is found in the literature is most likely caused by the complexity of lipid autoxidation reactions as well as by a great number of factors that can affect them.

1.3.2 Synthetic and natural antioxidants

Independently of their mode of action, all antioxidant substances can be classified as *synthetic antioxidants* (Figure 1.2 a) or as *natural antioxidants* (Figure 1.2 b)[31]. The term "synthetic" is not always correctly used when characterising antioxidants.

Ascorbyl palmitate, for instance, is often classified as a natural antioxidant[32]. Although ascorbic acid is an abundant compound in nature, this particular ester does not occur naturally[33]. A broad variety of antioxidative substances of plant origin will be reviewed later in this text.

Among the synthetic antioxidants, BHT, BHA, gallates (propyl, octyl and dodecyl), TBHQ, THBP, and ethoxyquin are the most used[26]. Relatively well-studied chemical and technological properties, stable supply and low prices of synthetic antioxidants are important aspects which, among others, still determine the choice of antioxidant.

1.3.3 Inhibitors of enzyme-initiated lipid oxidation

Rancidification of food lipids is partially caused by enzymes that are present in raw materials as well as in finished food products. Naturally present lipases and lipoxygenases play an important role in peroxidation processes and in the formation of secondary products. The lipase and lipoxygenase catalysed oxidation can be controlled technologically (e.g. thermal deactivation of enzymes, application of minimal processing methods) or by introduction of *enzyme inhibitors*, a special class of antioxidants.

It is thought that certain plant phenolics inhibit enzymatic oxidation by inactivating the intermediate free radicals of the enzymatic reactions, by reducing the iron in the active site of the enzyme or by affecting lipoxygenase activity due to specific structural features of their molecules[21,28]. In case of quercetin and fisetin, their high soybean lipoxygenase inhibitory activity is related to the catechol structure in ring B. Replacement of the hydroxyl groups in the catechol structure (e.g. apigenin, chrysin) as well as glycosidation of the molecule (e.g. rutin) generally reduce the lipoxygenase inhibitory potency of flavonoids[21].

1.3.4 Antioxidative enzymes

Enzymatic antioxidants affect lipid oxidation by consuming molecular oxygen present in food systems, by deactivating superoxide radicals, and by converting hydrogen peroxide. Molecular oxygen is consumed by the glucose oxidase catalysed oxidation of glucose. The reaction yields D-gluconic acid and hydrogen peroxide[34]. Superoxide dismutase catalyses the conversion of the superoxide anion to triplet oxygen and also produces hydrogen peroxide[28,34]:

$$2\ O_2^{\bullet -} + 2H^+ \longrightarrow {}^3O_2 + H_2O_2$$

Hydrogen peroxide is further converted into water and triplet oxygen via a reaction catalysed by catalase, a heme-containing enzyme:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + {}^3O_2$$

Another enzyme that reacts with peroxides is glutathione peroxidase (GSH). This selenium-containing enzyme catalyses the reduction of both hydrogen and lipid peroxides, producing, respectively, water or fatty acid alcohol. During the reaction, an oxidised form of gluthatione (GSSG) is formed:

$$H_2O_2 + 2\ GSH \longrightarrow 2\ H_2O + GSSG$$

$$ROOH + 2\ GSH \longrightarrow ROH + H_2O + GSSG$$

Endogenous enzymatic antioxidants that occur in raw materials are mostly lost during the various technological steps. If their presence is desirable in the finished product, these enzymes have to be added at the very end of the processing line in a pure form or as a microbial inoculum, since several of them (e.g. glucose oxidase, catalase and superoxide dismutase) can be produced by microbial fermentation[34,35].

1.3.5 Molecular structure and antioxidative efficiency

The majority of primary antioxidants in foods are of the phenolic type[10]. As mentioned before, primary antioxidants can donate hydrogen or an electron, which in most cases comes from a phenolic hydroxyl group. Standard one-electron reduction potentials can be used to predict the ability of an antioxidant to donate a hydrogen to a radical. For example, α-tocopherol ($E^{\circ\prime}$ = 500 mV) or catechol ($E^{\circ\prime}$ = 530 mV) are capable of donating a hydrogen atom to a peroxy radical ($E^{\circ\prime}$ = 1000 mV). The formed radical form of the antioxidant has a much lower reactivity due to the resonance delocalisation (Figure 1.3 [A]) by the aromatic ring[28]. The most efficient antioxidants produce the lowest energy radicals.

The activity of a primary phenolic antioxidant can be increased by alkyl substitution at the ortho and para positions, which increases the electron density on the hydroxyl group by an inductive effect[22]. Bulky groups that are present in the 2 and 6 positions of the aromatic ring of an antioxidant (e.g. 2,6-di-*t*-butyl-4-

methylphenol) increase the stability of its phenoxyl radical due to electron donation (inductive effect) and due to steric hindrance[10,22]. Introduction of a second hydroxy group at the ortho position (Figure 1.3 [B]) also increases the stability of the phenoxy radical due to formation of an intramolecular hydrogen bond which affords more resonance stabilisation. Dihydroxybenzene derivatives possess stronger antioxidative properties due to their ability to donate two hydrogen atoms via a two step oxidation mechanism.

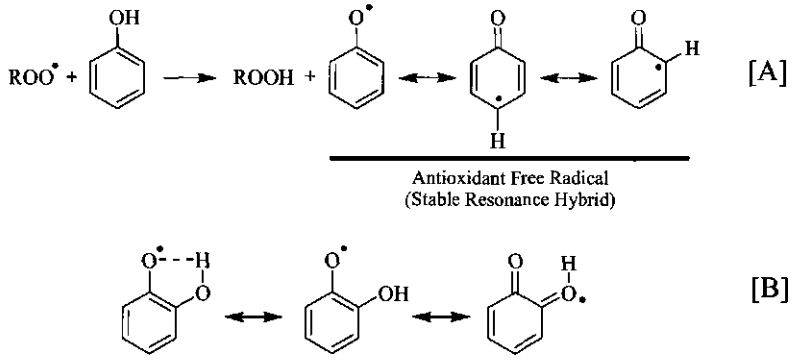


Figure 1.3 *Stabilisation of the phenoxyl radical: [A] formation of a stable resonance hybrid[2,28]; [B] stabilisation via an intramolecular hydrogen bond[22].*

Many known natural antioxidants that occur in fruits, vegetables, herbs and spices are flavonoids. Flavonoid aglycones are usually more effective antioxidants than the corresponding glycosides. A flavonoid molecule (Figure 1.2 b) with high antioxidant activity often has: (i) multiple hydroxyl groups in the B ring, especially at the 3' and 4' positions; (ii) 3- and 5-OH groups in rings A and C; and (iii) a 2,3 double bond in the C ring[21,33].

1.4 Methods of antioxidant activity assessment

1.4.1 Measuring lipid oxidation

The significance of lipid oxidation can be illustrated by the broad variety of techniques that are used to measure its extent in chemistry, food chemistry, biology, medicine and other sciences. The methods that are most relevant for the evaluation of food antioxidants will be discussed.

1.4.1.1 Evaluation of primary oxidation changes

Analysis of qualitative and quantitative changes in composition of reactants (i.e. oxygen and fatty acids) as well as monitoring formation and breakdown of hydroperoxides is important for studies of oxidative changes in food lipids. These tests can be used to evaluate oxidation in raw food products stored at low temperatures[35]. Many of them are used in model systems for the assessment of antioxidant activity, too.

- *Monitoring Oxygen Uptake.* Various stability tests employ this technique to evaluate oxygen consumption during substrate oxidation[36–38].
- *Determination of Changes in Fatty Acid Composition.* Fatty acids remaining in the lipid system under oxidation are analysed with GC according to the *AOCS Official Method Ce 1-62*[39].
- *Measuring Formation of Hydroperoxides.* Peroxide value/number (PV) can be determined by the *AOCS official Method Cd 8-53*[40]. The test is based on the ability of peroxides to liberate iodine from potassium iodide. Due to the instability of peroxides, care needs to be taken in interpretation of the results. The failure of this method to measure low peroxide levels has been reported in literature[41]. Alternative methods to monitor the formation of hydroperoxides involve spectrophotometric[42,43], chromatographic[44–46], polarographic[41], chemiluminescence[47,48] and other techniques.
- *Conjugated Diene Methods (Determination of Conjugated Diene Hydroperoxides – CDHP method; Determination of Conjugable Oxidation Products – C.O.P. value)* are based on the fact that conjugated dienes and trienes are formed during the oxidation of polyunsaturated fatty acids (Scheme 1.3). This technique is often used to replace/supplement peroxide value determination[49]. Fatty acids, hydroperoxides and other compounds with a conjugated diene structure absorb UV light at λ = 234 nm while a conjugated triene absorbs at λ = 268 nm. Although the degree of oxidation is not readily related to the magnitude of changes in the UV spectrum, these tests can be used in lipids of known fatty acid composition as a relative measurement[41].

1.4.1.2 Assessment of secondary oxidation changes

Aldehydes and ketones are the predominant volatile products resulting from the breakdown of hydroperoxides. These breakdown products can be effectively detected by sensory or instrumental methods.

- *Sensory methods* of rancidity determination often are ranked as the most useful ones. Although these tests are highly sensitive, they are expensive, dependent on the test panel and may vary from laboratory to laboratory[37,41,50].
- *GC analysis of static or dynamic headspace* of oxidised lipids: measurement of hexanal, which is the secondary product of linoleic acid oxidation[37,49,51–53], measurement of other volatile hydrocarbons, such as ethane, pentane, and aldehydes, such as 2-heptanal, 2,4-heptadienal, 2-decenal, 2,4-decadienal, etc[35,54–58].
- *Determination of fluorescent products*. Malonaldehyde and other carbonyl compounds can react with proteins that have free amino groups, deoxyribosenucleic acid, certain phospholipids, ribonuclease, and itself. Consequently, water and lipid soluble fluorescent products with the general structure of N,N^1-disubstituted 1-amino-3-iminopropene (R-NH-CH=CH-CH=N-R^1) can be formed. Such compounds are reported to have an excitation maximum around 360 nm and an emission maximum around 450 nm[41,54,59].
- *Thiobarbituric Acid Reactive Substances (TBARS)*. The method is based on the reaction of 2-thiobarbituric acid (TBA) with secondary oxidation products (malonaldehyde, alka-2,4-dienals, 2-alkenals, etc.), which results in a reddish-orange (λ = 532 nm) colour. The spectrophotometric analysis can by carried out (i) directly on the food product, (ii) on an extract of the food or (iii) on a portion of a steam distillate of the food[59]. The classical 2-thiobarbituric acid test is given by the *AOCS*[60]. The reactivity of TBA with compounds other than those derived from oxidising lipids (e.g. sucrose, wood smoke compounds) leads to poor correlations between the TBARS test and other oxidation tests[41].

Several other procedures that are based on reactions with anisidine, benzidine acetate, trichlorophenylhydrazine or hydroxylamine-hydrochloride can be used to determine *non-volatile carbonyl compounds* formed by degradation of hydroperoxides[41].

1.4.2 Stability tests and model systems for antioxidant activity evaluation

The period of time until rancidity of the product becomes apparent is generally accepted as a measurement of the stability of a fat or oil. According to the

definition of antioxidants used by food chemists[26], the main role of these additives is to slow down the rate of lipid peroxidation and to increase the shelf life of fats and oils. Thus, stability assessment techniques become of primary importance when evaluating the efficiency of antioxidants.

Shelf-storage tests under normal conditions take too much time, therefore the stability of fat containing foods is usually assessed by accelerated storage tests. These tests employ one or more accelerating factors such as temperature, oxygen pressure, light or metal catalysis to speed up the lipid peroxidation processes[61]. The advance of peroxidation in fats and fat containing foods under accelerated conditions is monitored by one or more of the methods that are normally used for the analysis of oxidised lipids. Care should be taken, however, with the interpretation of the results obtained, since many of these acceleration methods are reported to catalyse oxidation via other mechanisms than those of non-accelerated shelf-storage tests[50]. There is no universal stability test or 'ideal' acceleration system which would suit all purposes, that is why an antioxidant compound has to be tested by several techniques and under accelerated conditions which are as close as possible to those in real food systems[50,62]. Among the stability tests commonly used for antioxidant activity (AA) assessment of antioxidants are the Shelf-Storage Test, the Oven-Storage Test (or Schaal Oven Test); the Active-Oxygen Method (or Swift Stability Test); the Rancimat Test and the Oxygen-Absorption Method with its variations (i.e. Oxygen Bomb, Rarcroft-Warburg technique and Weight-Gain Technique).

Comparison of the AA of various compounds is often impossible when determined by a different stability test or when unequal oxidation conditions are used. Employment of model systems for antioxidant studies is often preferred, since they can simplify interpretation and facilitate comparison of the results as well as provide data on the antioxidation mechanisms involved.

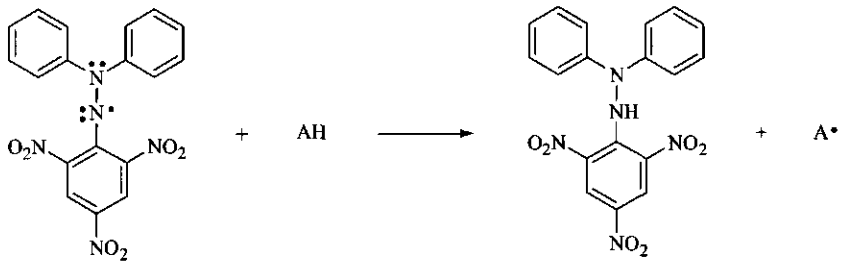
Most model systems are based on (i) competition between the antioxidant and an oxidisable indicator such as β-carotene for free radicals formed during the oxidation of simple lipids (e.g. linoleic acid); or on (ii) a direct reaction between a primary antioxidant and a radical. Such relatively stable radicals can be obtained commercially or can be easily produced from other commercial products. Radiolytic, photolytic, chemical and enzymatic systems are being used for the generation of oxygen radicals such as the superoxide anion $O_2^{\bullet -}$, or the hydroxyl $^{\bullet}OH$, peroxyl $ROO^{\bullet}$, and alkoxyl $RO^{\bullet}$ radicals[63]. Consumption of free radicals can be monitored by chemiluminescence, spectrophotometry, pulse radiolysis, electron

spin resonance (ESR) and other techniques. Some of the often used model systems for antioxidant evaluation are listed below.

- *Linoleic Acid Oxidation.* A variety of acceleration factors such as temperature[64], enzymes[65,66], heme[67], 2,2'-azobis (2-amidinopropane) dihydrochloride[68] can be used to catalyse peroxidation of linoleic acid. The effect of introduced antioxidants may be evaluated by monitoring the oxygen uptake[64,68], measuring the remaining linoleic acid concentration[65,67], determining the conjugated diene and/or the peroxide value[66], assessing the hexanal formation in headspace[66], or by a variety of other techniques reviewed earlier.
- *Ferric Thiocyanate Method.* This method also applies the oxidation of linoleic acid at 40°C in the dark in the presence of an antioxidant, ammonium thiocyanate, ferrous chloride and hydrochloric acid. The antioxidative effect is evaluated spectrophotometrically by monitoring the inhibition of red colour formation in the system[69–71].
- *Oxygen Electrode Method.* This procedure also uses linoleic acid as a substrate. Oxidation of linoleic acid in an atmosphere saturated with oxygen is initiated by 2,2'-azobis (2,4-dimethylvaleronitrile) at 37°C. The rate of oxygen consumption, which depends on antioxidant activity, is measured with an oxygen electrode[38].
- *Methyl Linoleate Oxidation.* This method involves accelerated oxidation (temperature, oxygen bubbling, etc.) of methyl linoleate in presence of antioxidants and GC monitoring of the residual methyl linoleate or the oxygen consumption[72-74].
- *Simultaneous Oxidation of Linoleic Acid and β-Carotene.* Three main variations of this method exist:
  - spectrophotometric assessment of β-carotene bleaching inhibition by introduced antioxidants[75–78];
  - visual monitoring of the intensity and persistence of the β-carotene colour in agar diffusion plates with added antioxidant[75,79].
  - applying a β-carotene-linoleic acid spray on thin layer and paper chromatograms of the antioxidant extracts[80–82].
- *DPPH$^{\bullet}$ Test.* This test involves spectrophotometric monitoring of the residual DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) radical (at λ = 515 nm) after a primary antioxidant is introduced[83–87]. Detection of antioxidant compounds on TLC plates using a DPPH$^{\bullet}$ spray[71] and a post-column

detection of antioxidant compounds in HPLC eluates[88] are two other modifications of this test.

Due to the extensive resonance, DPPH$^{\bullet}$ is a relatively stable radical under ambient conditions. The radical can accept an electron or a hydrogen radical losing in the process its strong absorption at 515 nm (Scheme 1.17)[88]:



Scheme 1.17 *Reduction of DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) by a radical scavenging antioxidant AH.*

Kinetics and reaction mechanisms depend on the molecular structure of antioxidants as was demonstrated by Brand-Williams *et al.*[85].

- *Chemiluminescence Test.* Inhibition of chemiluminescence caused by luminol/isoluminol oxidation in presence of a catalyst reflects the radical scavenging activity of introduced antioxidants[89–92].
- *Hydroxyl Radical Scavenging Assay.* Scavenging of hydroxyl radicals, which can be generated by the Fenton reaction, by γ-radiolysis or by microbial processes can be monitored by ESR, by chemiluminescence, by the 2-deoxyribose oxidation assay (detection of malondialdehyde) or by the benzoic acid hydroxylation method (fluorescence measurement)[93–95].
- *Metmyoglobin/H_2O_2/ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) Assay.* The test measures spectrophotometrically the ability of an antioxidant to scavenge the ABTS$^{\bullet+}$ radical, which has characteristic absorption maxima at λ = 660 nm, 734 nm and 815 nm, and compares it with the scavenging by Trolox, a synthetic water-soluble analogue of vitamin E ("Trolox Equivalent Antioxidant Capacity" or TEAC)[96–98]. A similar technique is the horseradish peroxidase/H_2O_2/ABTS assay, which monitors the decrease in ABTS$^{\bullet+}$ radical absorption at λ = 414 nm. The efficiency of an antioxidant is compared with that of ascorbic acid[99-101]. Some authors stress the lack of correlation between ABTS$^{\bullet+}$ results and results obtained with other radical species, particularly $^{\bullet}$OH and $O_2^{\bullet-}$ [102].

When testing an antioxidant for application purposes, it is important to choose relevant radical species, which are indeed involved in the oxidation mechanisms of a particular system. Usage of a simple oxidation substrate (e.g. linoleic acid, methyl linoleate) as well as monitoring scavenging efficiency towards a single radical should not be regarded as completely reliable, since they do not provide a complete knowledge on protective properties of a particular antioxidant in real foods. Additional activity studies in actual food systems under appropriate oxidation conditions are therefore strongly recommended. In case the introduced antioxidant possesses a 'carry-through' effect (an ability to survive processing steps), care should be taken to avoid possible negative interactions with other oxidisable targets that are present in a food matrix or *in vivo*[93,103].

1.5 Natural antioxidants

Living organisms are surrounded by an atmosphere that contains 20.9% of oxygen, which is a relatively reactive gas. Evolution of life has provided several tools to combat oxidation processes in procaryotic and eucaryotic cells. Therefore, it is not surprising, that compounds with various antioxidative properties occur in plant and animal tissues as well as in microorganisms. A number of initiators of oxidation can target lipids, proteins, carbohydrates or DNA in a cell. It may be noticed that the natural antioxidative defence mechanisms employed by a cell or an organism are oxidation substrate and oxidation initiator dependent. For example, plant seeds and germs naturally contain high concentrations of tocopherols and other antioxidants, which control the rate of autoxidation of polyunsaturated compounds. Leaves of most plants are protected with a layer of wax, which functions as an oxygen barrier. Various enzymatic systems, endogenous radical scavengers and quenchers of active oxygen species regulate autoxidation of animal cell wall lipids, proteins and DNA.

A very small number of naturally occurring plant antioxidants have been discovered and analysed so far. Even less of them are of commercial significance. Up to now, tocopherols, ascorbic acid and its derivatives as well as extracts from rosemary and sage have been industrially applied in foods. A strong tendency towards "all-natural" foods leads to exploration of other sources of food antioxidants. This can be illustrated by the appearance of a number of new products such as aromatised olive oil, butter, frying margarine, spreads, potato chips, frozen foods, patties and sauces that contain fresh or dried aromatic herbs (e.g. chives,

garlic, onion, thyme, tarragon, oregano, etc.) or herb extracts, which fulfil the double purpose of adding flavour and enhancing stability.

Many processed foods may contain residual tocopherols, phospholipids, enzymatic antioxidants, carotenoids and other natural antioxidants. Besides, some specific antioxidative compounds can actually be synthesised along diverse technological processes. Roasting, Maillard reaction, fermentation[104,105] (e.g. production of tempeh, natto, miso, soy sauce) or wood smoking are reported to generate various protective systems against oxidation[7,106]. Antioxidative compounds or their mixtures can also be obtained from certain microorganisms: uric acid, 3,4-dimethoxyphenol and 3-hydroxyindolin-2-one from various isolates of marine bacteria[71], resorstatin from *Pseudomonas* strain DC 165[107] and carazostatin and benthocyanin A from two *Streptomyces* species[108,109]. An effective antioxidant atrovenetin can be isolated from the mycelium of *Penicillium paraherquei*[110].

1.5.1 Tocopherols

Up to date, tocopherols are commonly recognised as the most important group of natural antioxidants[111]. Tocopherols and related substances (tocols and tocotrienols) are found in vegetable oils (e.g. soybean, sunflower oil), nuts (e.g. almonds, hazelnuts), wholemeal flour and green vegetables[112]. Natural tocopherols usually occur as a mixture of α-, γ- and δ-tocopherols. β-Tocopherol is found in only few vegetable oils. Tocopherols can be extracted from "sludges" which are obtained during the deodorization of refined vegetable oils[113]. There are some contradictory references concerning the radical scavenging activity of individual tocopherol isomers. The different oxidation substrates and the different evaluation techniques used are probably the reason for this. The majority of authors claim that δ-tocopherol is the most effective antioxidant, β- or γ-tocopherol have medium activity and α-tocopherol is the least active[113–115]. Others report that α-tocopherol is the most active and δ-tocopherol the least active[16,116].

Protection of animal fats is the primary field where tocopherols are used. Synergistic mixtures containing tocopherols together with phospholipids or other synergists (e.g. ascorbic acid, citric acid, some amino acids, gallate, or lecithin derivatives) are often applied[16,111,113].

α- and β- Tocopheryl radicals at low concentrations (50–500 mg/kg) undergo dimerisation, dismutation, addition or oxidation when reacting with lipid

radicals. In all these cases stable non-radical products are formed. However, a pro-oxidant activity can be detected at high levels of α- and β-tocopherol (> 600-700 mg/kg, at room temperature). The reason for this is that a significant amount of tocopheryl radicals participate in propagation of the chain-reaction by yielding new peroxy radicals when they react with lipid hydroperoxides[30].

1.5.2 Ascorbic acid

Ascorbic acid, or vitamin C, naturally occurs in rather high concentrations (up to 1% and more) in most fruits and vegetables: parsley, cabbage, onions, citrus fruits, paprika, apples, potatoes, blackcurrants, green peppers, kiwi fruit, etc, as well as in aqueous fractions of animal tissues[16,112]. Presently, ascorbic acid and its isomer, erythorbic acid (D-ascorbic acid) are synthesised in large quantities for application in foods[26,30].

Ascorbic acid is virtually insoluble in fats, while ascorbyl palmitate has a much better solubility in oils. As was already mentioned earlier, ascorbic acid acts as a multifunctional antioxidant and as a synergist for primary antioxidants. At higher concentrations and in certain food systems, this compound may exhibit pro-oxidant properties, especially in aqueous fat systems and when trace metals are present. It was suggested that an ascorbic acid-Me^{2+}-oxygen complex catalyses decomposition of hydroperoxides and accelerates the chain reaction of lipid oxidation[117].

1.5.3 Other antioxidant substances and their sources

A number of articles and reviews concerning plant sources of natural antioxidants have been published during the last two decades[7,31,102,104,113,118–122]. The majority refers to medicinal plants, herbs and spices as a source of phenolic antioxidants[79,97,111,121,123,124]. Many of these studies are focused on antioxidant compounds in sage[73,125–130] and rosemary[130-138]. Besides rosemary and sage, our screening studies of nine aromatic plants also revealed antioxidant activity in thyme and marjoram extracts[139].

Other important sources of natural antioxidants are cereals, olives and oilseeds, which are rich in tocopherols, tocotrienols, phenolic compounds, phospholipids and lignin derivatives[31,113,140]. Certain vitamins (vitamins C and E and provitamin A) as well as phenolic compounds such as flavonols,

anthocyanidins, flavones, isoflavones, flavanols, flavanones and hydroxycinnamates (e.g. caffeic, chlorogenic, ferulic, and *p*-coumaric acids) contribute to the antioxidative properties of fruits and vegetables[31,34]. Antioxidant activity or the presence of antioxidative compounds have also been reported in cocoa shells[141,142], grapes, tea[34,104], seaweeds[104], legumes[78], protein hydrolysates[31] as well as woods[118], bark[143], resins[31] and leaf waxes[144] (Table 1.2).

Table 1.2 *List of some higher plants containing known antioxidants*

Name	Latin name	Identified active compounds	Ref.
	Medicinal plants, spices and herbs		
Rosemary	*Rosmarinus officinalis* L.	Carnosol, carnosic acid, rosmarinic acid, rosmanol, epirosmanol, isorosmanol, rosmaridiphenol, rosmariquinone	128-135, 138, 139
Sage	*Salvia officinalis* L.	Carnosol, carnosic acid, rosmanol, epirosmanol, isorosmanol, rosmadial, methyl carnosate, phenolic glycosides	125, 128-130, 139, 145
Thyme	*Thymus vulgaris* L.	Carvacrol, thymol, *p*-cymene-2,3-diol, eriodictyol, caffeic acid, cirsineol, genkwanin and 4 other flavones, biphenyl compounds	139, 146-151
Oregano	*Origanum vulgare* L.	Rosmarinic acid, carvacrol, thymol, phenyl glycoside, protocatechuic acid, caffeic acid and 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy]phenylpropionic acid, apigenin and eriodictyol, dihydrokaempferol, dihydroquercetin	81, 139, 146, 152, 153
Chia seeds	*Salvia hispanica* L.	Caffeic acid, chlorogenic acid	75
Licorice	*Glycyrrhiza glabra* L.	3-Arylcoumarin, licochalchone A and B	154
Osbeckia	*Osbeckia chinensis* L.	Casuarinin, kaempferol, quercetin and quercetin glucosides, ellagic acid, gallic acid, methyl gallate, tannins	122
Summer savory	*Satureja hortensis* L.	Rosmarinic acid	155; 156
Red pepper	*Capsicum annum* L.	Capsicin, dihydrocapsicin	122

Clove	*Eugenia caryophyllata* Thunb.	Eugenol, gallic acid	171
Nutmeg	*Myristica fragrans* Houtt.	Eugenol, isoeugenol, linalool, limonene	146
Turmeric	*Curcuma longa* L.	Turmerin, curcumin	121
Ginger	*Zingiber officinalis* Roscoe	Curcumin, gingerols, diarylheptanoids, [6]-shogaol	70
Vanilla	*Vanilla planifolia* Andr.	Vanillin, vanillic acid	112
Cistanche or broomrape	*Cistanche deserticola* Y.C.Ma.	Acteoside, isoacteoside, 2'-acetylacteoside, tubulosides A and B, echinacoside, syringalide A 3'-α-rhamnopyranoside, cistanosides A and F	157
Safflower	*Cartamus tinctorius* L.	4,4"-Bis(*N*-*p*-coumaroyl)serotonin; 4-[*N*-(*p*-coumaroyl)serotonin-4"-yl]-*N*-feruloylserotonin; 4,4"-bis (*N*-feruloyl)serotonin	158
Gingko or maidenhair tree	*Ginkgo biloba* L.	Quercetin, kaempferol, isorhamnetin, other flavonoids and their glycosides	97, 122
	Oilseeds and oil crops		
Cocoa	*Theobroma cacao* L.	Catechin, epicatechin, chlorogenic acid	118
Soybean	*Glycine max* (L.) Merill	Genistein, daidzein, glycitein, phenolic acids, tocopherols, amino acids, peptides	159, 160
Sesame-seed	*Sesamum indicum* L.	Sesamol, sesaminol, tocopherol, sesamolinol	118, 122, 161
Cottonseed	*Gosypium herbaceum* L.	Quercetin, rutin, kaempferol, gossypetin, heracetin, dihydroquercetin, quercetrin, isoquercetrin	118, 144, 162
Peanuts	*Arachis hypogea* L.	Taxifolin	80, 144
Mustard seeds	*Sinapis alba* L., *Sinapis nigra* L.	Sinigrin, phenolic acids, sinapic acid methyl ester	94, 118
	Cereal crops		
Rice	*Oryza sativa* L.	Orizanol, isovitexin, cyanidine-3-*O*-β-D-glucopyranoside, pinoresinol, other phenolics	104, 122

Wild rice	*Zizania aquatica* L.	Phytic acid, luteolin glycoside, *p*-hydroxy acetophenone glycoside, 3,4,5-trimethoxycinnamic acid	104
Barley leaves	*Hordeum vulgare* L.	2'-*O*-Glucosylisovitexin	104
Oat	*Avena sativa* L.	Esters of caffeic and ferulic acids	122
	Fruits and vegetables		
Apples	*Malus silvestris* L.	Quercetin, epicatechin, chlorogenic acid, *p*-coumaric acid, phloridzin	120, 163, 164
Citrus fruits	*Citrus limon* (L.) Burm., *Citrus paradisi* Macf., *Citrus taitensis* Risso	Naringenin, hesperetin, hesperedin, eriocitrin, naringin, neoeriocitrin, narirutin, *p*-coumaric acid, caffeic acid, ferulic acid	34
Grapes	*Vitis vinifera* L.	Tannic acid, quercetin, procyanidines, other phenolics	104, 118
Onion	*Allium cepa* L.	Quercetin, myricetin	165
Carrots	*Daucus carota* L.	Lignin, carotene	118
Tomato	*Lycopersicum esculentum* L.	Quercetin, lycopene, rutin, prunin	112
Garlic	*Allium sativum* L.	*S*-Allylcysteine, *S*-allylmercaptocysteine	166
Horseradish	*Armoracia rusticana* Gaertn.	Sinigrin	118
Azuki beans	*Vigna angularis* Ohwi et Ohashi	Procyanidin dimers	78
	Other plant material		
Tea	*Camellia sinensis* (L.) O. Kuntze	Catechins, chlorogenic acid, theaflavins, caffein, quercetin, kaempferol, myricetin glycosides	167, 168
Gentiana	*Gentiana arisanensis* Hayata	Isooriontin-6"-O-glucoside, genistic acid	31, 169
Rooibos tea	*Aspalathus linearis* (Burm.f.) Dahlg	Vitexin, rutin, quercetin, aspalathin, luteolin, isoquercitrin, (+)-catechin; protocatechuic, caffeic, *p*-hydroxybenzoic, *p*-coumaric, ferulic and vanillic acids	170
Eucalyptus	*Eucalyptus globulus* Labill.	Tetrahydrocurcumin, *n*-tritriacontan-16,18-dione and their homologues	122
Wood and bark	various species	Tannins, dihydroquercetin, other phenolic compounds	118, 143, 144

1.6 Application of natural antioxidants

Due to the complexity of food products and the variety of possible oxidation mechanisms, it is not possible to find a single antioxidant that can control all factors involved in food oxidation[114,172]. Several questions have to be answered when designing an antioxidative protection system for a food product:

- Do the raw materials already possess natural antioxidants and at what concentrations?
- What kind of food processing will take place?
- Which oxidation mechanisms are expected? In case of free radical autoxidation, what are the main initiators and which free radicals are involved?
- What is the earliest stage at which an antioxidant can be added?
- What physical and chemical properties will the end-product have (type of lipids and their degree of unsaturation; polarity/apolarity of constituents; pH; humidity, presence of trace metals, enzymes, microorganisms; concentration of salt, etc.)?
- Will the product be submitted to further processing?
- Is a 'carry-through' effect desirable or not?
- Under which conditions (illumination, temperature, and type of packaging) and for how long will the product be stored?
- Where is the product going to be marketed and what legislation is valid there?

A comprehensive chemical analysis of raw materials is indispensable. The results will point out oxidation-sensitive constituents and provide information on already present indigenous antioxidants and synergists. Based on the data on the raw materials and thinking of the technological manipulations to which these materials will be submitted, the type of antioxidant, its amount, its possible synergists, and the stage of addition must be chosen.

Both synthetic and natural antioxidants can be employed as free radical scavengers or as secondary antioxidants. Synthetic antioxidants such as BHT, BHA, TBHQ and EDTA have dominated the market for decades, however, the appearance of publications on possible toxic properties of synthetic antioxidants and their breakdown products is presently shifting the preferences towards antioxidants of natural origin[111,173,174]. Most of the natural antioxidants are "generally recognised as safe" (GRAS) and many of them have been used by humans for centuries. Nevertheless, it would be wrong to claim that "natural =

safe" is always true. Nordihydroguaiaretic acid (NDGA) isolated from the *Larrea divaricata* bush as well as gossypol (a pigment in cottonseeds) can serve as examples of toxic natural compounds with antioxidant properties.

Regardless of the numerous reports on new natural compounds with antioxidative properties, their broad application is still hindered by a few important factors. Firstly, the price of purified and sufficiently active natural antioxidants is much higher than that of their synthetic counterparts. It was reported, for instance, that natural tocopherols can effectively prevent oxidation of β-carotene in butter and margarine, nevertheless the use of tocopherols is minimal since BHA and BHT are much cheaper (4.5 and 15 times, respectively)[10,175]. Synthetically produced nature-identical compounds such as taxifolin, quercetin or carnosic acid are not yet available[144]. Secondly, the functionality of natural antioxidant isolates is often rather limited by their low solubility in fats, low stability (in light and at elevated temperatures), and by imparted colour, off-flavour or aftertaste in the end-product[144,113]. Finally, the toxicological aspects of natural isolates and their breakdown products are often not known. Interactions between added natural antioxidants and other food constituents may also yield products of questionable properties. In case of a possible 'carry-through' effect, antioxidant/pro-oxidant as well as toxicity studies in biological systems are strongly advised and should be a precondition for their application[93,103,112].

1.7 Scope and content of this thesis

The major goal of this study was to evaluate aromatic plants, which are commonly cultivated in Lithuania, as a possible source of natural antioxidants for food use.

The use of synthetic antioxidants nowadays is strictly controlled in view of their possible negative effects on human health. Many species of aromatic and medicinal plants have been in use for centuries and, therefore, are generally considered as safe for human consumption. Some of them are known to possess antioxidant properties[73,79,111,121,123,124]. A general overview on natural antioxidants is given in **Chapter 1**.

Information on natural sources of food antioxidants available in Lithuania is scarce. Estimation of antioxidant properties of local herbs is mostly done on a basis of ethnobotanical knowledge or by drawing parallels with studies on similar herbs harvested elsewhere. Analytical screening for herbs with antioxidant activity was an indispensable primary step of this work. Furthermore, selection of adequate

extraction techniques for the isolation of antioxidants from plants is important for activity and yield of the product obtained. Thus, the preliminary study described in **Chapter 2** was aimed at broadening the knowledge on antioxidant activity of Lithuanian-grown herbs and at revealing the most promising extraction routes to obtain natural antioxidants.

One of the commonly used techniques for assessment of the antioxidant activity is based on monitoring oxidation in the β-carotene-linoleic acid model system (β-CLAMS). Two modifications of this procedure, namely the β-CLAMS agar-diffusion test and the β-CLAMS TLC proved to be unsuitable for the quantitative evaluation of the antioxidative effect. The third modification of the β-CLAMS test employs spectrophotometry to follow the bleaching rate of the β-carotene, and in this way a quantitative evaluation becomes possible. However, the used procedure of the spectrophotometric test did not allow the simultaneous assessment of several samples. Due to this, undesirable variations in the initial properties of the β-CLAMS occurred and inaccurate values of antioxidant activity were obtained. The scope of experimental work presented in **Chapter 3** was the development of a spectrophotometric multisample technique based on the β-CLAMS for faster and better evaluation of antioxidant activity.

A more detailed analysis of the active constituents in selected herb extracts was needed. The usual phytochemical procedures for isolation and purification of active compounds, prior to their antioxidant activity assessment, often yielded erroneous results due to chemical instability of the antioxidants. The study reported in **Chapter 4** was focused on the development of a convenient technique that allows HPLC separation and on-line chemiluminescence detection of radical scavenging compounds in crude herb extracts. Improvement and comparison of this method with another on-line technique (HPLC-DPPH$^{\bullet}$) of post-column detection of radical scavengers is presented in **Chapter 5**.

The isolation, purification and structure elucidation of the main radical scavenging constituents in *Thymus vulgaris*, one of the Lithuanian-cultivated herbs with high antioxidant activity, were the main objectives of the study described in **Chapter 6**. Isolation was carried out by various partitioning steps and was guided by simultaneous detection of radical scavenging activity. A number of analytical techniques such as TLC, HPLC-DAD, GC-MS, proton and carbon NMR were employed for the identification of the purified active compounds.

The main aspects of this experimental work are generalised in **Chapter 7.** The radical scavenging potential of various isolates from thyme and from other Lithuanian-grown aromatic/medicinal herbs is discussed. The advantage of solvent

extraction over other isolation techniques is stressed. Further, all presently known antioxidant compounds from thyme are listed. Ways to obtain better results from antioxidant activity tests are discussed as well. Finally, several important aspects for future research are mentioned.

1.8 References and notes

1. Frankel, E.N. 1996. Antioxidants in lipid foods and their impact on food quality. *Food Chem.*, 57, 51–55.
2. Sherwin, E.R. 1978. Oxidation and antioxidants in fat and oil processing. *J. Am. Oil Chem. Soc.*, 55, 809–814.
3. Ladikos, D. and Lougovois, V. 1990. Lipid oxidation in muscle foods: a review. *Food Chem.*, 35, 295–314.
4. Sanders, T. 1987. Toxicological considerations in oxidative rancidity of animal fats. *Food Sci. Technol. Today,* 1, 162–164.
5. Frankel, E.N. 1984. Lipid oxidation: mechanisms, products and biological significance. *J. Am. Oil Chem. Soc.*, 62, 1908–1917.
6. Davies, K.J.A. 1995. Oxidative Stress: the Paradox of Aerobic Life. p. 1-13. In: C. Rice-Evans, B. Halliwell and G.G. Lunt (eds). Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives, Biochemical Society, London.
7. Löliger, J. and Wille, H.J. 1993. Natural Antioxidants. *Oils Fats Int.,* 9, 18–22.
8. Nawar, W.W. 1996. Lipids. p. 225–319. In: O.R. Fennema (ed). Food Chemistry. Marcel Dekker, New York.
9. Hamilton, R.J., Kalu, C., Prisk, E., Padley, F.B. and Pierce, H. 1997. Chemistry of free radicals in lipids. *Food Chem.*, 60, 193–199.
10. Labuza, T.P. 1971. Kinetics of lipid oxidation in foods. *CRC Crit. Rev. Food Technol.*, 2, 355–404.
11. Min, D.B. 1998. Lipid oxidation of edible oil. p. 283–296. In: C.C. Akoh and D.B. Min (eds). Food Lipids. Marcel Dekker, New York.
12. Angelo, A.J.St. 1996. Lipid oxidation in foods. *Crit. Rev. Food Sci. Nutr.*, 36, 175–224.
13. Porter, N.A., Caldwell, S.E. and Mills, K.A. 1995. Mechanisms of free radical oxidation of unsaturated lipids. *Lipids,* 30, 277–290.

14. Larson, R.A. 1997. Naturally Occurring Antioxidants. CRC Press LLC, Boca Raton. 189 pp.

15. Swern, D. 1961. Primary products of olefinic autoxidations. p. 1–54. In: W.O. Lundberg (ed). Autoxidation and Antioxidants, Interscience, New York.

16. Kochhar, S.P. 1993. Oxidative pathways to the formation of off-flavours. p. 150-201. In: M.J. Saxby (ed). Food Taints and Off-Flavours. Blackie Academic & Professional, London.

17. Personal communication of de Groot, Ae. 2001. Laboratory of Organic Chemistry, Wageningen University, The Netherlands.

18. Hamilton, R.J. 1994. The chemistry of rancidity in foods. p. 1–20. In: J.C. Allen and R.J. Hamilton (eds). Rancidity in Foods. Applied Science Publishers, London.

19. http://class.fst.ohio-state.edu/FST605/lectures/Lect5.html; accessed on 20.03.2001.

20. Taylor, A.J. 1987. Effect of water quality on lipid oxidation. *Food Sci. Technol. Today,* 1, 158–159.

21. Hsieh, R.J. and Kinsella, J.E. 1989. Oxidation of polyunsaturated fatty acids: Mechanisms, products, and inhibition with emphasis on fish. *Adv. Food Nutr. Res.*, 33, 233–339.

22. Gordon, M.H. 1990. The mechanism of antioxidant action *in vitro*. p. 1–18. In: B.J.F. Hudson (ed). Food Antioxidants. Elsevier, London.

23. Frankel, E.N. 1991. Review. Recent advances in lipid oxidation. *J. Sci. Food Agric.*, 42, 495–511.

24. Frankel, E.N. 1985. Chemistry of autoxidation: mechanism, products and flavor significance. p. 1–37. In: D.B. Min and T.H. Smouse (eds). Flavor Chemistry of Fats and Oils. American Oil Chemists' Society, Champaign, IL.

25. Frankel, E.N. 1982. Volatile lipid oxidation products. *Prog. Lipid Res.*, 22, 1–33.

26. Reische, D.W., Lillard, D.A. and Eitenmiller, R.R. 1998. Antioxidants. p. 423–448. In: C.C. Akoh and D.B. Min (eds). Food Lipids. Marcel Dekker, New York.

27. Dziezak, J.D. 1986. Preservatives: antioxidants. The ultimate answer to oxidation. *Food Technol.*, 40, 94–97, 100–102.

28. Decker, E.A. 1998. Antioxidant mechanisms. p. 397–422. In: C.C. Akoh and D.B. Min (eds). Food Lipids. Marcel Dekker, New York.

29. Uri, N. 1961. Mechanism of antioxidation. p. 133–169. In: W.O. Lundberg (ed). Autoxidation and Antioxidants. Interscience, New York.

30. Schuler, P. 1990. Natural antioxidants exploited commercially. p. 99–170. In: B.J.F. Hudson (ed). Food Antioxidants. Elsevier, London.

31. Dugan, L.R. 1980. Natural antioxidants. p. 261–282. In: M.G. Simic and M. Karel (eds). Autoxidation in Food and Biological Systems. Plenum Press, New York.

32. Dorko, C. 1994. Antioxidants used in foods. Food Technol., 48, 33.

33. Rice-Evans, C. 1995. Plant polyphenols: free radical scavengers or chain-breaking antioxidants? p. 103-116. In: C. Rice-Evans, B. Halliwell and G.G. Lunt (eds). Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives, Biochemical Society, London.

34. Rice-Evans, C.A., Miller, N.J. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci.*, 2, 152–159.

35. Gray, J.I. and Monahan, F.J. 1992. Measurement of lipid oxidation in meat and meat products. *Trends Food Sci. Technol.*, 3, 315–319.

36. Cort, W.M. 1974. Hemoglobin peroxidation test screens antioxidants. *Food Technol.*, 28, 60–66.

37. Madsen, H.L., Andersen, L., Christiansen, L., Brockhoff, P. and Bertelsen, G. 1996. Antioxidative activity of summer savory (*Satureja hortensis* L.) and rosemary (*Rosmarinus officinalis* L.) in minced, cooked pork meat. *Z. Lebensm. Unters. Forsch.*, 203, 333–338.

38. Kumamoto, M. and Sonda, T. 1998. Evaluation of the antioxidative activity of tea by an oxygen electrode method. *Biosci. Biotechnol. Biochem.*, 62, 175–177.

39. Method Ce 1-62. 1986. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 3rd edn., R.O. Walker (ed). American Oil Chemists' Society, Champaign.

40. Method Cd 8-53. 1990. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., D. Firestone (ed). American Oil Chemists' Society, Champaign.

41. Gray, J.I. 1978. Measurement of lipid oxidation: a review. *J. Am. Oil Chem. Soc.*, 55, 539–546.

42. Hicks, M. and Gebicki, J.M. 1979. A spectrophotometric method for the determination of lipid hydroperoxides. *Anal. Biochem.*, 99, 249–253.

43. Akaza, I. and Aota, N. 1990. Colorimetric determination of lipid hydroperoxides in oils and fats with microperoxidase. *Talanta*, 37, 925–929.

44. Makinen, M., Piironen, V. and Hopia, A. 1996. Postcolumn chemiluminescence, ultraviolet and evaporative light-scattering detectors in high-performance liquid chromatographic determination of triacylglycerol oxidation products. *J. Chromatogr.*, 734, 221–229.

45. Yang, G.C. 1992. Detection of lipid hydroperoxides by HPLC coupled with post-column reaction. *Trends Food Sci. Technol.*, 3, 15–18.

46. Christensen, T.C. and Holmer, G. 1996. Lipid oxidation determination in butter and dairy spreads by HPLC. *J. Food Sci.*, 61, 486–489.

47. Matthaus, B.W. 1996. Determination of the oxidative stability of vegetable oils by Rancimat and conductivity and chemiluminescence measurements. *J. Am. Oil Chem. Soc.*, 73, 1039–1043.

48. Tena, M.T., Valcarcel, M., Hidalgo, P.J. and Ubera, J.L. 1997. Supercritical fluid extraction of natural antioxidants from rosemary: comparison with liquid solvent sonication. *Anal. Chem.*, 69, 521–526.

49. Frankel, E.N., Huang, S.W., Kanner, J. and German, J.B. 1994. Interfacial phenomena in the evaluation of antioxidants: bulk oils vs emulsions. *J. Agric. Food Chem.*, 42, 1054–1059.

50. Frankel, E.N. 1993. In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci. Technol.*, 4, 220–225.

51. Huang, S.W., Frankel, E.N. and German, J.B. 1994. Antioxidant activity of α- and γ-tocopherols in bulk oils and in oil-in-water emulsions. *J. Agric. Food Chem.*, 42, 2108–2114.

52. Fritsch, C.W. and Gale, J.A. 1977. Hexanal as a measure of rancidity in low fat foods. *J. Am. Oil Chem. Soc.*, 54, 225–228.

53. Warner, K., Evans, C.D., List, G.R., Dupuy, H.P., Wadsworth, J.I. and Goneen, G.E. 1978. Flavor score correlation with pentanal and hexanal contents of vegetable oil. *J. Am. Oil Chem. Soc.*, 55, 252–256.

54. Coxon, D. 1987. Measurement of lipid oxidation. *Food Sci. Technol. Today*, 1, 164–166.

55. Warner, K. and Frankel, E.N. 1985. Flavor stability of soybean oil based on induction periods for the formation of volatile compounds by gas chromatography. *J. Am. Oil Chem. Soc.*, 62, 100–103.

56. Warner, K., Evans, C.D., List, G.R., Boundy, B.K. and Kwolek, W.F. 1974. Pentane formation and rancidity in vegetable oils and in potato chips. *J. Food Sci.*, 39, 761–765.

57. Ulbert, F. and Roubicek, D. 1993. Evaluation of a static headspace gas chromatographic method for the determination of lipid peroxides. *Food Chem.*, 46, 137–141.

58. Evans, C.D., List, G.R., Hoffmann, R.L. and Moser, H.A. 1969. Edible oil quality as measured by thermal release of pentane. *J. Am. Oil Chem. Soc.*, 46, 501–504.

59. Melton, S.L. 1983. Methodology for following lipid oxidation in muscle foods. *Food Technol.*, 37, 105–111, 116.

60. Method Cd 19-90. 1990. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th ed., D. Firestone (ed). American Oil Chemists' Society, Champaign.

61. Sherwin, E.R. 1968. Methods for stability and antioxidant measurement. *J. Am. Oil Chem. Soc.*, 45, 632A, 634A, 646A, 648A.

62. Meyer, A.S. and Frankel, E.N. 1994. Methods of evaluating food antioxidants: reply. *Trends Food Sci. Technol.*, 5, 56–57.

63. Bors, W., Michel, C. and Saran, M. 1994. Flavonoid antioxidants: rate constants for reactions with oxygen radicals. *Methods Enzymol.*, 234, 420–429.

64. Poter, W.L., Levasseur, L.A. and Henick, A.S. 1977. Evaluation of some natural and synthetic phenolic antioxidants in linoleic acid monolayers on silica. *J. Food Sci.*, 42, 1533–1535.

65. Rogstad, A. and Reinskou, T. 1980. Evaluation of antioxidant activity: I. Application of an enzyme-catalyzed system. *J. Am. Oil Chem. Soc.*, 57, 189–191.

66. Roozen, J.P., Frankel, E.N. and Kinsella, J.E. 1994. Enzymic and autoxidation of lipids in low fat foods: model of linoleic acid in emulsified hexadecane. *Food Chem.*, 50, 33–38.

67. Rogstad, A. 1980. Evaluation of antioxidant activity: II. Application of a heme-catalyzed system. *J. Am. Oil Chem. Soc.*, 57, 191–193.

68. Pryor, W.A., Strickland, T. and Church, D.F. 1988. Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous sodium dodecyl sulfate micelle solutions. *J. Amer. Chem. Soc.*, 110, 2224–2229.

69. Jitoe, A., Masuda, T., Tengah, I.G.P., Suprapta, D.W., Gara, I.W. and Nakatani, N. 1992. Antioxidant activity of tropical ginger extracts and analysis of the contained curcuminoids. *J. Agric. Food Chem.*, 40, 1337–1340.

70. Kikuzaki, H. and Nakatani, N. 1993. Antioxidant effects of some ginger constituents. *J. Food Sci.*, 58, 1407–1410.

71. Takao, T., Kitatani, F., Watanabe, N., Yagi, A. and Sakata, K. 1994. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Biosci. Biotech. Biochem.*, 58, 1780–1783.

72. Cuvelier, M.E., Berset, C. and Richard, H. 1990. Use of a new test for determining comparative antioxidative activity of butylated hydroxytoluene, alpha-and gamma-tocopherols and extracts from rosemary and sage. *Sci. Aliments*, 10, 797–806.

73. Cuvelier, M.E., Richard, H. and Berset, C. 1996. Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. *J. Am. Oil Chem. Soc.*, 73, 645–652.

74. Roginsky, V.A., Barsukova, T.K, Remorova, A.A. and Bors, W. 1996. Moderate antioxidative efficiencies of flavonoids during peroxidation of methyl linoleate in homogeneous and micellar solutions. *J. Am. Oil Chem. Soc.*, 73, 777–786.

75. Taga, M.S., Miller, E.E. and Pratt, D.E. 1984. Chia seeds as a source of natural lipid antioxidants. *J. Am. Oil Chem. Soc.*, 61, 928–931.

76. Miller, H.E. 1971. A simplified method for the evaluation of antioxidants. *J. Am. Oil Chem. Soc.*, 48, 91.

77. Marco, G.J. 1968. A rapid method for evaluation of antioxidants. *J. Am. Oil Chem. Soc.*, 45, 594–598.

78. Ariga, T., Koshiyama, I. and Fukushima, D. 1988. Antioxidative properties of procyanidins B-1 and B-3 from Azuki beans in aqueous systems. *Agric. Biol. Chem.*, 52, 2717–2722.

79. Piccaglia, R., Marotti, M., Giovanelli, E., Deans S.G. and Eaglesham, E. 1993. Antibacterial and antioxidant properties of Mediterranean aromatic plants. *Ind. Crop. Prod.*, 2, 47–50.

80. Pratt, D.E. and Miller, E.E.A. 1984. Flavonoid antioxidant in Spanish peanuts. *J. Am. Oil Chem. Soc.*, 61, 1064–1067.

81. Vekiari, S.A., Oreopoulou, V., Tzia, C. and Thomopoulos, C.D. 1993. Oregano flavonoids as lipid antioxidants. *J. Am. Oil Chem. Soc.*, 70, 483–487.

82. Zegarska, Z., Rafalowski, R., Amarowicz, R., Karamac, M. and Shahidi, F. 1998. Stabilization of butter with deodorized rosemary extract. *Z. Lebensm. Unters. Forsch. A*, 206, 99–102.

83. Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature*, 181, 1199–1200.

84. Yoshida, T., Mori, K., Hatano, T., Okumura, T., Yehara, I., Komagoe, K., Fujita, Y. and Okuda, T. 1989. Studies on inhibition mechanism of autoxidation by tannins and flavonoids. V. Radical-scavenging effects of tannins and related polyphenols on 1,1-diphenyl-2-picrylhydrazyl radical. *Chem. Pharm. Bull.*, 37, 1919–1921.

85. Brand-Williams, W., Cuvelier, M.E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. u. - Technol.*, 28, 25–30.

86. Sanchez-Moreno, C., Larrauri, J.A. and Saura-Calixto, F. 1998. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.*, 76, 270–276.

87. Bondet, V., Brand-Williams, W. and Berset C. 1997. Kinetics and mechanisms of antioxidant activity using the DPPH$^{\bullet}$ free radical method. *Lebensm.-Wiss. u.-Technol.*, 30, 609–615.

88. Yamaguchi, T., Takamura, H., Matoba ,T. and Terao, J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Biosci. Biotechnol. Biochem.*, 62, 1201–1204.

89. Merfort, I., Heilmann, J., Weiss, M., Pietta, P. and Gardana, C. 1996. Radical scavenger activity of three flavonoid metabolites studied by inhibition of chemiluminescence in human PMNs (polymorphonuclear cels). *Planta Med.*, 62, 289–292.

90. Whitehead, T.P., Thorpe, G.H.G. and Maxwell, S.R.J. 1992. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal. Chim. Acta*, 266, 265–277.

91. Hirayama, O., Takagi, M., Hukumoto, K. and Katoh, S. 1997. Evaluation of antioxidant activity by chemiluminescence. *Anal. Biochem.*, 247, 237–241.

92. Arora, A. and Strasburg, G.M. 1997. Development and validation of fluorescence spectroscopic assays to evaluate antioxidant efficacy. Application to metal chelators. *J. Am. Oil Chem. Soc.*, 74, 1031–1040.

93. Halliwell, B., Aeschbach, R., Löliger, J. and Aruoma, O.I. 1995. The characterisation of antioxidants. *Food Chem. Toxic.*, 33, 601–617.

94. Chung, S.K., Osawa, T. and Kawakishi, S. 1997. Hydroxyl radical-scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*). *Biosci. Biotech. Biochem.*, 61, 118–123.

95. Reitberger, T. and Gierer, J. 1988. Chemiluminescence as a means to study the role of hydroxyl radicals in oxidative processes. *Holzforschung*, 42, 351–356.

96. Miller, N.J., Rice-Evans, C. and Davies, M.J. 1993. A new method for measuring antioxidant activity. *Biochem. Soc. Trans.*, 21, 95S.

97. Pietta, P., Simonetti, P. and Mauri, P. 1998. Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.*, 46, 4487–4490.

98. Strube, M., Haenen, G.R.M.M, van den Berg, H. and Bast, A. 1997. Pitfalls in a method for assessment of total antioxidant capacity. *Free Rad. Res.*, 26, 515–521.

99. Arnao, M.B., Cano, A., Hernández-Ruiz, J., García-Cánovas, F. and Acosta, M. 1995. Total antioxidant activity. A method for its estimation in plant material p.265–269. In: C. Garcia-Viguera, M. Castaner, M.I. Gil, F. Ferreres and F.A. Tomas-Barberan (eds). Current Trends in Fruit and Vegetables Phytochemistry. CSIC, Murcia.

100. Cano, A., Hernandez-Ruiz, J., Garcia-Cánovas, F., Acosta, M. and Arnao, M.B. 1998. An end-point method for estimation of the total antioxidant activity in plant material. *Phytochem. Anal.*, 9, 196–202.

101. Arnao, M.B., Cano, A., Hernandez-Ruiz, J., Garcia-Cánovas, F. and Acosta, M. 1996. Inhibition by L-ascorbic acid and other antioxidants of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) oxidation catalyzed by peroxidase: a new approach for determining total antioxidant status of foods. *Anal. Biochem.*, 236, 255–261.

102. Mantle, D., Anderton, J.G., Falkous, G., Barnes, M., Jones, P. and Perry, E.K. 1998. Comparison of methods for determination of total antioxidant status: application to analysis of medicinal plant essential oils. *Compar. Biochem. Physiol. B*, 121, 385–391.

103. Halliwell, B. 1995. How to characterize an antioxidant: an update. p. 73-102. In: C. Rice-Evans, B. Halliwell and G.G. Lunt (eds). Free Radicals and

Oxidative Stress: Environment, Drugs and Food Additives, Biochemical Society, London.

104. Ramarathnam, N., Osawa, T., Ochi, H. and Kawakishi, S. 1995. The contribution of plant food antioxidants to human health. *Trends Food Sci. Technol.*, 6, 75–82.

105. Scott, G. 1995. Antioxidants in food packaging: a risk factor? p. 235-246. In: C. Rice-Evans, B. Halliwell and G.G. Lunt (eds). Free Radicals and Oxidative Stress: Environment, Drugs and Food Additives, Biochemical Society, London.

106. Löliger, J. 1994. Natural antioxidants. p. 89–108. In: J.C. Allen and R.J. Hamilton (eds). Rancidity in Foods. Applied Science Publishers, London.

107. Kato, S., Shindo, K., Kawai, H., Matsuoka, M. and Mochizuki, J. 1993. Studies on free radical scavenging substances from microorganisms. III. Isolation and structural elucidation of a novel free radical scavenger, resorstatin. *J. Antibiot.*, 46, 1024–1026.

108. Kato, S., Kawai, H., Kawasaki, T., Toda, Y., Urata, T. and Hayakawa, Y. 1989. Studies on free radical scavenging substances from microorganisms. I. Carazostatin, a new free radical scavenger produced by *Streptomyces chromofuscus* DC 118. *J. Antibiot.*, 42, 1879–1881.

109. Bestmann, H.J., Erler, J. and Vostrowsky, O. 1985. Extraktion von Thymian mit flüssigem CO_2 im Labormaßstab. *Z. Lebensm. Unters. Forsch.*, 180, 491–493.

110. Ishikawa, Y., Morimoto, K. and Iseki, S. 1991. Atrovenetin as a potent antioxidant compound from *Penicillium* species. *J. Am. Oil Chem. Soc.*, 68, 666–668.

111. Löliger, J. 1991. Natural antioxidants. *Lipid Technol.*, 3, 58–61.

112. Aruoma, O.I. 1994. Nutrition and health aspects of free radicals and antioxidants. *Food Chem. Toxic.*, 32, 671–683.

113. Pokorny, J. 1991. Natural antioxidant for food use. *Trends Food Sci. Technol.*, 2, 223–227.

114. Six, P. 1994. Current research in natural food antioxidants. *Inform*, 5, 679–687.

115. Dougherty, M.E. 1993. Effectiveness of natural antioxidants compared to synthetic antioxidants. *IFI*, 3, 27–32.

116. Burton, G.W. and Ingold, K.U. 1986. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.*, 19, 194.

117. Grosch, W. 1977. Abbau von Linol- und Linolensäure Hydroperoxiden in Gegenwart von Ascorbinsäure. *Z. Lebensm. Unters. Forsch.*, 163, 4–7.

118. Gordon, M. 1987. Novel antioxidants. *Food Sci. Technol. Today*, 1, 172–173.

119. Larson, R.A. 1988. The antioxidants of higher plants. *Phytochemistry*, 27, 969–978.

120. Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M. and Pridham, J.B. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Rad. Res.*, 22, 375–383.

121. Madsen, H.L. and Bertelsen, G. 1995. Spices as antioxidants. *Trends Food Sci. Technol.*, 6, 271–277.

122. Namiki, M. 1990. Antioxidants / antimutagens in food. *Crit. Rev. Food Sci. Nutr.*, 29, 273–300.

123. Evans, R.J. and Reynhout, G.S. 1992. Alternates to synthetic antioxidants. p. 27–42. In: G. Charalambous (ed). Food Science and Human Nutrition. Elsevier Science Publishers, Amsterdam.

124. Yanishlieva, N.V. and Marinova, E.M. 1995. Antioxidant activity of selected species of the family Lamiaceae grown in Bulgaria. *Nahrung*, 39, 458–464.

125. Cuvelier, M.E., Berset, C. and Richard, H. 1994. Separation of major antioxidants in sage by high performance liquid chromatography. *Sci. Aliments*, 14, 811–815.

126. Djarmati, Z., Jankov, R.M., Schwirtlich, E., Djulinac, B. and Djordjevic, A. 1991. High antioxidant activity of extracts obtained from sage by supercritical CO_2 extraction. *J. Am. Oil Chem. Soc.*, 68, 731–734.

127. Ternes, W. and Schwarz, K. 1995. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. IV. Determination of carnosic acid in different foodstuffs. *Z. Lebensm. Unters. Forsch.*, 201, 548–550.

128. Schwarz, K. and Ternes, W. 1992. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. I. Determination of phenolic diterpenes with antioxidative activity amongst tocochromanols using HPLC. *Z. Lebensm. Unters. Forsch.*, 195, 95–98.

129. Schwarz, K. and Ternes, W. 1992. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. II. Isolation of carnosic acid and formation of other phenolic diterpenes. *Z. Lebensm. Unters. Forsch.*, 195, 99–103.

130. Brieskorn, C.H. and Domling, H.J. 1969. Carnosolsäure, der wichtige antioxydativ wirksame Inhaltsstoff des Rosmarin- und Salbeiblattes.

[Carnosic acid as antioxidant in rosemary- and sage-leaves]. *Z. Lebensm. Unters. Forsch.*, 141, 10–16.

131. Nakatani, N. and Inatani, R. 1984. Two antioxidative diterpenes from rosemary (*Rosmarinus officinalis* L.) and a revised structure for rosmanol. *Agric. Biol. Chem.*, 48, 2081–2085.

132. Nakatani, N. and Inatani, R. 1981. Structure of rosmanol, a new antioxidant from rosemary (*Rosmarinus officinalis* L.). *Agric. Biol. Chem.*, 45, 2385–2386.

133. Inatani, R., Nakatani, N., Fuwa, H. and Seto, H. 1982. Structure of a new antioxidative phenolic diterpene isolated from rosemary (*Rosmarinus officinalis* L.). *Agric. Biol. Chem.*, 46, 1661–1666.

134. Inatani, R., Nakatani, N. and Fuwa, H. 1983. Antioxidative effect of the constituents of rosemary (*Rosmarinus officinalis* L.) and their derivatives. *Agric. Biol. Chem.*, 47, 521–528.

135. Houlihan, C.M., Ho, C.T. and Chang, S.S. 1984. Elucidation of the chemical structure of a novel antioxidant, rosmaridiphenol, isolated from rosemary. *J. Am. Oil Chem. Soc.*, 61, 1036–1039.

136. Richheimer, S.L., Bernart, M.W., King, G.A., Kent, M.C. and Bailey, D.T. 1996. Antioxidant activity of lipid-soluble phenolic diterpenes from rosemary. *J. Am. Oil Chem. Soc.*, 73, 507–514.

137. Aruoma, O.I., Halliwell, B., Aeschbach, R. and Löligers, J. 1992. Antioxidant and pro-oxidant properties of active rosemary constituents: carnosol and carnosic acid. *Xenobiotica*, 22, 257–268.

138. Wu, J.W. 1981. Isolation and identification of the natural antioxidants from *Rosmarinus officinalis* L. *Dissert. Abstr. Intern.*, 42, 1379-B.

139. Dapkevicius, A., Venskutonis, R., van Beek, T.A. and Linssen, J.P.H. 1998. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J. Sci. Food Agric.*, 77, 140–146.

140. Caponio, F., Alloggio, V. and Gomes, T. 1999. Phenolic compounds of virgin olive oil: Influence of paste preparation techniques. *Food Chem.*, 64, 203–209.

141. Bracco, U., Löliger, J. and Viret, J.L. 1981. Production and use of natural antioxidants. *J. Am. Oil Chem. Soc.*, 58, 687–690.

142. Azizah, A.H., Ruslawati, N.M.N. and Tee, T.S. 1999. Extraction and characterization of antioxidant from cocoa by-products. *Food Chem.*, 64, 199–202.

143. Marinova, E.M., Yanishlieva, N.V. and Kostova, I.N. 1994. Antioxidative action of the ethanolic extract and some hydroxycoumarins of *Fraxinus ornus* bark. *Food Chem.*, 51, 125–132.

144. Pratt, D.E and Hudson, B.J.F. 1990. Natural antioxidants not exploited commercially. p. 171–192. In: B.J.F. Hudson (ed). Food Antioxidants. Elsevier, London.

145. Wang, M., Shao, Y., Li, J., Zhu, N., Rangarajan, M., La Voie, E.J. and Ho, C.T. 1999. Antioxidative phenolic glycosides from sage (*Salvia officinalis*). *J. Nat. Prod.*, 62, 454–456.

146. Dorman, H.J.D, Deans, S.G., Noble, R.C. and Surai, P. 1995. Evaluation in vitro of plant essential oils as natural antioxidants. *J. Essent. Oil Res.*, 7, 645–651.

147. Schwarz, K., Ernst, H. and Ternes, W. 1996. Evaluation of antioxidative constituents from thyme. *J. Sci. Food Agric.*, 70, 217–223.

148. Ternes, W., Gronemeyer, M. and Schwarz, K. 1995. Determination of *p*-cymene-2,3-diol, thymol, and carvacrol in different foodstuffs. *Z. Lebensm. Unters. Forsch.*, 201, 544–547.

149. Miura, K. and Nakatani, N. 1989. Antioxidative activity of flavonoids from thyme (*Thymus vulgaris* L.). *Agric. Biol. Chem.*, 53, 3043–3045.

150. Schulz, J.M. und Herrmann, K. 1980. Über das Vorkommen von Hydroxibenzoesäuren und Hydroxizimtsäuren in Gewürzen. IV. Über Gewürzphenole. *Z. Lebensm. Unters. Forsch.*, 171, 193–199.

151. Miura, K., Inagaki, T. and Nakatani, N. 1989. Structure and activity of new deodorant biphenyl compounds from thyme (*Thymus vulgaris* L.). *Chem. Pharm. Bull.*, 37, 1816–1819.

152. Lagouri, V., Blekas, G., Tsimidou, M., Kokkini, S. and Boskou, D. 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebensm. Unters. Forsch.*, 197, 20–23.

153. Kikuzaki, H. and Nakatani, N. 1989. Structure of a new antioxidative phenolic acid from oregano (*Origanum vulgare* L.). *Agric. Biol. Chem.*, 53, 519–524.

154. Gordon, M.H. and An, J. 1995. Antioxidant activity of flavonoids isolated from licorice. *J. Agric. Food Chem.*, 43, 1784–1788.

155. Yanishlieva, N.V., Marinova, E.M., Marekov, I.N. and Gordon, M.H. 1997. Effect of an ethanol extract from summer savory (*Satureja hortensis* L.) on the stability of sunflower oil at frying temperature. *J. Sci. Food Agric.*, 74, 524–530.

156. Madsen, H.L., Andersen, L., Christiansen, L., Brockhoff, P. and Bertelsen, G. 1996. Antioxidative activity of summer savory (*Satureja hortensis* L.) and rosemary (*Rosmarinus officinalis* L.) in minced, cooked pork meat. *Z. Lebensm. Unters. Forsch.*, 203, 333–338.

157. Xiong, Q., Kadota, S., Tani, T. and Namba, T. 1996. Antioxidative effects of phenylethanoids from *Cistanche deserticola*. *Biol. Pharm. Bull.*, 19, 1580–1585.

158. Zhang, H.L., Nagatsu, A. and Sakakibara, J. 1996. Novel antioxidants from safflower (*Cartamus tinctorius* L.) oil cake. *Chem. Pharm. Bull.*, 44, 874–876.

159. Pratt, D.E. 1980. Natural antioxidants of soybeans and other oil-seeds. p. 283–293. In: M.G. Simic and M Karel (eds). Autoxidation in Food and Biological Systems. Plenum Press, New York.

160. Hayes, R.E., Brookwater, G.N. and Bagley, E.B. 1977. Antioxidant activity of soybean flour and derivatives. *J. Food Sci.*, 42, 1527–1532.

161. Fukuda, Y., Osawa, T., Namiki, M. and Ozaki, T. 1985. Studies on antioxidative substances in sesame seeds. *Agric. Biol. Chem.*, 49, 301–306.

162. Whittern, C.C., Miller, E.E., and Pratt, D.E. 1984. Cottonseed flavonoids as lipid antioxidants. *J. Am. Oil Chem. Soc.*, 61, 1075–1078.

163. Lu, Y. and Foo, L. 1995. Polyphenolic antioxidants of apple. p. 3–8. In: C. Garcia-Viguera, M. Castaner, M.I. Gil, F. Ferreres and F.A. Tomas-Barberan (eds). Current Trends in Fruit and Vegetables Phytochemistry. CSIC, Murcia.

164. Miller, N.J. and Rice-Evans, C.A. 1997. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem.*, 60, 331–337.

165. Pratt, D.E. 1965. Lipid antioxidants in plant tissue. *J. Food. Sci.*, 30, 737–742.

166. Imai, J., Ide, N., Nagae, S., Moriguchi, T., Matsuura, H. and Itakura, Y. 1994. Antioxidant and radical scavenging effects of aged garlic extract and its constituents. *Planta Med.*, 60, 417–420.

167. Price, K.R., Rhodes, M.J.C. and Barnes, K.A. 1998. Flavonol glycoside content and composition of tea infusions made from commercially available teas and tea products. *J. Agric. Food Chem.*, 46, 2517–2522.

168. Vinson, J.A. and Dabbagh, Y.A. 1998. Tea phenols: Antioxidant effectiveness of teas, tea components, tea fractions and their binding with lipoproteins. *Nutr. Res.*, 18, 1067–1075.

169. Ko, F.N., Chu, C.C., Lin, C.N., Chang, C.C. and Teng, C.M. 1998. Isoorientin-6"-O-glucoside, a water-soluble antioxidant isolated from *Gentiana arisanensis*. *Biochim. Biophys. Acta*, 1389, 81–90.

170. von Gadow, A., Joubert, E. and Hansmann, C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-Tocopherol, BHT, and BHA. *J. Agric. Food Chem.*, 45, 632–638.

171. Kramer, R.E. 1985. Antioxidants in clove. *J. Am. Oil Chem. Soc.*, 62, 111–113.

172. Decker, E.A. 1998. Strategies for manipulating the prooxidative/antioxidative balance of foods to maximize oxidative stability. *Trends Food Sci. Technol.*, 9, 241–248.

173. Thompson, D.C., Cha, Y.M. and Trush, M.A. 1989. The peroxidase-dependent activation of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) to reactive intermediates. *J. Biol. Chem.*, 264, 3957–3965.

174. Barlow, S.M. 1990. Toxicological aspects of antioxidants used as food additives. p. 253–307. In: B.J.F. Hudson (ed). Food Antioxidants. Elsevier, London.

175. Personal communication of Airdo, K. 2001. Kraft Chemical Company, Melrose Park, IL.

ANTIOXIDANT ACTIVITY OF EXTRACTS OBTAINED BY DIFFERENT ISOLATION PROCEDURES FROM SOME AROMATIC HERBS GROWN IN LITHUANIA*

2.1 Introduction

The demand for natural additives, including natural antioxidants, has grown in Lithuania during the last years. This raises the need for new, local sources of natural antioxidants and new ways have to be developed to introduce them in food industries. Aromatic plants are thought to be relatively safe for human consumption due to the prolonged history of their usage as medicines and as food additives. Thus, the literature search for promising sources of natural food antioxidants was focussed on herbs and spices.

A number of aromatic herbs that can be grown in Lithuania have been mentioned to possess antioxidant activity (AA): thyme (*Thymus vulgaris* L.), marjoram (*Majorana hortensis* Moench.)[1], sage (*Salvia officinalis* L.)[2–4], oregano (*Origanum vulgare* L.)[5], lavender (*Lavandula angustifolia* Mill.), basil (*Ocimum basilicum* L.)[5–7], and catnip (*Nepeta cataria* L.)[8]. Unfortunately, the climatic conditions for the commercial cultivation of rosemary (*Rosmarinus officinalis* L.), which is so far regarded as the most effective plant in terms of antioxidant activity, are rather unfavourable in Lithuania.

Extraction with solvents is frequently used to obtain plant antioxidants, which often are physically and chemically labile substances. A multitude of solvents and extraction schemes has been employed. Chen *et al.*[9] found that the hexane extract of rosemary possessed a higher concentration of carnosic acid and carnosol, which are effective radical scavenging antioxidants, than extracts

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prepared with methanol or acetone. Chevolleau *et al.*[10] compared methanol and n-hexane oleoresins of some Mediterranean plants. Hexane extracts were reported to have higher antioxidant activity than the corresponding methanol extracts.

Extraction with supercritical carbon dioxide is another technique employed alone or in combination with solvent extraction for isolation of plant antioxidants. When analysing dried rosemary leaves, Tena *et al.*[11] compared SFE extraction with ultrasound-assisted acetone, methanol, hexane and dichloromethane extraction, and concluded, that SFE provided the highest recovery of carnosic acid, the lowest relative standard deviation in the yield of carnosic acid and the cleanest extract. SFE is regarded as an efficient, non-toxic, reliable, specific, but at the same time rather costly isolation technique.

Hydrodistillation of aromatic plants is still another way to obtain rather weakly active natural antioxidants. A number of publications indicated antioxidant activity in volatiles isolated from oregano, thyme, clove (*Eugenia caryophyllata* Thunb.), nutmeg (*Myristica fragrans* Houtt.), pimento (*Commiphora molmol*), bay (*Laurus nobilis* L.), cinnamon (*Cinnamomum zeylanicum* Breyne), and some other plant species[12–16]. Eugenol and isoeugenol (in clove and nutmeg)[12,14], *p*-cymene-2,3-diol, carvacrol and thymol (in thyme and oregano)[5,12–14] were reported to be among the antioxidants in essential oils that retard lipid oxidation. Studies on isolation of antioxidants in essential oils are scarce mostly for two reasons: (1) most plant antioxidants are diphenols or polyphenols, which are not volatile and cannot therefore be extracted *via* hydrodistillation; (2) the aromatic character and high volatility of essential oil constituents limit their use as antioxidants in foods.

The study presented in this chapter was aimed at assessing the antioxidant activity of some aromatic herbs which can be commercially cultivated in Lithuania and evaluating several isolation techniques in order to select the most promising sources of natural antioxidants and the most efficient routes to extract them.

2.2 Experimental

2.2.1 Plant material

The following aromatic herbs were collected in the experimental garden of the Lithuanian Institute of Horticulture on the second decade of July, 1994 (stage of vegetation defined):

- marjoram - blossom formation;

- catnip - full bloom;
- oregano - full bloom;
- lavender - full bloom;
- thyme - full bloom;
- hyssop (*Hyssopus officinalis* L.) - full bloom;
- anise hyssop (*Lophantus anisatus* Benth.) - full bloom;
- sage was collected from plants that did not form blossoms that year.

As a reference plant, dried rosemary obtained from the Superior Institute of Agronomy, Technical University of Lisbon, Portugal (harvested in Arrábida, Portugal, July, 1994) was used. The harvested herbs were dried in a drying cabinet with forced ventilation at ambient temperature (20°C) for 2–3 days. The decrease in mass during the drying process varied from 64 % (hyssop) to 80 % (thyme). The loss of essential oil under these conditions is negligible[17]. After the plants had been sorted out, only leaves and blossoms were used for the assessment of AA. The samples were packed in double walled paper bags and stored approx. for 3–4 months at ambient temperature before use.

2.2.2 Isolation techniques

Six different types of extracts were prepared from each of the dried herbs (Figure 2.1):

- essential oils (EO) hydrodistilled from 50 g of herb;
- deodorised acetone oleoresins (DAO) obtained by re-extracting all dried (forced ventilation cabinet, 100°C, 4 h) solid retentate remaining after hydrodistillation with 250 ml freshly redistilled acetone (99+%, Acros Organics, 17.717.63) with continuous shaking for 24 h;
- deodorised water extracts (DWE) concentrated from the liquid retentate remaining after hydrodistillation using a Vacuubrand Diaphragm Pump CVC 24, a Büchi rotory evaporator R-114 with a Büchi water bath B-480; the concentrate was subsequently freeze-dried at –40°C in a freeze-dryer;
- CO_2 extracts (CO_2E) extracted in a 10 ml extraction vessel from 2–3 g of herb material using a Suprex PrepMaster™ extractor coupled with an AccuTrap™ collecting device. The trap-collector was filled with Ottawa Sand Standard (20–30 mesh, Fisher Chemicals, S 23-3). The restrictor and the trap-collector were set at 35°C and –20°C, respectively;

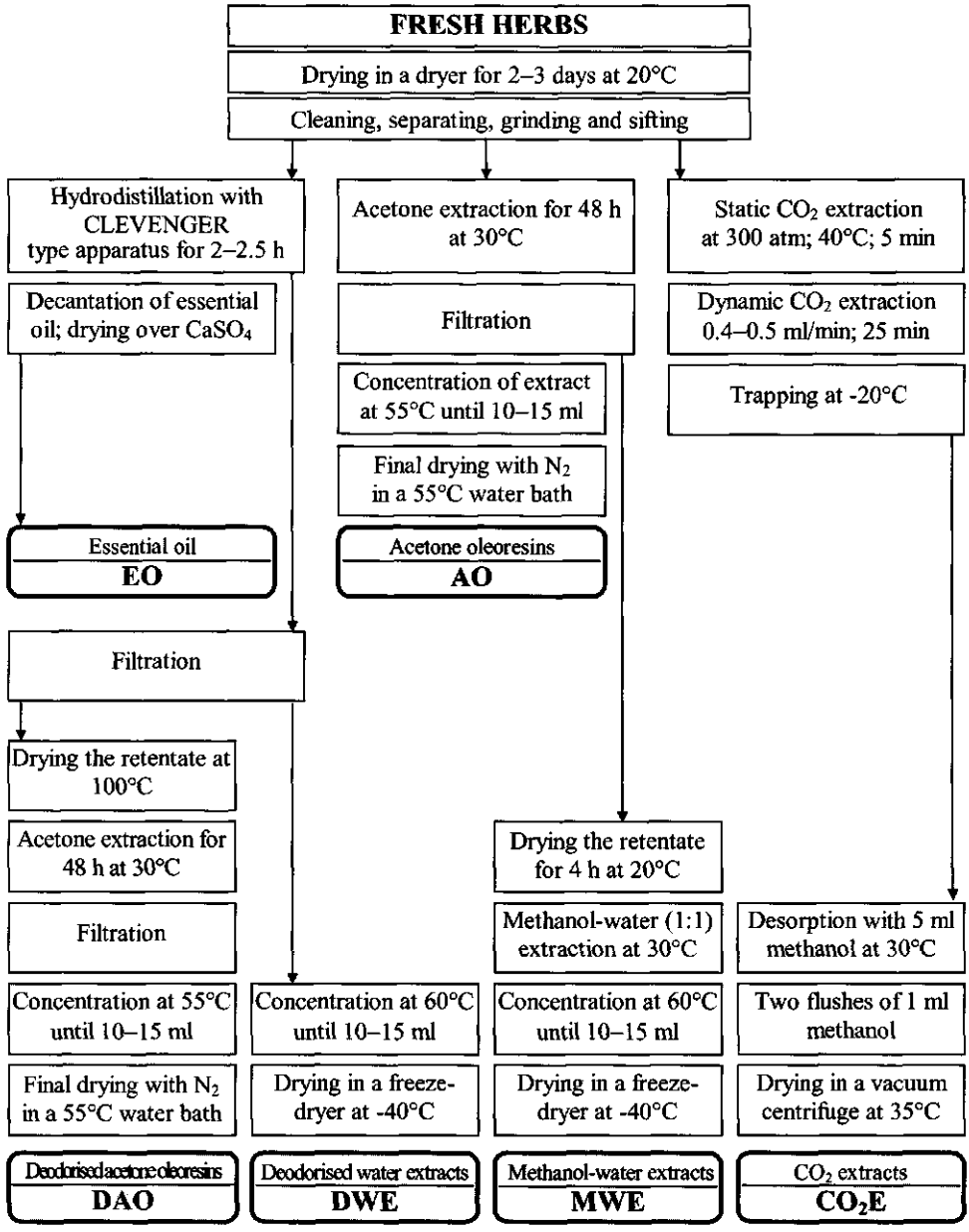


Figure 2.1 *Flow scheme of the isolation techniques used for the preparation of extracts from Lithuanian aromatic herbs for screening their antioxidant activity.*

- acetone oleoresins (AO) obtained by extracting 50 g of herb material with 250 ml freshly redistilled acetone by applying continuous shaking for 24 h;
- methanol-water extracts (MWE) obtained by re-extracting the plant material remaining after the acetone extraction by applying continuous shaking for 24 h.

2.2.3 Evaluation of antioxidant activity

2.2.3.1 β-Carotene bleaching (agar diffusion method)

The β-carotene bleaching test, diffusion method, (β-CBTD) was chosen for the initial screening of a large number of samples because of its simplicity and visual evidence of the results. The test procedure used was similar to the one described by Taga *et al.*[18] The extracts and oils obtained were dissolved in ethanol (1 g/l). The following materials were used for this test: ethanol (absolute p.a., Merck, 983.2500), acetone (p.a., Merck, 14.2500), Bacto Agar (Agar No.1, Oxoid L11; Unipath Ltd.), BHT (purum, Fluka EG, Buchs SG), β-carotene (synthetic, 95 %, Sigma Chemical Co. C9750), and linoleic acid (90 % tech., Janssen Chimica, 22.724.26).

Three grams of Bacto Agar were dissolved in 200 ml distilled water. The solution obtained was cooled to 50°C and 4 ml linoleic acid in ethanol at a concentration of 5 g/l as well as 20 ml acetone solution of β-carotene at a concentration of 1 g/l were added. Aliquots of 35 ml of this mixture were poured into 8.5 cm Petri dishes and allowed to solidify. Three wells of approximately 50 µl capacity were punched in the agar of each Petri dish (see Figure 2.2) and a drop (approx. 25–30 µl) of liquid agar was placed with a Pasteur pipette into each well. This modification was made to achieve a uniform diffusion of the test solutions into the agar. Twenty microlitres of each sample solution were injected into the wells, the wells were then closed with a microscopy cover glass and the Petri dishes were left at ambient temperature. Each sample was analysed in duplicate. The background colour around the control well filled with ethanol was bleached in approx. 28 h. The intensity of the colour remaining around each sample was determined visually and compared with the AA of a BHT solution in ethanol at a concentration of 1 g/l. The results were expressed in points from '0' (antioxidative efficacy of a blank sample) up to '5' (antioxidative efficacy of BHT).

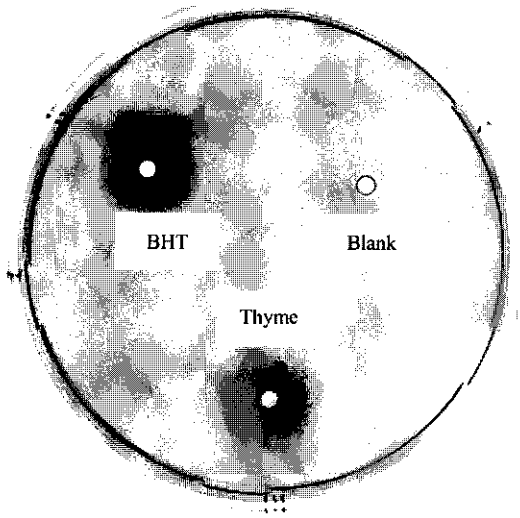


Figure 2.2 *Assessment of antioxidant activity by β-carotene bleaching (agar diffusion method).*

2.2.3.2 β-Carotene bleaching (spectrophotometric method)

The spectrophotometric method (β-CBTS) used to monitor instrumentally the bleaching of the carotene-linoleate solution was also similar to that described by Taga *et al.* (1984)[18]. The concentration of ethanolic solutions of isolates used in this test was 2 g/l. An ethanolic solution of BHT (2 g/l) was used as a reference. The model test mixture was prepared by dissolving 0.5 mg β-carotene in 1 ml chloroform (a.r. grade, 99.5%, Labscan Ltd., A 3505E). Twenty five microlitres of linoleic acid and 200 mg Tween 40 (Sigma, P-1504) were added to the β-carotene solution. Chloroform was removed using a rotary evaporator at 50°C. One hundred ml of distilled water saturated with oxygen during 30 min, flow rate 100 ml/min were added and the mixture was vigorously shaken. Two hundred fifty microlitres of this model test mixture were distributed in each of the wells of microtiter plates (Sigma, M-0156). Thirty five microlitres of an ethanolic solution of the appropriate antioxidant were added to each well. An equal amount of ethanol was used for the blank samples. Six replicates were prepared for each of the treatments (sage AO, thyme AO, oregano AO, BHT and blank). The microtiter plates were then placed in an incubator at 55°C. The absorbance was measured in an EAR 400 Microtiter Reader (SLT-Labinstruments, Austria) at $\lambda = 490$ nm.

ANOVAs (completely randomised design), means and standard deviations were calculated for the data of the β-CBTS experiment. Effects of treatments were

analysed separately for each sampling period. The Student-Newman-Keul's procedure was used to distinguish between treatments. All statistical analyses were performed as described by Dowdy[19].

2.3 Results and discussion

2.3.1 Yield of isolates

Quantitative results of the isolation of essential oils and extracted substances from the analysed herbs are summarised in Table 2.1. All plants were quite rich in essential oil compared with some published data[20,21]. For instance, in the present study, the concentration of EO in dried matter of thyme, anise hyssop and sage was approx. 60 g/kg. It should be noted, however, that only leaves and flowering parts were used for the hydrodistillation.

Table 2.1 *Yields of the isolates obtained from dried aromatic herbs (g/kg dry matter) and their antioxidant activity as evaluated by the β-carotene bleaching test (diffusion method)*[a]

Plant	EO	DAO	DWE	AO	MWE	CO_2E
Sage	58.0	**62.3 (4)**	**90.8 (1)**	**66.3 (4)**	**87.3 (2)**	**50.2 (4)**
Oregano	25.5	16.2	155.1	30.1	**71.7 (1)**	53.9
Rosemary	69.1	**39.7 (4)**	**58.8 (1)**	**113.3 (4)**	**60.2 (3)**	**71.5 (5)**
Catnip	28.2	29.6	119.5	82.7	126.1	22.8
Thyme	**62.7 (2)**	**4.4 (3)**	73.2	**35.0 (4)**	**73.9 (3)**	**54.6 (4)**
Lavender	42.4	9.8	135.1	105.3	105.5	26.7
Hyssop	45.0	13.8	127.9	30.9	96.0	37.1
Anise hyssop	60.4	36.2	141.6	44.7	123.8	59.6
Marjoram	36.4	6.0	115.3	**31.3 (2)**	**108.1 (1)**	37.4

[a] Abbreviations are explained in Figure 2.1. Yields of isolates possessing antioxidant activity are printed in bold. In parentheses: antioxidant activity, scale 0 (low, not shown) – 5 (high).

The total amount of deodorised extracts (DAO + DWE) in the dry mass of analysed herbs varied from 78 g/kg (thyme) to 178 g/kg (anise hyssop). In most of the herbs, DWE yield was considerably higher than that of DAO, i. E. From 19.2

times (marjoram) to 3.9 times (anise hyssop). In sage and rosemary, however, DAO and DWE yields were more similar to each other, with DWE 1.4 times and 1.5 times higher than DAO, respectively. These results indicate that the processes occurring during the hydrodistillation and distribution of extractives in filtrate and retentate are specific for each particular herb.

The yields of extracts obtained by acetone were lower than those isolated by methanol-water, except for lavender (almost equal) and rosemary (AO approx. 1.9 times > MWE). Extremely low yields of CO_2E were obtained for catnip and lavender.

In assessing the peculiarities of the extraction by different methods, it is worthwhile comparing the yield of EO, DAO and DWE (procedures involving hydrodistillation) with that of AO and MWE (no hydrodistillation). The results obtained show that the sums of all extractives isolated during and after hydrodistillation (EO + DAO + DWE) were generally higher than those obtained excluding the hydrodistillation step (AO + MWE). A striking example is oregano extracts in which EO + DAO + DWE yield was 1.9 times higher than that of AO + MWE. Boiling in water and the concomitant physical changes in the plant cell structure and chemical changes in constituents (e.g. hydrolysis of phenolic glycosides) as well as differences in extraction solvents used (water vs. Methanol + water) may account for these differences. In the case of catnip and lavender the yield of EO + DAO + DWE was slightly lower than that of AO + MWE. This could be attributed to a higher affinity of extractives from these two herbs for the methanol used in the latter extraction procedure.

The differences in the yields obtained using various solvents could be caused by several factors, i.e. composition of each particular herb, accessibility of endocellular metabolites, differences in the solubility of extractives and their polarity, extraction technique, etc. It is, however, difficult to give an exhaustive explanation for the results obtained within the scope of the present study and without more detailed experiments.

2.3.2 Results of the agar diffusion test

The products of linoleic acid oxidation affected the β-carotene-agar medium in 28 h in the blank. The discolouration process around the wells with antioxidant solutions was retarded and isolate solutions differed in their protective efficiency. The results of the visual assessment of antioxidant containing wells are

summarised in Table 2.1. In order to compare the efficiency of different isolation techniques and herb materials, both in terms of AA and yield of each particular isolate, BHT-equivalent yields Eq_{BHT} were introduced:

$$Eq_{BHT} = Y_i \times \frac{AA_i}{AA_{BHT}}$$

where: Y_i is the yield of the particular isolate (g/kg); AA_i is the antioxidant activity of the particular isolate according to β-CBT (scale 0–5 points); and AA_{BHT} is the antioxidant activity of BHT in β-CBT (5 points). Eq_{BHT} can be defined as the amount of isolate obtained from one kg of a particular herb possessing AA equal to that of BHT. As can be seen from Table 2.1, active isolates were obtained from five of the nine herb species tested. The Eq_{BHT} values for the different types of isolates obtained for each of these herbs are summarised in Figure 2.3.

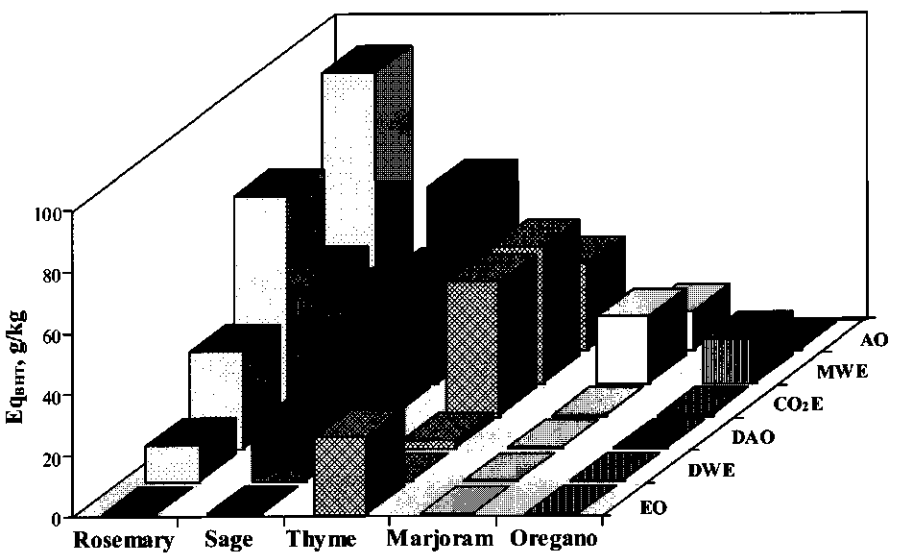


Figure 2.3 *Antioxidant efficiency (Eq_{BHT}) of different isolates obtained from Lithuanian aromatic herbs (see text for abbreviations).*

The results show that rosemary isolates possess the highest antioxidant efficiency, e.g. Eq_{BHT} of rosemary AO and MWE was 91 g/kg and 36 g/kg, respectively. The same type of isolates from two other plants, thyme and sage, was also very effective. These results agree with data published by other authors[1,22]. They also reported that methanolic oregano extract possessed high AA on lard. However, in this study the effect of oregano MWE on the β-carotene model system was not so evident (Eq_{BHT} = 14 g/kg, 1 point). AO (Eq_{BHT} = 13 g/kg, 2 points) and MWE (Eq_{BHT} = 22 g/kg, 1 point) solutions of marjoram also slightly retarded β-carotene bleaching. It is interesting to note that the isolates obtained from this herb with a hydrodistillation step involved (EO, DAO and DWE) were not effective in the test performed, possibly due to the breakdown of active constituents during the prolonged thermal stress. The same tendency for procedures involving hydrodistillation to result in isolates of lower Eq_{BHT} when compared with isolates obtained by cold extraction methods is observed in extracts from other herbs. The sum of Eq_{BHT} values for EO, DWE and DAO of thyme and rosemary was 28 and 44 g/kg, respectively, whereas the Eq_{BHT} values for AO and MWE of the same plants totalled 72 and 127 g/kg, respectively.

As expected, in most cases, EO of the assessed herbs did not retard oxidation of β-carotene-linoleate model system. Thyme EO, however, displayed some antioxidant activity (2 points, Table 2.1). Similar findings were also reported by other authors[10,14].

The activity of CO_2E was almost similar to that of AO, except for marjoram. However, the considerably lower yields of CO_2E when compared to the yields of AO + MWE indicate that a considerable part of the antioxidant substances may remain in the plant material after SFE. For instance, Eq_{BHT} of the sage CO_2E was more than twice as low as that of AO + MWE. Therefore, the conditions of CO_2 extraction need to be further revised.

The results of the preliminary β-CBTD pointed out sage and thyme as the most promising herbs among the ones that are commonly grown in Lithuania (Figure 2.3). For these herbs DAO, AO, MWE and CO_2E resulted in isolates with some antioxidant efficiency. Among these, AO and CO_2E displayed antioxidant activity scores $\geq$ 4 points (Table 2.1). When making the decision on the most effective way of processing a particular herb, not only yield and properties of the isolates, but also economical aspects should be considered. Having this in mind, AO of sage and thyme were used in subsequent experiments. Oregano AO and BHT were also included for comparative purposes.

2.3.3 *Results of the spectrophotometric test*

As a result of β-carotene bleaching caused by the oxidation of linoleic acid, the absorbance of the test solutions decreased with time (Figure 2.4). The discolouration process in the model system progressed differently for the various samples, which contained an equal amount (0.2 g/l) of an antioxidant (BHT or herb isolate) added.

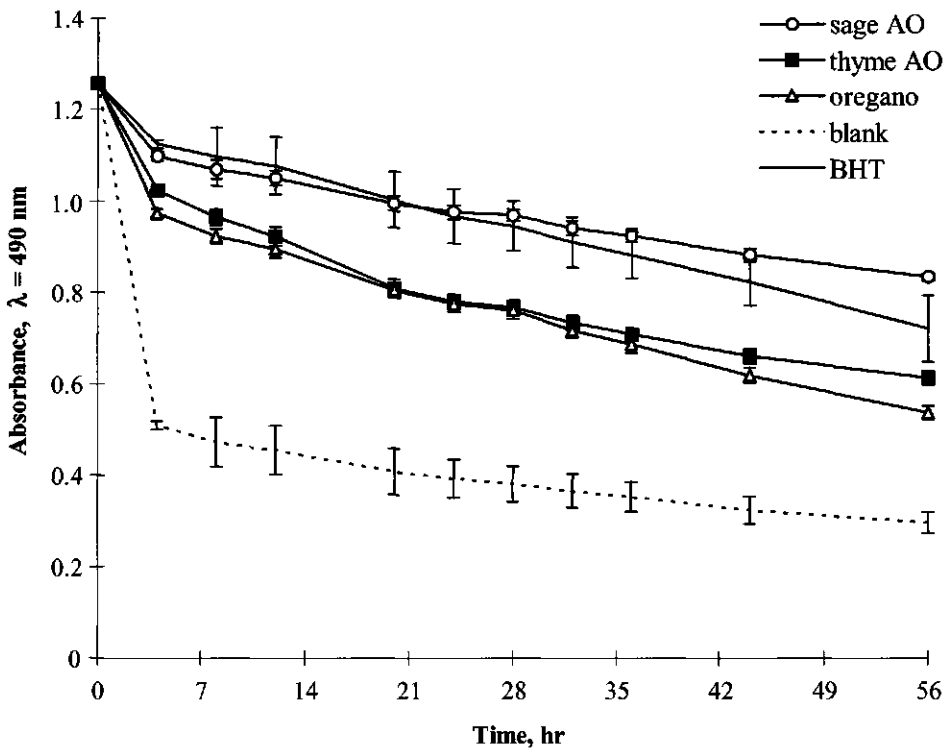


Figure 2.4 *Antioxidant effect of ethanolic solutions of herb acetone oleoresins and BHT in the β-carotene-linoleic acid model system at 55°C.*

For the purpose of the present discussion, relative antioxidant activities (RAA) were calculated for each sample as follows:

$$RAA = \frac{\text{Absorbance of sample}}{\text{Absorbance of BHT (reference)}}$$

RAA values (averages ± standard deviations) obtained after 24 and 56 h of oxidation are given in Table 2.2.

Table 2.2 *Relative antioxidant activity (RAA) of herb acetone oleoresins and BHT added at 0.2 g/l and assessed by the β-carotene bleaching test (spectrophotometric method) at 55°C* [a]

Sample	24 hr	56 hr
Sage AO	1.01 ± 0.05^{a}	1.16 ± 0.06^{a}
Thyme AO	0.81 ± 0.06^{b}	0.85 ± 0.06^{b}
Oregano AO	0.80 ± 0.06^{b}	0.75 ± 0.06^{c}
BHT (reference)	1.00^{a}	1.00^{d}
Blank	0.41 ± 0.04^{c}	0.41 ± 0.04^{e}

[a] The values shown represent the average of six determinations ± standard deviations. Within the same column, averages marked with the same index are not significantly ($P > 0.01$) different.

Initially, the performance of sage and BHT solutions in the model system was very similar (Figure 2.4). The RAA values for these samples after 24 h were not significantly different ($P > 0.01$). After the same period, thyme and oregano displayed significantly ($P < 0.01$) lower absorbance values than sage and BHT. After 56 h, however, all samples differed significantly ($P < 0.01$) among themselves. The sage AO solutions were the most efficient in inhibiting oxidation in the model system used at the end of the experimental period. The absorbance for BHT samples was, by then, 1.2 times lower than the corresponding value for sage AO. Although the performance of thyme AO and oregano AO did not differ after 24 h, at the end of the experiment, the absorbance of thyme samples exceeded that of oregano with 10%. These differences in antioxidant efficiency after 28 h and 56 h may be partly explained by the thermal instability of BHT and the active compounds in oregano AO. Thyme AO, although less effective in this model system than sage, is worth attention due to its more prolonged inhibitory effect on the discolouration of the reaction mixture than BHT and oregano (Figure 2.4).

When comparing the β-carotene bleaching results obtained by spectrophotometric and the diffusion methods, the higher activity is associated with sage and thyme AO in both tests. For both methods, the activity of sage AO is comparable to that of BHT. The only discrepancy observed was in the lack of

antioxidant activity of oregano AO when assessed by the diffusion method. Limitations of the agar diffusion test, such as the visual assessment and interference of the diffusion of antioxidants through the agar matrix, may account for this. Another explanation may be provided by the difference in experimental conditions between both tests (temperature, light, and reaction time).

2.4 Conclusions

The data on extraction procedures and antioxidant activity assessment obtained in this study on selected Lithuanian herbs single out thyme and sage as the most promising sources of natural antioxidants. Two solvent extractions, namely acetone and methanol-water extraction appear to be appropriate techniques for the isolation of substrates containing natural antioxidative compounds from the herbs studied. Antioxidant activity tests performed in these experiments brought out the need for a rapid, simple and reliable test method for evaluation of antioxidant substances. Purification and identification of the active compounds in selected herb isolates is required for a better understanding of the protective mechanisms involved and for possible application in food(s).

2.5 References

1. Banias, C., Oreopoulou, V. and Thomopoulos, C. D. 1992. The effect of primary antioxidants and synergists on the activity of plant extracts in lard. *J. Am. Oil Chem. Soc.*, 69, 520–524.
2. Chang, S.S., Ostric-Matijasevic, B., Hsieh, O.A.L. and Huang, C.L. 1977. Natural antioxidants from rosemary and sage. *J. Food Sci.*, 42, 1102–1106.
3. Schwarz, K. and Ternes, W. 1992. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. *Z. Lebensm. Unters. Forsch.*, 195, 95–98.
4. Svoboda, K. and Deans, S.G. 1992. A study of the variability of rosemary and sage and their volatile oils on the British market. *Flavour Fragr. J.*, 7, 81–87.
5. Lagouri, V., Blekas, G., Tsimidou, M., Kokkini, St. and Boskou, D. 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebensm. Unters. Forsch.*, 197, 20–30.

6. Economou, K.D., Oreopoulou, V. and Thomopolous, C.D. 1991. Antioxidant activity of some plant extracts of the family Labiatae. *J. Am. Oil Chem. Soc.*, 68. 109–112.

7. Vekiari, S.A., Oreopoulou, V. and Thomopolous, C.D. 1993. Oregano flavonoids as lipid antioxidants. *J. Am. Oil Chem. Soc.*, 70, 483–487.

8. Hirosue, T., Matsuzawa, M., Irie, I., Kawai, H. and Hosogai, Y. 1988. Antioxidative activities of herbs and spices. *J. Jap. Soc. Food Sci. Technol.*, 35, 630–633.

9. Chen, Q., Shi, H. and Ho, C.-T. 1992. Effects of rosemary extracts and major constituents on lipid oxidation and soybean lipoxygenase activity. *J. Am. Oil Chem. Soc.*, 69, 999–1002.

10. Chevolleau, S., Mallet, J.F., Ucciani, E., Gamisans, J. and Gruber, M. 1992. Antioxidant activity in leaves of some Mediterranean plants. *J. Am. Oil Chem. Soc.*, 69, 1269–1271.

11. Tena, M.T., Valcarcel, M., Hidalgo, P.J. and Ubera, J.L. 1997. Supercritical fluid extraction of natural antioxidants from rosemary: comparison with liquid solvent sonication. *Anal. Chem.*, 69, 521–526.

12. Dorman, H.J.D., Deans, S.G., Noble, R.C. and Surai, P. 1995. Evaluation in vitro of plant essential oils as natural antioxidants. *J. Essent. Oil Res.*, 7, 645–651.

13. Ternes, W., Gronemeyer, M. and Schwarz, K. 1995. Determination of *p*-cymene-2,3-diol, thymol, and carvacrol in different foodstuffs. *Z. Lebensm. Unters. Forsch.*, 201, 544–547.

14. Farag, R.S., Badei, A.Z.M.A., Hewedi, F.M. and El-Baroty, G.S.A. 1989. Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. *J. Am. Oil Chem. Soc.*, 66, 792–799.

15. Mantle, D., Anderton, J.G., Falkous, G., Barnes, M., Jones, P. and Perry, E.K. 1998. Comparison of methods for determination of total antioxidant status: application to analysis of medicinal plant essential oils. *Compar. Biochem. Physiol. B*, 121, 385–391.

16. Lagouri, V and Boskou, D. 1995. Screening for antioxidant activityof essential oils obtained from spices. p. 869–879. In: G. Charalambous (ed). Food Flavors: Generation, Analysis and Process Influence. Elsevier, London.

17. Venskutonis, R. 1997. Effect of drying on the volatile constituents of thyme (*Thymus vulgaris* L.) and sage (*Salvia officinalis* L.). *Food Chem.*, 59, 219–227.

18. Taga, M.S., Miller, E.E. and Pratt, D.E. 1984. Chia seeds as a source of natural antioxidants. *J. Am. Oil Chem. Soc.*, 61, 928–931.

19. Dowdy, S.M. 1983. *Statistics for Research*, John Wiley & Sons, New York, USA.

20. Cuvelier, M.E., Richard, H. and Berset, C. 1996. Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. *J. Am. Oil Chem. Soc.*, 73, 645–652.

21. Senatore, F. 1996. Influence of harvesting time on yield and composition of the essential oil of a thyme (*Thymus pulegiodes* L.) growing wild in Campania (southern Italy). *J. Agric. Food Chem.*, 44, 1327–1332.

22. Herrmann, K., Schütte, M. and Müller, H. 1981. Über die antioxidative Wirkung von Gewürze. *Dtsch. Lebensm – Rdsch.*, 77, 134–138.

RAPID SPECTROSCOPIC SCREENING FOR ANTIOXIDANT ACTIVITY IN HERB ISOLATES WITH THE β-CAROTENE-LINOLEIC ACID MODEL SYSTEM*

3.1 Introduction

Accelerated oxidation of lipids is often used instead of long lasting shelf-life tests when assessing food antioxidants. The most common method to speed up the chain reaction in foods is to increase the temperature. However simultaneously, a number of occurring chemical and physical processes in and among constituents of complex foods become more prominent at high temperatures[1]. These can alter the mechanism of lipid oxidation and interfere with measurement procedures.

Usage of simplified lipid systems for temperature accelerated oxidation tests for antioxidant activity screening purposes has several advantages: (i) the obtained results reflect well the actual effect of added antioxidants on the oxidation of lipids; (ii) the consistent composition of the reaction substrate provides good reproducibility of the results and the possibility to compare the efficiency of different antioxidants; (iii) simplification of the test system allows the use of sensitive methods for monitoring the course of oxidation; and (iv) the increased reaction rate shortens the testing time when compared with antioxidant activity assessment under real shelf-life conditions.

Linoleic acid (18 : 2ω6) is a commonly occurring polyunsaturated essential fatty acid, which is prone to oxidation at relatively low temperatures. Thus, this purified lipid can be used as a substrate in antioxidant activity tests. A model system that is based on co-oxidation of linoleic acid and β-carotene is capable of

* Dapkevicius, A., van Beek, T.A., Linssen, J.P.H. and Venskutonis, R. 1998. p. 295–366. In: P. Schreier, M. Herderich, H.U. Humpf and W. Schwab (eds). Natural Product Analysis: Chromatography, Spectroscopy and Biological Testing. Vieweg & Sohn Verlagsgesellschaft, Lengerich.

detecting protective properties for both chain breaking and preventive antioxidants. Secondary antioxidants introduced in the β-carotene-linoleic acid model system (β-CLAMS) interfere with the initiation of linoleic acid oxidation while primary antioxidants compete with carotene for free radicals formed during the oxidation of this fatty acid. In both cases, the bleaching of the yellow-orange colour that indicates oxidation of β-carotene is retarded. Three variations of the β-CLAMS oxidation test are being used for evaluating the efficacy of antioxidants.

The β-CLAMS agar-diffusion test of Araujo as described by Taga *et al.*[2] is carried out in agar plates containing linoleic acid and β-carotene. The antioxidant is added into small wells punched in the agar and the antioxidant activity of the tested compound is related to the intensity of the preserved β-carotene colour. This procedure was used with minor modifications in a preliminary screening for antioxidant activity in different herb extracts (Chapter 2). One of the major limitations of this technique is the lack of a reasonable method for quantitative assessment of the antioxidative effect. The proposed measurement of the zone where the carotene colour is retained using a vernier calliper[3,4] did not yield reliable and consistent results due to several reasons. Firstly, differences in physical properties of different samples caused unequal diffusion of active compounds into the agar. Secondly, the native colour of some extracts masked the orange-yellow colour of the remaining β-carotene. Finally, the limits of the coloured zone were usually diffuse, thus, the precision of the measurement was low. The agar diffusion test is labour-intensive, but does not require specific instruments and is relatively cheap. In conclusion, the β-CLAMS agar diffusion test may be employed in the initial screening of samples with similar physical properties when only a semi-quantitative assessment of the antioxidant activity is needed.

The β-CLAMS oxidation on TLC plates was used for the first time by Philip[5]. The sample under assessment is applied on a TLC plate and optionally submitted to chromatographic elution. Spots with antioxidant activity are visualised by spraying with a β-carotene- linoleic acid solution in chloroform followed by exposure to UV[6] or daylight[7–10]. A remaining yellow colour on a pale background indicates antioxidant activity. The intensity of colour corresponds to the amount of antioxidant activity. This method suffers from similar difficulties in quantifying antioxidant activity of active compounds as the agar diffusion test. Besides, the oxidation processes taking part in this test are promoted by an excess of light and oxygen, which are not the main accelerating factors in real shelf-life storage conditions. However, the combination of β-CLAMS spraying with prior

chromatography on TLC plates of herb extracts or their fractions is a useful tool in final screening steps. This fast and simple technique can separate naturally occurring but non-active coloured constituents and provides preliminary qualitative information on the number and nature of active constituents present in a herb extract.

The spectrophotometric assessment of β-CLAMS oxidation, which is capable of an instrumental evaluation of β-carotene bleaching, was proposed by Marco[11] and later modified by Miller[12]. Linoleic acid is emulsified in oxygenated water in the presence of an emulsifier and β-carotene. The reaction mixture is vigorously shaken or sonicated to increase the stability of the emulsion. Oxidation in the β-CLAMS takes place in spectrophotometric cuvettes, which are thermostated in a water bath at 50°C. Oxidation of β-carotene is followed by measuring the absorbance at λ = 470 nm at regular time intervals (e.g. 10[11], 15[8,13] or 30[14] min). The final absorbance values for calculating antioxidant activity are determined at the end-point of the experiment. Three different ways for the end-point determination have been suggested by different authors. The end-point is considered at: (i) a certain point of the reaction time (e.g., 105[8], 120[15, 16], or 180 min[14], Figure 3.1 a); (ii) the time were the blank sample reaches a pre-determined absorbance value[2] (Figure 3.1 b); or (iii) a pre-determined absorbance value[11] (e.g., 70 or 50% of the initial absorbance, Figure 3.1 c).

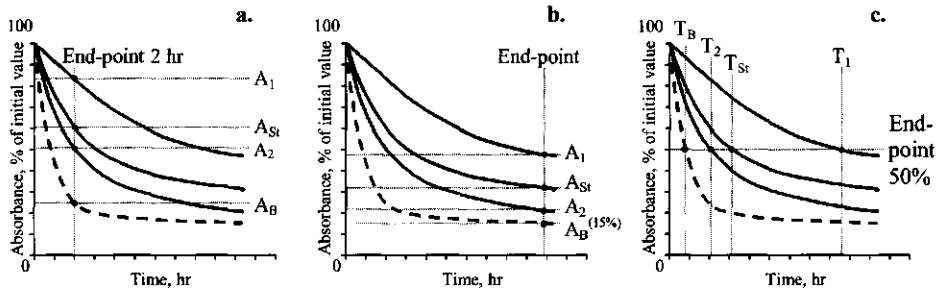


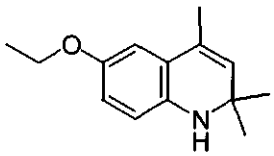
Figure 3.1 *The end-point determination and the estimation of absorbance and time values in the β-CLAMS. A_1, A_2, A_{St} and A_B are absorbance values at the end-point of samples with antioxidant 1, antioxidant 2, standard antioxidant and blank, respectively. T_1, T_2, T_{St} and T_B are time values at the end-point of samples as above.*

Two types of formulas are used to express the antioxidant activity. One of them involves comparison of the efficacies to inhibit β-carotene bleaching of the

sample and a known antioxidant (e.g. 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline[11] – also known as Ethoxyquin or Santoquin®; BHA[16]; BHT or α-tocopherol). Marco[11] used the following formula to calculate antioxidant response as per cent of that of Santoquin® ($AR_{Santoquin}$):

$$AR_{Santoquin} = 100 \times [(T_I - T_B)/(T_{St} - T_B)]\ \%$$

in which T_I is time for the sample with the antioxidant under assessment to reach the end-point absorbance value; T_B is time for the blank sample to reach the end-point absorbance value; and T_{St} is time for the sample containing Santoquin® to reach the end-point absorbance value. Negative $AR_{Santoquin}$ values can be calculated for compounds with pro-oxidants properties.



Santoquin® or Ethoxyquin

Another type of formula compares the β-carotene bleaching rates of a blank sample and a sample with added antioxidant. Hidalgo *et al.*[17] calculated the antioxidant activity (AA) of the added compound as:

$$AA = 100\ [1 - (A_{1,t=0} - A_1)/(A_{B,t=0} - A_B)]$$

where $A_{1,t=0}$ is the absorbance of the sample with antioxidant at time = 0; A_1 is the absorbance of the sample with antioxidant at the end point set as a time value; $A_{B,t=0}$ is the absorbance of the blank at time = 0; A_B is the absorbance of the blank at the end point. The maximal effect of antioxidant gives AA = 100, when pro-oxidant activity is observed with AA < 0.

Chevolleau et al.[18] proposed to use the antioxidant activity coefficient (AAC) to characterise the antioxidant activity of herb extracts:

$$AAC = 1000 \times [(A_I - A_B)/(A_{B,t=0} - A_B)]$$

(abbreviations as above).

Since a sample with antioxidant is compared with a blank sample, inadequate evaluation of the AAC can occur due to possible variations in the preparation of the β-CLAMS in different laboratories. Purity and freshness of reagents used, level of water saturation with oxygen, stability of the prepared emulsions, remaining amount of chloroform in the model system, and several other

factors may cause errors in the AAC determination. Comparison of newly identified antioxidative substances at the same molar concentration with a well-known pure antioxidant as a reference is more valuable[19].

Another important bottleneck of the antioxidant activity assessment using β-CLAMS is that new batches of fresh reaction mixture have to be prepared quite often when a high number of samples have to be screened. This causes variations in initial properties of the β-CLAMS, slows down the screening procedure and adds extra labour and reagents costs. The main objective of the work presented in this chapter was to simplify the monitoring of absorbance in the β-CLAMS during the oxidation test and to evaluate the antioxidative properties of some aromatic herb isolates.

3.2 Experimental

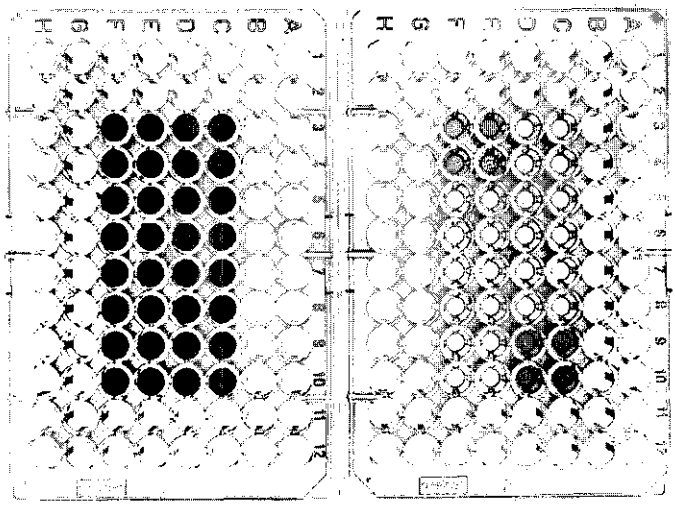
3.2.1 Preparation of herb isolates

Oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.) and sage (*Salvia officinalis* L.) were collected in the early stages of vegetation (Lithuanian institute of Horticulture, May 25, 1994). Acetone oleoresins (AO) were obtained by shaking 50 g of dried herb material with 250 ml of freshly redistilled acetone for 24 h. Extracts were filtered and concentrated under reduced pressure at 55°C. Essential oils (EO) were hydrodistilled from 50 g of herb during 2.5 hr in a Clevenger type apparatus.

3.2.2 Spectrophotometric evaluation of β-carotene bleaching in the β-CLAMS

The spectroscopic method used to monitor the bleaching of the model system was similar to that described in Chapter 2. The β-CLAMS was prepared by dissolving 0.5 mg of β-carotene in 1 ml of chloroform. Twenty-five microlitres of linoleic acid and 200 mg of Tween 40 were added to the β-carotene solution. The chloroform was removed using a rotary evaporator at 50°C. One hundred millilitres of oxygenated distilled water were added and the mixture was vigorously shaken. Two hundred fifty microlitres of this mixture were distributed in the central 32 wells of microtiter plates (SIGMA, M-0156). Thirty-five microlitres of an ethanolic solution of each isolate (2 g/l) were added to four wells (Figure 3.2a). An equal amount of ethanol was used for the blank samples. An ethanolic solution (2 g/l) of

2,6-di-*tert*-butyl-4-methylphenol (BHT) was used as a reference. The microtiter plates were then placed in an incubator at 55°C. The absorbance was measured in an EAR 400 Microtiter Reader (SLT-Labinstruments, Austria) at $\lambda = 490$ nm. The last measurement was carried out at $t = 72$ hr (Figure 3.2b).



a. **b.**

Figure 3.2 *Oxidation of β-CLAMS with herb isolates (0.25 g/l) in microtiter plate (a) at time = 0 and (b) time = 72 hr.*

3.3 Results and discussion

The discolouration process in the β-CLAMS with the added herb isolates advanced in three different ways (Figure 3.3): acceleration of bleaching (sage EO, oregano EO), short and weak inhibition of bleaching (oregano AO, thyme EO), strong and prolonged inhibition of bleaching (sage AO, thyme AO).

Prolonged oxidation (72 hr) in the β-CLAMS at 55°C revealed some aspects of antioxidant activity evaluation, which are normally overlooked when performing the usual oxidation in 2 or 3 hr at 50°C. Firstly the evaluation of antioxidative properties of volatile antioxidants such as essential oils and BHT by measuring oxidative changes in β-CLAMS over a short period of time can be inaccurate. As can be seen from Figure 3.3, the initially similar performance of sage AO and BHT started to show differences after approx. 10 hr of accelerated oxidation. The antioxidative effect of BHT in this test completely disappeared after

approx. 70 hr, while the sample with sage AO at that moment still maintained approx. 35% of its initial β-carotene absorbance. Secondly the evaluation of the antioxidant activity in short term oxidation tests of certain herb isolates can be misleading. This can be illustrated by the sample containing oregano AO, which demonstrated relatively high antioxidant activity during the initial 2 hr of testing, but started to act as a pro-oxidant after approx. 22 hr.

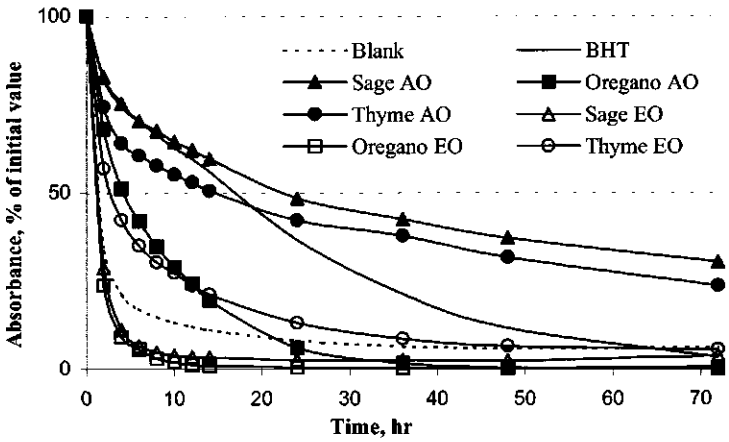


Figure 3.3 *Inhibitory effect of herb isolates and BHT on the bleaching of β-carotene.*

For comparison purposes, the half-reaction time value (HRT) of each sample was graphically estimated. HRT is defined as the period of time required to reach 50 % of the initial absorbance value. HRT's of herb isolates were compared with that of BHT and expressed as the relative antioxidant activity (RAA):

$$RAA = HRT_{Sample}/HRT_{BHT}$$

The RAA values (averages ± SD) are presented in Figure 3.4. The highest RAA value of 1.24 ± 0.13 was found for sage AO. Thyme AO was another extract which effectively retarded bleaching of β-carotene (RAA = 0.88 ± 0.04). Moderate RAA values were found for thyme EO and oregano AO, while the RAA of oregano and sage EO did not differ significantly from the blank ($P > 0.05$). The reasons for the different RAA's are: (i) differences in chemical composition, namely the presence of antioxidative (i.e. phenolic compounds) and pro-oxidative substances (e.g. chlorophyll); (ii) loss of active constituents due to evaporation; and (iii)

differences in polarity of the antioxidants, which cause the so called 'polar paradox'[20].

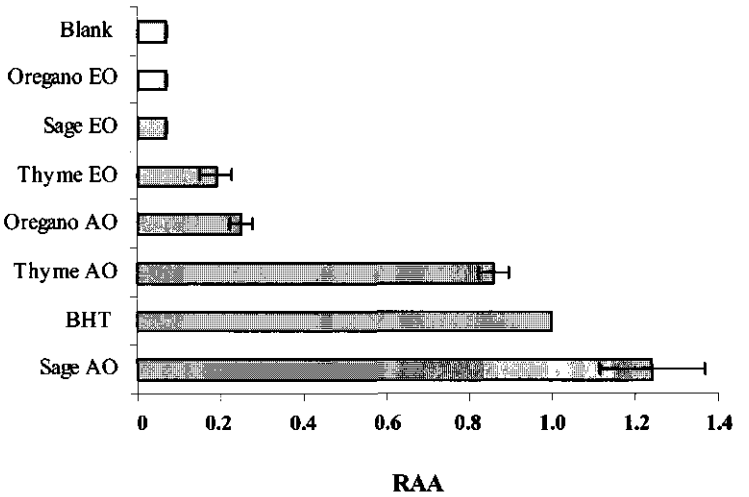


Figure 3.4 *Relative antioxidant activity (RAA) of samples assessed by β-CLAMS.*

The prolonged oxidation in the β-CLAMS raised some doubts about the suitability of BHT for the evaluation of unknown antioxidants. This widely used, pure and cheap, but rather volatile, synthetic antioxidant could be replaced by the less-volatile TBHQ or α- tocopherol.

3.4 Conclusions

The modified spectrophotometric version of the β-CLAMS proved to be adequate for screening large numbers of samples. A minimal amount of sample is required (0.07 mg) for this analytical procedure. Several microtiter plates (4-6), each containing 96 samples, can be easily prepared from a single batch of the reaction mixture by an experienced laboratory technician in approximately one hour. Data processing can be automated using common spreadsheet programs such as Lotus 1-2-3 or Microsoft Excel.

When assessed by the modified β-CLAMS method, the essential oils of sage and oregano did not show significant antioxidant activity. Possible reasons for this were discussed in Chapter 2. A low antioxidant activity was observed for the

essential oil of thyme. This data confirmed the results obtained by the diffusion method of the β-CLAMS test (Chapter 2). All three acetone oleoresins showed AA in the test system used. Sage oleoresin possessed the highest inhibitory effect in the β-CLAMS, comparable with that of BHT. When comparing with earlier spectrophotometric β-CLAMS experiments (Chapter 2), a decrease in antioxidant activity of acetone oleoresins, especially from oregano, can be noticed. This can be attributed to the ageing (oxidation) of crude herb extracts, which were stored at +4°C for 22 months.

3.5 References

1. Ragnarsson, J.O. and Labuza, T.P. 1977. Accelerated shelf-life testing for oxidative rancidity in foods - a review. *Food Chem.*, 2, 291–308.
2. Taga, M.S., Miller, E.E. and Pratt, D.E. 1984. Chia seeds as a source of natural lipid antioxidants. *J. Am. Oil Chem. Soc.*, 61, 928–931.
3. Piccaglia, R., Marotti, M., Giovanelli, E., Deans, S.G. and Eaglesham, E. 1993. Antibacterial and antioxidant properties of Mediterranean aromatic plants. *Ind. Crop. Prod.*, 2, 47–50.
4. Svoboda, K.P. and Deans, S.G. 1992. A study of the variability of rosemary and sage and their volatile oils on the British market: their antioxidative properties. *Flav. Fragr. J.*, 7, 81–87.
5. Philip, F. 1974. An investigation of the antioxidants in a texture vegetable protein product for soy flour. Thesis: Purdue University, W. Lafayette IN.
6. Hostettmann, K., Terreaux, C., Marston, A. and Potterat, O. 1997. The role of planar chromatography in the rapid screening and isolation of bioactive compounds from medicinal plants. *J. Planar Chromatogr.*, 10, 251–257.
7. Pratt, D.E. and Miller, E.E. 1984. A flavonoid antioxidant in Spanish peanuts. *J. Am. Oil Chem. Soc.*, 61, 1064–1067.
8. Pratt, D.E. 1980. Natural antioxidants of soybeans and other oil-seeds. p. 283-293. In: M.G. Simic and M. Karel (eds). Autoxidation in Food and Biological Systems. Plenum Press, New York.
9. Lagouri, V., Blekas, G., Tsimidou, M., Kokkini, S. and Boskou, D. 1993. Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebensm. Unters. Forsch.*, 197, 20–23.

10. Vekiari, S.A., Oreopoulou, V., Tzia, C. and Thomopoulos, C.D. 1993. Oregano flavonoids as lipid antioxidants. *J. Am. Oil Chem. Soc.*, 70, 483–487.

11. Marco, G.J. 1968. A rapid method for evaluation of antioxidants. *J. Am. Oil Chem. Soc.*, 45, 594–598.

12. Miller, H.E. 1971. A simplified method for the evaluation of antioxidants. *J. Am. Oil Chem. Soc.*, 48, 91.

13. von Gadow, A., Joubert, E. and Hansmann, C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-Tocopherol, BHT, and BHA. *J. Agric. Food Chem.*, 45, 632–638.

14. Lagouri, V. and Boskou, D. 1995. Screening for antioxidant activity of essential oils obtained from spices. p. 869–879. In: G. Charalambous (ed). Food Flavors: Generation, Analysis and Process Influence. Elsevier, Amsterdam.

15. Bevenue, A. 1974. Thin-layer chromatographic examination of various seed oils. *J. Chromatogr.*, 95, 235–237.

16. Shahidi, F., Wanasundara, U.N. and Amarowicz, R. 1994. Natural antioxidants from low-pungency mustard flour. *Food Res. Int.*, 27, 489–493.

17. Hidalgo, M.E., Fernandez, E., Quilhot, W. and Lissi, E. 1994. Antioxidant activity of depsides and depsidones. *Phytochemistry*, 37, 1585–1587.

18. Chevolleau, S., Mallet, J.F., Ucciani, E., Gamisans, J. and Gruber, M. 1992. Antioxidant activity in leaves of some Mediterranean plants. *J. Am. Oil Chem. Soc.*, 69, 1269–1271.

19. Frankel, E.N. 1993. In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci. Technol.*, 4, 220–225.

20. Porter, W.L. 1980. Recent trends in food applications of antioxidants. p. 295–366. In: M.G. Simic and M. Karel (eds). Autoxidation in Food and Biological Systems. Plenum Press, New York.

ON-LINE DETECTION OF ANTIOXIDATIVE ACTIVITY IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ELUATES BY CHEMILUMINESCENCE*

4.1 Introduction

The introduction of new antioxidative substances requires reliable methods for the evaluation of their antioxidative activity and a number of studies have been published on this subject[1–3]. Radical scavenging activity of primary antioxidants can be assessed directly in foods (oil, lard, meat products, etc.)[4–6], by employing model systems (methyl linoleate, β-carotene bleaching, and ferric thiocyanate tests)[7–9] or by monitoring the reduction reaction of a stable radical ($ABTS^{+\bullet}$, $DPPH^{\bullet}$) when antioxidant is added[10,11]. Methods for antioxidant activity assessment have been summarised in Chapter 1.

Chemiluminescence (CL) measurements can be used to determine initial amounts of radical products of lipid oxidation[12–16] and the radical scavenging activity of antioxidants[17,18]. These techniques most often employ the chemiluminescence reaction of phthalic hydrazide derivatives such as luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) or isoluminol (6-amino-2,3-dihydrophthalazine-1,4-dione)[19]. In protic media, this reaction requires a potent oxidising agent such as hydroperoxide, a catalyst such as heme-containing proteins, transition-metal ions or peroxidases and pH 10–13. The exact mechanism of hydrazide chemiluminescence is difficult to clear up due to the complexity of the reaction products and a high number of quickly progressing side-reactions[20]. The present knowledge suggests that α-hydroxy hydroperoxides are key intermediates in the oxidation of cyclic hydrazides[21,22] (Figure 4.1). Decomposition of α-hydroxy hydroperoxides

* Parts of this chapter have been published:
Dapkevicius, A., van Beek, T.A., Niederländer, H.A.G. and de Groot, Ae. 1999. *Anal. Chem.*, 71, 736–740.

leads to the production of excited dicarboxylate molecules and eventually to emission of light at 425 nm. It is assumed that radical scavengers inhibit the CL reaction by quenching the available radical intermediates such as lipid oxyradicals[18], radicals derived from luminol[23] and oxygen[23–26].

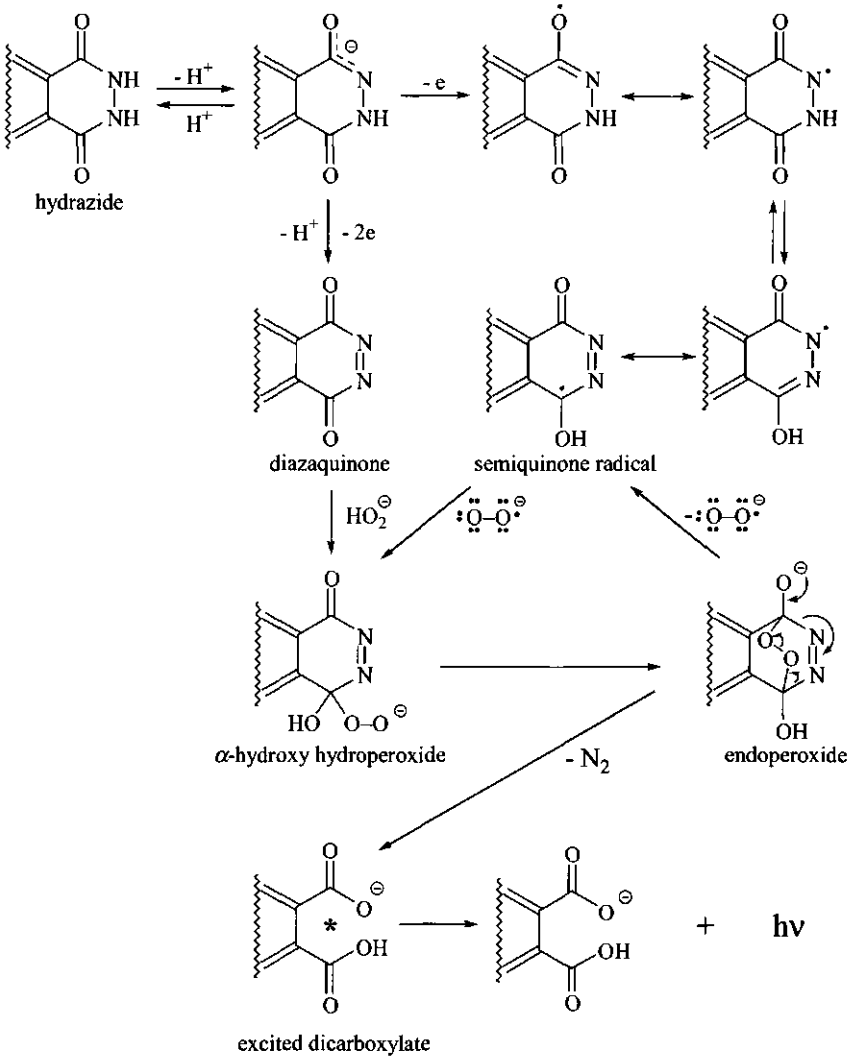


Figure 4.1 *Chemical pathways of chemiluminescence via oxidation of phthalic hydrazide derivatives.*

Detection of natural radical scavenging compounds in plant extracts by CL can be carried out either on-line after a prior HPLC separation or off-line without any prior separation. Heilmann *et al.* detected radical scavenging activity of 3',4'-

dihydroxyflavonols from *Arnica montana* and *Buphthalmum salicifolium* using an off-line CL technique[27]. With this technique laborious, time and material consuming isolation and purification procedures are necessary for the assessment of antioxidant activity and this makes it not attractive for screening for antioxidant compounds.

More recently, Ogawa and co-workers presented an on-line method for detecting antioxidant compounds in HPLC eluents[24]. The proposed technique is useful for screening purposes due to its simple operation and high sensitivity. The majority of other HPLC methods with on-line CL detection were developed for the analysis of lipid peroxides[13,14,15,25,28]. In some of these studies, naturally occurring tocopherols known as potent antioxidants interfered with the CL detection of peroxides[15,25,28] producing negative CL signals.

A novel on-line CL screening method for the detection of radical scavenging constituents in herb isolates separated by HPLC is presented in this chapter.

4.2 Experimental

4.2.1 HPLC–CL conditions and instrumental setup

The CL detection of radical scavenging compounds was carried out with the HPLC system represented in Figure 4.2. The linear binary gradient was formed in a Star 9012 Solvent Delivery System (Varian Chromatography Systems, Walnut Creek, California) at a constant total flow rate of 1.0 ml/min. Solvent A decreased in 25 min from 70 to 30% while solvent B increased from 30 to 70%. During the following 5 min, the eluent was isocratic. After this, the gradient returned to its initial settings in 5 min. Analytes were injected with a Must HP 6 multiport stream switch (Spark Holland, Emmen, The Netherlands) equipped with a 10-µl injection loop and separated on a Spherisorb C_{18} 5 µm analytical column (25 cm × 4.6 mm, Phase Separations, Waddinxveen, The Netherlands) which later was replaced by an Alltima C_{18} 5 µm analytical column (25 cm × 4.6 mm, Alltech Associates, Deerfield, IL.). The compounds eluting from the column were detected with a model 759A absorbance detector (Applied Biosystems, Foster City, CA) at $\lambda = 280$ nm (range 0.02 AU, rise time 1.0 s). On-line post-column addition of CL reagents was performed with an infusion only syringe pump (model 980532, Harvard Apparatus, South Natick, MA) equipped with two 30-ml Plastipak® syringes

(Becton Dickinson, Dublin, Ireland). A 60-cm mixing coil was installed between the hydrogen peroxide addition junction and a Spectroflow 980 fluorescence detector (Kratos Analytical, Ramsey, NJ). The fluorescence detector was used as a photon detector with the excitation lamp switched off. Both the CL signal and the signal from the UV detector were recorded on a BD 41 double-pen recorder (Kipp & Zonen, Delft, The Netherlands). Stainless steel tubing of 0.2-mm i.d. was used to interconnect the components of the HPLC–CL system.

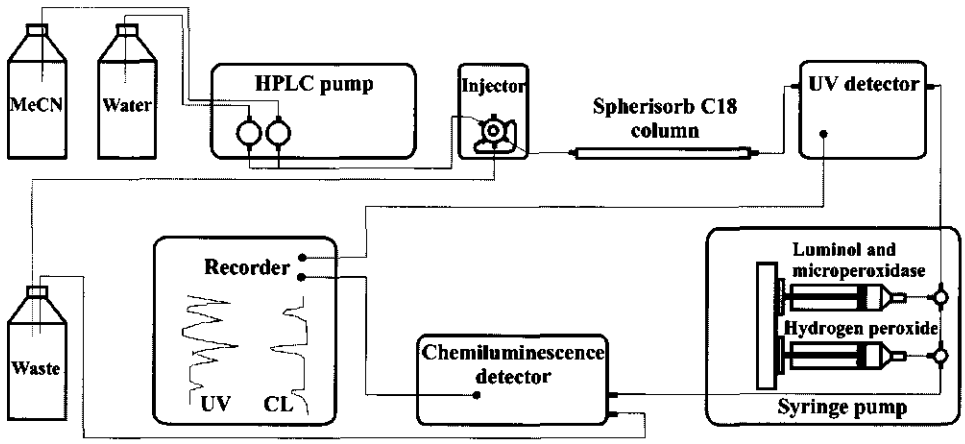


Figure 4.2 *Instrumental setup for the on-line chemiluminescence detection of radical scavenging compounds.*

Optimisation of the CL signal was carried out under off-line conditions with an LS 50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, U.K.) supplemented with Instrument Control Software version 4.00.

4.2.2 HPLC–CL reagents and tested antioxidants

Membrane-filtered (0.45 μm) and sonified "Milli-Q" water (Millipore, Bedford, MA) was used as solvent A in the HPLC gradient program. It was mixed with freshly redistilled HPLC grade acetonitrile (Labscan, Dublin, Ireland), introduced as eluent B. Horseradish microperoxidase sodium salt MP-11 (90%, Sigma–Aldrich, Steinheim, Germany) was dissolved in sodium tetraborate buffer (pH 10) at 5.7 mg/l. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione, 97%, Sigma–Aldrich) was dissolved in HPLC grade methanol (Labscan) at 1.6 mM.

Stock solutions of MP-11 and luminol were prepared in advance and stored at 4°C. Prior to use MP-11 and luminol solutions were mixed in a ratio of 7:3. The mixture was added to the HPLC eluate at the primary T-junction. At the secondary T-junction, an aqueous solution of hydrogen peroxide (35 wt. %, Sigma–Aldrich) at a concentration of 0.2 ml/l was added. Both streams of the CL reagents were introduced into the HPLC eluate at a flow rate of 0.05 ml/min.

Extracts of thyme (*Thymus vulgaris* L.) and sage (*Salvia officinalis* L.) were prepared by a Soxhlet extraction of 10 g of ground samples with redistilled acetone during 6 hr. The extracts were concentrated in a rotary evaporator at 55°C.

Some commonly used antioxidants such as α-tocopherol, *tert*-butylhydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), thymol, and carvacrol – all purchased from Sigma–Aldrich – as well as rosmarinic acid (Extrasynthèse, Genay, France) and carnosic acid (extracted from dried rosemary[29]) were dissolved in methanol (Labscan) in various concentrations and used in CL detection sensitivity tests.

4.3 Results and discussion

4.3.1 Initial tests

It is assumed that superoxides and other active radicals formed during the reaction of microperoxidase with hydrogen peroxide bring luminol into its excited state[13]. Thus, the intensity and duration of the CL background required for a sufficient detection of radical scavengers are greatly dependent on the concentration of luminol and on the conditions of this enzymatic reaction. Concentrations of the MP-11 and hydrogen peroxide solutions as well as pH value and ratio of buffer/methanol in the reaction mixture have to provide a CL signal in a certain range of intensity. Too low and too high CL signals can adversely affect the sensitivity of the CL detector. The sensitivity is also dependent on the duration of the CL signal. This defines the length of the tubing from the last T-junction to the CL detection cell.

A common substrate used to obtain chemiluminescence in many analytical procedures is luminol[12,14]. Microperoxidase (MP-11), which is a proteolytic fragment of cytochrome C, is reported to be the most effective catalyst for the oxidation of luminol[25]. Therefore, solutions of luminol, MP-11, and hydrogen

peroxide were prepared according to previous experience[30] and literature data[13–16,31] for the initial tests of the CL detection.

The first syringe was filled with a freshly prepared 3:1 mixture of MP-11 and luminol. The amount of MP-11 dissolved in sodium tetraborate buffer was 0.4 mg/l. The concentration of luminol in methanol was 0.6 mM. A 0.5 ml/l aliquot of 35% hydrogen peroxide solution in distilled water was used to fill the second syringe of the syringe pump. The CL signal registered by the fluorescence detector was of a high intensity (~1200 mV). Nevertheless, the sensitivity and reproducibility of the radical scavenging as well as the low signal-to-noise ratio (S/N=11.8) were not satisfactory. It was necessary to readapt the experimental conditions in order to suit the requirements of the HPLC–CL system and to improve the parameters of the radical scavenging detection.

4.3.2 Off-line CL experiments

It was assumed that the variables defining the CL process are independent within a narrow range of concentrations. Based on this, the concentrations of the CL reagents and the pH of the reaction were optimised using a luminescence spectrometer. The off-line CL signal was monitored in a 1-cm path length quartz cuvette. All measurements were carried out in triplicate. Figure 4.3 illustrates the kinetics of the signal obtained. Chemiluminescence in the cuvette reaches its maximum in 0.5–1.0 s after introduction of the hydrogen peroxide solution. The reaction end-point was set to a value of 10 mV.

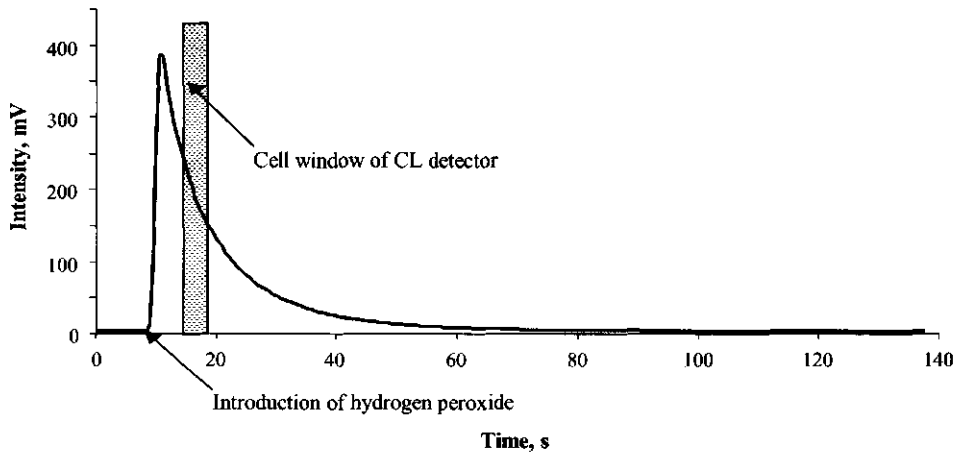


Figure 4.3 *Schematic representation of kinetics of the chemiluminescence reaction.*

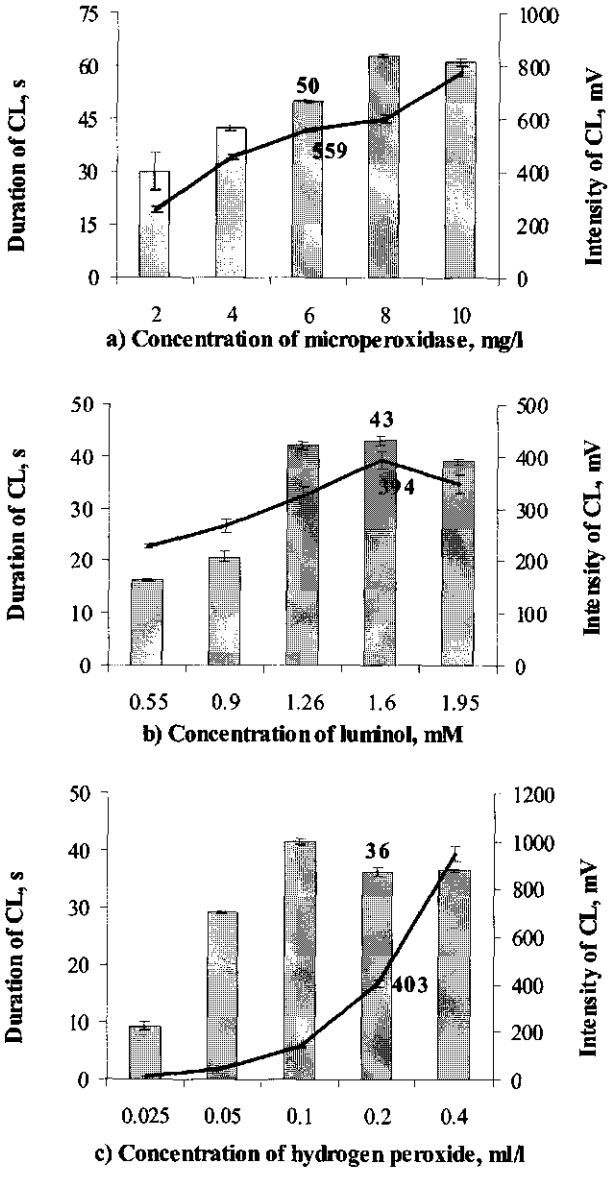


Figure 4.4 *Optimisation of concentrations of chemiluminescence reagents: (a) microperoxidase when [luminol] = 0.6 mM and [H_2O_2] = 0.5 ml/l; (b) luminol when [MP-11] = 5.7 mg/l and [H_2O_2] = 0.5 ml/l; (c) hydrogen peroxide when [MP-11] = 5.7 mg/l and [luminol] = 1.6 mM. Duration of CL signal bars, intensity lines.*

Figure 4.4 shows the intensity and duration of the CL signal as a function of the MP-11, luminol, and hydrogen peroxide concentrations.

The data collected confirm the already reported finding[15,23] that the amount of MP-11 has a considerable effect on the response of the CL detector. The duration of the signal reaches its maximum value at an MP-11 concentration of 8 mg/l. Due to the high price of MP-11, it is not reasonable to use concentrations of this enzyme above 6 mg/l. The activity of the enzyme slowly decreases in time upon storage; thus an occasional readjustment of the enzyme concentration is necessary. The CL intensity does not change much at the luminol concentrations tested. However, the duration of the CL signal significantly increases at luminol concentrations above ≈1.0 mM. The range of suitable concentrations is limited by the low solubility of luminol in methanol (≈2 mM). The concentration of hydrogen peroxide showed the most marked effect on the CL intensity. A linear dependence of the signal intensity on the hydrogen peroxide concentration was observed at the concentrations tested. However, it is not possible to obtain a CL signal for a longer time than ~40 s by increasing the amount of this reagent. Higher concentrations of hydrogen peroxide decrease the sensitivity of the CL detection and are therefore not desirable. Based on these experimental data, the concentrations of MP-11, luminol and H_2O_2 were set to 5.7 mg/l, 1.6 mM and 0.2 ml/l respectively, for further experimental work.

The effect of the sodium tetraborate buffer / methanol ratio in the mixture of CL reagents on the CL signal was evaluated, and the results are presented in Table 4.1.

Table 4.1 *Optimisation of the ratio of solvents for the preparation of CL reagents*[a]

Buffer/MeOH ratio	Duration, s	Intensity, mV
1/9	18.2 ± 0.29	123 ± 10.50
3/7	30.8 ± 1.44	695 ± 7.52
1/1	35.0 ± 0.50	1333 ± 9.66
7/3	42.5 ± 1.32	1656 ± 33.21
9/1	52.2 ± 1.04	1975 ± 28.06

[a] Averages of three measurements (± SD).

It was observed that an increasing amount of methanol in the mixture decreased the strength and length of the CL signal. This may be explained by the

degree of dilution of the sodium tetraborate buffer and by a decrease in apparent pH value. On the other hand, it is important to have a sufficient amount of methanol in the mixture of CL reagents in order to completely dissolve the luminol. Satisfactory results can be obtained by mixing buffer and methanol in a ratio of 7:3.

As mentioned earlier, the characteristics of the CL signal are dependent on the pH. Different buffers with pH values varying from 9 to 13 were used as solvents for MP-11. As can be seen in Figure 4.5, the kinetics of the photochemical reaction greatly differ, most likely due to the optimum pH for microperoxidase activity. Under less alkaline reaction conditions (pH 9–10) an intensive and short CL signal is seen. While at pH 12–13, chemiluminescence is prolonged and of low intensity with the maximum occurring later in time. A buffer solution with pH 10 value was considered to be optimal and chosen for further experiments.

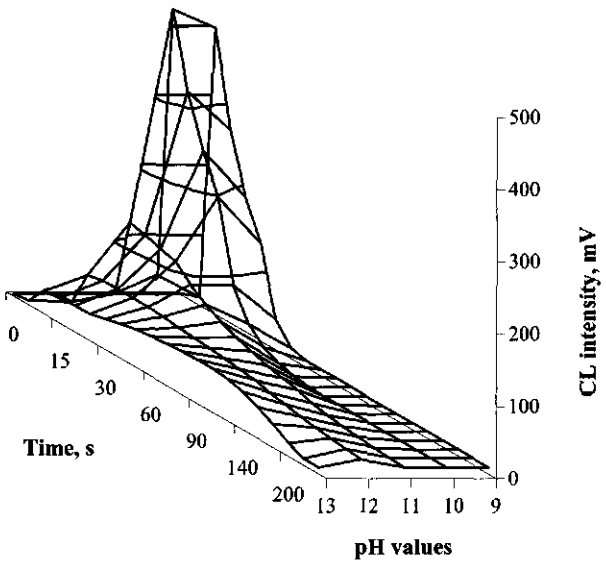


Figure 4.5 *Effect of pH value of buffer on the kinetics of CL signal.*

4.3.3 Optimisation of the HPLC–CL system

The starting composition of the HPLC gradient contained 30% acetonitrile (solvent B). Lower concentrations could not be used due to an unequal CL level in polar and non-polar parts of the gradient. Therefore, it is not possible to monitor the

whole gradient run without changing the sensitivity of the CL detector. The eluents used in the HPLC–CL system had to be of a neutral nature. Addition of acids to solvent A reduced the sensitivity of the CL detection.

The signal intensity and S/N value was determined in order to optimise the length of the tubing between the last T-junction and the CL detector cell. The intensity of the CL signal constantly increased when shortening the tubing from 100 to 30 cm. The highest S/N was calculated with 90 cm of tubing. The best combination of these two parameters (S/N = 12.1, intensity 261–268 mV) was registered when the length of tubing was 60 cm. The flow rate of the CL reagents during this experiment was 0.1 ml/min.

The S/N ratio of CL was also monitored while the flow of the CL reagents was changed from 0.025 to 0.5 ml/min. The highest S/N values (28.0 ± 0.5) were recorded when the flow was 0.05–0.07 ml/min. Exchanging the position of the post-column reagents (first hydrogen peroxide and then MP-11 + luminol) did not influence the CL signal or the sensitivity of the antioxidant detection.

4.3.4 Detection of radical scavenging activity

Various concentrations of methanolic solutions of several antioxidative compounds were tested to determine their detection limit (L_d). The negative peak on the CL signal was regarded as detectable when its height was exceeding the L_d value calculated according to the formula:

$$L_d = [\text{average blank signal}] - 2t[\text{standard deviation of blank signal}]$$

The coefficient $t = 1.725$ for 20 measurements of blank signal with a confidence interval of 90%.

A combined UV–CL chromatogram of seven dilutions of carvacrol is presented in Figure 4.6. Detection limits for all compounds tested are summarised in Table 4.2. It was found that injecting 0.5 ng of rosmarinic acid still caused a detectable CL signal. This is 142 times lower than the minimal detectable quantity of thymol. As expected, the detection limits of antioxidative compounds greatly depend on their ability to donate a hydrogen atom in the reaction with a free radical. The decrease in CL signal caused by carnosic acid and BHT was still linear within the dilution range tested. The linearity interval was determined graphically as the range of concentrations over which the response of the CL detector was constant to within 5%[32]. The smallest linear response of the CL detector was observed for rosmarinic acid.

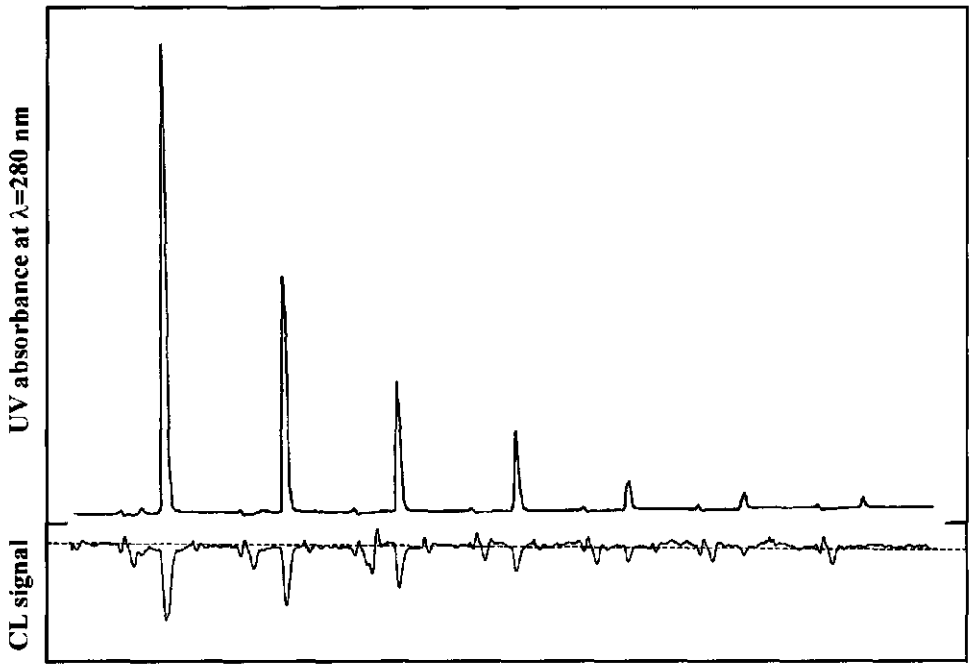


Figure 4.6 *UV and CL response to sequentially (left to right) injected carvacrol (220, 110, 55, 27.5, 13.8, 6.9, and 3.4 μg/ml) under isocratic conditions. The CL detection limit L_d for carvacrol is 3.6 μg/ml.*

Table 4.2 *Detection limit, mass sensitivity and linearity of detection of some antioxidants by HPLC–CL system under isocratic conditions*

Compound tested	Detection limit L_d, μg/ml[a]	Minimum amount still detectable with CL, ng	Linearity interval, μg/ml
Rosmarinic acid	0.05	0.5	0.3 – 2.6
Carnosic acid	0.51	5.1	0.5 – 10.0[b]
α-Tocopherol	0.96	9.6	10.0 – 40.0
BHT	2.77	27.7	2.7 – 80.0[b]
TBHQ	3.54	35.4	10.0 – 40.0
Carvacrol	3.60	36.0	13.8 – 110.0
Thymol	7.10	71.0	40.0 – 160.0

[a] Detected with 10-μl injection loop.
[b] Linear for all concentrations tested.

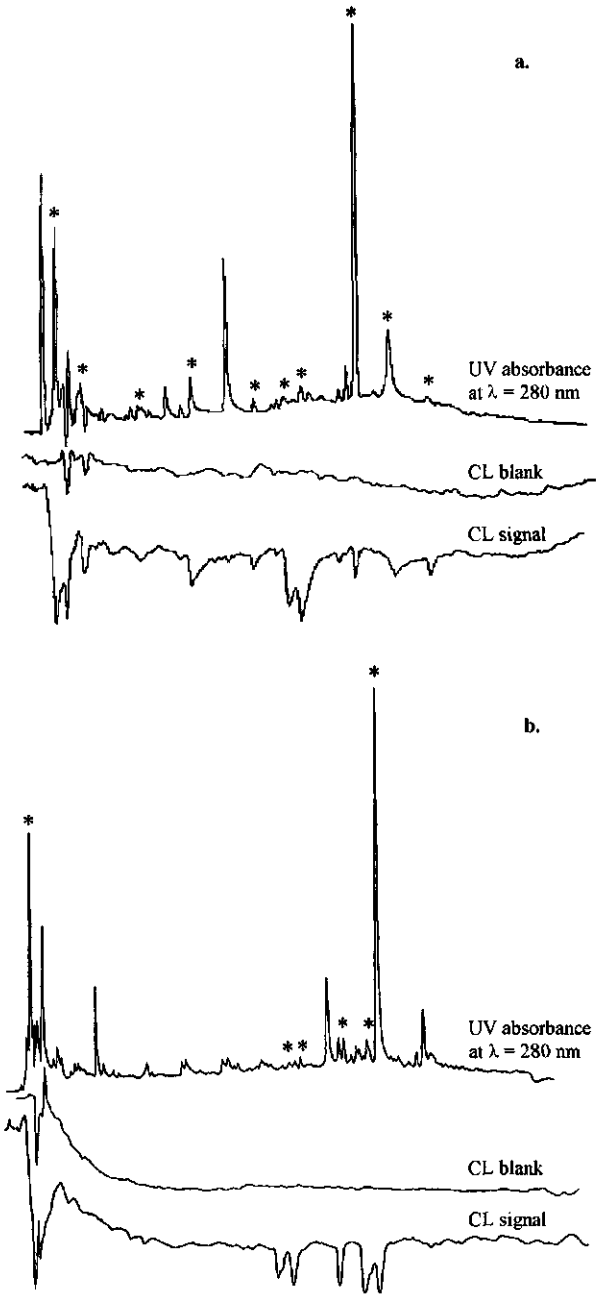


Figure 4.7 *UV and CL response to thyme (a) and sage (b) samples with gradient elution. Compounds/compound groups possessing radical scavenging activity and appearing reproducibly in 10 chromatograms of the same sample are marked with an *.*

Subsequently, the method was tested on thyme and sage acetone oleoresins. The herb isolates were dissolved in MeOH at concentrations of 0.2 g/l prior to injection into the HPLC–CL system. Ten eluted compounds in the thyme sample and six in the sage sample gave a detectable CL signal as can be observed in Figure 4.7. When the concentration of the herb samples was tripled the CL signal of some peaks – in contrast to the UV signal – did not show a 3-fold increase in area. This was particularly true for the stronger signals where quenching was reaching its maximum, i.e., where the concentration is clearly outside of the linear range. The variable sensitivity of the CL detector for different antioxidative compounds that elute from the HPLC column with some tailing is causing unequally prolonged CL quenching and broadening of some CL peaks. Therefore, an individual calibration is indispensable for the quantitative analysis of each antioxidative analyte. To evaluate more polar constituents of the herb extracts, the HPLC solvents and the gradient have to be modified.

4.4 Conclusions

The results of this study confirm the possibility of assessing radical scavenging activity of antioxidants using the chemiluminescence reaction of luminol. The combination of an HPLC system and on-line CL detection allows a rapid detection of natural antioxidative substances in herb extracts in the presence of many inactive constituents with a minimum of preparatory manipulations. This technique could also be used to obtain quick information on the presence of antioxidative additives in complex mixtures such as drugs or fat-containing foods. Not all antioxidative compounds quench radicals equally well, therefore calibration of each antioxidant present in a sample mixture has to be carried out prior to any quantitative assessments.

The HPLC–CL system can be used for the direct evaluation and comparison of the antioxidative efficiency of primary antioxidants. Such information is crucial for the establishment of reliable structure-antioxidant activity relationships. This test can be a useful tool in activity-guided isolation of natural antioxidants.

4.5 References

1. Sherwin, E.R. 1968. Methods for stability and antioxidant measurement. *J. Am. Oil Chem. Soc.*, 45, 632A; 634A; 646A; 648A.
2. Gray, J.I. 1978. Measurement of lipid oxidation: a review. *J. Am. Oil Chem. Soc.*, 55, 539–546.
3. Coxon, D. 1987. Measurement of lipid oxidation. *Food Sci. Technol. Today,* 1, 164–166.
4. Gray, J.I. and Monahan, F.J. 1992. Measurement of lipid oxidation in meat and meat products. *Trends Food Sci. Technol.*, 3, 315–319.
5. Banias, C., Oreopoulou, V. and Thomopoulos, C.D. 1992. The effect of primary antioxidants and synergists on the activity of plant extracts in lard. *J. Am. Oil Chem. Soc.*, 69, 520–524.
6. Pokorný, J., Nguyen, H.T.T. and Korczak, J. 1997. Antioxidant activities of rosemary and sage extracts in sunflower oil. *Nahrung*, 41, 176-177.
7. Kikuzaki, H. and Nakatani, N. 1993. Antioxidant effects of some ginger constituents. *J. Food Sci.*, 58, 1407–1410.
8. Hidalgo, M.E., Fernandez, E., Quilhot, W. and Lissi, E. 1994. Antioxidant activity of depsides and depsidones. *Phytochemistry*, 37, 1585–1587.
9. Cuvelier, M.E., Richard, H. and Berset, C. 1996. Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. *J. Am. Oil Chem. Soc.*, 73, 645–652.
10. Brand-Williams, W., Cuvelier, M.E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Z. Lebensm.-Wiss. Technol.*, 28, 25–30.
11. von Gadow, A., Joubert, E. and Hansmann, C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. *J. Agric. Food Chem.*, 45, 632–638.
12. Yang, G.C., Qiang, W., Morehouse, K.M., Rosenthal, I., Ku, I. and Yurawecz, P. 1991. Determination of hydroperoxides in edible oil by electron spin resonance, TBA, and liquid cromatography-chemiluminescence techniques. *J. Agric. Food Chem.*, 39, 896–898.
13. Yang, G.C. 1992. Detection of lipid hydroperoxides by HPLC coupled with post-column reaction. *Trends Food Sci. Technol.*, 3, 15–18.

14. Miyazawa, T., Kunika, H., Fujimoto, K., Endo, Y. and Kaneda, T. 1995. Chemiluminescence detection of mono-, bis-, and tris-hydroperoxy triacylglycerols present in vegetable oils. *Lipids*, 30, 1001–1006.

15. Mäkinen, M., Piironen, V. and Hopia, A. 1996. Postcolumn chemiluminescence, ultraviolet and evaporative light-scattering detectors in high-performance liquid chromatographic determination of triacylglycerol oxidation products. *J. Chromatogr. A*, 734, 221–229.

16. Christensen, T.C. and Holmer, G. 1996. Lipid oxidation determination in butter and dairy spreads by HPLC. *J. Food Sci.*, 61, 486–489.

17. Cash, G.A., George, G.A. and Bartley, J.P. 1988. A chemiluminescence study of the oxidation of vegetable oils and model compounds and the effects of antioxidants. *J. Sci. Food Agric.*, 43, 277–287.

18. Hirayama, O., Takagi, M., Hukumoto, K. and Katoh, S. 1997. Evaluation of antioxidant activity by chemiluminescence. *Anal. Biochem.*, 247, 237–241.

19. Hage, D.S. 1992. Chemiluminescent detection in high-performance liquid chromatography. p. 57–75. In: G. Patonay (ed). HPLC Detection: Newer Methods. VCH Publishers, New York.

20. Steijger, O.M. 1998. Chemiluminescence and electrochemiluminescence: generation, optimization and application. Thesis: University Utrecht. 199 pp.

21. Merényi, G. and Lind, J. 1980. Role of peroxide intermediate in the chemiluminescence of luminol. A. mechanistic study. *J. Am. Chem. Soc.*, 102, 5831–5835.

22. Merényi, G., Lind, J. and Eriksen, T.E. 1986. Nucleophilic addition to diazaquinones. Formation and breakdown of tetrahedral intermediates in relation to luminol chemiluminescence. *J. Am. Chem. Soc.*, 108, 7716–7726.

23. Whitehead, T.P., Thorpe, G.H.G. and Maxwell, S.R.J. 1992. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal. Chim. Acta,* 266, 265–277.

24. Ogawa, A., Arai, H., Tanizawa, H., Miyahara, T. and Toyooka, T. 1999. On-line screening method for antioxidants by liquid chromatography with chemiluminescence detection. *Anal. Chim. Acta*, 383, 221–230.

25. Yamamoto, Y., Brodsky, M.H., Baker, J.C. and Ames, B.N. 1987. Detection and characterization of lipid hydroperoxides at picomole levels by high-performance liquid chromatography. *Anal. Biochem.*, 160, 7–13.

26. Larson, R.A. 1997. Naturally Occurring Antioxidants. CRC Press LLC, Boca Raton. 189 pp.

27. Heilmann, J., Merfort, I. and Weiss, M. 1995. Radical scavenger activity of different 3',4'-dihydroxyflavonols and 1,5-dicaffeoylquinic acid studied by inhibition of chemiluminescence. *Planta Med.*, 61, 435–438.

28. Frei, B., Yamamoto, Y., Niclas, D. and Ames, B.N. 1988. Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. *Anal. Biochem.*, 175, 120–130.

29. Richheimer, S.L., Bernart, M.W., King, G.A., Kent, M.C. and Bailey, D.T. 1996. Antioxidant activity of lipid-soluble phenolic diterpenes from rosemary. *J. Am. Oil Chem. Soc.*, 73, 507–513.

30. Niederländer, H.A.G., de Jong, M.M., Gooijer, C. and Velthorst, N.H. 1994. Flow injection system for determination of singlet oxygen quenching efficiencies utilizing on-line dioxetane chemiluminescence detection. *Anal. Chim. Acta,* 290, 201–214.

31. Matthäus, B.W. 1996. Determination of the oxidative stability of vegetable oils by Rancimat and conductivity and chemiluminescence measurements. *J. Am. Oil Chem. Soc.*, 73, 1039–1043.

32. Poole, C.F. and Poole S.K. 1991. Chromatography today; Elsevier, Amsterdam.

EVALUATION AND COMPARISON OF TWO IMPROVED TECHNIQUES FOR THE ON-LINE DETECTION OF ANTIOXIDANTS IN HPLC ELUATES*

5.1 Introduction

The methods of antioxidant activity evaluation in model systems that were discussed in Chapter 1 have, in most cases, a batch-like character. This means that each previously isolated sample or a defined number of samples have to be analysed in separate experiments, which include preparation and calibration of equipment, preparation of reagents, etc. Due to this, detection, identification and comparison of active compounds in complex mixtures (e.g. plant extracts, drugs and food systems) are rather problematic.

Some recent publications demonstrate the potential of merging the efficiency of an HPLC separation with the convenience of on-line post-column detection of radical scavenging compounds based on a model oxidation system[1–3], i.e. luminol chemiluminescence inhibition, or DPPH$^{\bullet}$ reduction. The on-line luminol chemiluminescence (CL) assay is based on the scavenging of free radical species (including some reactive oxygen species) involved in the reaction sequence leading to luminol chemiluminescence[4–7]. The resulting inhibition of chemiluminescence is registered at 425 nm. The on-line DPPH$^{\bullet}$ quenching assay is based on a decrease in DPPH$^{\bullet}$ absorbance at 517 nm, upon reduction of this relatively stable radical by an antioxidant[8]. The theoretical background of these two detection techniques was outlined in more detail in Chapters 1 and 4.

* Parts of this chapter have been published:
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Improvement and evaluation of two on-line methods[1,3] are presented in this chapter. Both methods are compared with respect to their sensitivity, selectivity, compatibility with common HPLC solvents, range of pH and some technical aspects.

5.2 Experimental

5.2.1 HPLC–CL instrumental set-up

The HPLC system with on-line chemiluminescence (CL) detection of radical scavengers is shown in Figure 5.1. The binary gradient was formed in a Waters 600E multisolvent delivery system (Millipore, Waters Chromatography Division, Milford, MA). A second pulse damper (toroid mixer, Scientific Systems, State College, PA) was introduced to improve pressure stability. Samples were injected using a Gilson 401 dilutor (Gilson Medical Electronics, Middleton, WI) combined with a Gilson 231 auto-sampling injector equipped with a 10-µl injection loop. Analytes were separated on an Alltima C_{18} 5U analytical column (250 × 4.6 mm, Alltech Associates, Deerfield, IL) and detected with a Waters 990 Series photodiode array detector from λ = 210–450 nm. Data were processed on a PC with original Waters software (version LCA–6.22a) and printed on a Waters 5200 printer plotter. On-line post-column addition of CL reagents was performed with two 45-ml laboratory-made syringe pumps (Free University, Amsterdam, The Netherlands). The CL was monitored with a fluorescence detector (Kratos Analytical, Ramsey, NJ) with the excitation lamp switched off. The CL signal obtained was recorded on a BD 40 recorder (Kipp & Zonen, Delft, The Netherlands). A 15-m long mixing/neutralisation coil of 0.25-mm I.D. PEEK tubing was introduced between two T-junctions. A 60-cm reaction coil of 0.25-mm I.D. PEEK tubing was used to connect the syringe pump B with the fluorescence detector. All other parts of the system were interconnected with stainless steel tubing of 0.2 mm I.D.

5.2.2 HPLC–CL reagents and gradient conditions

Ultra-pure water (0.05 µS/cm) was prepared with a combined Seradest LFM 20 and Serapur Pro 90C apparatus (Seral, Ransbach-Baumbach, Germany) and mixed with HPLC grade acetonitrile (Lab-Scan Analytical Sciences, Dublin, Ireland) in a

ratio of 3:1. The mixture was acidified with 2.5 ml/l glacial acetic acid (Merck, Darmstadt, Germany), filtered through a 0.45 μm membrane filter (Schleicher & Schuell, Dassel, Germany) and used as solvent A in the HPLC gradient program. HPLC grade filtered acetonitrile acidified with glacial acetic acid (2.5 ml/l) was introduced as solvent B. In linear binary gradient runs, solvent A decreased in 40 min from 95 to 10%, was held during the following 15 min, and then returned from 10 to 95% in 5 min. The combined flow of HPLC eluents was kept constant at 0.85 ml/min.

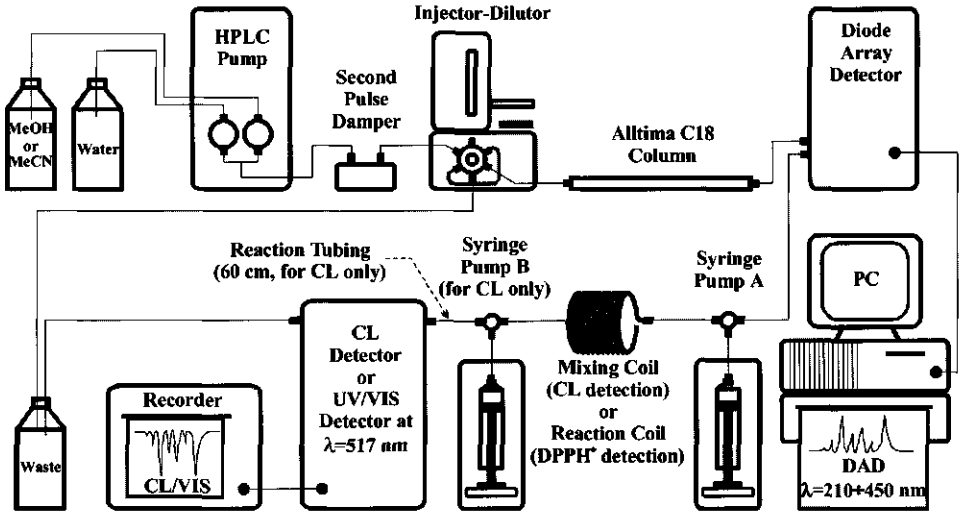


Figure 5.1 *Improved instrumental set-up for the on-line chemiluminescence/DPPH• detection of radical scavenging compounds.*

An aqueous 0.6-ml/l hydrogen peroxide solution was mixed with a 1 M NaOH solution at a ratio 2:5, filtered and stored in a refrigerator prior to use with the first syringe pump (pump A). Horseradish microperoxidase sodium salt MP-11 (90%, Sigma–Aldrich) was dissolved in disodium tetraborate buffer at 5.7 mg/l. This buffer, which had been brought to pH 10 by addition of 0.1 M NaOH, contained 50 ml of 0.025 M disodium tetraborate decahydrate and 18.3 ml of 0.1 M NaOH solutions prepared in ultra-pure water. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 97%, Sigma–Aldrich) was dissolved in HPLC grade methanol (Lab-Scan) at 1.6 mM. Stock solutions of MP-11 and luminol were stored at 4°C. MP-11 and luminol solutions were mixed at a ratio of 7:3 prior to use with the second syringe pump (pump B). The pumps A and B introduced streams of CL reagents at flow rates of 0.02 and 0.22 ml/min, respectively.

5.2.3 HPLC–DPPH• instrumental set-up

The HPLC system adapted for DPPH• on-line detection of radical scavenging compounds differed from the HPLC–CL system in that pump B and the 60-cm reaction coil were removed and the fluorescence detector was replaced by a UV/VIS model 759A absorbance detector (Applied Biosystems, Foster City, CA) equipped with a tungsten lamp. Thus, the 15-m PEEK coil was connected directly to the absorbance detector working at λ = 517 nm (0.05 range; 5.0 rise time) and functioned here as a reaction coil instead of a mixing coil.

5.2.4 HPLC–DPPH• reagents and gradient conditions

Two binary HPLC gradients based on methanol (MeOH) or acetonitrile (MeCN) were tested with the HPLC–DPPH• set-up.

For the MeOH/H_2O gradient, solvent A was prepared by mixing HPLC grade MeOH in ultra-pure water (MeOH/H_2O = 35:65, v/v) and acidifying with glacial acetic acid (0.5 ml/l). Pure MeOH with 0.5 ml/l glacial acetic acid was used as solvent B. The percentage of solvent A in the gradient was as follows: 0–4 min 85%, then from 85 to 50% in 10 min, hold for 3 min, then, from 50 to 0% in 9 min, hold for 9 min, then return from 0 to 85% in 3 min. The combined total flow of HPLC solvents was 0.78 ml/min. The DPPH• reagent (2,2'-diphenyl-1-picrylhydrazyl radical, 95%, Sigma–Aldrich) was dissolved in MeOH at a concentration of 5.8 mg/l. This solution was mixed with citric acid–disodium hydrogen phosphate buffer at a ratio of 3:1. The buffer, pH 7.6, contained 6.8 ml of 0.01 M citric acid and 93.2 ml of 0.02 M disodium hydrogen phosphate solutions prepared in ultra-pure water. The buffered DPPH• solution was introduced by the syringe pump at 0.5 ml/min.

For the MeCN/H_2O gradient, solvent A was a mixture of HPLC grade MeCN with ultra-pure water prepared at the ratio 1:4 and acidified with glacial acetic acid at 2.5 ml/l. Pure MeCN mixed with 2.5 ml/l acetic acid was used as solvent B. The percentage of solvent A in the gradient was as follows: 0–3 min 100%, then to 65% in 11 min, hold for 7 min, then from 65 to 5% in 7 min, hold for 12 min, then from 5 to 0% in 3 min, hold for 2 min, then back from 0 to 100% in 5 min. The buffered DPPH• solution was prepared similarly to that used in MeOH/H_2O gradient runs with the only difference being that the reagent was dissolved in MeCN. The flow-rates of HPLC eluents and the DPPH• mixture were identical to those of the MeOH/H_2O gradient. Prior to use, all solvents as well as

$DPPH^{\bullet}$ mixtures were filtered through a 0.45-μm membrane filter and during the runs, they were sparged with helium at 25 ml/min.

5.2.5 Sample preparation

Samples of thyme (*Thymus vulgaris* L.) were obtained from the Lithuanian Institute of Horticulture (Babtai, Lithuania) and dried in a drying cabinet with forced ventilation at ambient temperature for 2 days. A total of 50 g of herb material was ground and extracted with 500 ml of redistilled MeOH in an Erlenmeyer flask with a magnetic stirrer under N_2 during 72 hr. After filtration, the extract obtained was vacuum-dried at 45°C with a Büchi RE 11 rotary evaporator (Büchi Laboratoriums-Technik, Flawil, Switzerland) combined with a Vacuubrand CVC2 vacuum pump (Vacuubrand, Wertheim, Germany) and a Büchi 461 water bath. Obtained extracts were dissolved in $MeOH/H_2O$ (1:1) at 2.5 mg/ml for the HPLC–$DPPH^{\bullet}$ analysis and in $MeCN/H_2O$ (1:1) at 0.5 mg/ml for the HPLC–CL assessment. Extract solutions were membrane filtered before injection into the HPLC system.

Standards of some antioxidative compounds were purchased from the following suppliers: eugenol, isoeugenol, quercetin dihydrate, α-tocopherol, ascorbic acid, Trolox, 2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), thymol, and carvacrol from Sigma–Aldrich; rosmarinic acid from Extrasynthèse (Genay, France); rutin trihydrate from Fluka (Buchs, Switzerland); chlorogenic acid from Koch-Light (Colnbrook, England); (±)-taxifolin and eriodictyol from Rotichrom® (Carl Roth, Karlsruhe, Germany). Carnosic acid was isolated in our laboratory.

5.2.6 Detection limits, minimum detectable amounts (MDA), and minimum detectable concentrations (MDC)

The HPLC column was connected between the pulse damper and the injector-dilutor. Unbuffered $MeOH/H_2O$ eluents were used with the $DPPH^{\bullet}$ detection, while with chemiluminescence unbuffered $MeCN/H_2O$ elution was used. For each compound the percentage of eluent A under isocratic conditions is given in Table 5.1.

Table 5.1 *HPLC eluent composition, calculated minimum detectable amount (MDA) and minimum detectable concentration (MDC) values for on-line DPPH$^{\bullet}$/CL detection of some antioxidants*

Compound	HPLC–DPPH$^{\bullet}$ detection[a]			HPLC–CL detection[b]		
	Eluent A, %	MDC, μmol/l[c]	MDA, ng[c,d]	Eluent A, %	MDC, μmol/l[c]	MDA, ng[c,d]
α-Tocopherol	0	1.3 ± 0.1	5.6 ± 0.4	3	1.7 ± 0.1	7.2 ± 0.6
Ascorbic acid	90	2.0 ± 0.2	3.6 ± 0.4	90	0.204 ± 0.033	0.36 ± 0.06
BHT	0	130 ± 10	280 ± 16	5	8.7 ± 2.4	19 ± 5
Carnosic acid	5	0.13 ± 0.02	0.43 ± 0.06	5	1.7 ± 0.2	5.8 ± 0.8
Carvacrol	10	1600 ± 60	2400 ± 83	5	4.2 ± 1.3	6.4 ± 1.9
Chlorogenic acid	90	0.15 ± 0.05	0.54 ± 0.2	90	0.087 ± 0.01	0.31 ± 0.02
Eriodictyol	40	1.5 ± 0.1	4.4 ± 0.4	40	0.62 ± 0.21	1.8 ± 0.6
Eugenol	10	27 ± 3	44 ± 5	5	0.14 ± 0.02	0.23 ± 0.02
Isoeugenol	10	0.84 ± 0.3	1.4 ± 0.5	5	2.1 ± 0.4	3.5 ± 0.6
Quercetin	0	0.31 ± 0.09	1.03 ± 0.3	5	1.2 ± 0.8	4.1 ± 2.6
Rosmarinic acid	40	0.017 ± 0.006	0.062 ± 0.023	40	0.104 ± 0.011	0.38 ± 0.04
Rutin	10	0.69 ± 0.02	4.6 ± 0.1	40	2.5 ± 0.2	16 ± 1.2
Taxifolin	40	5.8 ± 0.3	18 ± 1	40	0.54 ± 0.17	1.6 ± 0.5
TBHQ	30	9.1 ± 0.3	15 ± 0.5	40	2.5 ± 0.9	4.1 ± 1.4
Thymol	10	1500 ± 120	2200 ± 200	5	4.7 ± 0.4	7.03 ± 0.66
Trolox	20	0.34 ± 0.1	0.86 ± 0.29	40	2.2 ± 0.5	5.4 ± 1.2

[a] Eluent A 35% MeOH in water; eluent B 100% MeOH.
[b] Eluent A 25% MeCN in water; eluent B 100% MeCN.
[c] Mean (n = 3) ± standard deviation.
[d] Determined with a 10-μl injection loop.

From eight to twelve serial dilutions of antioxidant compounds were prepared in the corresponding mixture of eluents and tested in triplicates. Detection limits (L_d, in arbitrary units) were calculated for each compound and method as described by Koleva *et al.*[3]:

$$L_d = -2t[\text{standard deviation of blank signal}]$$

in which $t = 1.725$ for 20 measurements of blank signal with a confidence interval of 90%.

MDC (in µmol/l) values were calculated for each antioxidant and each detection method by regression using SPSS 8.0 for Windows software (SPSS, Chicago, IL). Equations were fitted to the different antioxidant concentrations (x) and the corresponding detector responses (y) by the least-squares method. These equations were used to calculate the MDC using the previously calculated L_d value as y. Minimum Detectable Amounts (in ng) were calculated from the MDA values taking into account the loop size used.

5.2.7 Statistical analysis

The data obtained for both detection methods under study were submitted to ANOVA using the SPSS 8.0 for Windows software.

5.3 Results and discussion

5.3.1 Improvements in DPPH• and luminol CL detection

The sensitivity of both on-line detection methods[1,3] was largely dependent on flow stability of both HPLC eluents and post-column reaction reagents. In this study, the instrumental set-up underwent several alterations in order to improve flow stability and to get better L_d values than those reported in Chapter 4. This could be achieved by: (i) the introduction of an additional pulse damper between the eluent pump and the injector; (ii) the use of more stable syringe pumps for post-column reagent addition in the luminol chemiluminescence method; and (iii) increasing detector lamp intensity at the detection wavelength (517 nm) for the DPPH• method.

Other changes that were applied in this study to both radical scavenger detection techniques are discussed below in more detail.

5.3.2 The luminol chemiluminescence quenching method

Several alterations in the CL detection method presented in Chapter 4 were made in order to improve the chromatographic separation of herb extract constituents and to increase the detection sensitivity of their antioxidant activity. Chromatographic parameters for two unidentified and closely eluting compounds of the crude thyme extract were calculated from new and previously obtained chromatograms. As can be observed from Table 5.2, the chromatographic separation of these two mid-polarity compounds greatly improved.

Table 5.2 *Improvement on chromatographic parameters of two thyme extract constituents*

Chromatographic parameter	Previous chromatographic conditions (Chapter 4)		New chromatographic conditions	
	Compound X	Compound Y	Compound X	Compound Y
Capacity factor, k'	0.5	0.3	4.6	3.8
Number of theoretical plates, N	623	1643	12451	3310
Number of effective theoretical plates, N_{eff}	73	109	8378	2071
Asymmetry factor	n.a.[a]	n.a.[a]	0.96	0.95
Resolution of X & Y, R_s	0.8		2.4	

[a] Parameter not established due to insufficient separation of peaks.

Changing the acetonitrile/water gradient program and lowering the pH of HPLC solvents resulted in a better separation efficiency especially for polar (acidic) analytes. However, partial pH neutralisation was required to allow for the post-column CL reaction, which needs alkaline conditions. This was accomplished by adding 1 M NaOH to the H_2O_2 solution at a ratio of 2:5. Attempts to combine a methanol/water gradient with post-column luminol CL detection were not successful due to unacceptable chemiluminescence baseline drift. This was caused by a rising backpressure during the gradient runs, which in turn resulted from viscosity differences of the methanol/water mixtures.

Modification of the instrumental set-up and introduction of acidified eluents made it necessary to change the concentrations and flow-rates of the CL reagents. The H_2O_2 concentration was increased three times, whereas the H_2O_2 solution flow-rate was lowered three times. The composition of MP-11 and luminol solutions was not changed, although the flow rate was increased four times. This was carried out to improve the signal-to-noise ratio and to produce a CL signal of similar intensity to that given in Chapter 4.

After introducing the above changes in set-up and flows, the sensitivity of the CL detection improved on average 4.2 times. The biggest change was observed for thymol, carvacrol and TBHQ, whose MDA decreased approximately ten, six and nine times, respectively.

The separation of a thyme extract with combined UV/CL detection also improved (Figure 5.2a vs. Figure 4.7a). A variable sensitivity of CL detection for individually eluting compounds was observed, i.e. the UV response did not correlate well with CL quenching. Due to its sensitivity, the CL method registered broadened peaks. This is possibly caused by a minimal tailing from the HPLC column and/or a certain inertia of the CL reaction. The CL detection of active compounds yielded qualitatively and quantitatively different results when compared with those from the DPPH$^{\bullet}$ quenching method. A greater number of non-polar compounds of the thyme extract were detected by CL.

5.3.3 The DPPH$^{\bullet}$ quenching method

Chromatographic separation efficiency for a thyme extract in the "mid-polarity range" was improved by applying both acetonitrile/water as well as methanol/water gradients and lowering the eluent pH to 3.0–3.2. The corresponding drop in the DPPH$^{\bullet}$ quenching detection sensitivity was compensated for by buffering the DPPH$^{\bullet}$ reagent flow with citric acid-disodium hydrogen phosphate buffer (pH 7.6). In order to obtain a higher detection sensitivity than that reported by Koleva *et al.*[3], the concentration of the DPPH$^{\bullet}$ solution was increased by 10% to 11 µmol/l and its flow-rate was lowered to 0.5 ml/min (from 0.7 ml/min). Purging the DPPH$^{\bullet}$ solution with He gas before use further reduced earlier observed baseline drift. This is probably due to elimination of interferences in the detection process, caused by dissolved oxygen. The result is shown in Figure 5.2b. An acetonitrile gradient was preferred over a methanol gradient for reasons of diminished pressure variations during gradient runs, stability of the DPPH$^{\bullet}$ solution and separation efficiency.

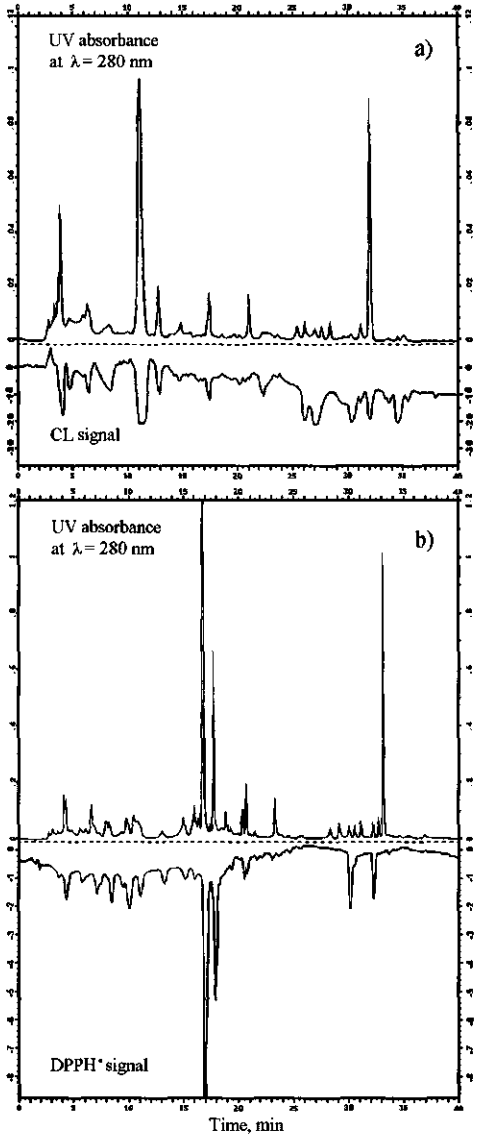


Figure 5.2 On-line assessment of radical scavengers from *(T. vulgaris) methanolic thyme extract: (a) modified UV-CL detection with a post-column neutralisation of acidic HPLC eluents by mixing 1 M NaOH with the H_2O_2 solution (pump A) at a ratio of 2:5; (b) modified UV-DPPH• detection using acidified HPLC eluents and a buffered (pH 7.6, citric acid–disodium hydrogen phosphate buffer) DPPH• solution. Differences in the UV trace are caused by different acidic eluents, i.e. MeCN/H_2O and MeOH/H_2O, used for UV-CL and UV-DPPH• detection, respectively.*

5.3.4 Consequences of eluent composition and eluent and reagent solution pH for MDA values with DPPH• detection

Minimum detectable amounts (MDA's) for the two model compounds carvacrol and rosmarinic acid, which differ in their polarity were calculated by: (i) applying methanol/water and acetonitrile/water eluents that were individually prepared for each model compound as indicated in Table 5.1; (ii) assessing neutral eluents and eluents with added glacial acetic acid (0.5 ml/l for methanol/water and 2.5 ml/l for acetonitrile/water); and (iii) using non-buffered DPPH• solution and DPPH• solution buffered with citric acid–disodium hydrogen phosphate buffer (pH 7.6).

Significantly different MDA values ($P < 0.05$) were obtained (Figure 5.3), and these differences do not show a similar trend for both model compounds (Figure 5.3a vs. 5.3b). However, when the eluent is acidified, a general tendency towards less favourable MDA values can be observed. In combination with a methanol/water gradient, the MDA values for carvacrol and rosmarinic acid increased almost nine-fold and five hundred-fold respectively upon acidification of the eluent to pH 3.0–3.2. These findings as well as herb extract analyses demonstrated that DPPH• quenching should preferably be carried out under neutral (i.e. unbuffered) conditions either by operating the whole system under neutral conditions, or by post-column neutralisation of the eluent. However, care should be exercised, whenever a buffered DPPH• solution is added into a stream of an acetonitrile/water eluent. When this eluent contains 96% (v/v) of acetonitrile, a clogging of the reaction coil occurs, which in turn adversely affects the MDA values.

With carvacrol the most favourable MDA (0.23 µg) was obtained with an acetonitrile/water gradient acidified with acetic acid (pH 3.2), and a DPPH• reagent flow buffered with citric acid–disodium hydrogen phosphate buffer (pH 7.6). In contrast, with rosmarinic acid the most favourable MDA (62 pg) was obtained with a methanol/water gradient (eluent- and reagent-flow pH unadjusted). However, it should be noted that the MDA with an acetonitrile/water gradient (eluent pH unadjusted and reagent flow buffered), was only slightly higher (101 pg).

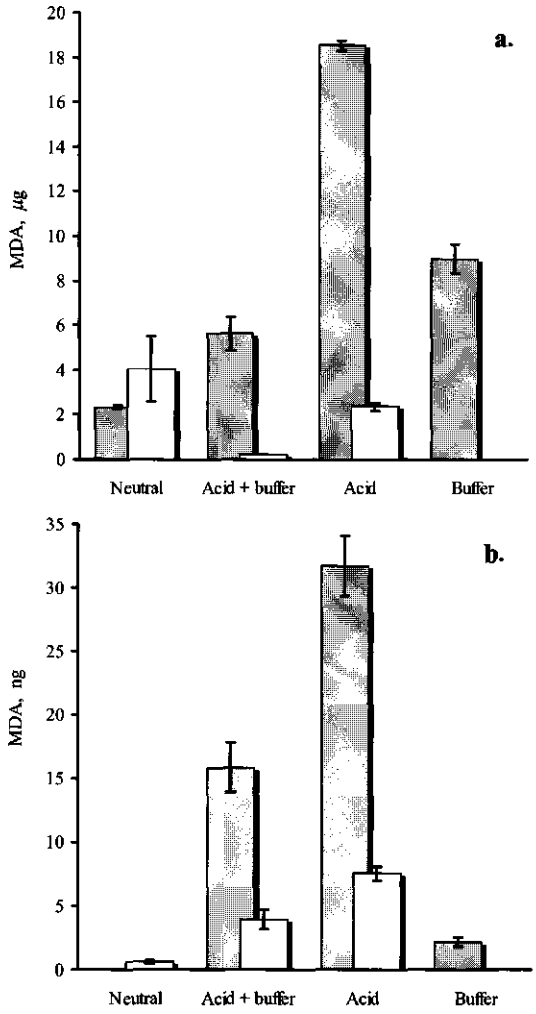


Figure 5.3 *Sensitivity of DPPH• detection assessed for (a) carvacrol and (b) rosmarinic acid with neutral eluents, acidified eluents + buffered DPPH• solution, acidified eluents, and buffered DPPH• solution. Acidification of HPLC eluents with glacial acetic acid to pH 3.0–3.2; buffering DPPH• solution with pH 7.6 citric acid-disodium hydrogen phosphate buffer. Methanol/water elution is represented by columns in squares; acetonitrile/water by blank columns. Data on MDA of carvacrol eluted with acetonitrile/water and detected with buffered DPPH• solution are not available due to the reaction coil clogging.*

5.3.5 Comparison of MDC and MDA values for both methods

It is clear that the observed MDA and MDC values are strongly pH dependent, as was shown above and as was observed when the thyme extracts were screened with both methods. This is in accordance with earlier findings[1,3,13]. It was therefore concluded that a comparison of MDA and MDC values for both methods should be performed under neutral conditions (i.e. when eluents and reagent-flows were unbuffered and not pH adjusted). Under these conditions MDA and MDC values were calculated for 16 antioxidative compounds.

The MDA value for carvacrol was almost two times lower with methanol/water than with acetonitrile/water, while for rosmarinic acid methanol/water elution showed a ten times lower MDA. Therefore a methanol/water eluent was chosen with DPPH$^{\bullet}$ quenching detection. With luminol chemiluminescence quenching detection, acetonitrile/water elution was preferred, based on a better detector baseline stability during gradient runs. The MDA and MDC values are summarised in Table 5.1.

No correlation between MDA and MDC values measured with either detection method was observed. In general, lower MDA values were observed with CL than with DPPH$^{\bullet}$ detection (nine vs. seven) However, the DPPH$^{\bullet}$ quenching system demonstrated significantly higher sensitivity for certain radical scavenging compounds (e.g. rosmarinic acid, carnosic acid, Trolox, isoeugenol, and rutin). Various explanations can be given for the observed lack of correlation:

- A broader range of antioxidant mechanisms may be involved in the quenching of luminol chemiluminescence (see Chapter 4)[4,6,7].
- Differences in reactivity between the DPPH$^{\bullet}$ radical and the radical species involved in luminol chemiluminescence may lead to differences in the rate of hydrogen abstraction, depending on the antioxidant involved. The activity of phenolic radical scavengers depends on the O–H bond dissociation energy, resonance delocalisation of the phenolic radical and on steric effects[14].
- Less reactive compounds with more than one electron involved in the reaction with DPPH$^{\bullet}$ may not have sufficient time to complete their reaction in an on-line system. In such a case, the incomplete detection of the radical scavenging activity would give non-representative MDA values.

The reproducibility of MDA and MDC values, obtained with both methods, was similar: RSD values larger than 20% were only obtained for 5 compounds with DPPH$^{\bullet}$ quenching and for 7 compounds with luminol

chemiluminescence quenching. These larger standard deviations were mainly caused by instrumental parameters, related to the performance of the auto-sampler and the HPLC pump. Furthermore, with DPPH$^{\bullet}$ quenching detection, gradual deterioration of the reagent solution during the measurements may have played a negative role.

Table 5.3 *Advantages and disadvantages of CL and DPPH$^{\bullet}$ on-line detection techniques for radical scavengers*

HPLC–DPPH$^{\bullet}$	HPLC–CL
+ Determination of radical scavengers in HPLC eluates	+ Determination of scavengers that react with some oxygen species and other radicals in HPLC eluates
+ Compatible with common HPLC eluents at pH 3–7	+ Good reproducibility, low reagent flow rates
+ Simple instrumental set-up and easy to perform	+ Applicability in determination of Total Antioxidant Capacity in physiological fluids
+ Applicable in kinetic studies of radical scavengers	+ Applicability in studies of lipid oxidation/antioxidation mechanisms
+ Stable baseline	– Incompatible with water/methanol gradients
– Lower reproducibility due to high flow rates of reagents, frequent re-filling	– Two syringe pumps necessary

5.4 Conclusions

The two improved on-line HPLC-radical scavenging detection methods, based on luminol chemiluminescence quenching and DPPH$^{\bullet}$ reduction, allow the direct qualitative determination of radical scavengers in complex mixtures, using isocratic or gradient elution. Both methods are applicable for on-line screening of herb and spice extracts, oils, juices, wine, or hydrolysed protein products for natural radical scavengers. Furthermore, both methods can be applied for the elucidation of lipid oxidation/antioxidation mechanisms and radical scavenging processes in general. Most of these analytical tasks can be carried out with minimal sample preparation,

thus saving time and costs. It was found that the HPLC–CL system is more sensitive but also more prone to disequilibration, which leads to baseline stability problems[15]. Other disadvantages of the CL method are its incompatibility with water/methanol gradients and the more complex set-up. The advantages and disadvantages of the two methods have been summarised in Table 5.3. Both techniques can greatly speed up the identification of radical scavengers in complex samples. The $DPPH^{\bullet}$ method seems to be the more robust.

5.5 References

1. Dapkevicius, A., van Beek, T.A., Niederlander, H.A.G. and de Groot, Ae. 1999. On-line detection of antioxidative activity in high-performance liquid chromatography eluates by chemiluminescence. *Anal. Chem.*, 71, 736–740.
2. Ogawa, A., Arai, H., Tanizawa, H., Miyahara, T. and Toyooka, T. 1999. On-line screening method for antioxidants by liquid chromatography with chemiluminescence detection. *Anal. Chim. Acta*, 383, 221–230.
3 Koleva, I.I., Niederländer, H.A.G. and van Beek, T.A. 2000. An on-line HPLC method for detection of radical scavenging compounds in complex mixtures. *Anal. Chem.*, 72, 2323–2328.
4. Whitehead, T.P., Thorpe, G.H.G. and Maxwell, S.R.J. 1992. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal. Chim. Acta*, 266, 265–277.
5. Larson, R.A. 1997. Naturally Occurring Antioxidants. CRC Press, Boca Raton. 189 pp.
6. Glazener, J.A., Orlandi, E.W., Harmon, G.L. and Baker, C.J. 1991. An improved method for monitoring active oxygen in bacteria-treated suspension cells using luminol-dependent chemiluminescence. *Physiol. Mol. Plant Pathol.*, 39, 123–133.
7. Hirayama, O., Takagi, M., Hukumoto, K. and Katoh, S. 1997. Evaluation of antioxidant activity by chemiluminescence. *Anal. Biochem.*, 247, 237–241.
8. von Gadow, A., Joubert, E. and Hansmann, C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. *J. Agric. Food Chem.*, 45, 632–638.
9. Yamamoto, Y., Brodsky, M.H., Baker, J.C. and Ames, B.N. 1987. Detection and characterization of lipid hydroperoxides at picomole levels by high-performance liquid chromatography. *Anal. Biochem.*, 160, 7–13.

10. Reitberger, T. and Gierer, J. 1988. Chemiluminescence as a means to study the role of hydroxyl radicals in oxidative processes. *Holzforschung*, 42, 351–356.

11. Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature*, 181, 1199–1200.

12. Brand-Williams, W., Cuvelier, M.E. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensm.- Wiss. u. - Technol.*, 28, 25–30.

13. Zhou, Y., Nagaoka, T., Li, F. and Zhu, G. 1999. Evaluation of luminol-H_2O_2-KIO_4 chemiluminescence system and its application to hydrogen peroxide, glucose and ascorbic acid assays. *Talanta*, 48, 461–467.

14. Sanchez-Moreno, C., Larrauri, J.A. and Saura-Calixto, F. 1998. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.*, 76, 270–276.

15. Mäkinen, M., Piironen, V. and Hopia, A. 1996. Postcolumn chemiluminescence, ultraviolet and evaporative light-scattering detectors in high-performance liquid chromatographic determination of triacylglycerol oxidation products. *J. Chromatogr. A*, 734, 221–229.

ISOLATION AND STRUCTURE ELUCIDATION OF RADICAL SCAVENGERS FROM *THYMUS VULGARIS* LEAVES*

6.1 Introduction

The majority the natural herb antioxidants that are presently used are partially purified rosemary extracts. The screening of extracts from several Lithuanian aromatic plants for their antioxidant activity[1] presented in Chapter 2 and the further antioxidant activity tests described in Chapter 3 and 4 revealed the potential of thyme (*Thymus vulgaris* L.) to scavenge free radicals. This herb is commonly grown in Lithuania and could act as a substitute for rosemary. Rosemary is difficult to cultivate in Lithuania due to unfavourable climatic conditions. Knowledge on non-volatile radical scavenging compounds from thyme extracts is scarce. Their structure elucidation is of importance for safe and adequate implementation of thyme as a food antioxidant.

This chapter presents the results of the activity-guided fractionation and isolation of two different extracts from thyme leaves. For the antioxidant activity assessment, TLC as well as on-line and off-line techniques based on scavenging of $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radicals[2] were used.

6.2 Experimental

6.2.1 General experimental procedures

All solvents used for extraction, partitioning and fractionation purposes were redistilled, with the exception of MeOH and MeCN, which were HPLC grade. UV

* Parts of this chapter are submitted as:
Dapkevicius, A., van Beek, T.A., Lelyveld, G.P., van Veldhuizen, A., de Groot, Ae., Linssen, J.P.H. and Venskutonis, R.. *J. Nat. Prod.*, submitted for publication.

spectra were recorded on a Perkin Elmer Lambda 18 UV/VIS spectrophotometer. Optical rotations were recorded on a Perkin Elmer 241 polarimeter with a 10-cm cell with a sodium lamp in MeOH. CD spectra were recorded on a Jasco J-715 spectropolarimeter in MeOH. One-dimensional NMR spectra were recorded on a Bruker AC200 or Bruker AM-400 spectrometer using the residual solvent peaks as internal standard. Two-dimensional spectra (COSY, HETCOR and COLOC) were all recorded on a Bruker AM-400. FD and CI (positive and negative mode, reaction gas CH_4) mass spectra were obtained on a MAT 711 mass spectrometer. Solvents were removed with a rotary evaporator at 12–35 mm Hg pressure with a Büchi RE 11 rotary evaporator combined with a Vacuubrand CVC2 vacuum pump and Büchi 461 water bath. Freeze-drying was carried out in a Christ Alpha 1-2 freeze dryer equipped with a Vacuubrand rotary vane vacuum pump. Column chromatography was performed using Fluka Si gel (Kieselgel 60, 230–400 mesh) and Pharmacia Sephadex LH-20. MPLC was carried out on a Jobin Yvon axial compression system equipped with a Gilson 303 pump and 802 C manometric module. The column was filled with Bakerbond 40 μm RP-18 stationary phase. Eluted fractions were collected with a LKB Bromma 2111 multirac sample collector. Semi-preparative RP-HPLC was conducted with a Waters 600 E pump combined with a 250 × 10 mm Rainin Dynamax RP-HPLC column filled with Microsorb 5 μm C_{18} stationary phase, a Waters 990 photodiode array detector, and a Gilson 231 auto-sampling injector equipped with a 100-μl injection loop.

The instrumental set-up used for analytical HPLC was similar to that used for semi-preparative purposes except for the introduction of a Scientific Systems pulse damper and a 250 × 4.6 mm Alltima Associates RP-HPLC analytical column filled with a C_{18} 5 μm stationary phase. On-line post-column addition of the DPPH$^{\bullet}$ (2,2'-diphenyl-1-picrylhydrazyl radical, 95%, Sigma-Aldrich) reagent was performed with a 45-ml laboratory-made syringe pump (Free University, Amsterdam, The Netherlands).

An isocratic elution with 50.5% (v/v) acetonitrile solution in water was used in semi-preparative runs, while for analytical RP-HPLC a binary MeCN/H_2O gradient was used. Solvent A was 25% (v/v) HPLC grade MeCN in ultra-pure water, which was acidified with glacial acetic acid (2.5 ml/l). Pure MeCN mixed with 0.25% (v/v) acetic acid was used as solvent B. During the initial 3 min the eluent consisted of 100% A. Then, the concentration of solvent A decreased to 65% in 11 min and remained at this percentage during the next 7 min. During the following 7 min solvent A further decreased to 5% where it remained during 12

min. In the next 3 min solvent A reached the concentration of 0% and remained so during 2 min. Finally, solvent A returned to its initial concentration in 5 min.

Standards of thymol, and carvacrol were purchased from Sigma-Aldrich, (±)-taxifolin and eriodictyol from Roth (Carl Roth, Karlsruhe, Germany), and rosmarinic acid from Extrasynthèse (Genay, France).

On-line HPLC–DPPH$^{\bullet}$ radical scavenging detection was carried out following the procedure described in Chapter 5.

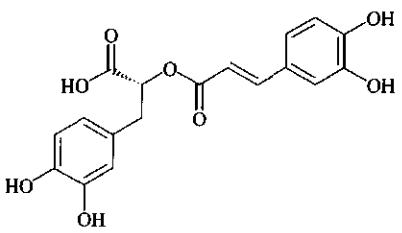
6.2.2 Plant material

The thyme leaves (*Thymus vulgaris* L.) were collected in the last decade of September, 1997 in the experimental garden of the Lithuanian Institute of Horticulture, Babtai, Lithuania. A voucher specimen has been deposited in the Lithuanian Institute of Horticulture, no. TV-97-20. The plant material was dried in a drying cabinet with forced ventilation at 40°C for 2-3 days. The samples were packed in double walled paper bags and stored at ambient temperature before use.

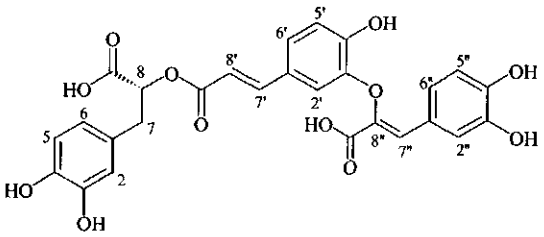
6.2.3 Extraction and isolation procedures

6.2.3.1 Procedure I

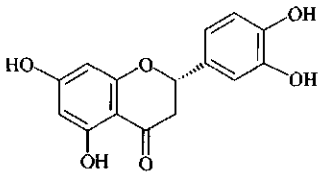
Dried ground thyme leaves (100 g) were extracted at 20°C with 1 l of EtOH-H_2O-HOAc (80:19:1) under N_2 for 5 days. The obtained crude extract was filtered and successively extracted in a separatory funnel with petrol ether (40°–60°) and EtOAc. The petrol ether layer was not further processed. After removal of the EtOH with a rotary evaporator, the aqueous layer was extracted with EtOAc. The EtOAc layer was concentrated *in vacuo* at 45°C yielding 5.6 g of extract. This was fractionated on Sephadex LH-20 with EtOH and Me_2CO-H_2O (1:1) resulting in 35 fractions of 15 ml. Fractions were checked for their capacity to bleach DPPH$^{\bullet}$ on TLC. After evaporation, active fractions were re-fractionated on Sephadex with the same eluents. Active fractions were pooled, concentrated and successively fractionated on a Si gel column with hexane-EtOAc-HCO_2H (60:39:1) and hexane-EtOAc-HCO_2H (40:59:1). Three pure radical scavenging compounds were isolated and identified as eriodictyol **3** (15 mg), taxifolin **4** (63 mg) and rosmarinic acid **1** (390 mg). Chemical structures of isolated compounds are given in Figure 6.1.



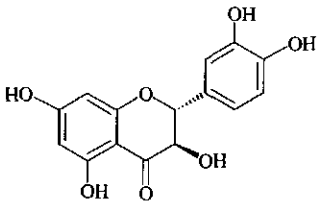
1 Rosmarinic acid



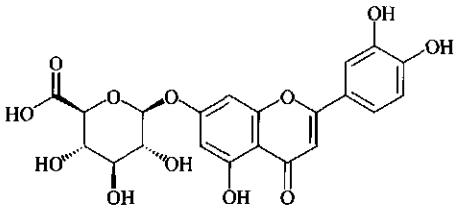
2 3'-O-(8"-Z-caffeoyl)-rosmarinic acid



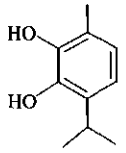
3 Eriodictyol



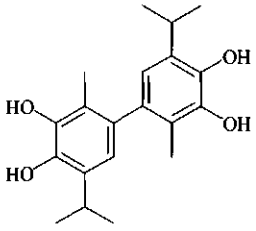
4 Taxifolin (syn. Dihydroquercetin)



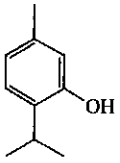
5 Luteolin 7-glucuronide



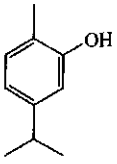
6 *p*-Cymene 2,3-diol



7 *p*-Cymene 2,3-diol 6,6'-dimer



8 Thymol



9 Carvacrol

Figure 6.1 *Radical scavenging compounds from T. vulgaris leaves.*

The aqueous layer remaining after extraction with EtOAc was concentrated *in vacuo* at 65°C. Any residual water was removed by freeze drying for 48 hrs. This extract was partitioned between H_2O and BuOH. The aqueous extract was inactive and discarded. The BuOH layer, after drying and concentration, was separated by RP-MPLC on C_{18} with THF-H_2O-HCO_2H (30:69:1) as eluent. Fractions with antioxidant activity were pooled and separated by CC on Sephadex with EtOH-H_2O (1:1). Again, active fractions were pooled and concentrated (141 mg). This material was washed 4× with 50 ml of EtOAc-MeOH-H_2O-HCO_2H

(87:10:2:1). The insoluble residue was centrifuged and dried *in vacuo* resulting in a yellow powder (38.5 mg) identified as luteolin 7-glucuronide **5**.

6.2.3.2 Procedure II

Dried ground herb material (50 g) was extracted for 70 hr under N_2 with 500 ml of MeOH. The extract was filtered and concentrated *in vacuo* at 40°C yielding 8.9 g of crude methanolic extract. The extract was partitioned between hexane and warm H_2O (45°C). The hexane layer was dried over Na_2SO_4 and concentrated *in vacuo* yielding 2.45 g of hexane extract. This extract was separated by CC on Si gel with toluene-Me_2CO-HCO_2H (184:15:1). Two active fractions A and B were obtained which were both further purified by CC on Si gel with toluene-Me_2CO-HCO_2H (190:19:1) and on Sephadex with MeOH. Purified fraction A was further purified to homogeneity by crystallisation from MeOH at –18°C and semipreparative RP-HPLC on a C_{18} column with MeCN-H_2O (1:1). It was identified as *p*-cymene 2,3-diol **6** (11 mg). Purified fraction B was further purified to homogeneity by dissolving it in benzene, filtration and evaporation of the benzene solubles. It was identified as *p*-cymene 2,3-diol 6,6'-dimer **7** (11 mg).

The aqueous layer remaining after the hexane partitioning was successively extracted with tBuMeO, EtOAc and BuOH. The remaining aqueous layer was evaporated to dryness (3.1 g). This fraction was chromatographed on a Si gel column with toluene-Me_2CO-HCO_2H (133:66:1). In total 110 fractions were collected. Pooled fractions 69-100 contained 33 mg of 3'-O-(8''-Z-caffeoyl)-rosmarinic acid **2**.

6.2.4 Assessment of radical scavenging activity of fractions

A fast screening method that involved spotting 8–10 µl of each collected fraction on a Merck Si gel 60 F_{254} 0.25 mm thickness TLC plate and spraying it with 0.1% DPPH$^{\bullet}$ solution in MeOH without chromatographic development was used to detect isolates with radical scavenging activity. Plates were examined after 15 min. Radical scavengers of active fractions bleached the purple background of DPPH$^{\bullet}$ giving a white-yellowish spot. Before pooling, active fractions were submitted to a procedure similar to that described by Takao *et al.*[3], which combines the TLC separation of isolates followed by the DPPH$^{\bullet}$ detection of active spots. The TLC chromatography was carried out on Merck Si gel or RP-18 F_{254S} TLC plates with suitable eluents. After development and drying, active compounds were detected

with 0.1% methanolic DPPH$^{\bullet}$ solution. Purity of combined fractions and radical scavenging activity of individual constituents was also evaluated with the on-line HPLC–DPPH$^{\bullet}$ system described in Chapter 5.

6.2.5 Off-line ABTS$^{\bullet+}$ radical scavenging assay

A 2.0 mM ABTS$^{\bullet+}$ stock solution in PBS buffer (8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl dissolved in 1 l ultra pure water, the pH was adjusted to 7.4 with NaOH) and a 70 mM $K_2S_4O_8$ solution in ultra pure water were prepared. Radical cation solution was produced by mixing 50 ml of ABTS$^{\bullet+}$ stock solution with 200 ml $K_2S_4O_8$ solution and leaving the mixture to stand in the dark at room temperature for 16–17 hr before use. The radical was stable under these conditions for more than two days. For the radical scavenging assay the ABTS$^{\bullet+}$ solution was diluted with PBS buffer to an absorbance of 0.800 ± 0.030 at λ = 734 nm. Methanolic stock solutions of the compounds to be investigated were diluted with PBS in such a way, that a 10-µl aliquot of each dilution introduced into 990 µl of the ABTS$^{\bullet+}$ solution produced a 10-80% reduction of the initial absorbance. Final concentrations of 0-2 µM of test compounds or Trolox standard in EtOH or PBS were obtained. The readings were taken exactly 1 and 6 min after the initial mixing. The experiment was carried out at ambient temperature (20°C). Appropriate solvent blanks were run with each assay. All determinations were carried out in triplicate at each separate concentration of the standard and samples. In most cases, 6 concentrations were measured (e.g., 8, 4, 2, 1, 0.5, and 0.25 µM). The percentage inhibition of the absorbance at 734 nm was calculated and plotted as a function of the concentration of radical scavengers and of the Trolox standard. The concentration of test substance giving the same decrease of ABTS$^{\bullet+}$ absorbance at 734 nm as Trolox was calculated in terms of the Trolox Equivalent Antioxidant Capacity (TEAC) at two time points (1 and 6 min). The TEAC value is calculated by dividing the slope of the plot of the test substance by that of Trolox.

6.2.6 Off-line DPPH$^{\bullet}$ radical scavenging assay

Radical scavenging activity against the stable radical DPPH$^{\bullet}$ was measured using the method of von Gadow *et al.*[4], which was modified as described below. 10^{-4} M methanolic solutions of DPPH$^{\bullet}$ (0.375 g/l) and a test compound (3.75 g/l) were mixed in a 1-cm path length disposable plastic half-micro cuvette (Greiner

Labortech, The Netherlands). Final molar ratios between the test compound and DPPH$^{\bullet}$ of 1:10 as well as 1:2 were obtained. The mixtures were kept for 15 min in the dark at room temperature (20°C) and the decrease of absorbance at λ = 515 nm was measured against methanol using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The absorbance of a blank sample containing the same amount of methanol and DPPH$^{\bullet}$ solution was prepared and measured daily. DPPH$^{\bullet}$ solution was freshly prepared prior each experiment and kept in a dark at 4°C between the measurements. All determinations were performed in triplicates. The radical scavenging activity of the tested samples expressed as % of inhibition ($I_{DPPH^{\bullet}}$) was calculated by the following formula[2]:

$$I_{DPPH^{\bullet}} = [(AB - AA)/AB] \times 100\%$$

where AB = absorbance of the blank sample (t = 0), and AA = absorbance of tested antioxidant after 15 minutes.

6.3 Results and discussion

6.3.1 Activity-guided isolation of radical scavenging compounds

To compare the influence of the initial extraction solvent, a methanolic and an acidic aqueous alcoholic extract were prepared and both extracts were fractionated by a combination of solvent partitioning, normal phase chromatography, size exclusion chromatography and reversed phase MPLC or HPLC. Between the two types of solvents no major differences in terms of radical scavengers present in the initial crude extract were observed. However, the presence of 20% water and 1% acetic acid in one of the solvents significantly decreased the amount of chlorophyll present in the initial extract, thus facilitating further fractionation steps. For this reason the acidic aqueous solvent will be used for leaves in any future studies of this type. Whenever possible, fractions of similar composition were pooled after analysis by TLC or HPLC. The radical scavenging activity of each fraction was determined either by spraying the TLC plate with a solution of the blue coloured stable radical DPPH$^{\bullet}$ or by HPLC analysis with on-line detection of radical scavenging activity[5]. When radical scavengers are present, the DPPH$^{\bullet}$ is reduced to a colourless product. The bleaching can be observed visually on TLC plates as a yellowish spot on a purple background or after an HPLC separation by a visible wavelength detector as a decrease in absorption at λ = 517 nm.

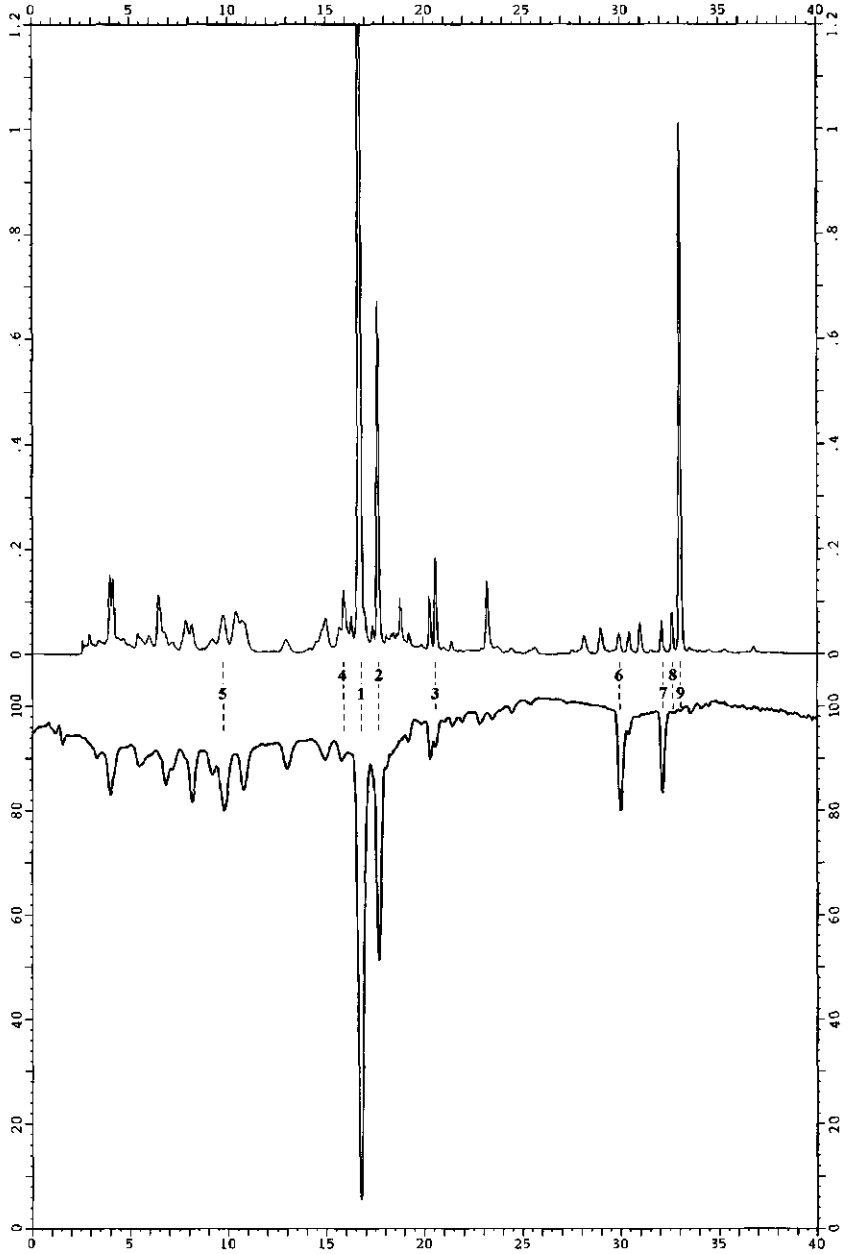


Figure 6.2 *RP-HPLC profiles of a total methanolic extract of thyme leaves with UV = 280 nm (upper trace) and DPPH$^{\bullet}$ radical scavenging activity detection (lower trace). With the latter detector, negative peaks indicate activity. For chromatographic conditions see experimental.*

Although radical scavenging activity should not be considered as being synonymous with antioxidant activity, powerful natural antioxidants like rosmarinic acid, tocopherol, carnosol and ascorbic acid are also strong scavengers of the DPPH$^{\bullet}$ radical. Thus, good activity in this test is a first indication of the presence of possible antioxidants. The UV and DPPH$^{\bullet}$ bleaching profiles of a crude methanolic thyme extract presented in Figure 6.2 exemplify an application of the on-line HPLC–DPPH$^{\bullet}$ radical scavenging detection. Two strongly active peaks in the middle polar-apolar region are clearly detectable. It is worth noticing that two minor apolar constituents in the UV profile (**6** and **7** in Figure 6.2) demonstrated pronounced radical scavenging activity in the DPPH$^{\bullet}$ profile, which might otherwise have been overlooked. The on-line assay significantly speeded up the fractionation process. Several weakly active polar constituents elute in the beginning of the chromatogram.

6.3.2 Structure elucidation

Compounds **1** and **3-7** were identified by a combination of UV, MS, ^{1}H NMR and ^{13}C NMR as rosmarinic acid **1**, eriodictyol **3**, taxifolin **4**, luteolin 7-glucuronide **5**, *p*-cymene 2,3-diol **6** and *p*-cymene 2,3-diol 6,6'-dimer **7**. The identity of compounds **1**, **3** and **4** was further confirmed by comparison of their R_f values on TLC and their retention time on HPLC with those of reference compounds. Additionally the weakly active volatile compounds carvacrol **8** and thymol **9** were identified by GC-MS and by comparison of their GC and HPLC retention times and R_f value on TLC with those of standards. Thymol corresponds with the large active peak at the end of the UV chromatogram (**9** in Figure 6.2). In spite of its high concentration, this compound showed negligible activity in the on-line DPPH$^{\bullet}$ assay. This is in contrast to their considerable activity on TLC plates, indicating a slow reaction with DPPH$^{\bullet}$. All compounds except **4** and **5** had been reported before in thyme[6-12] and the presence of one or more phenolic groups explains their radical scavenging activity. The significant antioxidant activity of **3**, **6** and **7** has been described earlier[10-12]. The highly active compound **2** was new and its structure elucidation is described below.

The chromatographic behaviour of **2** on RP-HPLC was very similar to that of rosmarinic acid **1** and only an acidified water-acetonitrile gradient gave two baseline-separated peaks. Also the UV spectrum of **2** was reminiscent to that of rosmarinic acid with only a small shift of the high wavelength maximum, thus

suggesting a structure similar to that of **1**. FD mass spectrometry gave a molecular weight of 538 with a most likely elemental composition of $C_{27}H_{22}O_{12}$. This finding corresponds with the attachment of an additional caffeic acid moiety to rosmarinic acid (Figure 6.1 **1**). A literature survey showed the existence of two independent reports on the same compound fitting the above description. Agata *et al.* reported melitric acid **10** (Figure 6.3) from *Melissa officinalis*[13] while Zhang and Li isolated the same compound from *Salvia cavaleriei*[14]. They named it salvianolic acid.

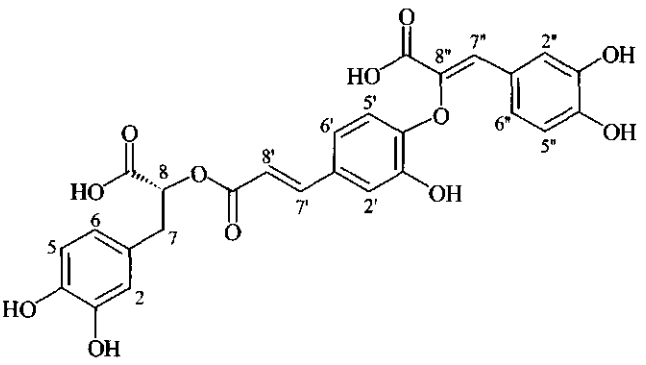


Figure 6.3 *Melitric[13]/salvianolic[14] acid [10].*

When the spectral data of **2** were compared with those of **10**, it became apparent that they were almost identical. Next all protons and carbons in **2** were assigned by comparison with literature and our own data for **2** and **10** respectively, COSY and direct and long range ^{1}H-^{13}C correlated spectra (see Table 6.1). The correlations in the long-range COLOC spectrum are given in Figure 6.4. The assignment showed that there were only significant differences in chemical shifts for the middle caffeic acid unit in **2** and **10**. As both the ^{1}H-^{1}H coupling constants and ^{1}H-^{13}C couplings unambiguously proved the presence of a caffeic acid unit, the only possible difference between **2** and **10** is the position of the attachment of the second caffeic acid unit. The only alternative attachment is to the other phenolic hydroxyl of the middle caffeic acid moiety, i.e. position 3' instead of 4'. Application of ^{13}C substituent parameter rules to the values reported by Agata *et al.* for **10** leads to significant upfield shifts for C-4' and C-6', moderate downfield shifts for C-1', C-2' and C-3' and no or small changes for C-5', C-7', C-8', C-8" and C-9". Except for C-5' the expected changes were indeed observed confirming the different mode of attachment. The shift of H-7" indicated that the stereochemistry of the C7"-C8" double bond is *Z*. If the stereochemistry would be E which *inter*

alia would correspond with the rare Z-caffeic acid as one of the building blocks, the H-7" proton would be shifted more upfield[13]. The absolute configuration of **2** was determined by CD spectroscopy. As the chiral centre and its immediate environment is identical to that of rosmarinic acid **1**, one would expect a similar CD spectrum if the configuration around C-8 is the same as in **1**. Indeed the CD spectra were only quantitatively different. Thus structure **2** is proposed for this new phenylpropanoid trimer.

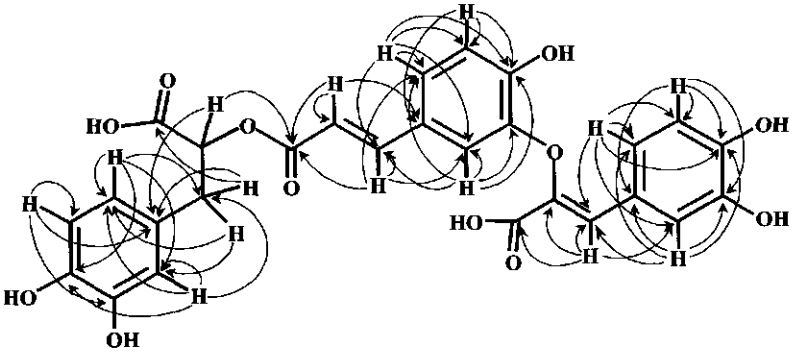


Figure 6.4 *Observed COLOC correlations in 3'-O-(8"-Z-caffeoyl)-rosmarinic acid [2].*

Rosmarinic acid 1: identification by UV (MeOH) λ_{max} 217, 231 (sh), 290, 329 nm, $[\alpha]_D$ = +81.6° (c = 0.41 in MeOH), CD λ ($\Delta\varepsilon$) 332 (+3.2), 297 (+4.0), 233 (–2.2), 218 (+2.0), 215 (0.0), EI-MS *m/z* (rel. int. %) 201 (12), 200 (17), 199 (13), 126 (32), 125 (80), 124 (100), 123 (50), 113 (14), 78 (10), 77 (9), FAB-MS 362 (19), 361 (M^+ + 1, 27), 187 (26), 186 (67), 181 (36), 180 (34), 164 (25), 163 (83), 115 (19), 94 (100), ^{1}H NMR and ^{13}C NMR see Table 6.1, R_F value on TLC and R_t with HPLC identical to those of an authentic reference. NMR data are in agreement with those published[15,16].

3'-O-(8"-Z-caffeoyl)-rosmarinic acid 2: identification by UV (MeOH) λ_{max} 217 (sh), 231 (sh), 291, 322 nm, $[\alpha]_D$ = +40.3° (c = 1.2 in MeOH), CD λ ($\Delta\varepsilon$) 327 (+7.9), 297 (+8.0), 275 (+1.0), 252 (+1.8), 233 (–1.4), 221 (+0.9), 217 (0.0), FD-MS *m/z* 538, pos. CI-MS (CH_4) *m/z* (rel. int. %) 391 (80), 113 (24), 73 (22), 57 (100), neg. CI-MS (CH_4) *m/z* (rel. int. %) 346 (88), 176 (100), ^{1}H NMR and ^{13}C NMR see Table 6.1, COLOC spectrum see Figure 6.4.

Table 6.1 *^{1}H and ^{13}C NMR data for compounds **1**, **2** and **10***

Position	$^{1}H^{a}$				$^{13}C^{b}$			
	2	**2**	**10**[14]	**1**	**2**	**2**	**10**[14]	**1**
1					129.0	128.1	129.4	129.2
2	6.72	6.79	6.79	6.76	117.2	117.2	117.6	117.5
3					146.1	145.1	146.1	146.1
4					145.2	144.2	145.2	145.2
5	6.67	6.71	6.76	6.68	116.0	115.7	116.4	116.2
6	6.58	6.61	6.68	6.59	121.5	121.1	121.7	121.8
7a	2.96	2.98	3.04	2.97	36.9	36.9	37.9	37.8
7b	3.06	3.09	3.14	3.08				
8	5.13	5.18	5.22	5.13	73.9	73.3	74.9	74.5
9					172.6	170.7	173.7	173.5
1'					127.2	126.8	130.8	127.6
2'	6.94	7.14	7.18	7.02	114.5	116.7	116.7	114.3
3'					146.2	145.4	146.4	146.7
4'					151.0	150.1	148.3	149.7
5'	6.92	6.99	6.79	6.76	117.7	115.9	116.5	116.4
6'	7.17	7.27	7.01	6.93	125.3	124.4	122.2	123.2
7'	7.48	7.54	7.60	7.53	146.7	145.5	146.7	147.7
8'	6.17	6.26	6.39	6.25	114.8	114.6	116.4	115.1
9'					167.2	166.2	168.2	168.4
1"					125.5	125.1	125.8	
2"	7.30	7.40	7.30		117.8	117.2	118.2	
3"					146.4	145.3	146.4	
4"					148.8	147.6	148.8	
5"	6.75	6.83	6.80		116.1	115.1	115.7	
6"	7.11	7.15	7.12		124.7	125.0	125.0	
7"	7.34	7.34	7.37		129.1	128.6	129.2	
8"					139.2	138.5	139.3	
9"					166.0	164.4	167.0	
Solvent[c]	Me-d4	Ac-d6	Ac-d6	Me-d4	Me-d4	Ac-d6	Ac-d6	Me-d4

[a] Couplings constants: all 1,3,4-trisubstituted benzene rings showed similar coupling constants of 8 (J H5-H6) and 2 Hz (J H2-H6), J (H7'-H8') 16 Hz, J (H7a-H7b) = 14.3 Hz, J (H7a-H8) = 4.3 Hz, J (H7b-H8) = 8.6 Hz.

[b] ^{13}C-NMR assignments in methanol have been confirmed by 2-dim. NMR techniques.

[c] Me-d4 = deuterated methanol, Ac-d6 = deuterated acetone.

Eriodictyol 3: HR-EI-MS *m/z* 288.0620 (calcd for $C_{15}H_{12}O_6$, 288.0634); R_f value on TLC and R_t with HPLC identical with those of an authentic reference; UV, EI-MS and ^1H NMR data are in agreement with those published[17,26].

Taxifolin (syn. dihydroquercetin) 4: HR-EI-MS *m/z* 304.0556 (calcd for $C_{15}H_{12}O_7$, 304.0583); R_f value on TLC and R_t with HPLC identical with those of an authentic reference; UV, EI-MS and ^1H NMR data are in agreement with those published[17,26].

Luteolin 7-glucuronide 5: identification by UV (MeOH) λ_{max} 255, 268 (sh) and 348 nm, EI-MS *m/z* (rel. int. %) 286 (100), FAB-MS *m/z* (rel. int. %) 463 ($M^+ + 1$, 25), 449 (15), 287 (35), 263 (20), 243 (30), 207 (80), 183 (40), 115 (100), FD-MS *m/z* (rel. int. %) 463 ($M^+ + H$, 30), 449 (22), 447 (3), 288 (12), 287 (83), 286 (100), ^{13}C NMR (CD_3OD) δ 183.0 (C4), 171.3 (C6''), 165.8 (C2), 163.2 (C7), 161.6 (C5), 157.7 (C9), 150.0 (C4'), 145.8 (C3'), 122.3 (C1'), 119.7 (C6'), 115.8 (C5'), 113.2 (C2'), 105.1 (C10), 103.1 (C3), 100.2 (C1''), 100.2 (C6), 95.0 (C8), 76.1 (C5''), 75.5 (C3'') 73.3 (C2''), 71.9 (C4''); UV, EI-MS and ^1H NMR data are in agreement with those published[17-20].

***p*-Cymene 2,3-diol 6:** identification by GC-MS *m/z* (rel. int. %) 166 (M^+, 30), 151 (100), 133 (10), 105 (10), 77 (5); ^1H NMR and ^{13}C NMR ($CDCl_3$) spectra are in agreement with those published[11].

***p*-Cymene 2,3-diol 6,6'-dimer (systematic name: 3,4,3',4'-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl) 7:** UV, EI-MS ^1H NMR and ^{13}C NMR data are in agreement with those published[12].

Carvacrol 8: R_f value on TLC and R_t with HPLC and capillary GC are identical to those of an authentic reference; UV and GC-MS data are in agreement with those published[22,23].

Thymol 9: R_f value on TLC and R_t with HPLC and capillary GC are identical to those of an authentic reference; UV and GC-MS data are in agreement with those published[22,23].

6.3.3 Radical scavenging activity detected with HPLC–DPPH•

The activity guided fractionation of Lithuanian thyme leaves led to the isolation of in total nine radical scavenging compounds. Thymol **9** and carvacrol **8** are two well-known simple phenolic monoterpenes, which occur in apolar extracts and in the essential oil of thyme at relatively high concentrations. The on-line HPLC–DPPH• assay of the total methanol extract (Figure 6.2) and its hexane-

soluble fraction from Lithuanian thyme hardly showed any radical scavenging activity. In contrast, two minor apolar constituents identified as p-cymene 2,3-diol **6** and p-cymene 2,3-diol 6-6'-dimer **7** (Figure 6.2) demonstrated potent radical scavenging activity in this on-line assay. Their presence in thyme leaf as well as antioxidant activity has been reported earlier[10-12]. The two most important radical scavenging compounds eluting between 16th and 18th minutes (Figure 6.2) were identified as rosmarinic acid **1** and 3'-O-(8"-Z-caffeoyl)-rosmarinic acid **2**.

Table 6.2 *Radical scavenging activity of rosmarinic acid [1] and 3'-O-(8''-Z-caffeoyl)-rosmarinic acid [2] as assessed by off-line $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ techniques. The Trolox Equivalent Antioxidant Capacity (TEAC) values were estimated at t = 1 min and t = 6 min. The decrease in absorbance at λ = 515 nm was evaluated after 15 min with the molar concentration of $DPPH^{\bullet}$ [M_{DPPH}] exceeding that of the test compound [M_{TC}] two and ten times*

Compound	TEAC value at:		$I_{DPPH^{\bullet}}$ % at:	
	1 min	6 min	$[M_{DPPH}]/[M_{TC}]=2$	$[M_{DPPH}]/[M_{TC}] = 10$
Rosmarinic acid **1**	1.5	1.54	95.0	n.a.[a]
3'-O-(8''-Z-caffeoyl)-rosmarinic acid **2**	0.58	0.69	96.2	55.2

[a] Value not available.

6.3.4 $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging efficiency of compounds 1 and 2

Two off-line techniques based on evaluation of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging efficiency were used to compare the antioxidant activity of the newly identified compound **2** with that of rosmarinic acid **1**. 3'-O-(8"-Z-caffeoyl)-rosmarinic acid **2** demonstrated 2.6 and 2.2 times lower TEAC values than rosmarinic acid **1** when measured at t = 1 min and t = 6 min, respectively (Table 6.2). In contrast, rosmarinic acid **1** was a less potent scavenger of the $DPPH^{\bullet}$ radical, when measured at t = 15 min. Several reasons can cause the observed difference in antioxidant activity of these two compounds. Firstly, the ability of compounds **1** and **2** to quench two different radicals (i.e., $DPPH^{\bullet}$ and $ABTS^{\bullet+}$) can be different.

Mantle *et al.* arrived at a similar conclusion when comparing the abilities of antioxidants to react with $ABTS^{\bullet+}$ and with radicals formed during the catalysed oxidation of luminol[24]. Secondly, two basically different types of calculations were performed to obtain the TEAC and the $I_{DPPH^\bullet}$ values. For the TEAC values, a graphical estimation of a slope of the line that represents inhibition as a function of concentration is carried out, while for the $I_{DPPH^\bullet}$, a percentage of quenched $DPPH^\bullet$ is arithmetically calculated. Finally, the speed of the radical quenching reaction for two analysed compounds may also differ. As can be seen from Table 6.2, the TEAC value for rosmarinic acid **1** during the last five reaction minutes increased approx. 2.5%, while for 3'-O-(8''-Z-caffeoyl)-rosmarinic acid **2** this value during the same time increased approx. 16%. This suggests that the reaction time of 6 min was not sufficient for complete estimation of the reactivity of compound **2** with $ABTS^{\bullet+}$. It should be noted that the $DPPH^\bullet$ reaction with antioxidants lasted almost three times longer. When analysing the $ABTS^{\bullet+}$ quenching by various antioxidants, van den Berg *et al.* also concluded that most antioxidants, except Trolox, exhibit a slow reaction[25]. Some further experimental work employing different techniques is needed to complete the comparison of antioxidant activity of rosmarinic acid **1** and 3'-O-(8''-Z-caffeoyl)-rosmarinic acid **2.**

6.4 Conclusions

Acidic aqueous methanolic/ethanolic solvents proved to be suitable for the initial extraction of dried thyme leaves since relatively low concentrations of chlorophyll were observed in the crude extracts. The fractionation process was significantly simplified and speeded up by the on-line HPLC–$DPPH^\bullet$ assay.

The presence of several potent antioxidants could have been overlooked if only diode array UV detection had been used. Numerous fractionation steps followed by structure elucidation of the purified compounds confirmed the presence of earlier reported antioxidant compounds in *T. vulgaris*, namely, rosmarinic acid **1**, eriodictyol **3**, *p*-cymene 2,3-diol **6**, *p*-cymene 2,3-diol 6,6'-dimer **7**, carvacrol **8** and thymol **9**. Two other known radical scavenging compounds taxifolin **4** and luteolin 7-glucuronide **5** were identified for the first time in *T. vulgaris*. A new phenylpropanoid trimer 3'-O-(8"-Z-caffeoyl)-rosmarinic acid **2** was isolated from the aqueous layer, which remained after the hexane/water partitioning of a crude methanolic extract. This compound demonstrated higher $DPPH^\bullet$ scavenging activity than rosmarinic acid **1**. Approximately 2.6 and 2.2

times lower TEAC values were obtained for this new compound **2** when its $ABTS^{\bullet+}$ scavenging activity was compared with that of the rosmarinic acid **1**. More studies on antioxidant activity of 3'-O-(8"-Z-caffeoyl)-rosmarinic acid **2** are needed to complete the evaluation of its radical scavenging properties.

6.5 References

1. Dapkevicius, A., Venskutonis, R., van Beek, T.A. and Linssen, J.P.H. 1998. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J. Sci. Food. Agric.*, 77, 140–146.
2. Koleva, I.I., Niederländer, H.A.G. and van Beek, T.A. 2000. An on-line HPLC method for detection of radical scavenging compounds in complex mixtures. *Anal. Chem.*, 72, 2323–2328.
3. Takao, T., Kitatani, F., Watanabe, N., Yagi, A. and Sakata, K. 1994. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Biosci. Biotech. Biochem.*, 58, 1780–1783.
4. von Gadow, A., Joubert, E. and Hansmann, C.F. 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (*Aspalathus linearis*), α-Tocopherol, BHT, and BHA. *J. Agric. Food Chem.*, 45, 632–638.
5. Dapkevicius, A., van Beek, T.A. and Niederländer, H.A.G. 2001. Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in liquid chromatography eluates. *J. Chromatogr. A*, 912, 73–82.
6. Kwok, D. and Shetty, K. 1996. *Pseudomonas spp*-mediated regulation of total phenolics and rosmarinic acid in thyme (*Thymus vulgaris*) clonal lines. *J. Food Biochem.*, 20, 365–377.
7. Shetty, K. 1997. Biotechnology to harness the benefits of dietary phenolics; focus on Lamiaceae. *Asia Pacific J. Clin. Nutr.*, 6, 162–171.
8. Aruoma, O.I., Spencer, J.P.E., Rossi, R., Aeschbach, R., Khan, A., Mahmood, N., Munoz, A., Murcia, A., Butler, J. and Halliwell, B. 1996. An evaluation of the antioxidant and antiviral action of extracts of rosemary and Provençal herbs. *Food Chem. Toxic.*, 34, 449–456.
9. Deighton, N., Glidewell, S. M., Goodman, B.A. and Deans, S. G. 1994. The chemical fate of the endogenous plant antioxidants carvacrol and thymol

during oxidative stress. Proceedings of the Royal Society of Edinburgh Section B (Biological Sciences), 102, 247–252.

10. Ternes, W., Gronemeyer, M. and Schwarz, K. 1995. Determination of *p*-cymene-2,3-diol, thymol, and carvacrol in different foodstuffs. *Z. Lebensm. Unters. Forsch.*, 201, 544–547.

11. Schwarz, K., Ernst, H. and Ternes, W. 1996. Evaluation of antioxidative constituents from thyme. *J. Sci. Food Agric.*, 70, 217–223.

12. Haraguchi, H., Saito, T., Ishikawa, H., Date, H., Kataoka, S., Tamura, Y. and Mizutani, K. 1996. Antiperoxidative components in *Thymus vulgaris*. *Planta Med.*, 62, 217–221.

13. Agata, I., Kusakabe, H., Hatano, T., Nishibe, S. and Okuda, T. 1993. Melitric acids A and B, new trimeric caffeic acid derivatives from *Melissa officinalis*. *Chem. Pharm. Bull.*, 41, 1608–1611.

14. Zhang, H.J. and Li, L.N. 1994. Salvianolic acid: a new depside from *Salvia cavaleriei*. *Planta. Med.*, 60, 70–72.

15. Kelley, C.J., Mahajan, J.R., Brooks, L.C., Neubert, L.A., Breneman, W.R. and Carmack, M. 1975. Polyphenolic acids of *Lithospermum ruderale* Dougl. ex Lehm. (Boraginaceae). I. Isolation and structure determination of lithospermic acid. *J. Org. Chem.*, 40, 1804–1815

16. Kelley, C.J., Harruff, R.C. and Carmack, M. 1976. The polyphenolic acids of *Lithospermum ruderale*. II. Carbon-13 nuclear magnetic resonance of lithospermic and rosmarinic acids. *J. Org. Chem.*, 41, 449–454.

17. Markham, K.R. and Geiger, H. 1994. ^{1}H Nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulfoxide. p. 441–497. In: J.B. Harborne (ed). The Flavonoids – Advances in Research since 1986. Chapman & Hall, London.

18. Klimek, B. 1995. Flavonoid glucoronides from *Verbascum lychnitis* L. and *V. nigrum* L. *Acta Poloniae Pharmaceutica – Drug Research*, 52, 53–59.

19. Feeney, P., Sachdev, K., Rosenberry, L., Lorraine, C. and Carter, M. 1988. Luteolin 7-O-(6''-O-malonyl)-β-D-glycoside and trans-chlorogenic acid: oviposition stimulants for the black swallowtail butterfly. *Phytochemistry*, 27, 3439–3448.

20. Agrawal, P.K. 1989. Carbon-13 NMR of Flavonoids. Elsevier, Amsterdam.

21. Miura, K., Inagaki, T. and Nakatani, N. 1989. Structure and activity of new deodorant biphenyl compounds from thyme (*Thymus vulgaris* L.). *Chem. Pharm. Bull.*, 37, 1816–1819.

22. Dev, S., Narula, A.P.S. and Yadav, J.S. 1982. CRC Handbook of Terpenoids – Monoterpenoids Vol. II. CRC Press, Boca Raton.

23. Jennings, W.G. and Shibamoto, T. 1980. Quantitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary GC. Academic Press, New York.

24. Mantle, D., Anderton, J.G., Falkous, G., Barnes, M., Jones, P. and Perry, E.K. 1998. Comparison of methods for determination of total antioxidant status: application to analysis of medicinal plant essential oils. *Compar. Biochem. Physiol. B*, 121, 385–391.

25. van den Berg, R., Haenen, G.R.M.M., van den Berg, H. and Bast, A. 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.*, 66, 511–517.

26. NIST 98 spectral database, NIST, Gaithersburg, USA.

GENERAL DISCUSSION

Lithuanian-grown thyme as possible source of natural antioxidants

Economical and social changes that took place in Lithuania during the last decade influenced changes in eating habits of an average Lithuanian family. More and more people are consuming frozen/dried/canned or otherwise processed foods, and its manufacture requires the usage of additives, including antioxidants. When looking for natural sources of antioxidants, attention is often focused on various isolates of aromatic and medicinal plants. A number of studies on the antioxidant properties of aromatic herbs grown in Lithuania have been published. Povilaitytė and Venskutonis have demonstrated the antioxidant activity of the acetone oleoresin from Roman chamomile (*Anthemis nobilis* L.) and of the deodorised acetone extract from Moldavian dragonhead (*Dracocephalum moldavica* L.)[1]. A moderate antioxidant activity of acetone oleoresins from Lithuanian-grown sage (*Salvia officinalis* L.) and catnip (*Nepeta cataria* L.) was detected using the spectrophotometric β-CLAMS test[2]. The acetone extract and the deodorised acetone extract prepared from horehound (*Marrubium vulgare* L.) harvested in Lithuania possessed similar antioxidant activity as that isolated from sage[3]. Various techniques employed for the initial screening tests (Chapter 2) and for the assessment of antioxidant activity (Chapters 3 and 4) in this study indicated that Lithuanian-grown thyme (*Thymus vulgaris* L.) has the potential to be used as a source of natural radical scavenging compounds.

So far, thymol and carvacrol have been the two most often referred to antioxidant compounds in thyme[4-9]. Together with thymol and carvacrol, Schwarz *et al.* for the first time identified *p*-cymene-2,3-diol in *n*-hexane and CO_2 extracts from thyme[7]. When using electrochemical detection for radical scavenging compounds in HPLC eluates, these authors noticed, but did not identify, the presence of another antioxidant compound 3,4,3'4'-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl (a dimer of *p*-cymene-2,3-diol). This compound was isolated and identified for the first time by a group of Japanese scientists[10]. A few years later, another group of Japanese authors[11] isolated and confirmed the

structure of this biphenyl compound. The antioxidant properties of this dimer and of eriodictyol were reported. Miura and Nakatani isolated and identified six flavones in the weakly acidic fraction of the non-volatile part of the acetone extract of thyme[12]. Three of these compounds, namely, 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone; 5,4'-dihydroxy-6,7,3'-trimethoxyflavone and 5,4'-dihydroxy-7-methoxyflavone showed similar antioxidant activity to that of BHT. Caffeic acid was isolated from a hot water extract of thyme and identified as the most abundant phenolic acid with antioxidant properties[13]. Rosmarinic acid, an effective radical scavenger present in many Labiatae plants, was also identified in extracts from various *Thymus* species[14,15]. Isolation and structure elucidation of the radical scavenging compounds presented in Chapter 6 confirmed the presence of thymol, carvacrol, eriodictyol, rosmarinic acid, *p*-cymene-2,3-diol and its dimer. Two known antioxidant compounds, luteolin 7-glucuronide and taxifolin (syn. dihydroquercetin) were identified in *Thymus vulgaris* for the first time (Chapter 6). Implementation of a new on-line technique for detection of radical scavenging compounds and the improved HPLC separation of thyme extracts permitted the discovery of a new phenylpropanoid trimer. This compound was identified as 3'-O-(8"-Z-caffeoyl)-rosmarinic acid and demonstrated radical scavenging activity similar to that of rosmarinic acid.

Once the source of natural antioxidants is chosen, the correct extraction technique, which would maximise the yield of active compounds, is required. There are a number of publications on the antioxidant activity of thyme essential oil, with thymol or carvacrol (dependently on herb chemotype) as its main radical scavenging constituent[4,5,16,17]. However, the specific aroma that is imparted by thyme volatile constituents is usually not desirable in the finished product. Due to this and to the volatility of thymol and carvacrol, the essential oil constituents from thyme clearly have a limited use as food antioxidants.

The screening experiments (Chapter 2) and the results published by other authors[6,7,18] indicate that potent thyme antioxidant extracts can be obtained using supercritical fluid extraction (SFE) with CO_2. Manipulation of extraction parameters such as pressure, temperature and co-solvents defines the polarity of the extracted constituents. Typically, thymol and carvacrol along with *p*-cymene-2,3-diol and other apolar antioxidant compounds are isolated[6,7]. Milder SFE conditions allow for a better extraction of temperature sensitive antioxidant compounds. However, the application of crude thyme supercritical CO_2 extracts still bears similar problems as those of the essential oil.

Based on the various extraction techniques used in this study (Chapters 1 and 6) it can be stated, that the most cost- and time-effective way to obtain food grade isolates from thyme is to apply solvent extraction with acidified aqueous alcoholic solutions. Such an extraction, when performed under mild conditions and when combined with an adequate auxiliary technique (e.g. heating, mixing, shaking, or percolation), yields a crude thyme extract with a high concentration of radical scavenging compounds and with a relatively low concentration of chlorophylls. However, such crude extract still bears a significant amount of undesirable flavour constituents which have to be removed by further refinement steps.

Antioxidant activity of extracts is often carried out in real lipids systems, i.e. vegetable oils and fats of animal origin. It is known that in refined vegetable oils a relatively high concentration of endogenous antioxidants such as tocopherols still remain. When these oils are used as oxidation substrates in stability tests, the effect of the added antioxidant adds to that of the tocopherols. Very often the efficiency of antioxidants under assessment is compared to the efficiency of BHT or BHA. The initial concentration of these reference antioxidants is often altered during accelerated stability tests due to the volatility of BHA and especially BHT. Furthermore, these two commonly used synthetic antioxidants are very effective in animal fats, but much less potent in vegetable oils[19]. Due to these reasons the results of antioxidative activity assessment in vegetable oils have to be considered critically.

To overcome problems related with oil stability tests, model systems employing bleaching of β-carotene-linoleic acid (β-CLAMS), reduction of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ as well as quenching of luminol chemiluminescence (CL) were used in this study (Chapters 2 to 6). Although off-line antioxidant activity tests performed on model systems are very useful for screening proposes, their application for an activity-guided isolation of active compounds is problematic due to the chemical instability of purified compounds. A new dynamic and accurate on-line HPLC-CL technique, which allowed for a direct detection of radical scavengers in HPLC eluates with a chemiluminescence detector, was developed (Chapter 4). In spite of being very sensitive, the HPLC–CL detection lacked simplicity and reproducibility. Therefore, for the final steps of activity-guided isolation, the HPLC–$DPPH^{\bullet}$ on-line detection method developed by Koleva *et al.*[20] was preferred. This method, based on the on-line reaction of the eluted antioxidant with the $DPPH^{\bullet}$ radical, was more robust, easier to operate but less sensitive than the HPLC–CL method. After improvement (Chapter 5), the on-line HPLC–$DPPH^{\bullet}$

technique allowed for a higher sensitivity towards radical scavengers and for a better HPLC separation of plant constituents.

Studies on other *Thymus* species as well as on their cultivation conditions may be useful for the final selection of raw material for the preparation of a natural antioxidant extract. The solubility of isolated natural antioxidants, their stability, "carry through" potential and their effect on other food components have to be assessed. Although the Commission E has approved thyme preparations for various medicinal purposes[21], toxicity studies should be considered prior to the application of thyme extracts as antioxidant preparations for the food industry.

References

1. Povilaitytė, V. and Venskutonis, P.R. 2000. Antioxidant activity of purple peril (*Perilla frutescens* L.), Moldavian dragonhead (*Dracocephalum moldavica* L.), and Roman chamomile (*Anthemis nobilis* L.) extracts in rapeseed oil. *J. Am. Oil Chem. Soc.*, 77, 951–956.

2. Dapkevičius, A., Venskutonis, P.R. and Baranauskienė, M. 1995. Evaluation of antioxidant activity of some aromatic plants by β-carotene bleaching test. p 16–19. In: Chemistry and Technology of Food. Technologija, Kaunas.

3. Weel, K.G.C., Venskutonis, P.R., Pukalskas, A., Gruzdienė, D. and Linssen J.P.H. 1999. Antioxidant activity of horehound (*Marrubium vulgare* L.) grown in Lithuania. *Fett/Lipid*, 101, 395–400.

4. Dorman, H.J.D, Deans, S.G., Noble, R.C. and Surai, P. 1995. Evaluation in vitro of plant essential oils as natural antioxidants. *J. Essent. Oil Res.*, 7, 645–651.

5. Farag, R.S., Badei, A.Z.M.A., Hewedi, F.M. and El-Baroty, G.S.A. 1989. Antioxidant activity of some spice essential oils on linoleic acid oxidation in aqueous media. *J. Am. Oil Chem. Soc.*, 66, 792–799.

6. Ternes, W., Gronemeyer, M. and Schwarz, K. 1995. Determination of *p*-cymene-2,3-diol, thymol, and carvacrol in different foodstuffs. *Z. Lebensm. Unters. Forsch.*, 201, 544–547.

7. Schwarz, K., Ernst, H. and Ternes, W. 1996. Evaluation of antioxidative constituents from thyme. *J. Sci. Food Agric.*, 70, 217–223.

8. Aruoma, O.I., Spencer, J.P.E., Rossi, R., Aeschbach, R., Khan, A., Mahmood, N., Munoz, A., Murcia, A., Butler, J. and Halliwell, B. 1996. An evaluation of the antioxidant and antiviral action of extracts of rosemary and Provençal herbs. *Food Chem. Toxic.*, 34, 449–456.

9. Botsoglou, N.A., Yannakopoulos, A., Fletouris, D.J., Tserveni-Goussi, A.S. and Fortomaris, P. 1997. Effect of dietary thyme on the oxidative stability of egg yolk. *J. Agric. Food Chem.*, 45, 3711–3716.

10. Miura, K., Inagaki, T. and Nakatani, N. 1989. Structure and activity of new deodorant biphenyl compounds from thyme (*Thymus vulgaris* L.). *Chem. Pharm. Bull.*, 37, 1816–1819.

11. Haraguchi, H., Saito, T., Ishikawa, H., Date, H., Kataoka, S., Tamura, Y. and Mizutani, K. 1996. Antiperoxidative components in *Thymus vulgaris*. *Planta Med.*, 62, 217–221.

12. Miura, K. and Nakatani, N. 1989. Antioxidative activity of flavonoids from thyme (*Thymus vulgaris* L.). *Agric. Biol. Chem.*, 53, 3043–3045.

13. Schulz, J.M. und Herrmann, K. 1980. Über das Vorkommen von Hydroxibenzoesäuren und Hydroxizimtsäuren in Gewürzen. IV. Über Gewürzphenole. *Z. Lebensm. Unters. Forsch.*, 171, 193–199.

14. Kurkin, V.A., Zapesochnaya, G.G., Krivenchuk, P.E. and Plaksina, T.I. 1988. Flavones and rosmarinic acid of *Thymus zheguliensis*. *Khimiya Prirodnykh Soedinenii*, 4, 594–595.

15. Shetty, K., Carpenter, T.L., Kwok, D., Curtis, O.F. and Potter, T.L. 1996. Selection of high phenolics-containing clones of thyme (*Thymus vulgaris* L.) using *Pseudomonas* sp. *J. Agric. Food Chem.*, 44, 3408–3411.

16. Dac-Vinh-Nguyen, Takacsova, M., Jakubik, T. and Minh-Nhat-Dang. 2000. Antioxidative effect of thyme in rape-seed oil. *Biologia-Bratislava*, 55, 277–281.

17. Dorman, H.J.D., Surai, P. and Deans, S.G. 2000. *In vitro* antioxidant activity of a number of plant essential oils and phytoconstituents. *J. Ess. Oil Res.*, 12, 241–248.

18. Bestmann, H.J., Erler, J. and Vostrowsky, O. 1985. Extraktion von Thymian mit flüssigem CO_2 im Labormaßstab. *Z. Lebensm. Unters. Forsch.*, 180, 491–493.

19. Coppen, P.P. 1994. The use of antioxidants. p. 67–87. In: J.C. Allen and R.J. Hamilton (eds). Rancidity in Foods. Applied Science Publishers, London.

20. Koleva, I.I., Niederländer, H.A.G. and van Beek, T.A. 2000. An on-line HPLC method for detection of radical scavenging compounds in complex mixtures. *Anal. Chem.*, 72, 2323–2328.

21. http://www.degenie.com/Naturaopathic/T/Thyme.html; accessed on 24.07.2001.

SUMMARY

Oxidative spoilage of lipid-rich foods decreases their shelf-life and leads to undesirable chemical and physical changes. Nowadays natural antioxidants are generally preferred. The major part of industrially used antioxidants consists of radical scavengers, which inhibit the oxidative chain reaction by inactivating free radicals formed during peroxidation of lipids. Aromatic and medicinal herbs are rich sources of natural radical scavenging compounds. The research described in this thesis focuses on the evaluation of several aromatic plants grown in Lithuania as a possible source of food antioxidants. Various aspects of lipid oxidation, of antioxidative mechanisms and of natural sources of radical scavenging antioxidants are outlined in Chapter 1.

Employment of model systems for antioxidant studies is often preferred over lipid stability tests due to their speed and the simplified comparison and interpretation of the obtained results. Preliminary screening of extracts from aromatic plants was carried out using the β-carotene-linoleic acid model system (β-CLAMS). The method is based on a competition between the plant antioxidant and an oxidisable indicator (β-carotene) for free radicals, which are generated during temperature-accelerated oxidation of linoleic acid. The agar-diffusion and spectrophotometric β-CLAMS tests singled out thyme (*Thymus vulgaris* L.) and sage (*Salvia officinalis* L.) as the most promising sources of natural antioxidant compounds (Chapter 2). The screening results also revealed that supercritical fluid extraction (SFE) as well as solvent extraction with acetone and methanol-water were the two most efficient techniques for isolation of antioxidant constituents from sage and thyme (Chapter 2). In further antioxidant activity tests, the spectrophotometric β-CLAMS technique was adapted to microtiter plates, which gave a better reproducibility and a shorter analysis time. The modified and improved spectrophotometric β-CLAMS confirmed the preliminary screening results. Sage and thyme acetone extracts again demonstrated the highest radical scavenging activity with relative antioxidant activities similar to those of 2,6-di-*tert*-butyl-4-methylphenol (BHT) (Chapter 3).

The activity of primary antioxidants can be evaluated directly by monitoring the reduction of free radicals. Such experiments can be carried out off-line, i.e. by the introduction of previously isolated antioxidant compound(s) into an equilibrated free radical containing model system, or on-line, by adding a solution of a free radical to the eluate of an HPLC column. The on-line techniques allow for a rapid and selective detection of radical scavenging substances in the presence of many inactive constituents with a minimum of preparatory manipulations. Two on-line model systems based on the reduction of radical intermediates of the luminol chemiluminescence (CL) reaction and on the reduction of the stable 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were used for the detection of antioxidant compounds in thyme and sage isolates. Ten compounds in the thyme acetone oleoresin and six in sage gave an inhibition signal on the CL detector (Chapter 4).

An adequate HPLC separation and a sensitive detection of radical scavenging are two key-elements that determine the success of on-line experiments. Improved separation efficiency especially for polar analytes was observed after an acetonitrile/water gradient program was introduced and after lowering of the pH of the HPLC solvents (Chapter 5). A stable, pulse-free flow, appropriate instrumental set-up and optimised compositions of the CL and the DPPH$^{\bullet}$ reagents improved the sensitivity of the on-line detection. The minimum detectable amounts registered in this study varied from 0.06 ng for rosmarinic acid to 2400 ng for carvacrol (HPLC–CL detection) and from 0.2 ng for eugenol to 19 ng for BHT (HPLC–DPPH$^{\bullet}$ detection) (Chapter 5). It was found that the HPLC–CL system was on average more sensitive, however the HPLC–DPPH$^{\bullet}$ method was more robust.

The on-line HPLC–DPPH$^{\bullet}$ method as well as TLC plates sprayed with DPPH$^{\bullet}$ solution were used for the activity-guided isolation of radical scavenging compounds from a leaf extract of thyme. Nine active compounds were isolated from the methanolic and the acidic aqueous alcoholic extract by means of solvent partitioning, normal phase chromatography, size exclusion chromatography and reversed phase MPLC or HPLC (Chapter 6). Rosmarinic acid, eriodictyol, taxifolin, luteolin 7-glucuronide, *p*-cymene 2,3-diol and *p*-cymene 2,3-diol 6-6′-dimer were identified by a combination of UV, CD, mass spectrometry and ^{1}H and ^{13}C NMR. Two weakly active, volatile compounds, thymol and carvacrol, were identified by GC-MS and their structures confirmed by GC and TLC analysis with reference compounds. The structure of a highly active new phenylpropanoid trimer 3'-O-(8"-Z-caffeoyl)-rosmarinic acid was elucidated from its UV and CD spectra, FD mass spectrum, and ^{1}H–,^{13}C-, COSY and direct and long range ^{1}H–^{13}C NMR

spectra (Chapter 6). In off-line Trolox Equivalent Antioxidant Capacity (TEAC) and $DPPH^{\bullet}$ assays, this compound was a weaker and stronger radical scavenger than rosmarinic acid, respectively.

The presence in *T. vulgaris* of radical scavenging compounds of different polarity and with distinctive reactivity to different radicals offers the possibility to use extracts from this plant as natural antioxidants for various lipid-containing food systems.

SAMENVATTING

Oxidatieve processen in vetrijke levensmiddelen leiden vaak tot ongewenste chemische en fysische veranderingen, zoals het ontstaan van ransheid, waardoor hun houdbaarheid afneemt. Dergelijke reacties kunnen tegengegaan worden door antioxidanten toe te voegen. Veel industriëel gebruikte antioxidanten zijn zogenaamde radicaalafvangers die de oxidatieve kettingreactie kunnen stoppen. Tegenwoordig wordt in het algemeen de voorkeur gegeven aan toepassing van natuurlijke antioxidanten. Aromatische en geneeskrachtige kruiden zijn een goede bron van natuurlijke antioxidanten die meestal werken als radicaalafvangers. Het in dit proefschrift beschreven onderzoek richt zich op de aanwezigheid van antioxidanten in verschillende aromatische planten afkomstig uit Litouwen. Het doel was om de activiteit van de verschillende extracten te bepalen, de verantwoordelijke antioxidatieve verbindingen te isoleren en te identificeren en te bezien of deze stoffen inderdaad als antioxidanten in voedsel gebruikt zouden kunnen worden. Verschillende aspecten van vetoxidatie in voedsel en het voorkomen van natuurlijke antioxidanten in planten en hun werkingsmechanismen zijn beschreven in hoofdstuk 1.

Het gebruik van modelsystemen om het effect van antioxidanten te testen is eenvoudiger, eenduidiger en sneller dan testen in levensmiddelen. Diverse Litouwse kruiden werden onderzocht met een modelsysteem gebaseerd op de gecombineerde oxidatie van beta-caroteen en linolzuur door zuurstof. Als radicaalafvangers aanwezig zijn, wordt het gele beta-caroteen minder snel geoxideerd hetgeen met het oog goed waar te nemen is. Het resultaat van dit experiment was, dat thijm (*Thymus vulgaris*) en salie (*Salvia officinalis*) de meeste potentiële antioxidatieve activiteit vertoonden. Dit wordt beschreven in hoofdstuk 2. De invloed van de bereiding op de antioxidatieve activiteit van het ruwe plantenextract is eveneens onderzocht. Extractie met de oplosmiddelen aceton en een mengsel van methanol en water bleek zeer geschikt. Ook extractie met superkritisch koolzuur leverde actieve extracten op (hoofdstuk 2).

Na aanpassing kon de beta-caroteen - linolzuur test op veel kleinere schaal uitgevoerd worden, en wel op microtiterplaten met automatische uitlezing,

waardoor de test beter reproduceerbaar en sneller uitgevoerd kon worden. De nieuwe versie van deze test, zoals beschreven in hoofdstuk 3, bevestigde andermaal, dat thijm- en salie-extracten het meest actief waren met een werkzaamheid die vergelijkbaar was met die van het synthetische antioxidant BHT (bis-*tertiair*-butyl-hydroxy-tolueen).

De activiteit van antioxidanten kan ook gemeten worden door het volgen van hun reactie met vrije radicalen. Zulke experimenten kunnen zowel uitgevoerd worden in een op zichzelf staand modelsysteem als direct na een vloeistofchromatografische scheiding. In een modelsysteem wordt het geïsoleerde zuivere antioxidant of het extract toegevoegd aan een vrij (stabiel) radicaal en het verdwijnen van het vrije radicaal wordt gevolgd. Na een hogedruk-vloeistofchromatografie-scheiding (HPLC) wordt in het "directe" systeem een radicaaloplossing toegevoegd aan de veelal gescheiden zuivere stoffen. Na ongeveer 30 sec kan voor elke stof bekeken worden of deze gereageerd heeft met het vrije radicaal, d.w.z. of deze antioxidatieve activiteit bezit. Het voordeel van deze "directe" methodes is dat in korte tijd veel informatie beschikbaar komt over de antioxidatieve activiteit van individuele stoffen in een ingewikkeld mengsel. Elke stof hoeft niet meer apart geisoleerd te worden. Twee "directe" methodes zijn gebruikt voor het meten van de antioxidatieve activiteit van thijm- en salie-extracten: de luminol chemiluminescentie (CL) reactie en de 2,2'-diphenyl-1-picrylhydrazyl (DPPH) reactie. Tien stoffen in acetonextracten van thijm- en zes stoffen in salieëxtracten vertoonden antioxidative activiteit in de "directe" CL methode (hoofdstuk 4).

In hoofdstuk 5 wordt beschreven hoe de "directe" CL en DPPH methodes verder werden geoptimaliseerd. Door aanpassingen aan het HPLC systeem werd de scheiding van zeer polaire zure stoffen verbeterd. Door een betere pulsvrije toevoeging van de radicaaloplossingen en een andere samenstelling werd de gevoeligheid aanzienlijk verbeterd. In de praktijk betekende dit dat meer stoffen gescheiden konden worden en dat van kleinere hoeveelheden stof de antioxidant activiteit nog aantoonbaar was. De kleinst aantoonbare hoeveelheden van een aantal standaard antioxidanten varieerden van 60 picogram voor rozemarijnzuur tot 2,4 microgram voor carvacrol. Van de meeste antioxidanten was 1 - 10 nanogram nog aantoonbaar. Het HPLC-CL systeem was gemiddeld iets gevoeliger, terwijl het HPLC-DPPH systeem iets gemakkelijker te gebruiken en minder storingsgevoelig was.

Het HPLC-DPPH systeem en dunnelaag-chromatografie, gevolgd door besproeien met een DPPH oplossing, werden gebruikt bij de isolatie van de meest

actieve radicaalafvangende stoffen in thijmbladeren. In totaal werden negen stoffen geisoleerd uit een methanol- en een waterig alcoholisch extract. De scheiding vond plaats middels uitschudden, lage druk vloeistofchromatografie met silica gel en Sephadex LH20 en middel- en hogedruk vloeistofchromatografie met RP C18 fasen (hoofdstuk 6). De stoffen rozemarijnzuur, eriodictyol, taxifoline, luteoline 7-glucuronide, *para*-cymeen 2,3-diol en para-cymeen 2,3-diol 6,6'-dimeer werden gekarakteriseerd door middel van ultraviolet- en proton en koolstof-13 kernspinresonantie-spectroscopie (NMR) en massaspectrometrie. De twee weinig actieve stoffen thymol en carvacrol werden geidentificeerd middels gecombineerde gaschromatografie en massaspectrometrie en vergelijking met referentiestoffen. De structuur van een nieuwe zeer actieve verbinding bestaande uit drie fenylpropaaneenheden (3'-(O)-(8"-cis-koffiezure enol ether) van rozemarijnzuur) werd mede opgehelderd met de eerder genoemde technieken en met behulp van circulair dichroïsme spectroscopie en een aantal twee-dimensionale kernspinresonantietechnieken (hoofdstuk 6). In aparte antioxidant testen met het DPPH en het ABTS radicaal was deze nieuw beschreven stof respectievelijk een sterkere en een zwakkere radicaalafvanger dan het nauwverwante rozemarijnzuur. De aanwezigheid van diverse radicaalafvangers in thijmbladeren kan het in de toekomst mogelijk maken dat specifieke bladextracten van deze plant toegepast zouden kunnen worden als natuurlijke antioxidant ter verlenging van de houdbaarheid van levensmiddelen.

TRUMPAS DISERTACIJOS TURINYS

Dėl lipidais turtingų maisto produktų oksidacinio gedimo vyksta nepageidaujami cheminiai ir fizikiniai pokyčiai bei sutrumpėja produktų saugojimo trukmė. Šiuo metu vis populiaresni tampa natūralūs antioksidantai. Didesnioji maisto pramonėje naudojamų antioksidantų dalis yra sintetiniai laisvųjų radikalų surišėjai. Šios medžiagos reaguoja su lipidų peroksidacijos metu susiformavusiais radikalais tokiu būdu stabdydami grandininę oksidacijos reakciją. Aromatiniai ir medicininiai augalai yra svarbus natūralių radikalus surišančių junginių šaltinis. Šioje disertacijoje pateikti tiriamojo darbo rezultatai įvertina keleto Lietuvoje auginamų aromatinių augalų antioksidacines savybes. Įvairūs lipidų oksidacijos ir antioksidacinio mechanizmo aspektai bei natūralių antioksidantų šaltinių sąrašas yra pateikti 1 skyriuje.

Tiriant antioksidantus, vis dažniau yra naudojamos eksperimentinės modelinės sistemos, o ne įprasti lipidų stabilumo bandymai. Tokiu būdu supaprastinamas gautų rezultatų palyginimas ir interpretavimas. Šio darbo pradžioje aromatinių augalų ekstraktų tyrimai buvo atlikti panaudojant β-karotino ir linolio (C18:2) rūgšties modelinę sistemą (β-KLRMS). Šis metodas yra paremtas antioksidanto ir oksiduojamo indikatoriaus (β-karotino) reakcijų su linolio rūgšties oksidacijos metu susidariusiais radikalais kinetikos skirtumais. Difuzijos į agarą ir spektrofotometrinio β-KLRMS bandymų rezultatai parodė, kad čiobrelis (Thymus vulgaris L.) ir šalavijas (Salvia officinalis L.) yra potencialiausi natūralių antioksidacinių junginių šaltiniai (2 skyrius). Ekstrakcija acetonu, metanolio ir vandens mišiniu ir CO_2 superkrizinėmis sąlygomis buvo pripažinti efektyviausiais čiobrelių ir šalavijų antioksidacinių junginių išskyrimo būdais (2 skyrius). Kituose β-KLRMS eksperimentuose buvo panaudotos mikrotitravimo plokštelės, ir tai pagerino gautų rezultatų tikslumą bei sutrumpino eksperimento trukmę. Šiuo spektrofotometriniu β-KLRMS metodu gauti rezultatai patvirtino pirminių tyrimų metu sukauptus duomenis. Šalavijų ir čiobrelių acetono ekstraktai vėlgi buvo efektyviausi surišant laisvuosius radikalus. Panaudojus minėtus ekstraktus, buvo gauti reliatyvaus antioksidacinio pajėgumo dydžiai, kurie buvo artimi analogiškiems 2,6-di-tert-butil-4-metilfenolio (BHT) rodikliams (3 skyrius).

Pirminių antioksidantų aktyvumas gali būti įvertintas tiesiogiai stebint laisvų radikalų redukcijos reakciją. Tokie eksperimentai gali būti atliekami neišskirstant junginių chromatografijos būdu (*off-line*), t.y. įvedant iš anksto išskirtus antioksidacinius junginius į pusiausvyroje esančią laisvųjų radikalų modelinę sistemą; arba išskirstant chromatografijos būdu (*on-line*), kada laisvaisiais radikalais turtinga modelinė sistema yra įvedama į eliuentų srautą išeinantį iš skysčių chromatografijos (HPLC) kolonėlės. Metodais, kuriuose yra naudojamas chromatografinis išskirstymas, galima greitai, selektyviai ir su trumpiausiu mėginių paruošimu aptikti neaktyvių medžiagų mišinyje esančius radikalų surišėjus. Du tokie metodai paremti luminolio chemoliuminescencine (CL) reakcija ir stabilaus 2,2`-difenil-1-pikrilhidrazilo radikalo ($DPPH^{\bullet}$) redukcijos reakcija buvo panaudoti čiobrelių ir šalavijų ekstraktų antioksidacinių komponentų aptikimui. Dešimt aktyvių junginių čiobrelių acetono oleorezine ir šeši junginiai šalavijų acetono oleorezine buvo aptikti CL detektoriumi (4 skyrius).

Adekvatus išskirstymas chromatografijos būdu ir jautrus radikalus surišančių junginių aptikimas yra du esminiai elementai nulemiantys antioksidacinių tyrimų sėkmę chromatografijos būdu. Pagerintas išskirstymo efektyvumas, ypač polinių junginių, buvo pasiektas pritaikius acetonitrilo ir vandens mišinio koncentracijos gradiento programą ir sumažinus tirpiklių pH (5 skyrius). Stabilus ir be pulsacijų tirpiklių tekėjimas, patobulinta prietaisų schema bei optimizuota CL ir $DPPH^{\bullet}$ reagentų sudėtis pagerino chromatografinių antioksidacinių tyrimų jautrumą. Šiame darbe užregistruoti minimalūs aptinkami dydžiai svyravo tarp 0,06 ng rozmarinų rugščiai ir 2400 ng karvakroliui (CL detektorius) bei tarp 0,2 ng eugenoliui ir 19 ng BHT ($DPPH^{\bullet}$ detektorius) (5 skyrius). Buvo nustatyta, kad daugeliui atvejų HPLC–CL detektorius pasižymėjo didesniu jautrumu, kai tuo tarpu HPLC–$DPPH^{\bullet}$ detektorius buvo paprastesnis ir atsparesnis įvairiems eksperimentų trigdžiams.

Chromatografinis HPLC–$DPPH^{\bullet}$ bei plonasluoksnės chromatografijos TLC-$DPPH^{\bullet}$ metodai buvo panaudoti čiobrelių lapų ekstrakte esančių radikalus surišančių junginių aptikimui. Devyni antioksidaciniu aktyvumu pasižymintys junginiai buvo išskirti iš čiobrelių ekstraktų, gautų metanoliu bei parūgštintu vandens ir etanolio mišiniu. Tai buvo atlikta išskirstant frakcijas skirtingo poliškumo tirpikliais, normalių fazių, dydžio eksliuzijos, o taip pat vidutinio bei aukšto slėgio atvirkštinių fazių chromatografijos metodais (6 skyrius). Rozmarinų rūgštis, eriodiktiolis, taksifolinas, luteolin-7-glukuronidas, *p*-cimen-2,3-diolis ir *p*-cimen-2,3-diolio 6,6´-dimeras buvo identifikuoti UV, CD, masių spektrometrijos bei ^{1}H- ir ^{13}C-BMR metodais. Timolis ir karvakrolis, du mažo aktyvumo lakūs

junginiai, buvo identifikuoti masių spektrometrijos (GC-MS) būdu. Jų molekulinės struktūros buvo patvirtintos dujų ir plonasluoksnės chromatografijos metodais lyginant su etalonais. Naujo ir labai aktyvaus fenilpropanoidinio trimero 3′-O-(8′′-Z-kafeoil)-rozmarinų rūgšties struktūra buvo nustatyta pagal UV ir CD spektrus, FD masės spektrus, ^{1}H-, ^{13}C-, COSY bei tiesioginio ^{1}H-^{13}C BMR ir tolimosios srities ^{1}H-^{13}C BMR spektrus (6 skyrius). Lyginant naujojo junginio troloksui ekvivalentišką antioksidacinį pajėgumą (TEAC dydis) bei pajėgumą reaguoti su $DPPH^{\bullet}$, buvo nustatyta, kad identifikuotas fenilpropanoidinis trimeras yra silnesnis $ABTS^{+\bullet}$ ir stipresnis $DPPH^{\bullet}$ radikalų surišėjas nei rozmarinų rūgštis.

Gauti eksperimentų rezultatai parodo, kad *T. vulgaris* antioksidaciniai junginiai pasižymi skirtingu poliškumu bei skirtingu pajėgumu surišti įvairius laisvuosius radikalus. Iš to galima daryti išvadą, kad yra galimybė panaudoti šio augalo ekstraktus kaip natūralius antioksidantus skirtingų lipidais turtingų maisto produktų kokybės išsaugojimui.

RESUMO

A deterioração oxidativa de alimentos ricos em lípidos decresce a sua vida de prateleira e conduz a alterações físico-químicas indesejáveis. Geralmente, preferem-se antioxidantes naturais aos sintéticos. A maior parte dos antioxidantes utilizados industrialmente consiste em *scavengers* de radicais livres, que inibem a reacção em cadeia da oxidação inactivando os radicais livres formados durante a peroxidação dos lípidos. As ervas medicinais e aromáticas são fontes naturais ricas em compostos que são *scavengers* de radicais livres. A pesquisa descrita nesta tese tem como foco a avaliação de várias plantas aromáticas cultivadas na Lituânia como possíveis fontes de antioxidantes alimentares. Os diversos aspectos da oxidação dos lípidos, de mecanismos antioxidantes e de fontes naturais de antioxidantes *scanvengers* de radicais são apresentados no Capítulo 1.

O uso de sistemas-modelo para estudos sobre antioxidantes é frequentemente preferido em relação aos testes de estabilidade de lípidos pela sua rapidez e por simplificar a comparação e interpretação dos resultados obtidos. A testagem preliminar de extractos de plantas aromáticas foi levada a cabo utilizando o sistema modelo do β-caroteno-ácido linoleico (β-CLAMS). Este método baseia-se na competição entre o antioxidante da planta e um indicador oxidável (β-caroteno) pelos radicais livres gerados durante a oxidação termicamente acelerada do ácido linoleico. Os testes do β-CLAMS (método da difusão em agar e método espectrofotométrico) apontaram para o tomilho (*Thymus vulgaris* L.) e sálvia (*Salvia officinalis* L.) como sendo as fontes mais promissoras de compostos naturais de acção antioxidante (Capítulo 2). Os resultados destes testes também revelaram que a extracção por fluidos supercríticos (SFE) e a extracção por solventes com acetona e metanol-água eram as duas técnicas mais eficientes para o isolamento de constituintes antioxidantes a partir da sálvia e do tomilho (Capítulo 2). Em testes de actividade antioxidante realizados posteriormente, a técnica do β-CLAMS espectrofotométrico foi adaptada a microplacas, o que resultou em melhor reprodutibilidade e reduziu o tempo de análise. O β-CLAMS espectrofotométrico modificado e melhorado confirmou os resultados dos testes preliminares. Os extractos em acetona da sálvia e do tomilho voltaram a demonstrar a actividade

scavenger de radicais mais elevada com actividades antioxidantes relativas semelhantes às apresentadas pelo 2,6-di-*tert*-butil-4-metilfenol (BHT) (Capítulo 3).

A actividade dos antioxidantes primários pode ser avaliada directamente através da redução dos radicais livres. Tais ensaios podem ser realizado *off-line*, i.e. introduzindo o(s) composto(s) antioxidante(s) previamente isolado(s) num sistema-modelo equilibrado contendo radicais livres, ou *on-line*, através da adição de uma solução de radical livre ao eluído de uma coluna de HPLC. As técnicas *on-line* permitem uma detecção rápida e selectiva de substâncias que sejam *scavengers* de radicais em presença de muitos constituintes inactivos, com o mínimo de manipulações preparatórias. Dois sistemas-modelo *on-line*, baseados na redução de radicais intermediários da reacção de quimioluminescência do luminol (CL) e na redução do radical estável 2,2´-difenil-1-picril hidrazil (DPPH$^{\bullet}$) foram utilizados na detecção de compostos antioxidantes em isolados de tomilho e sálvia. Dez compostos presentes na oleoresina obtida por extracção de tomilho com acetona e seis da sálvia deram sinal de inibição no detector de CL (Capítulo 4).

A separação cromatográfica adequada e a detecção sensível da acção *scavenger* de radicais são dois elementos chave que determinam o sucesso dos ensaios *on-line*. Observou-se uma melhoria da eficiência da separação por HPLC, em particular dos compostos polares, após a introdução de um programa de gradiente acetonitrilo/água e após decrescer o pH dos solventes da HPLC (Capítulo 5). A sensibilidade da detecção *on-line* aumentou com a aplicação de um esquema de montagem apropriado, com um fluxo estável e isento de flutuações e com a optimização das condições dos reagentes para o CL e para o DPPH$^{\bullet}$. As quantidades detectáveis mínimas registadas neste estudo, com estes detectores, variaram entre os 0.06 ng para o ácido rosmarínico e os 2400 ng para o carvacrol e desde 0.2 ng para o eugenol até 19 ng para o BHT, respectivamente (Capítulo 5). Verificou-se que o sistema HPLC–CL era em média mais sensível. O método HPLC–DPPH$^{\bullet}$ era, contudo, mais robusto.

O método de HPLC–DPPH$^{\bullet}$ *on-line*, bem como placas para cromatografia em camada fina (TLC) reveladas com solução de DPPH$^{\bullet}$, foram utilizados para o isolamento por actividades de compostos *scavengers* de radicais a partir de um extracto foliar de tomilho. Isolaram-se nove compostos activos a partir dos extractos metanólico e aquoso-alcoólico acidificado de tomilho por intermédio de partição por solventes, cromatografia de fase normal, cromatografia de exclusão de dimensões e cromatografias de fase reversa, de média ou pressão (Capítulo 6). Foram identificados ácido rosmarínico, eriodictiol, taxifolina, luteolina 7-glucorónido, *p*-cimeno 2,3-diol e o dímero 6-6´ do *p*-cimeno 2,3 diol usando uma

combinação de UV, espectroscopia de dicroísmo circular (CD), espectrometria de massas e ressonância magnética nuclear em ^{1}H e ^{13}C. Dois compostos voláteis com fraca actividade, o timol e o carvacrol, foram identificados por GC-MS e as suas estruturas confirmadas por análise por GC e TLC com compostos de referência. A estrutura de um novo trímero fenilpropanóide, o ácido 3'-O-(8"-Z-cafeoil)-rosmarínico foi elucidada com base nas técnicas anteriormente mencionadas e no seu espectro obtido através de CD (Capítulo 6). Em ensaios *off-line* de Capacidade Antioxidante em Equivalentes Trolox (valor TEAC) e $DPPH^{\bullet}$, este composto era, respectivamente, mais fraco e mais forte como *scavenger* de radicais do que o ácido rosmarínico.

A presença no *T. vulgaris* de compostos *scavengers* de radicais com diferentes polaridades e reactividades distintas a diferentes radicais oferece a possibilidade de utilizar extractos desta planta como antioxidantes naturais em diversos sistemas alimentares contendo lípidos.

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CURRICULUM VITAE

Airidas Dapkevicius was born on April 25, 1966 in Klaipėda, Lithuania. He attended primary school at the 18th Secondary School of Klaipėda from 1972-1980. From 1980 he attended secondary school at the 14th Secondary School of Klaipėda, from where he obtained the Secondary School Accomplishment Diploma in June 1984. He then started studies at the Kaunas University of Technology, Faculty of Chemical Technology. Studies were interrupted from 1984 to 1986 by obligatory recruitment to the Soviet Army. In June 1991 he obtained an Engineer degree in Catering Technology at the Kaunas University of Technology. He started doctorate studies at the Department of Food Chemistry, Kaunas University of Technology.

On August 1994 he was admitted as a Ph.D. candidate for a so-called "sandwich" research project between the Department of Food Technology of the Kaunas University of Technology, and two sections of the Department of Agrotechnology and Food Sciences of Wageningen University, namely the Laboratory of Organic Chemistry and the Food Chemistry Group. Simultaneously with the Ph.D. studies, he took part in several research projects at Kaunas University of Technology and at Wageningen University. At present, he cooperates on a voluntary basis with the Department of Agrarian Sciences (University of the Azores, Azores, Portugal).

LIST OF PUBLICATIONS

1. Dapkevičius, A., Venskutonis, P.R., Sudžienė, S. and Bražaitė, R. 1992. Vietinių kvapniųjų augalų panaudojimas saldainių aromatizavimui. [Use of local aromatic plants in the flavouring of confectionery]. *Organinių medžiagų ir maisto chemija ir technologija*, 54–57.

2. P.R. Venskutonis, Dapkevičius, A. and Baranauskienė, M. 1993. Kvapniųjų augalų eterinio aliejaus kiekio tyrimai. [Investigation of essential oil in aromatic plants]. *Maisto produktų chemija ir technologija*, 85–87.

3. Venskutonis, P.R., Dapkevičius, A. and Baranauskienė, M. 1994. Phenylpropenes in tarragon (*Artemisia dracunculus*). *Maisto produktų chemija ir technologija*, 3–11.

4. Dapkevičius, A. and Venskutonis, P.R. 1994. Vietiniai kvapnieji augalai – natūralūs antioksidantai. [Local aromatic plants - natural antioxidants]. *Maisto produktų chemija ir technologija*, 37–39.

5. Dapkevičius, A., Venskutonis, P.R. and Baranauskienė, M. 1994. Analysis of aroma composition of anise hyssop (*Agastache foeniculum* [Pursch] Kuntze) by capillary gas chromatography. *Maisto chemija ir technologija*, 28, 75–81.

6. Venskutonis, P.R., Dapkevičius, A. and Baranauskienė, M. 1995. Flavour composition of some lemon-like aroma herbs from Lithuania. p. 833–847. In: G. Charalambous (ed). Food Flavors: Generation, Analysis and Process Influence. Elsevier Science Publishers, Amsterdam.

7. Venskutonis, P.R. and Dapkevičius, A. 1995. Flavour of anise hyssop (*Agastache foeniculum* [Pursch] Kuntze). p. 660–664. In: M. Rothe and H.P. Kruse (eds). Aroma: Perception, Formation, Evaluation. Eigenverlag Deutsches Institut für Ernährungsforschung, Potsdam-Rehbrücke.

8. Dapkevičius, A., Venskutonis, P.R. and Baranauskienė, M. 1995. Evaluation of antioxidant activity of some aromatic plants by β-carotene bleaching test. *Maisto produktų chemija ir technologija,* 16–19.

9. Venskutonis, P.R., Dapkevičius, A. and Baranauskienė, M. 1995. Flavour of costmary (*Balsamita major* L.) from Lithuania. p. 85. In: K.H.C. Baser (ed). Flavours, Fragrances and Essential Oils. vol. 1. Proceedings of the 13th International Congress of Flavours, Fragrances and Essential Oils, Istanbul. Turkey, 15-19 October 1995. AREP Publ., Istanbul.

10. Venskutonis, P.R. and Dapkevičius, A. 1995. Some aspects of herb aroma research. *Food Chemistry and Technology*, 29, 28–32.

11. Venskutonis, P.R., Dapkevičius, A. and Baranauskienė, M. 1996. Kvapniųjų augalų eterinio aliejaus antioksidacinio aktyvumo įvertinimas. [Evaluation of antioxidant activity of essential oils and extracts isolated from aromatic herbs]. *Maisto produktų chemija ir technologija*, 26–29.

12. Venskutonis, P.R., Dapkevicius, A. and Baranauskiene, M. 1997. Composition of the essential oil of lavender (*Lavandula angustifolia* Mill.) from Lithuania. *J. Essent. Oil Res.*, 9, 107–110.

13. Dapkevicius, A., Venskutonis, P.R., van Beek, T. A. and Linssen, J.P.H. 1998. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J. Sci. Food Agric.*, **77**, 140–146.

14. Dapkevicius, A., van Beek, T.A., Linssen, J.P.H. and Venskutonis, P.R. 1998. Rapid spectroscopic screening for antioxidant activity in sage, thyme and oregano isolates with the β-carotene-linoleic acid model system. p. 235–237. In: P. Schreier, M. Herderich, H.U. Humpf, and W. Schwab (eds). Natural Product Analysis: Chromatography, Spectroscopy and Biological Testing. Vieweg & Sohn Verlagsgesellschaft, Lengerich.

15. Dapkevicius, A., van Beek, T.A., Niederländer, H.A.G. and de Groot, Ae. 1999. On-line detection of antioxidative activity in high-performance liquid chromatography eluates by chemiluminescence. *Anal. Chem.*, 71, 736–740.

16. Dapkevicius, A., van Beek, T.A. and Niederländer, H.A.G. 2001. Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in liquid chromatography eluates. *J. Chromatogr. A*, 912, 73–82.

17. Dapkevicius, A., van Beek, T.A., Lelyveld, G.P., van Veldhuizen, A., de Groot, Ae., Linssen, J.P.H. and Venskutonis, R. 2001. Isolation and structure elucidation of radical scavengers from *Thymus vulgaris* leaves. *J. Nat. Prod.*, (submitted for publication).