New methods

for the screening of antioxidants

in three *Sideritis* species

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“If you have love,
you don’t need anything else,
If you have not love,
It does not matter what else you have.”

James Barry

<u>To all I love!</u>

Content

Introduction

1.1 Lipid oxidation and free radicals

"An adult at rest may produce close to 2 kg of $O_2^{\bullet-}$ per year."
(Halliwell 1994)

The nickname "Dr. Jekyll-Mr. Hyde" can reasonably be attributed to oxygen. It is the first life-essential element, needed for normal functioning of all aerobic systems (Namiki 1990) but it is also responsible for a number of oxidation processes with harmful consequences such as weathering of non-biological materials (Larson 1997a), oxidative stress in plants (Dalton 1995; Garcia-Plazaola & Becerril 1999), food quality deterioration (Finley & Otterburn 1993; Frankel 1996; Hiramatsu et al. 1997) and human health disorders that are related to oxidative damage of important biological molecules (Bermond 1990; Ramarathnam et al. 1995; Tyrrel 1995; Cook & Samman 1996; Arouma 1998; Papas 1999; Pokorny et al. 2001; Halliwell & Whiteman 2004). Target substances of oxidation encompass a multitude of chemical structures occurring in nature − proteins, DNA, (poly)unsaturated fatty acids (PUFA), cholesterol, phospholipids, carbohydrates, etc. (for abbreviations see the Appendix). Next to water and air, food is the third fundamental element of our life. Lipids are major components of many foods and subject of intense protection against oxidation since they are important building elements of cell membranes. Lipid oxidation in food causes a number of problems such as deterioration of food safety, nutritional value and flavour- and texture aspects or appearance, which has serious economical consequences (Shahidi & Wanasundara 1992).

Due to their unsaturated chemical nature lipids are very susceptible to oxidation. This takes place through reactions involving triplet oxygen ($^3O_2$) and singlet oxygen ($^1O_2$). The main mechanism of lipid oxidation in foods is a free radical chain process called *autoxidation*. This process comprises three main phases – initiation (start), propagation and termination, whose kinetics are illustrated in Figure 1.1. Some authors have divided this process into four steps instead of three and between propagation and termination they include branching. A schematic presentation is given in Figure 1.2 (Hamilton 1983; Belitz et al. 2004). The onset of oxidation of a lipid molecule (RH) starts with an initiator. Such an

initiator can be UV light irradiation, heat, pro-oxidative metal ions or enzymes, or the presence of a photosensitiser.

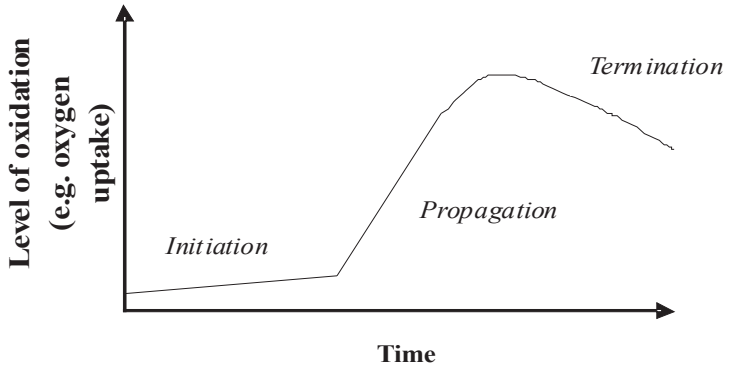


Figure 1.1 Kinetics of free-radical lipid oxidation (Larson 1997b).

Start or **Initiation:** Formation of peroxy ($ROO^{\bullet}$), alkoxy ($RO^{\bullet}$) or alkyl ($R^{\bullet}$) radicals.

Chain propagation:

$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$ *(fast)* (1.1)

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$ *(slow, rate-limiting)* (1.2)

$RO^{\bullet} + RH \rightarrow ROH + R^{\bullet}$ (1.3)

Chain branching:

$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH$ (1.4)

$2\ ROOH \rightarrow ROO^{\bullet} + RO^{\bullet} + H_2O$ (1.5)

Chain termination:

$R^{\bullet} + R^{\bullet} \rightarrow$ *stable products* (1.6)

$R^{\bullet} + ROO^{\bullet} \rightarrow$ *stable products* (1.7)

$ROO^{\bullet} + ROO^{\bullet} \rightarrow$ *stable products* (1.8)

Figure 1.2 Schematic presentation of autoxidation (based on Belitz et al. 2004).

Most foods contain traces of metals as a result of processing (e.g. Ni from oil hydrogenation), storage (Cu or Fe from packaging), or as a food component. Traces of metals in foods are unavoidable and concentrations of down to 0.5 ppm are sufficient to catalyze lipid oxidation (Labuza 1971; Larson 1997c). The most reactive are metals whose capable of one-electron-transfer redox reactions.

$$RH + M^{(n+1)+} \rightarrow R^{\bullet} + Me^{n+} + H^{+} \qquad (1.9)$$

The main "consequence" of all initiation mechanisms is the formation of free lipid radicals and this stage of the process is called the *Initiation* phase of the oxidation. The reaction is slow and the radical concentration at the beginning is low. The detailed mechanisms through which free radicals are formed from lipids with the participation of initiators are not yet fully understood. Allyl radicals will be formed preferentially because of resonance stabilization of the radical.

The *Propagation* phase of the oxidation begins after the initiation has occurred. The generated free radicals easily react with the biradical oxygen to produce peroxyl radicals ($ROO^{\bullet}$) (reaction 1.1). Reaction 1.1 is fast and the concentration of peroxyl radicals rapidly increases at the expense of alkyl or allyl radicals. Peroxyl radicals attack other lipid molecules, which leads to the formation of lipid hydroperoxides (ROOH) and proliferation of free radicals in the system (reactions 1.2 and 1.3). Peroxyl radicals are considered to be key species in the lipid oxidation as they are the main chain-carrying radical species. Reaction 1.2 is the rate-limiting reaction, it is much slower than reaction 1.1, which basically is a rapid radical-radical coupling. The relatively moderate reactivity of $ROO^{\bullet}$ determines their selectivity for the most weakly bound hydrogen in the unsaturated fatty acid molecule (Larson 1997c; Frankel 1998a; Min 1998), which are the allylic hydrogen atoms in (poly)unsaturated fatty acids (PUFA). The type of the formed hydroperoxide (ROOH) depends on the type and degree of unsaturation of the fatty acid. The formed ROOHs can disproportionate through a monomolecular route to hydroxyl ($HO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals (reaction 1.4). This phase is called *Branching*. Hydroxyl radicals are extremely reactive (half-life of 10^{-9} s) (Pieta 2000). They attack every species in their vicinity and thus, lead to intensive free radical formation and a fast acceleration of the oxidation. For comparison, the rate of abstraction of an allylic hydrogen atom from a lipid molecule by $HO^{\bullet}$ is 10^{9} times higher than by $ROO^{\bullet}$ (Noguchi & Niki 1999; Sanchez-Moreno 2002). Alkoxyl radicals, though much less reactive than $HO^{\bullet}$, also contribute to propagation and branching of the oxidation as they can either abstract hydrogen from lipid

molecules (reaction 1.3) or from the formed hydroperoxides, or further decompose through β-fission to an allylic alkyl radical and an aldehyde. Hydroperoxide decomposition may also occur through a bimolecular pathway which is exothermic (reaction 1.5), unlike the endothermic monomolecular decomposition (reaction 1.4). However, reaction 1.5 is of no relevance since oil oxidation makes a food unpalatable well before reaching the necessary hydroperoxide level for this reaction step to occur (Belitz et al. 2004).

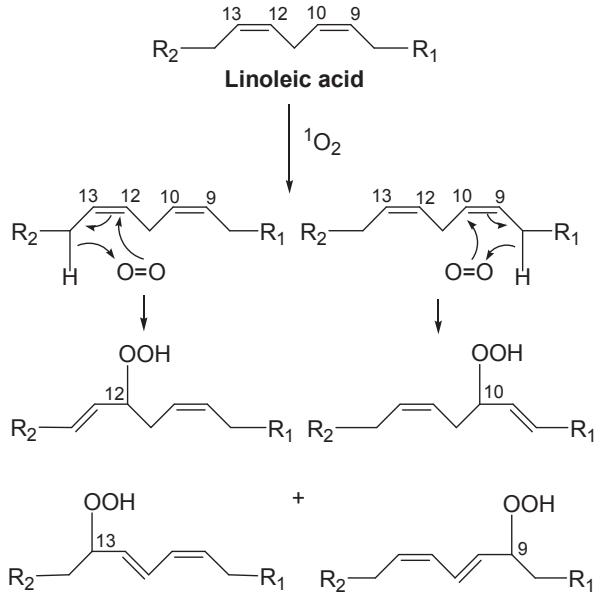


Figure 1.3 Oxidation of linoleic acid by singlet oxygen (Frankel 1998b).

Foods usually contain natural pigments or artificial dyes and are often exposed to light. This brings about the possibility of another oxidation mechanism with the participation of singlet oxygen. Singlet oxygen ($^1\Delta g\ O_2$) (O=O) is a non-radical species with sufficient energy and a relatively long lifetime. Being a very electrophilic compound it readily attacks electron-rich compounds such as double bonds in unsaturated fatty acids. For comparison, it reacts $10^3 – 10^4$ times faster with methyl linoleate than triplet oxygen (Labuza 1971). Figure 1.3 represents oxidation of linoleic acid by singlet oxygen (Frankel 1998b). Consequently, hydroperoxides different from those produced by the radical autoxidation mechanism are formed. Singlet oxygen reacts faster but stoichiometrically (mol per mol) with the lipid molecule (Noguchi & Niki 1999). Therefore, it is generally agreed that once the initial hydroperoxides are formed, the radical chain reactions prevail as the main oxidation mechanism (Benavente-Garcia et al. 2000). Singlet oxygen can be formed through photochemical, chemical, enzymatic or physical processes. The photochemical production of $^1O_2$ is initiated by sensitisers (Sens) and light and is the most common way to produce $^1O_2$ in foods. The sensitisers can be food dyes (e.g. erythrosine, FD and C red No 3) (Hamilton et al. 1997) or natural pigments (e.g.

chlorophylls, hemeproteins and flavins), which are able to absorb UV/Vis light and become electronically excited (^{3}Sens). There are two types of sensitizers (Fig. 1.4). Type I sensitisers (e.g. riboflavin) are those which, once activated by light, react directly with the substrate (RH), generating substrate radicals ($R^{\bullet}$) and form hydroperoxides, which are the same as those formed via free radical autoxidation. These trigger the autoxidation process. Type II sensitisers (e.g. chlorophylls) activate the ground state of oxygen by energy transfer to produce $^1O_2$. This attacks the lipid molecules to produce hydroperoxides through an entirely different mechanism (as shown in Fig. 1.3) (Hamilton et al. 1997; Frankel 1998b; Min 1998; Belitz et al. 2004). Free radical scavengers cannot interfere with the formation of this type of hydroperoxides. Figure 1.4 represents the photooxidation by sensitizers (Frankel 1998b).

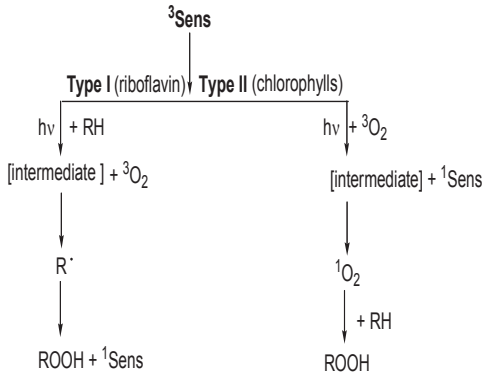


Figure 1.4 Photooxidation by sensitizers (Frankel 1998b).

In the presence of both types of sensitisers, the favoured mechanism depends on the structure of the sensitizer, on the substrate polarity and structure, and on the oxygen concentration. Pathway II is favoured in non-polar media such as lipids where the solubility of $^1O_2$ is higher than in water. In water-containing solutions, where the concentration of oxygen is lower, pathway I is preferred (Min 1998).

Hydroperoxide decomposition may occur in the presence of transition metal ions through reactions 1.10 and 1.11. It is assumed that first a metal-hydroperoxide complex is formed before decomposition to free radicals occurs. The oxidation step of the redox cycle of the metal ions is faster (the metal itself is reduced, reaction 1.10) than the reduction (reaction 1.11) and it controls the overall catalytic activity of the metal ion (Labuza 1971).

$$ROOH + M^{n+} \rightarrow [ROOH–M^{n+}] \rightarrow ROO^{\bullet} + H^{+} + M^{(n-1)+} \quad 1.10$$

$$ROOH + M^{(n-1)+} \rightarrow RO^{\bullet} + OH^{-} + M^{n+} \quad 1.11$$

The solvent may either inhibit or enhance the catalytic action of the metal. For instance, water may complex with metals, thus preventing the peroxides from doing so, or facilitate the formation of insoluble metal hydroperoxides. In some cases, the solvent may increase the solubility and hence, the mobility of the metal, which results in a stronger catalytic effect on the oxidation (Labuza 1971).

The main route for hydroperoxide decomposition occurs through cleavage reactions leading to the formation of low molecular volatile products such as aldehydes, ketones, hydrocarbons, acids and epoxides. In Figure 1.5 the homo/heterolytic mechanism for the decomposition of 13-OOH methyl linoleate hydroperoxides is shown. A "mixed" mechanism (homolytic-heterolytic) is proposed to avoid the formation of unfavourable vinylic radical intermediates (de Groot 2001).

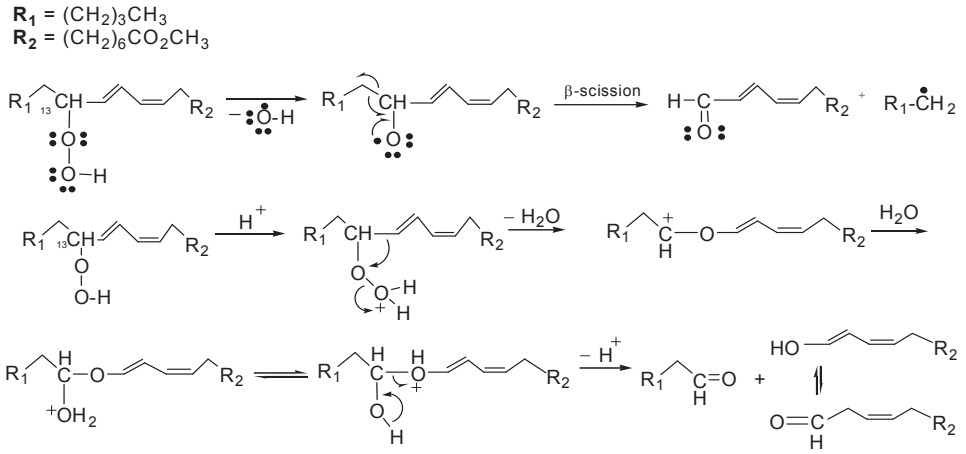


Figure 1.5 Homo/heterolytic mechanism for the decomposition of 13-OOH methyl linoleate hydroperoxides (de Groot 2001).

These secondary products of oxidation are very important in food industry, as they cause the rancid off-flavour and odour of lipid-containing products. They have low threshold values. A few ppb are often sufficient to give an unacceptable odour to foods (Labuza 1971). Secondary oxidation products may themselves participate in further reactions such as oxidation, decomposition, polymerization, etc. The obtained products may negatively alter the colour, texture and safety of foods (Bermond 1990; Finley & Otterburn 1993; Frankel 1998c). They may also interact with important proteins and cause enzyme inactivation (Decker 1998b) and/or with other food components, which may lead to

Lipid oxidation is a process, which involves different types of reactions, a variety of chemical intermediates and a multitude of other factors. Therefore, various approaches to control lipid oxidation are possible. In general, they impact on all phases of lipid oxidation. In terms of protection and preservation of foods, different measures for control and minimization of lipid oxidation are undertaken in industry. These include: blending of polyunsaturated fats with more stable monounsaturated lipids; partial hydrogenation of the polyunsaturated fats; control of processing parameters such as limited aeration; minimizing traces of impurities and loss of endogenous antioxidants such as tocopherols; suitable temperature regime; air-free packaging or encapsulation; and appropriate storage conditions (e.g. lack of direct light, cold or frozen storage, etc.) (Decker 1998b; Frankel 1998f; Yanishlieva-Maslarova 2001). Nevertheless, it is neither possible nor practical to remove all factors that may provoke oxidation. Therefore, the use of added substances (antioxidants), able to prevent, delay or retard the oxidative processes is indispensable in contemporary food preservation.

An antioxidant is a reductant, but a reductant is not necessarily an antioxidant (Prior & Cao 1999). The term "*antioxidant*" has been generally formulated as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell 1995a). *'Food antioxidant*" is specially formulated as "any substance that in small quantities is able to prevent or retard the oxidation of easily oxidizable materials such as fats" (Frankel & Meyer 2000). However, a broader meaning is hidden in this simple description, which is rooted in the complex phenomena involved in oxidation – antioxidation. Antioxidants exercise their protection at different stages of lipid oxidation and by different mechanisms. A distinction must be made between short- and long-term protection of antioxidants owing to the reaction kinetics (Antolovich et al. 2002). Compounds with antioxidant properties belong to various chemical groups. Therefore, an orderly classification of all existing antioxidants is difficult. Usually, they are classified either by their mechanism of action or by their chemical nature, the first approach being more comprehensive and theoretically more sound. A general division of antioxidants into two main groups according to their mode of action is commonly accepted. Labuza (1971) has proposed a third group, which does not comprise specific compounds but concerns the control of environmental factors such as temperature, oxygen level, or moisture content.

1.2.1 Division of antioxidants by the mechanism of action

Group I Antioxidants. Primary, true or chain-breaking antioxidants or free radical scavengers are the names attributed to antioxidants from this group. They are able to inhibit the initiation and propagation of oxidation by inactivating the free radicals, participating in oxidative chain reactions by converting them into stable non-radical products or more stable radicals. Pryor et al. (1993) have defined another group of antioxidants as "retarders" (e.g. hydroxytetronic acids - see the structure on the right in Fig. 1.6). In contrast to the true antioxidants, retarders slow down the oxidation propagation and after their consumption, the rate of oxidation does not return to its initial value. In Figure 1.6 the difference in the action of the two types of oxidation inhibitors is illustrated.

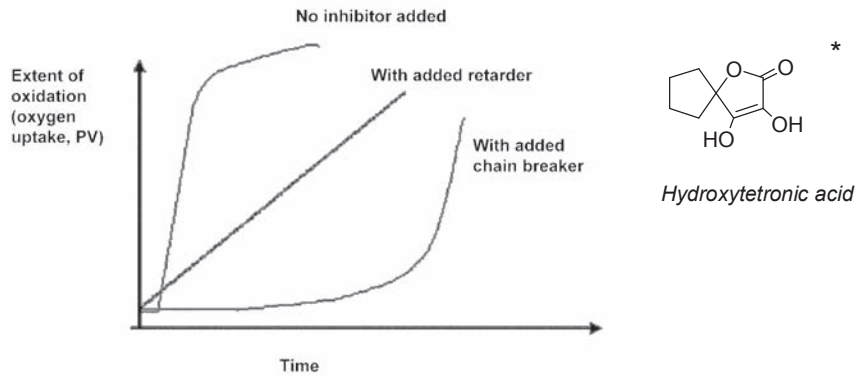


Figure 1.6 Autoxidation with and without inhibitors (Larson 1997d).
(* - the structure on the right is an example of a retarder)

Primary antioxidants are mainly phenolic compounds (AH) able to donate a hydrogen atom or an electron to the free radical and to convert it into a stable non-radical product (chain-breaking electron donors). Free radical scavengers predominantly react with peroxyl radicals – reaction 1.12 − for two reasons: the higher concentration (reaction 1.1 is faster than reaction 1.2) and the lower energy of the $ROO^{\bullet}$ in comparison to other radicals such as $RO^{\bullet}$, and the low concentration of the free radical scavengers in food, which make them not competitive with highly reactive species such as $HO^{\bullet}$ (Decker 1998a). A good free radical scavenger, even at low concentrations, efficiently competes with lipids to deactivate the free radicals by donation of an electron, followed by deprotonation (Frankel & Meyer 2000). Furthermore, it itself forms a low-energy stable radical, which does not promote further chain propagation and does not react with oxygen to produce peroxides (Decker 1998a). The latter is due to antioxidant radical stabilization through resonance

delocalization, intramolecular hydrogen bonding or by further oxidation (Fig. 1.7) (Frankel & Meyer 2000).

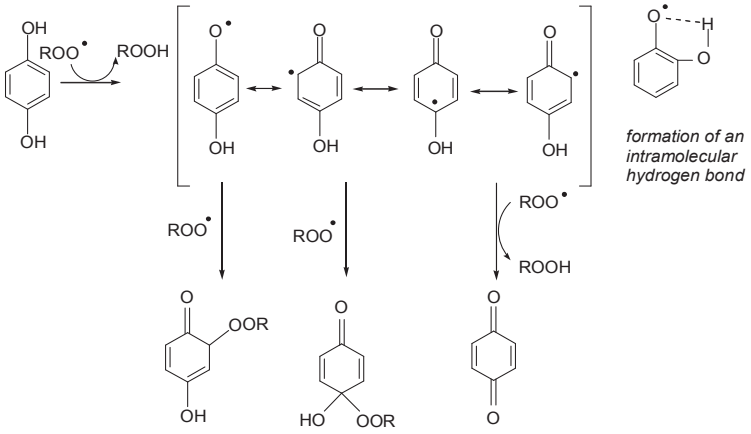


Figure 1.7 Antioxidant radical stabilization through resonance delocalization, intramolecular hydrogen bonding or by further oxidation.

The most stable antioxidant radicals are formed by phenolics with *o*-dihydroxy groups and a bulky alkyl substituent at the *p*-position (Robards et al. 1999). Free radical scavengers may also block free radicals by coupling (termination) reactions (chain-breaking electron acceptors) − reactions 1.13, 1.15, 1.17. Thus, one molecule of the free radical scavenger can deactivate at least two free radicals as seen from reactions 1.12 − 1.17 and Figure 1.7 (Noguchi & Niki 1999).

$$AH + ROO^{\bullet} \rightarrow ROOH + A^{\bullet} \quad 1.12$$

$$A^{\bullet} + ROO^{\bullet} \rightarrow ROO\text{-}A \quad 1.13$$

$$AH + RO^{\bullet} \rightarrow ROH + A^{\bullet} \quad 1.14$$

$$A^{\bullet} + RO^{\bullet} \rightarrow RO\text{-}A \quad 1.15$$

$$AH + R^{\bullet} \rightarrow RH + A^{\bullet} \quad 1.16$$

$$A^{\bullet} + R^{\bullet} \rightarrow RA \rightarrow R_1H + AH \quad 1.17$$

Antioxidant radicals may interrupt the chain-carrying reaction further by forming non-radical peroxide-antioxidant compounds such as: ROO-A, RO-A and RA (reactions 1.13,

1.15, 1.17) (Rajalakshmi & Narasimhan 1995). The homolytic decomposition of ROOH to alkoxyl radicals further leads to the formation of secondary oxidation products (see Fig. 1.5) producing rancid flavour (Frankel & Meyer 2000). Therefore, reactions 1.14 and 1.15 are very important as they represent the mechanism by which free radical scavengers may inhibit the formation of rancid products.

$$AH + O_2 \rightarrow A^{\bullet} + HOO^{\bullet} \quad 1.18$$

$$AH + ROOH \rightarrow RO^{\bullet} + H_2O + A^{\bullet} \quad 1.19$$

$$A^{\bullet} + RH \rightarrow AH + R^{\bullet} \quad 1.20$$

At high antioxidant concentrations or elevated temperatures antioxidants, especially less substituted phenolics, may participate in chain-propagating reactions and exert pro-oxidant effect (reactions 1.18 − 1.20) (Cillard et al. 1980; Husain et al. 1987; Gordon 1990; Larson 1997e; Frankel 1998g; Robards et al. 1999).

Group II Antioxidants. Compounds from this group are referred to as preventive or secondary antioxidants. They encompass a large group of chemically different substances, which inhibit lipid oxidation by different mechanisms and do not convert free radicals to non-radical products. With some exceptions, secondary antioxidants are generally related to inhibition of the factors initiating oxidation. Group II antioxidants include: chelators of pro-oxidative metals, quenchers of singlet oxygen, scavengers of molecular oxygen, UV light absorbers, inhibitors of pro-oxidative enzymes, enzymatic antioxidants, and hydroperoxide decomposers. Synergists, which enhance the effect of primary antioxidants but by themselves do not possess antioxidant activity, are also categorised as a type of secondary antioxidants

- Deactivators (chelators) of pro-oxidative metals

They either alter metal solubility or its redox potential, or sterically hinder the formation of metal-hydroperoxide complexes (reaction 1.10) and thus prevent their further decomposition (Decker 1998a; Frankel 1998g; Reische et al. 1998). The most efficient metal chelators are compounds that possess lone electron pairs to donate to the metal ion and orbitals suitably arranged in space so that vacant metal orbitals can be accommodated. Typical examples include EDTA and citric acid (Mahoney & Graf 1986; Arora & Strasburg 1997) but also some flavonoids exert such properties. For the latter a catechol moiety in the B-ring (Fig. 1.8), a 3-hydroxy and a 4-oxo group in the heterocyclic ring C, and a 4-oxo and a 5-hydroxyl group in the C- and A rings, respectively is necessary (Pietta 2000).

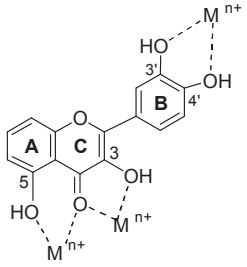


Figure 1.8 Binding sites for trace metals (Pietta 2000). Depending on pH and the exact metal ion present, one or more phenolic groups can be deprotonated.

- Quenchers of singlet oxygen

Singlet oxygen can be inactivated by physical (collisional) or chemical quenching (it can add to antioxidants to form endoperoxides) (Huang et al. 2005), the first mechanism being predominant. Collisional quenching takes place by energy transfer from the $^1O_2$ to the quenching molecule. The latter dissipates the acquired energy as emission of heat into the surrounding medium (Larson 1997c; Beutner et al. 2001). This process allows one molecule of the quencher to inactivate many moles of $^1O_2$. Carotenoids are the most efficient quenchers of $^1O_2$ by this mechanism (Burton & Ingold 1984). They are also able to chemically incorporate the $^1O_2$ molecule or to act as UV absorbers but the physical quenching is prevalent. Tocopherol, ascorbate and some phenolics are able to chemically quench $^1O_2$ (Decker 1998a). Quercetin may also scavenge singlet oxygen (Kanner et al. 1994; Larson 1997e).

- Scavengers of molecular oxygen and reducing agents

Strong reducing agents such as ascorbate and sulfur dioxide are able to scavenge molecular oxygen and are especially useful in foods with headspace or dissolved oxygen (Reische et al. 1998).

- UV light absorbers

Substances such as carbon black that are able to absorb the harmful UV light promoting oxidation are used in non-food formulations such as polymers (Larson 1997a; Frankel 1998g). The presence of conjugated systems in phenolic antioxidants may explain their use as UV absorbers in this way protecting foods and beverages from oxidative deterioration (Larson 1997e; Andersen et al. 2003).

- Inhibitors of pro-oxidative enzymes

Phenolics may inactivate pro-oxidative enzymes such as lipoxygenases by reducing the metal ion in their active site to its inactive reduced form (e.g. Fe^{3+} to Fe^{2+}) (Decker 1998a). Anthocyanins may inhibit oxidative enzymes (Kanner et al. 1994; Larson 1997e).

- Enzymatic antioxidants

These antioxidants deactivate oxidation intermediates such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or lipid hydroperoxides (ROOH). Superoxide dismutase (SOD) converts $O_2^{\bullet -}$ to H_2O_2, which in turn is decomposed to water by catalase (Munday & Winterbourn 1989) – reactions 1.21a and 1.21b. More detailed information can be found in Table 1.1. Some phenolics are also able to deactivate the superoxide anion (Robards et al. 1999).

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad 1.21a$$

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2 \qquad 1.21b$$

Table 1.1 Enzymes with antioxidant activity.

Superoxide dismutase (SOD)	Converts $O_2^{\bullet -} \rightarrow H_2O_2$
Glucosoxidase	Converts glucose → glucuronic acid
Catalase	Breaks down $2H_2O_2 \rightarrow 2H_2O + O_2$
Peroxidase (PH_2)	Breaks down H_2O_2 and ROOH $ROOH + PH_2 \rightarrow ROH + H_2O + P$ $H_2O_2 + PH_2 \rightarrow 2H_2O + P$
Glutathione peroxidase (cell)	Breaks down H_2O_2 and ROOH $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ $ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$
Glutathione peroxidase (plasma)	Breaks down H_2O_2 and phospholipid hydroperoxides (PhROOH) $PhROOH + 2GSH \rightarrow PhROH + H_2O + GSSG$
Phospholipid hydroperoxide glutathione peroxidase	Breaks down phospholipid hydroperoxides (PhROOH)

- Hydroperoxide destroyers

The accumulation of lipid hydroperoxides, which decompose to generate new radicals or unwanted secondary oxidation products represents a key feature in lipid oxidation. Antioxidants that are able to induce decomposition of these hydroperoxides through non-radical routes or "bind" the generated secondary products will favourably affect the ultimate antioxidant effect. Specific enzymes such as catalases and peroxidases (Table 1.1), and strong reducers such as reduced sulfur and phosphorous compounds (Larson 1997f), melanoidins (Obretenov et al. 1986; Schuler 1990; Bailey et al. 1996) are effective hydroperoxide destroyers.

Synergistic interaction between antioxidants enhances the total antioxidant potential either by regeneration of one of the antioxidants or by "sparing" effects. A good synergistic combination generally combines two free radical scavengers such as tocopherol and

ascorbate (Fig. 1.9) (Frankel 1998g) or a free radical scavenger with a metal chelator (Miller et al. 1996). In the first case, ascorbate regenerates tocopherol by donation of a hydrogen atom to the tocopheryl radical. Amino acids, amines, proteins, phospholipids are another example of synergistic action (Schuler 1990).

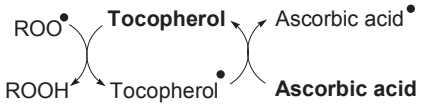


Figure 1.9 Synergistic combination between tocopherol and ascorbate (Frankel 1998g).

The metal chelator diminishes the formation of free radicals and hence, the consumption of a free radical scavenger will be slower and its total concentration will increase i.e. the free radical scavenger will be "spared" (Banias et al. 1992; Wada & Fang 1992; Baldioli et al. 1996). Also, some flavonoids like quercetin and tea catechins (Pietta 2000), regenerate tocopherol from tocopheroxyl radical, thus ensuring a "sparing" effect. A specific synergistic interaction is the one between tocopherol and β-carotene (Fig. 1.10) (Frankel 1998b). Tocopherol enforces β-carotene by taking up part of its $^1O_2$-quenching "duties" i.e. exhibiting a "sparing" effect.

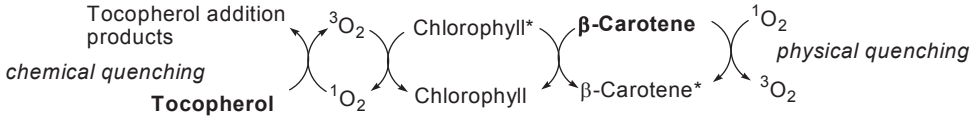


Figure 1.10 Synergistic combination between tocopherol and β-carotene (Frankel 1998b).

Some antioxidants exert multifunctional antioxidative properties. For example, ascorbic acid can act as a free radical scavenger, deactivates molecular oxygen, scavenges singlet oxygen in aqueous solutions and effectively regenerates tocopherol i.e. acts as a synergist (Halliwell 1994). Many flavonoids are good free radical scavengers, metal chelators or synergists (Miller et al. 1996; Arora & Strasburg 1997). Phospholipids and Maillard reaction products are also antioxidants with multiple functions (Obretenov et al. 1986; Gordon 1990; Bailey et al. 1996).

The generally accepted classification of antioxidants presented above, however can be somewhat misleading as the majority of the antioxidants termed as "secondary" act, in fact, on the first defense line of oxidation i.e. inhibition of the factors and agents that initiate oxidation. The "primary" antioxidants start to be effective on the next stages when the free radicals are already formed and propagated.

A mere mechanical addition of an antioxidant to food cannot ensure a sufficiently good protection. Additional considerations should be borne in mind. The antioxidant and oxidation agent should be in the same location. For example, a water-soluble metal chelator may not be effective if the pro-oxidant metal is located in the lipid phase (Decker 1998a). Tocopherol will be more effective against radicals generated in lipophilic media, while ascorbate will scavenge aqueous radicals more efficiently despite having similar reactivities toward oxygen radicals (Noguchi & Niki 1999). Different radical scavengers are effective toward different radicals. For example, tocopherol ($E^{0'}$ = 480 mV) scavenges $ROO^{\bullet}$ ($E^{0'}$ = 1000 mV) but not $^{\bullet}OH$ ($E^{0'}$ = 2300 mV) and $RO^{\bullet}$ ($E^{0'}$ = 1600 mV) radicals the latter ones being too reactive (Noguchi & Niki 1999; Pietta 2000). The potential of a free radical scavenger to compete with the lipid molecule for donation of a hydrogen atom to the free radical can be primarily predicted from their standard redox potentials ($E^{0'}$) (Pietta 2000; Andersen et al. 2003). If there are no kinetic restrictions, any species having a redox potential lower than that of the oxidizing free radical will be able to donate hydrogen to the free radical. For example, α-tocopherol can react more rapidly with $ROO^{\bullet}$ than a PUFA because $E^{0'}_{\alpha\text{-Tocopherol}}$ = 480 mV, $E^{0'}_{PUFA}$ = 600 mV, and $E^{0'}_{ROO^{\bullet}}$ = 1000 mV (Decker 1998a; Pietta 2000). But redox potentials are not always correlated to the hydrogen atom donating ability. Thus, the reducing capacity of a sample is not related to its scavenging capacity (Lucarini et al. 1999).

Interfacial phenomena are another factor which have been proven to be important for the oxidizable substrate and antioxidant partitioning. The so-called “polar paradox” explains why lipophilic antioxidants perform better in polar media such as emulsions whereas hydrophilic antioxidants are more efficient in bulk oil systems (Porter 1993; Frankel & Meyer 2000). In emulsions apolar antioxidants concentrate in the oil and the oil-water interface thus ensuring a better protection of the lipid while polar antioxidants “dilute” in the aqueous phase. In bulk oil systems the reverse is observed i.e. by being present near the oil-air interface hydrophilic antioxidants efficiently protect against lipid oxidation (Fig. 1.11). Possible interactions between the antioxidant and medium components, as well as pH and polarity of the medium should be considered (Fujimori et al. 1994; Huang et al. 1996; Moure et al. 2001; Huang et al. 2005).

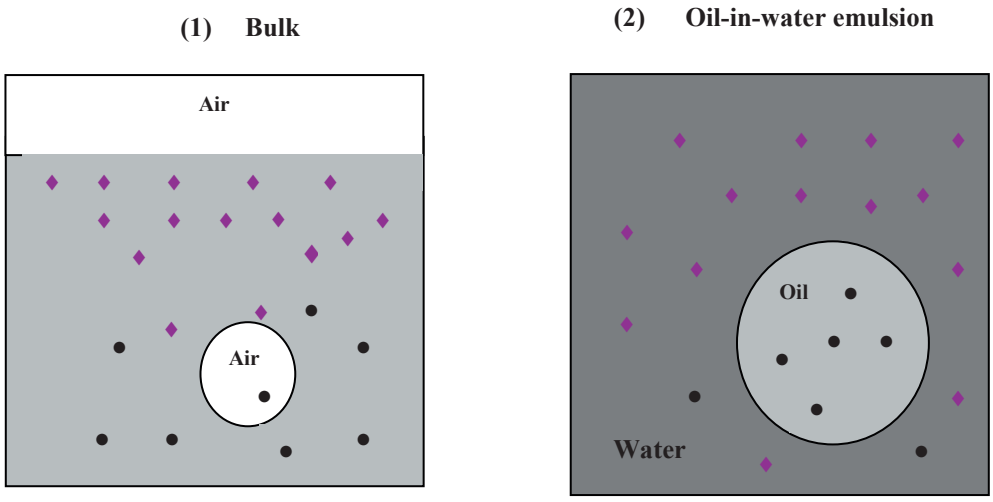


Figure 1.11 Partitioning of hydrophilic (♦) and lipophilic (●) antioxidants in bulk oils and oil-in-water emulsions (from Frankel & Meyer 2000). **(1)** Hydrophilic (♦), more protective > Lipophilic (●); **(2)** Lipophilic (●), more protective > Hydrophilic (♦).

Apparently, no single antioxidant is able to ensure a total "security" for all type of lipids and through all the stages of oxidation i.e. a "universal" antioxidant cannot be found. It is clear that the key for an efficient protection lies in the balance between oxidative and antioxidative species and other factors. In Figure 1.12, a schematic presentation of the "HACCP" (**H**azard **A**nalysis of **C**ritical **C**ontrol **P**oints) (Danchev 1997) in oxidation-antioxidation processes is presented. Nature has created efficient mechanisms in living organisms to inhibit oxidation by multicomponent antioxidant systems (Decker 1998a). Following the examples presented by biological systems, efficient food-protection systems can be developed by combining different types of antioxidants.

1.2.2 Division of antioxidants by chemical nature (synthetic and natural antioxidants)

In foods, the most widely used antioxidants are the phenolic chain-breaking antioxidants. Several synthetic [BHT (**1**), BHA (**2**), TBHQ (**3**) and PG (**4**); Fig. 1.13(a)] and a few natural compounds [tocopherol (**5**), ascorbic acid (**6**), β-carotene (**7**); Fig. 1.13(b)] have been officially allowed for use in foods (Schuler 1990).

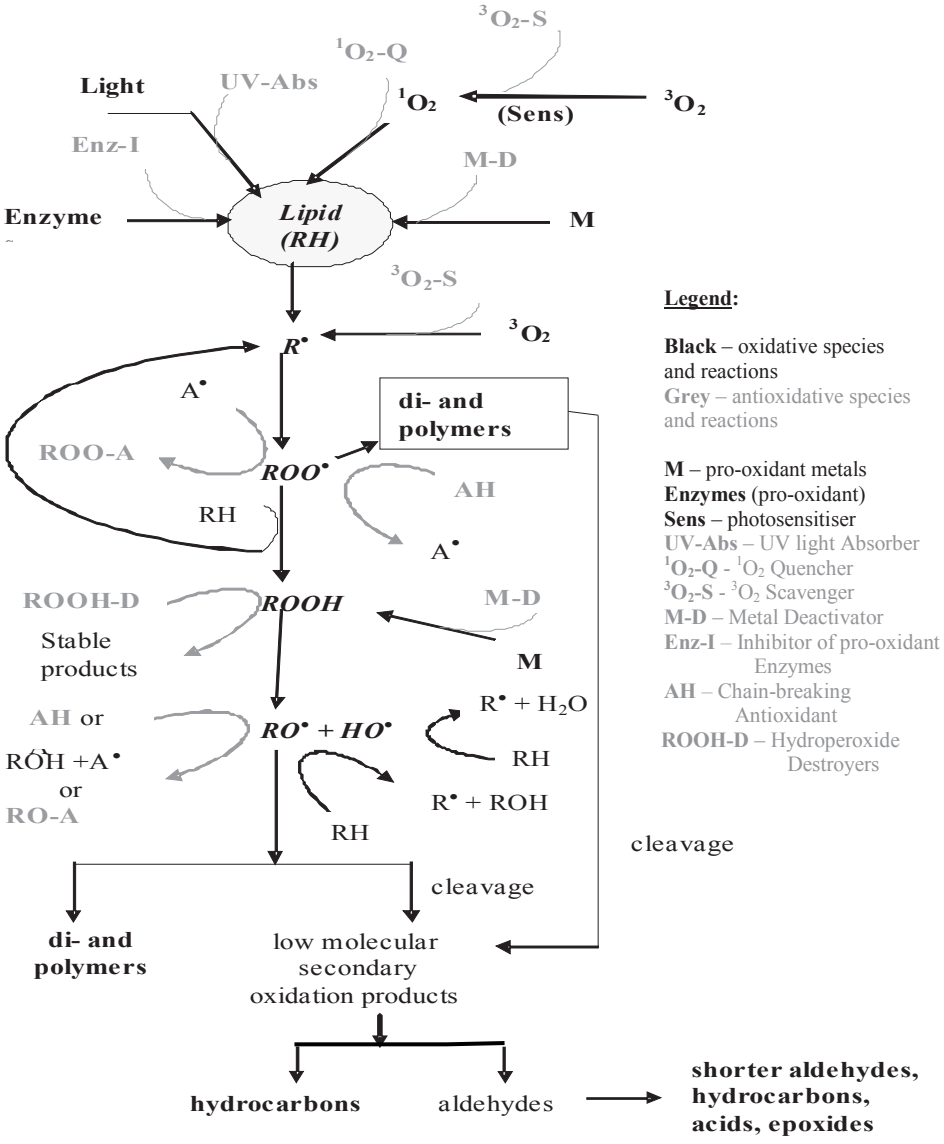
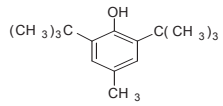


Figure 1.12 The "HACCP" in oxidation-antioxidation processes (the abbreviation "HACCP" is taken to show hazards and measures to inhibit them).

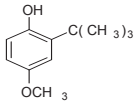
However, toxicological studies have implicated synthetic antioxidants as a possible health hazard (Barlow 1990; Evans & Reynhout 1992; Nieto et al. 1993; Madhavi & Salunkhe 1995). Superiority of "all-natural" antioxidants has intensified the search for novel antioxidants of natural origin. A great deal of natural substances and mixtures have been investigated and recognized as antioxidants. A huge amount of papers and extensive reviews can be found elsewhere (Larson 1988; Namiki 1990; Lőliger 1991; Shihidi & Wanasundara 1992; Okuda et al. 1994; Madsen & Bertelsen 1995; Rice-Evans et al. 1996; Decker 1998b; Robards et al. 1999; Pietta 2000; Moure et al. 2001). Some natural products have already been exploited commercially (Schuller 1990). Examples include not only ascorbic acid (**6**) and tocopherols (**5**) but also spices like rosemary, sage, thyme, oregano, turmeric, and curcuma (Schuller 1990; Shihidi & Wanasundara 1992; Madsen & Bertelsen 1995; Nakatani 1996; Madsen et al. 1997); soya beans (Pratt 1980; Schuler 1990; Shihidi & Wanasundara 1992); seeds such as grape seed, sesame, canary, cottonseed, rapeseed, chia, olive (Pratt 1980; Taga et al. 1984; Shukla et al. 1996; White & Xing 1996; Yamaguchi et al. 1999; Altarejos et al. 2005; Perez-Bonila et al. 2006); different teas (Cao et al. 1996; Ho et al. 1996; von Gadow et al. 1997a,b; Zandi & Gordon 1999) and wine (Kanner et al. 1994; Vinson & Hontz 1995; Williams & Elliot 1996; Sanchez-Moreno et al. 1999; Pellegrini et al. 2000). Others still require toxicological studies to prove their safety (Pratt & Hudson 1990; Madhavi & Salunkhe 1995).

Meanwhile, intensive research with various plants is on-going and many active compounds have been isolated and evaluated as antioxidants. In the majority of cases, the active ingredients are of a phenolic nature – *phenolic acids* (**8** − **12**) (Dziedzic & Hudson 1984; Cuvelier et al. 1992; Marinova & Yanishlieva 1992; Marinova & Yanishlieva 1994; Heilmann et al. 1995; Rice-Evans et al. 1996; Chen & Ho 1997), *flavonoids* (**14**, **15**) (Heilmann et al. 1995; Rice-Evans et al. 1996; Velioglu et al. 1998; Pietta 2000; van der Sluis et al. 2000; Moure et al. 2001), *isoflavones* (**16**) (Dziedzic & Hudson 1984; White & Xing 1996; Ruiz-Larrea et al. 1997; Hendrich et al. 1999), *catechins* (**17**) (Kanner et al. 1994; Sawai & Sakata 1998), *phenylpropanoids* (**19**) (Gao et al. 1999), *anthocyanidins* (Rice-Evans et al. 1995; Wang et al. 1999) and *chalcones* (Fig. 1.14) (Mathiesen et al. 1997). Other classes include hydrolyzable tannins, proanthocyanidins, lignans as well as biflavones, aurones, and coumarins (**18**) (Pietta 2000). Some compounds of terpenoid nature are also antioxidants (carnosic acid (**13**), carnosol) (Frankel et al. 1996). Because phenolics are widespread in the plant kingdom (approx. 5000 − 8000) (Cao et al. 1997; Robards et al. 1999; Pietta 2000), they are currently attracting the biggest attention. The chemical structures of selected synthetic and natural antioxidative compounds are given in Figures 13(a) and 13(b).

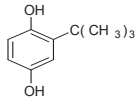
(a)



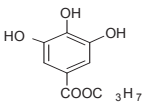
2,6-di-*tert*-Butyl-*p*-hydroxytoluene (BHT) **(1)**



2-*tert*-Butyl-*p*-methoxyanisole (BHA) **(2)**

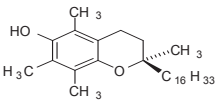


tert-Butylhydroquinone (TBHQ) **(3)**

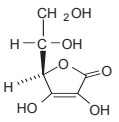


Propyl gallate (PG) **(4)**

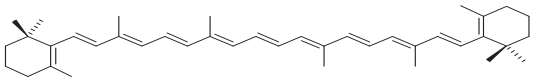
(b)



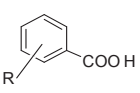
α-Tocopherol **(5)**



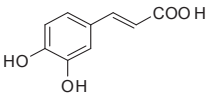
Ascorbic acid **(6)**



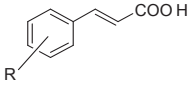
β-Carotene **(7)**



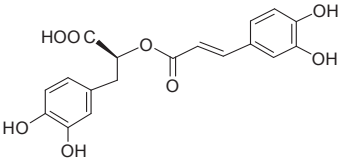
Benzoic acids **(8)**



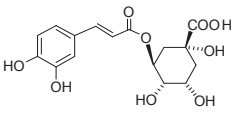
Caffeic acid **(9)**



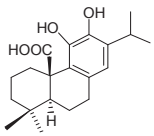
Cinnamic acids **(10)**



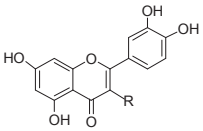
Rosmarinic acid **(11)**



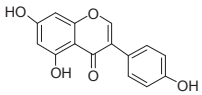
Chlorogenic acid **(12)**



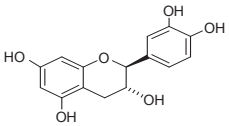
Carnosic acid **(13)**



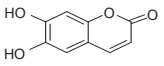
Quercetin **(14)**; R = OH
Rutin **(15)**; R = Rutinose



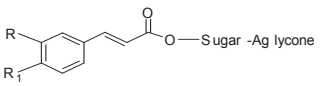
Genistein (isoflavonoid) **(16)**



(+)-Catechin **(17)**



Aesculetin **(18)**



general phenylpropanoid structure **(19)**

Figure 1.13 Selected **(a)** synthetic and **(b)** natural antioxidants.

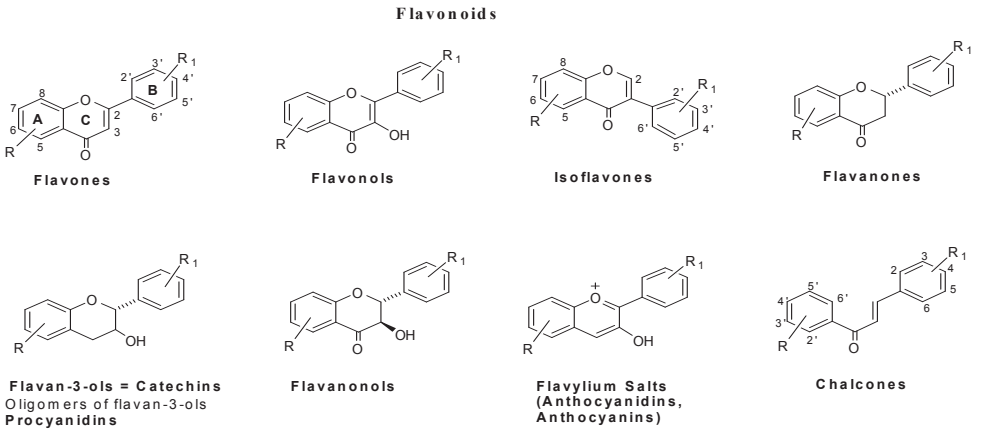


Figure 1.14 General structures of flavonoids (adapted from Frankel 1998g; Robards et al. 1999; Pietta 2000; Pannala et al. 2001).

The strong antioxidant properties of phenolics are determined by their electron-rich nature, which implies an easy electron donation followed by deprotonation of their phenolic hydroxyl groups or the other way around, first deprotonation and then electron donation (Frankel & Meyer 2000). The formed phenoxyl radical is stabilized by electron delocalization (Fig. 1.7), which is eased by the presence of conjugated systems such as the 2,3-double bond in conjugation with a 4-oxo function in the C-ring of flavonoids (Hudson & Lewis 1983, Rice-Evans 1995; Rice-Evans et al. 1996; Lien et al. 1999; Fukumoto & Mazza 2000; Pietta 2000) or the conjugated side chain in cinnamic acid derivatives (**10**) (Larson 1997e). The latter explains why cinnamic acid derivatives are stronger antioxidants than hydroxybenzoic acid derivatives (**8**) (Hudson & Mahgoub 1980; Fukumoto & Mazza 2000). In general, both the position and degree of hydroxylation (catechol structure in the B-ring) are important for the exhibited activity of flavonoids or phenolic acids (Robards et al. 1999; Fukumoto & Mazza 2000; Pietta 2000; Pannala et al. 2001). The presence of a 3-hydroxyl group in the heterocyclic ring increases the radical scavenging activity of flavonoids, like flavonols being more active than flavones. Flavanonols and flavanones are weak antioxidants because of the lack of conjugation with the 4-oxo group (see Fig. 1.14) (Fukumoto & Mazza 2000; Pietta 2000). Methylation or esterification (e.g. chlorogenic acid (**12**)) and glycosylation (e.g. rutin (**15**)) of one or more of the hydroxyl groups generally reduce the antioxidant abilities (Larson 1997e; Robards et al. 1999; Fukumoto & Mazza 2000; Pietta 2000). The opposite for glycosylation has also been observed (Robards

et al. 1999; Fukumoto & Mazza 2000). *Ortho*-dihydroxy groups like in some phenolic acids (**8 − 12**) and in the A- and B-rings of flavonoids, or hydroxyl groups at C-3 and C-5 with a 4-oxo function in the A- and C-rings of flavonoids enhance the antioxidant potential and enable metal chelation (Figs. 8 and 14) (Rice-Evans 1995; Larson 1997e; Robards et al. 1999; Pietta 2000).

In the plant kingdom, natural substances exist as mixtures, which ensure an efficient protection for the plant from oxidative damage or stress due to synergetic or additive effects (Pratt & Hudson 1990; Moure et al. 2001). Utilizing plant extracts or enriched fractions may present an attractive alternative for preservation of foods. In addition, many natural products (real antioxidants) have been found to exhibit other wholesome properties such as anticarcinogenic, immunostimulating or antiinflammatory properties (Alcaraz & Ferrandiz 1987; Bermond 1990; Madhavi et al. 1995). Indirect evidence is the established relationship between the low incidence of diseases such as cancer, atherosclerosis and cardiovascular diseases and the increased dietary in-take of fruits and vegetables rich in phenolics (e.g. the so-called "French paradox") (Renaud & de Lorgeril 1992; Cook & Samman 1996; Robards et al. 1999; Moure et al. 2001). However, it should be kept in mind that "natural" is not necessarily equivalent to "harmless". For example, some flavonoids have been found *in vitro* to be mutagenic (Sahu & Gray 1993; Sahu & Gray 1994). The biological and pharmacological properties of flavonoids depend on their antioxidant or pro-oxidant behaviour (Cao et al. 1997). It should be noted that, in general, the more hydroxyl substituents, the stronger the antioxidant activity but also the pro-oxidant activity.

Moreover, in *in vivo* studies more factors such as metabolic transformation and dietary interactions play a role than in *in vitro* studies in foods (Ghiselli et al. 2000). Any natural substance aimed to be used as food preservative should be toxicologically tested for its safety. Nowadays the authorities in many countries only allow antioxidants included in the so-called "Positive list" and approval of any new antioxidant requires intensive toxicological studies, including mutagenic, teratogenic and cancerogenic ones (Mikova 2001). For commercial application, a natural antioxidant mixture should also be "compatible" with the food i.e. not change its appearance, taste, flavour, odour or texture.

1.3 Evaluation of antioxidant activity and determination the level of lipid oxidation

A process or phenomenon is not of much value if it cannot be adequately measured and evaluated. Five main questions should be answered when oxidation and antioxidant activity is evaluated: which, what, how, when and where.

(This thesis; Halliwell 1995a)

In literature numerous methods and techniques for assessment of lipid oxidation and evaluation of antioxidant activity have been reported (Shervin 1968; Ragnarsson & Labuza 1977; Khal & Hilderbrant 1986; Namiki 1990; Frankel 1993b; Frankel 1998d,e; Tian & Dasgupta 1999; Frankel & Meyer 2000). In principle, if a compound is a poor antioxidant *in vitro,* it is unlikely to be any better *in vivo* (Hanasaki et al. 1994). That is why, assays concerning *in vitro* lipid oxidation and antioxidant effectiveness will be discussed here. Mechanisms of oxidation and its prevention in *in vivo* systems are different due to the cellular permeability of antioxidants and transport processes (Antolovich et al. 2002) and are not discussed in this thesis. The majority of the methods rely on objective chemical or physical processes and instrumentation and include: chromatographic like TLC, GC, HPLC and all varieties of these techniques; spectroscopic like UV/Vis, IR, fluorimetry, MS, NMR and electrochemical ones like polarography and voltammetry. Almost any analytical procedure requires a substantial pre-treatment of the sample before the actual measurement. The methods cover primary and secondary antioxidants.

Except for sensory analysis of foods, most methods measure a particular parameter at a single time (e.g. DPPH, ABTS, static head-space GC), other monitor the dynamics of oxidation (such as oxygen by AOM, Rancimat, oxygen uptake and bomb, weight gain; fatty acids by GC and HPLC; or free radicals by EPR, chemiluminescence). To speed up the analysis, elevated temperatures and/or increased access to air are applied. Storage at ambient temperatures is also used, as it is most representative for "real-life" circumstances. To simplify the experimental protocol or to facilitate theoretical studies, model or simplified test systems are used instead of authentic food: linoleic acid (Mahoney & Graf 1986; Roozen et al. 1994; Lavelli et al. 1999) and its methyl ester (Yamauchi et al. 1988; Bertelsen et al. 1995; Roginsky et al. 1996), β-carotene bleaching, citronellal (Bocco 1998), model radicals, liposome and microsome systems (Aruoma et al. 1989; Kansci et al. 1997; Plumb et al. 1997; Heinonen et al. 1998). Some analyses measure the amount of final or intermediate products generated through the oxidation (conjugated dienes or PV, TBARS test, total or selected carbonyls, anisidine value, head-space GC or sensory analysis). Because radical scavenging properties are of primary importance for an antioxidant

defense, a number of methods have been developed to evaluate the radical-trapping efficiency of antioxidants (ABTS, DPPH, DMPD, DBO, automated ORAC, TRAP, FRAP, cyclic voltammetry (Chevion et al. 1997), $O_2^{\bullet-}$ (Thornalley & Bannister 1985; Hanasaki et al. 1994; Quick et al. 2000; Toyo'oka et al. 2003). Different parameters have been elaborated for expressing the efficiency of antioxidants [e.g. TEAC (Miller et al. 1993; Cao & Prior 1998; Prior & Cao 1999; Re et al. 1999; Arnao 2000), % Inhibition (Yoshida et al. 1989; Rice-Evans & Miller 1994; Strube et al. 1997), EC_{50} (Brand-Williams et al. 1995), Antiradical Efficiency (Sanchez-Moreno et al. 1998), AUC (area-under-curve) (Cao & Prior 1998), lag-time (Anotolovich et al. 2002; Huang et al. 2005) and TAA (Arnao et al. 1996)]. A universal classification of all existing methods is not possible and different classifications for assays have been proposed (Frankel 1998d,e; Shahidi & Wanasundara 1998; Huang et al. 2005).

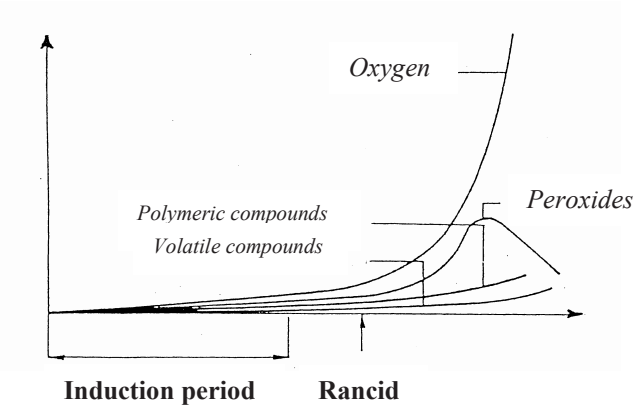


Figure 1.15 Dynamics of oxidation as a function of time, expressed by different oxidation products (based on Labuza 1971).

Depending on what we want to measure and evaluate in a given sample, the appropriate methods should be chosen, which is illustrated by the dynamics of oxidation in time, expressed for different oxidation products in Figure 1.15 (Labuza 1971). For example, the amount of accumulated hydroperoxides is to be measured before they start to break down. After the hydroperoxide decomposition has commenced, the rancidity of a sample has to be estimated on the basis of volatiles or other secondary oxidation products formed. Each assay possesses a range of abilities and suffers from specific drawbacks. The advantages and limitations of some of the methods together with relevant references are

presented in Table 1.2. It is clear that it is impossible to develop a test method, which will satisfy all requirements and provide for a comprehensive picture of the entire oxidation/antioxidation phenomena, and be applicable to all types of oxidisable substrates and antioxidants. Furthermore, one has to keep in mind that a pro-oxidant behaviour is often possible (Fukumoto & Mazza 2000). The need of using combinations of complementary methods has been established by many researchers and many relevant studies have been published (Sherwin 1968; Khal & Hilderbrant 1986; Frankel 1993b; Halliwell 1995b; Larson 1995; Robards et al. 1999; Frankel & Meyer 2000; Halliwell & Whiteman 2004; Huang et al. 2005

Table 1.2 Advantages and limitations of methods for estimation of lipid stability and evaluation of antioxidant activity (references in this Table are arranged according to year of publication).

Method	Advantages	Limitations	References
Stability tests of oils/fats			
Storage: • *at ambient temperature*	+The most relevant to real-life conditions; +No equipment is needed;	–Very slow; –Not very reproducible;	Ragnarsson & Labuza 1977; Ragnarsson et al. 1977; Economou et al. 1991; Wada & Fang 1992; Vekiari et al. 1993; Kim et al. 1994; Frankel 1998e.
• *at elevated temperature*	+Speeded up in comparison to the above technique; +Common equipment.	–Possible loss of volatile or thermally labile antioxidants; –Changed oxidation mechanism; –Thermal alterations of lipids that are not relevant to the real storage conditions may occur.	
Weight gain	+Simple performance; +Common equipment; +Satisfactory reproducibility.	–Not very sensitive; –Formation of volatile products affect the obtained results; –Accurate control of the experimental parameters to minimize their influence on the	Wanasundara & Shahidi 1994; Pokorny et al. 1997; Ackoh & Min 1998; Frankel 1998e.

		results is needed; –Laborious.	
Rancimat and Active Oxygen Method (AOM)	+Rancimat is automated; +Measure the rate of oxidation.	–Questionable end-point; –Rancimat requires high levels of oxidation; –Loss of volatile or thermally labile antioxidants; –Unreliable results due to hydroperoxide decomposition and side reactions of the lipid → not suitable for easily oxidized lipids whose hydroperoxides readily decompose; –Sensitive to small variations in oxygen pressure; –Large sample size is needed; –Low relevance to flavour scores; –No relation to the oxidation under normal storage conditions; –Time-consuming; –Requires specific equipment (Rancimat).	Ragnarsson & Labuza 1977; Ragnarsson et al. 1977; Laubli & Bruffel 1986; Obretenov et al. 1986; Matthaus 1996; Mendez et al. 1996; Ackoh & Min 1998; Frankel 1998e; Schwarz et al. 2001.
Schaal oven	+Relatively mild oxidation and fewer problems in comparison to the other accelerated tests; +Good relevance to the real shelf-life; +No need of special equipment.	–Slow; –Large sample size is needed.	Ragnarsson & Labuza 1977; Pazola et al. 1990; Mendez et al. 1996; Frankel 1998e; Kovatcheva et al. 2001.
Oxygen uptake	+Small samples can be studied; +Measures the rate of oxidation; +Good correlation between the rate of oxygen consumption and	–Requires high level of oxidation; –Not very sensitive; –Requires special instrumentation.	Ragnarsson et al. 1977; Pryor et al. 1988; Mikkelsen & Skibsted 1992; Jorgensen & Skibsted 1993; Pryor et al. 1993; Frankel 1998e; Tian &

	storage time → can be used for shelf-life prediction.		Dasgupta 1999; Huang et al. 2005.
Oxygen bomb	+Excellent reproducibility; +Measures the rate of oxidation; +Smaller sample required.	–The temperature is too high (99 °C); –The degree of oxidation depends on the % PUFA in the lipid; –Unwanted contaminants from the tissue paper used for the sample.	
Methods for oxidative changes & evaluating antioxidants			
Sensory panel tests	+Best related to the quality and consumer acceptability of the food; +Sensitive.	–Requires specially trained panelists and suitable premises; –Results depend on the experience and sensitivity of the panelists; –Results are subject of misunderstanding due to the lack of common descriptive vocabulary; –Results depend on the threshold values of the analysed volatiles; –Poor reproducibility; –Low precision.	Burkow et al. 1992; Frankel 1998d,e; Madsen et al. 1998; Mahrour et al. 1998.
HPLC analysis of the changes in fatty acid composition or HPLC of formed hydroperoxides	+Very sensitive; +Reproducible; +Provides qualitative and quantitative information for the fatty acids involved in the oxidation or the formed hydroperoxides; +Oxidation of easily oxidized lipids may be monitored; +No need of preliminary derivatization of fatty acids or sample preparation.	–Good knowledge about the nature and retention characteristics of the analysed compounds is needed; –Requires relatively expensive instrumentation.	Gebicki & Guille 1989; Christensen & Holmer 1996; Hopia et al. 1996; Guo et al. 1997; Frankel 1998e; Yamaguchi et al. 1998.

GC analysis of the changes in fatty acid composition. Headspace GC of secondary volatiles	+Very sensitive; +Reproducible; +Provides qualitative and quantitative information for the fatty acids involved in the oxidation; +Oxidation of easily oxidized lipids may be monitored; +Oxidation may be followed in static or dynamic model; +Very good correlation with the results from sensory analysis.	–Requires sophisticated instrumentation, especially for the analysis of volatiles (a headspace device); –Preliminary sample preparation such as fatty acid derivatization is needed, which may affect the precision and accuracy of the quantitative analysis; –Low precision.	Roozen 1987; Frankel & Gardner 1989; Frankel et al. 1989; Frankel 1993a; Frankel et al. 1994; Takacsova et al. 1995; Hopia et al. 1996; Frankel 1998d,e; Anotolovich et al. 2002; Koleva et al. 2003.
• *static*	+Relatively rapid → automatic performance → routine analysis of a large number of samples;	–Not suitable for compounds, that decompose during the equilibration step in the static headspace GC;	
• *dynamic*	+Direct analysis of complex samples without special pretreatments; +Capable to detect trace amounts; +Sample subjected to lower-temperature heating than in the static version.	–Dynamic headspace GC is slow and not suitable for routine analyses → requires much time- and labour input.	
Peroxide value (PV)	+Provides information about the concentration of the formed hydroperoxides; +Suitable for bulk oils.	–Not suitable to study the advanced oxidation phase when hydroperoxides start to decompose → not very suitable for easily oxidized lipids; –Not suitable for emulsions or biological materials; –Not very sensitive and selective; –Low results due to possible addition of iodine across	Gebicki & Guille 1989; Takao et al. 1994; Yen & Chen 1995; Zhang et al. 1996; Akoh & Min 1998; Frankel 1998d,e; Kovatcheva et al. 2001; Anotolovich et al. 2002.
• *Iodometric titration*	+Routine analytical protocol and common reagents; +Relatively precise; +Relatively		

• *Ferric thiocyanate colorimetry*	sensitive. +More sensitive than iodometric titration; +Requires smaller-size sample than for iodometric titration.	unsaturated bonds; –Oxidation of iodide by dissolved oxygen; –Variations of reactivity of peroxides; –Requires relatively high level of oxidation → not suitable for the very first moments of oxidation when the hydroperoxide concentration is low; –The correlation between the PV and the onset of rancidity is not always good → needs cautiousness in shelf-life prediction; –Provides information only for the current oxidative status of the sample but not about its potential to oxidise; –Requires relatively large samples; –Experimental conditions (e.g. light, operator's skillfulness, time) may strongly affect the results; –Chemical structure and reactivity of ROOH may influence the results.	
Conjugated dienes	+Sensitive; +Reproducible; +Results do not depend on chemical reactions; +Faster than PV; +Simple; +Selective if using HPLC or matrix subtraction using second-derivative spectroscopy; +Measures only early stage in the oxidation; +Requires small sample size; +Uses common spectrophotometric	–Other chromophores absorbing in the same spectral region (e.g. carotenes) present in the sample may interfere; –Requires mild oxidation of the sample in order to prevent hydroperoxide decomposition; –Not suitable for hydrogenated or deodorized oils since they contain conjugated di- or three-enes; –Generic	Pryor et al. 1993; Kanner et al. 1994; Akoh & Min 1998; Frankel 1998d,e; Madsen et al. 1998; Wettasinghe & Shahidi 1999; Anotolovich et al. 2002.

	equipment; +Suitable for both bulk oils and emulsions.	measurement → little info about the structure of the compounds.	
TBARS. Total or selected carbonyl compounds	+Simple; +Sensitive, especially for polyunsaturated lipids; +Selective if using HPLC → characterization the individual species; +Precise; +Uses common spectrophotometric equipment; +Good correlation with the sensory analysis of some vegetable oils.	–Not very sensitive for mono- and diunsaturated lipids; –Not specific as other sample components may form red-coloured products with the TBA; –Reaction conditions have effected color development; –Strong influence of the experimental conditions (light, temperature, time of heating, presence of metal ions or certain compounds such as amines, etc.); –Some experimental protocols can be time-consuming or provoke formation of artifacts.	Yang et al. 1991; Jacobson 1993; Akoh & Min 1998; Frankel 1998d,e; Miura et al. 1998; Moller et al. 1999; Anotolovich et al. 2002.
p-Anisidine value	+Precise; +Both volatile or non-volatile carbonyls can be analysed; +Good correlation with the flavour acceptability scores.	–Less sensitive than GC; –Other sample compounds may interfere either with the reaction or the absorption.	Akoh & Min 1998; Frankel 1998d,e; Zandi & Gordon 1999.
TOTOX (2PV + AnV)	+Present state i.e. PV + past history i.e. *p*-AnV.	–Lacks sound scientific basis → 2PV + TBA.	Akoh & Min 1998; Frankel 1998d,e.
β-Carotene bleaching	+Simple; +Relatively rapid; +Sensitive; +Uses common spectrophotometric instrumentation.	–Not specific; –Strong influence of the experimental conditions and other sample components; –Oxidizable substrate is not representative for real lipids → the obtained data are not directly related to the actual oxidation; –Mechanism of oxidation-antioxidation is not	Marco 1968; Miller 1971; von Gadow et al. 1997a,b; Dapkevicius et al. 1998a,b; Frankel 1998e; Fukumoto & Mazza 2000; Koleva et al. 2003.

		clear; –Reliable results are limited to less polar antioxidants.	
Fluorescent analysis of ROOH or secondary oxidation products	+Very sensitive; +Suitable for complex food and biological systems.	–Not specific; –Requires special instrumentation.	Porter et al. 1989; Miyagawa et al. 1991; Arora & Strasburg 1997; Akoh & Min 1998; Frankel 1998d.
Other methods for evaluation of antioxidants			
Chemiluminescence	+Very sensitive; +Ability to detect low levels of oxidation → oils can be easily studied with no need of elevated temperature; +Suitable for complex food and biological systems; +Reproducible; +The scavenging activity toward radical species and reactive oxygen species that are generated in real systems can be evaluated; +Can be combined on-line with HPLC for large-scale screening of complex samples.	–Requires special instrumentation; –Uses relatively expensive and not very stable reagents (e.g. enzymes).	Yamamoto et al. 1985; Cash et al. 1988; Yang et al. 1991; Burkow et al. 1992; Whitehead et al. 1992; Nakano 1994; Niederländer 1994; Popov & Lewin 1994; Matthaus 1996; Navas & Jimenez 1996; Escobar et al. 1997; Kalitchin et al. 1997; Akoh & Min 1998; Dapkevicius et al. 1999.
Radical-trapping methods employing stable model radicals (e.g. DPPH$^{\bullet}$, ABTS$^{\bullet+}$, DMPD, DBO, etc.)	+Sensitive; +Reproducible; +Selective; +Rapid; +Simple and routinely manageable; +Robust; +Easy; +Use common spectrophotometric instrumentation and easily available reagents; +Can be combined on-line with HPLC for large-scale screening of	–Employs model radical species not encountered in real systems → no direct correlation with the oxidation-antioxidation mechanisms in real systems; –Does not employ lipid substrate → no information about which lipid system a tested antioxidant will be efficient for; –DPPH$^{\bullet}$ → depends on compounds's structural	Blois 1958; Arnao et al. 1995; Brand-Williams et al. 1995; Rice-Evans et al. 1995; Campos & Lissi 1996; Cao et al. 1996; Bondet et al. 1997; Miller & Rice-Evans 1997; Strube et al. 1997; Cao & Prior 1998; Cano et al. 1998; Nau 1998; Sanchez-Moreno et al. 1998; Fogliano et al. 1999; Prior & Cao 1999; Re et al. 1999; Gil et al 2000; Koleva et

	complex samples; +Kinetic studies are possible; +High resolution for DBO; +Non-invasive measurements; +High photostability for DBO; +DBO provides direct experimental info on the absorbance reactivity or concentration of antioxidants; +Applicable for heterogeneous biological systems.	conformation; –More expensive - ABTS-commercial kits (~ 9 times than ORAC).	al. 2000; Koleva et al. 2001; Anotolovich et al. 2002; Sanchez-Moreno 2002; Schwarz et al. 2001; Koleva et al. 2003; Huang et al. 2005.
TRAP (total radical trapping antioxidant parameter)	+Precise if using CL; +Automated if using CL.	–Oxygen electrode will not maintain its stability till the end-point; –Time-consuming (up to 2 hours/sample).	Wayner et al. 1985; Ghiselli et al. 1995; Anotolovich et al. 2002.
FRAP (ferric ion reducing antioxidant power)	+Simple; +Convinient; +Inexpensive.	–Does not measure the SH-group-containing antioxidants; –Measuring reducing capacity does not necessarily reflect antioxidant activity.	Benzie & Strain 1996; Cao & Prior 1998; Benzie & Strain 1999; Anotolovich et al. 2002.
ORAC (oxygen radical absorbance capacity)	+Specific; +Responds to numerous antioxidants.	–Requires fluorescence detector → long time of analysis (~ 60 min.); –Not linear with time; –Oxygen electrode endpoint → imprecision.	Glazer 1990; Cao et al. 1993; Pieri et al. 1994; Cao et al. 1996; Wang et al. 1996; Cao et al. 1997; Guo et al. 1997; Wang et al. 1997; Cao & Prior 1998; Sanchez-Moreno 2002; Anotolovich et al. 2002; Huang et al. 2005.
EPR (ESR) [electron para(spin) resonance]	+The only analytical technique that can detect the free radicals involved in autoxidation; +Detects and evaluates short-	–Requires sophisticated instrumentation; –Specialist nature; –Direct application to lipid oxidation system is hampered by the short life of many	Yang et al. 1991; Dodd 1995; Bors et al. 1996; Milic et al. 1998; Akoh & Min 1998; Sawa et al. 1999; Wettasinghe & Shahidi 1999; Yamaguchi 1999;

	lived radical species; +Selective.	radical species; –Special techniques such as freezing and spin trapping are needed to achieve measurable concentration of free radicals → trapping efficiencies differ for various radicals; –Spin traps can have oxidant and antioxidant action, while spin adducts can act as antioxidants; –Insensitive → only fairly unreactive radicals (in biomolecules).	Anotolovich et al. 2002.
IR (infrared)	+Monitors formation/disappearance of certain oxidation products (ROOH, carbonyls, acids, etc.); +Requires very small amount of sample; +Simple; +Rapid; +After calibration, it may serve for determination of PV or anisidine value.	–Not very specific; –Not very sensitive.	Sedman et al. 1996; Akoh & Min 1998.
^{1}H-NMR (nuclear magnetic resonance)	+Non-destructive; +Requires very small amount of sample; +Rapid; +Reliable; +Data from ^{1}H-NMR correlate with TOTOX.	–The changes of fatty-acid profiles reflect both primary and secondary oxidation products; –Not very specific; –Not very sensitive.	Shahidi & Wanasundara 1996; Akoh & Min 1998.

1.4 Genus *Sideritis* - a brief botanical, chemical and pharmacological profile.

"The sigh of Euridice you are.
Or the kiss of song -
A sip of you tames the heart,
Opens chest with no words
And swiftly flies the mind
To distant worlds……."
("Pirin tea", Alikovski 1999)

1.4.1 Botanical characteristics.

The genus *Sideritis* was first described by C. Linnaeus in 1737 (Gonzales et al. 1979). It belongs to the Labiatae (Lamiaceae) family and counts about 150 species (Tomas-Barberan et al. 1988a; Barber et al. 2002). *Sideritis* plants are either annual or perennial xerophite, thermophyte, calciphyte, chasmophyte or heliophyte plants, and grow on dry, sunny mountainous or flat areas (Evstatieva & Vasilev 1995). They are mainly distributed throughout the Mediterranean region, the Balkan peninsula and North Africa. Four species are encountered in Bulgaria: *S. scardica* Griseb. and *S. syriaca* L. (perennial), *S. montana* L. and *S. lanata* L. (annual). *S. lanata* is a very rare species. *S. scardica* is endemic in the Balkan peninsula and is known by the trivial name "Pirin mountain" or "Mursalski" tea and is under governmental protection since its natural resources are almost exhausted (Evstatieva 2002). *S. syriaca* (known before 1989 as *S. taurica* Steph. ex Wild.) (Evstatieva & Vasilev 1995) has limited distribution in Bulgaria. In 1984 it has been included in the "Red Book of Bulgaria" as a species, threatened by extinction (Evstatieva & Vasilev 1995).

The taxonomy of the genus is rather complex because of the interspecific hybridization and therefore it has not been satisfactorily resolved (Koedam 1986; Tomas-Lorente et al. 1989). On the basis of pollen features, Heywood has divided the Labiatae into two subfamilies – Lamioideae and Nepetoideae (Koedam 1986; Tomas-Barberan & Wollenweber 1990). The position of the genus *Sideritis* is illustrated in Figure 1.16 (only the species encountered in Bulgaria are mentioned). Plants from the subfamily Lamioideae are characterized by a low concentration of essential oils, a lack of rosmarinic acid and the presence of iridoid glycosides, whereas Nepetoideae plants are rich in essential oils, contain some rosmarinic acid and lack iridoids (Tomas-Barberan et al. 1988b; Tomas-Barberan & Wollenweber 1990).

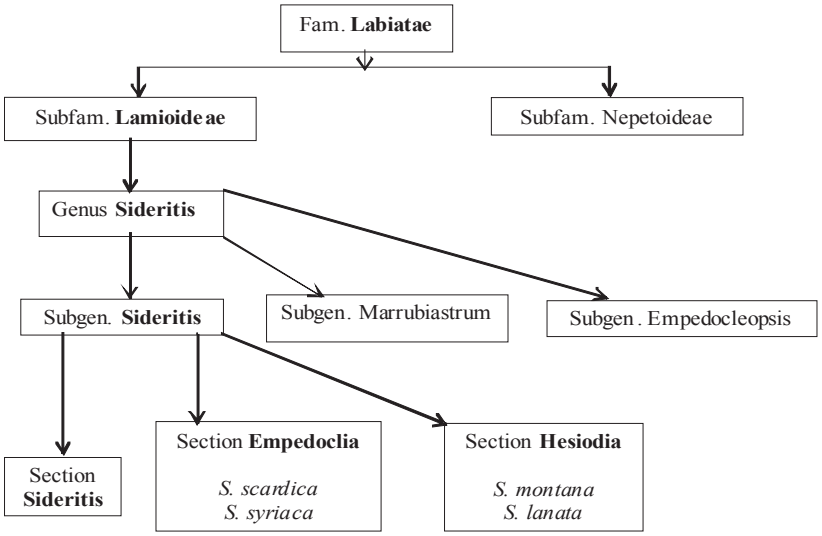


Figure 1.16 "Botanical tree" of *Sideritis*.

1.4.2 Chemical composition.

On a worldwide basis there is a rich literature concerning investigations on components in *Sideritis* plants. The reports usually concern Spanish, Italian, Greek and Turkish populations. The investigations on Bulgarian *Sideritis* populations are limited to a few reports (Sattar et al. 1993; Evstatieva & Vasilev 1995; Sattar et al. 1995a; Sattar et al. 1995b; Koleva & Handjieva 1997; Evstatieva & Koleva 2000; Todorova et al. 2000; Evstatieva 2002).

The main secondary metabolites are either diterpenoids or flavonoids. The variety of **diterpenoids** isolated from different *Sideritis* species is enormous. In *Sideritis* species growing in Bulgaria, only siderol (*ent*-7α-acetoxy-kaurene-15-en-18-ol) (**20**) has been found (Sattar et al. 1995b). From *S. syriaca* ucriol, siderone and some other diterpenoids of the *ent*-kaurene-isokaurene type have been isolated although not isolated from Bulgarian species (Venturella et al. 1983a,b). From *S. scardica* siderol (**20**) and five other diterpenoids, which have not yet been found in Bulgarian *Sideritis* species, have been isolated (Venturella & Bellino 1979) (also see for more information Table 1.3).

Triterpenoids, such as ursolic (**21**) / oleanolic acid, squalene, α- and β-amyrin and glutinol, have been isolated and identified in *Sideritis*. **Sterols** have been found in some

Sideritis species, including those distributed in Bulgaria but so far they have not been isolated from Bulgarian species. A few **iridoid glycosides** − ajugol (**22**), ajugoside (**23**), harpagide (**24**), 8-acetyl harpagide (**25**), melittoside (**26**), have been found in *Sideritis* species including *S. syriaca, S. scardica* and *S. montana* (Koleva & Handjieva 1997; also see for more information Table 1.3).

Plants from the *Sideritis* genus possess pleasant odours, which indicate the presence of **essential oils**. The content differs depending on the species and on other factors that have influence on the essential oil composition. In general, the essential oil content of *Sideritis* species varies from 0.01% – 0.85%. A great number of mono- and sesquiterpenes have been identified in these oils. In *S. syriaca* α-pinene, carvacrol, thymol, caryophylene, palmitic acid and oleic alcohol have been found. In *S. scardica,* besides the previously mentioned, also β-pinene and *p*-cymene have been found. *S. syriaca* and *S. scardica* possess β- and α-pinene-rich oils (Kirimer et al. 2004). The only study on the essential oil from Bulgarian *Sideritis* populations (Todorova et al. 2000) shows that myrcene is the major constituent of *S. syriaca* whereas sesquiterpenes dominate in the essential oils from *S. montana* and *S. scardica.* Plants containing high concentrations of essential oils generally do not contain iridoids or their concentration is very low (Tomas-Lorente et al. 1988) (also see for more information Table 1.3).

The literature abounds with reports on the flavonoid composition of different *Sideritis* species which is partially due to the importance of flavonoids as chemotaxonomic markers (Harborne et al. 1986; Ferreres et al. 1987; Tomas-Barberan et al. 1988a; Tomas-Barberan et al. 1990; Gil et al. 1993; Fraga et al. 1995). Aglycones and glycosides, mainly of the flavone type have been isolated. Only two papers report the isolation of flavonols, flavanones or their glycosides (Tomas-Barberan 1986; Fernandez et al. 1988).

Flavone aglycones can be described as lipophilic, externally located hydroxylated aglycones (Gil et al. 1993). They provide for UV screening, are a chemical barrier against pathogens and serve to ensure an adaptation mechanism for life in (semi-)arid or alpine habitats (Tomas-Barberan et al. 1988a; Gil et al. 1993). The latter is especially applicable to *Sideritis* plants. However, an interesting relationship has been established between the occurrence of externally located flavonoid aglycones or terpenoids and the morphology of plant leaves. Plants, which are covered with a dense mat of hairs, are devoid of external flavonoids and terpenoids since the hairs play the same role as the aglycones, namely a better adaptation to dry habitats (Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Barberan & Wollenweber 1990). The reports concerning the presence of external flavonoids in species encountered in Bulgaria are limited to *S. montana*. This is expected as its leaves are not hairy, this in contrast to the other two species. Plants from the *Sideritis*

genus produce 5-OH-6,7-OMe flavones and 5-OH-6,7,8-OMe flavones (Tomas-Barberan & Wollenweber 1990). Flavonoid aglycones were presented as the most suitable of all plant constituents as taxonomic markers, since they are chemically stable and very little affected by physiological conditions (Tomas-Barberan et al. 1985a). Tomas-Barberan et al. (1985b) have established that *Sideritis* plants can be divided qualitatively according to their aglycones into two groups depending on the solvent used in extraction (polar or apolar). Apolar solvents such as petrol extract highly methoxylated flavones, EtOH extracts highly and/or less methoxylated flavones, and aqueous-alcohol mixtures extract the more polar flavones. Thus, the division is as follows: *Group I* – with sideritoflavone as principal component and trace amounts of phloroglucinol-based flavones; *Group II* – with phloroglucinol-based flavones as the major constituents. With regard to the evolutionary status of the flavonoid characters Group II, to which belongs *S. montana,* appears to be more primitive than Group I (also see for more information Table 1.3).

Flavonoid glycosides are located in plant vacuoles. Under stress conditions, specific enzymes are released, which liberate the protective aglycones by hydrolysis of the glycosidic part. The presence of specific flavonoid aglycones and glycosides has been extensively studied for chemotaxonomic purposes. Tomas-Barberan et al. (1988a) and Gil et al. (1993) have suggested to divide the *Sideritis* genus into three subgenera, which would cover the Mediterranean and Atlantic region on the basis of the flavonoids present: *(1)* species, containing 5,7-dihydroxy flavone 7-O-glycosides; *(2)* species, containing 8-hydroxy flavone 7-allosylglucosides and *(3)* species, containing flavonoid *p*-coumaroyl glucosides (relatively lipophilic). Sattar et al. (1993, 1995a) have reported the isolation of four flavonoid glycosides from Bulgarian populations of *S. scardica* and *S. syriaca* (see for more information Table 1.3).

Only four reports concern the isolation of **phenylpropanoid glycosides** (e.g. verbascoside (acteoside) (**29**), leucosceptoside A, martynoside, etc.) from *Sideritis* species. The presence of **phenolic acids** is minor and limited to caffeic (**9**), chlorogenic (**12**), *p*-hydroxybenzoic (**8**) and ferulic acids (**33**). Isolated reports concern the occurrence of a **lignan** ((+)-sesamin (**27**) - attracts insects) and **coumarins** (siderin (**28**), xanthotoxin) in some Spanish *Sideritis* species (for the structures see Figs. 1.13(b) and 1.17). By chemical screening the presence of catechuic **tannins** and **saponins** has been established. **Polyhydroxynaphtoquinone** and **lactones** have also been found. Some *Sideritis* species including *S. montana* have been studied for **fatty acids**. In *S. scardica* the presence of **minerals** has been reported and that is why the English name for this drug is “ironwort”. **Alkaloids** and other basic N-substances have been found only in *S. montana* but the data are from 1969 and need to be confirmed (see for more information Table 1.3).

Since the variety in chemical structures and the number of isolated ones is enormous, summarised information on the types of compounds found in *Sideritis* is presented together with the relevant references in Table 1.3. The chemical structures of selected compounds, encountered in species present in Bulgarian flora and some other important structures are presented in Figure 1.17.

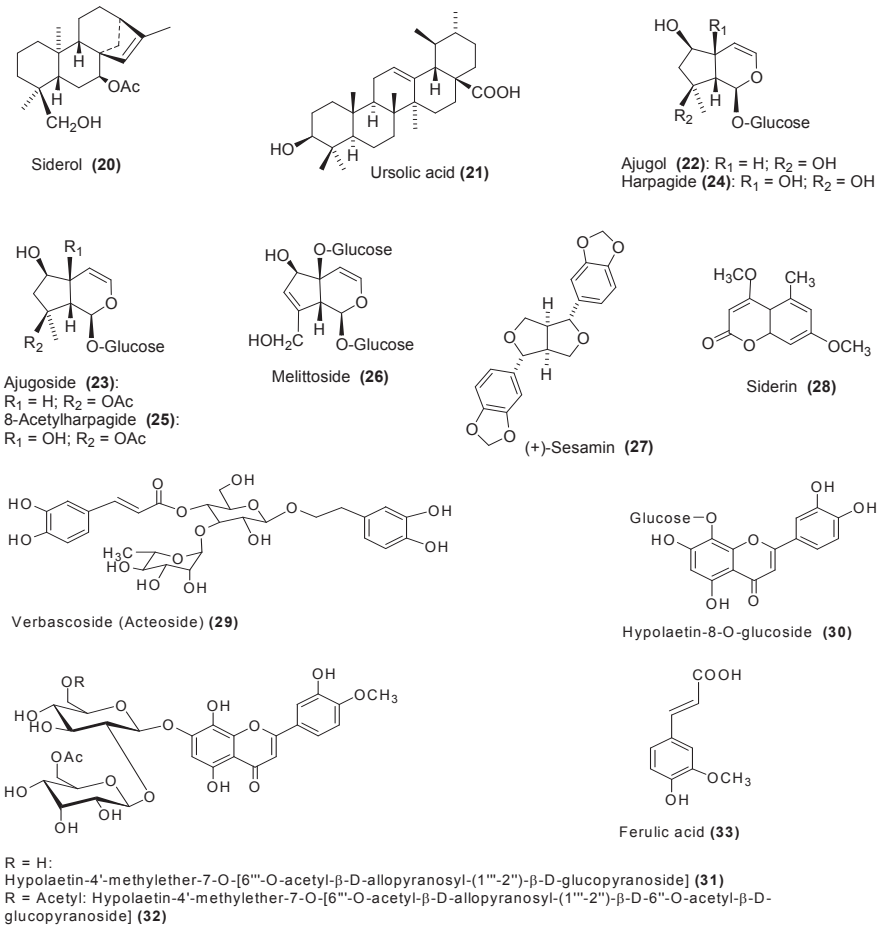


Figure 1.17 Chemical structures of selected compounds.

1.4.3 Biological properties of Sideritis components and extracts.

Sideritis plants have been used in folk medicines in many countries for a long time. Wholesome properties of the investigated *Sideritis* species can be summarized into three main beneficial categories: **antimicrobial**, **antiinflammatory** and **antioxidant**.

Antimicrobial activity of *Sideritis* plants is attributed to either the essential oil or the terpenoids. The relationship antimicrobial activity − essential oil composition has been studied (Villar et al. 1986; Gergis et al. 1991). Sesquiterpenic alcohols or carvacrol are the major constituents of those volatile fractions with the greatest antimicrobial potency. In general, the antimicrobial activity has been greater against Gram−positive than against Gram−negative bacteria. Aligiannis et al. (2001) have investigated the antimicrobial and antifungal activity of some species among them *S. syriaca* and found that carvacrol was the main constituent and responsible for the activity. The relationship antimicrobial activity − diterpenoid structure has been studied (Diaz et al. 1988a; Rodriguez-Linde et al. 1994) (data in Table 1.4). Not only pure compounds have been tested for such activity but different extracts – in petrol, C_6H_{14}, $CHCl_3$, EtOEt, EtOAc, CH_3COCH_3, BuOH, EtOH, MeOH, H_2O (Table 1.5). With regard to Bulgarian species and some other varieties of *S. syriaca,* distributed in the Balkan peninsula, all of them showed moderate antimicrobial activity (Sattar et al. 1995b).

Antiinflammatory activity is in most cases assigned to diterpenoids or flavonoids. The antiinflammatory properties of hypolaetin 8-glucoside (**30**) have been studied in detail (see Table 1.4). In addition it has analgesic, antiarthritic and antiulcer potencies. Antiinflammatory activity has also been studied for various extracts and fractions from *Sideritis* species (Table 1.5). Usually it concerns extracts or fractions that contain terpenoids (see for more information Tables 1.4 & 1.5).

The presence of flavonoids in *Sideritis* plants suggests they might possess **antioxidant activity**. At the start of this research in 1998 only few reports were available. Recently, intensive research has been carried out into this direction (see Tables 1.4 & 1.5). Demo et al. (1998) have investigated the content of tocopherols in some Mediterranean plants including *S. syriaca.* In all of them except *S. syriaca* tocopherols have been found.

Although *Sideritis* plants growing in Bulgaria are used in Bulgarian folk medicine, their pharmacological properties have not been well investigated and their active principles have not been isolated and determined. With exception of one report on the antimicrobial/antiviral activities of leaf exudates from several Lamiaceae plants including *Sideritis* species growing in Bulgaria (Sattar et al. 1995b), no other data could be found. Recently, Ivanova et al. (2005) have investigated aqueous extracts of 25 medicinal plants from several genera including *S. scardica* using ABTS and TEAC. There is a strong need for investigations of antioxidant activity of extracts and of pure compounds by multifunctional methods of Bulgarian species.

Table 1.3 Chemical constituents of *Sideritis* plants (references in this Table are arranged in alphabetical order).

Chemical type of compounds[a]	References
Terpene type	
Essential oils	Adzet et al. 1989a; Adzet et al. 1989b; Adzet et al. 1990; Aligiannis et al. 2001[sy]; Baser et al. 1996a; Baser et al. 1997[sc]; Burzaco et al. 1992; Ezer et al. 1995a; Ezer et al. 1996; Flamini et al. 1994; Galati et al. 1996; Gergis et al. 1989; Kirimer et al. 1992a; Kirimer et al. 1992b; Kirimer et al. 1994; Kirimer et al. 1996; Kirimer et al. 1999; Kirimer et al. 2000[sy,sc,m]; Kirimer et al. 2001a; Kirimer et al. 2001b; Kirimer et al. 2003; Kirimer et al. 2004[sc,sy]; Kocabas & Karaman 2001[sy]; Koedam 1986; Kokkalou 1987[sc]; Komaitis et al. 1985; Komaitis et al. 1992[sc]; Laer et al. 1996[sy]; Manez et al. 1991; Mateo et al. 1983; Mateo et al. 1988; Menkovic et al. 1991[sc]; Moron et al. 2005; Ozcan 2005; Ozcan et al. 2001; Ozek et al. 1993; Pulatova & Aminov 1969[m]; Rodriguez-Garcia et al. 2004; Schulz et al. 2005; Tabanca et al. 2001; Tabanca et al. 2001; Tirillini et al. 2001[sy]; Todorova et al. 2000[sy,sc,m]; Tumen et al. 1995; Tzakou 2002; Villar et al. 1984a; Villar et al. 1984b; Villar et al. 1985a; Zafra-Polo & Blazquez 1989
Iridoids	Pakaln et al. 1976; Pulatova & Aminov 1969[m]; Taskova et al. 1997
8-Acetyl harpagide **(25)**	Koleva & Handjieva 1997[sy,sc,m]; Kooiman 1972[sy,m]; Zinchenko et al. 1972[m]; Hegnauer & Kooiman 1978
Ajugol **(22)**	Koleva & Handjieva 1997[sy,sc,m]; Akcos et al. 1998
Ajugoside **(23)**	Koleva & Handjieva 1997[sy,sc,m]; Ezer et al. 1995a
Harpagide **(24)**	Koleva & Handjieva 1997[sy,sc,m]; Kooiman 1972[sy,m]; Zinchenko et al. 1972[m]; Hegnauer & Kooiman 1978
Melittoside **(26)**	Koleva & Handjieva 1997[sy,sc,m]; Kooiman 1972[sy,m]; Hegnauer & Kooiman 1978
Diterpenoids [bicyclic, tetracyclic (kaurene type), trachylobane, pentacyclic]	Aboutabl et al. 2002; Alcaraz & Ferrandiz 1990; Alcaraz et al. 1989a; Algarra et al. 1983; Ayer et al. 1974, Baser et al. 1996b; Breton et al. 1969; Bruno et al. 2002; Bruno et al. 2005; Cabrera et al. 1983; Cabrera et al. 1988; de Quesada et al. 1972; de Quesada et al. 1974; Diaz et al. 1988a; Disli et al. 2002; Escamilla & Rodriguez 1980; Fernandez et al. 1985; Fernandez et al. 1986a; Fernandez et al. 1986b; Fraga et al. 1987; Fraga et al. 1990; Fraga et al. 1991a; Fraga et al. 1995; Fraga et al. 2001a; Fraga et al. 2001b; Fraga et al. 2003a; Garcia-Alvares & Rodriguez 1976; Garcia-Alvares & Rodriguez 1980; Garcia-Granados & Molina 1989; Garcia-Granados et al. 1980; Garcia-Granados et al. 1984; Garcia-Granados et al 1985a; Garcia-Granados et al. 1985b; Garcia-Granados et al. 1985c; Garcia-Granados et al. 1986; Ghoumari et al. 2005; Gomez-Serranilos et al. 1997; Gomez-Serranilos

	et al. 1998; Gomez-Serranilos et al. 2004; Gonzalez et al. 1971; Gonzalez et al. 1973a; Gonzalez et al. 1973b; Gonzalez et al. 1973c; Gonzalez et al. 1975; Gonzalez et al. 1979; Kilic et al. 2003; Kilic et al. 2005; Kilic 2006; Lopez et al. 1977; Marquez et al. 1975; Oktemer & Logoglu 2003; Piozzi et al. 1968; Rodriguez & Valverde 1973; Rodriguez & Valverde 1975; Sezik et al. 1985; Taskova et al. 1997; Tomas-Lorente et al. 1988; Topcu et al. 1999; Topcu et al. 2001; Topcu et al. 2002a; Topcu et al. 2002b; Venturella et al. 1975; Venturella et al. 1978; Venturella et al. 1989; Villar et al. 1993; von Carstenn-Lichterfelde et al. 1974
Siderol (**20**) (kaurene diterpene)	Baser et al. 1996b; Bruno et al. 2005; Gomez-Serranilos et al. 2004; Kilic 2006; Sattar et al. 1995[sy,sc,m]; Topcu et al. 2001; Topcu et al. 2002a; Topcu et al. 2002b; Venturella et al. 1978; Venturella & Bellino 1979[sc]
Siderone (keto diterpene)	Venturella et al. 1983a[sy]
Tetracyclic kaurene type	Gonzalez et al. 1979[sy]
Ucriol (epoxy diterpene)	Venturella et al. 1983b[sy]
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Triterpenoids	Gonzalez et al. 1974; Villar et al. 1984b
α- and β-Amyrin	Aboutabl et al. 2002; Gonzalez et al. 1979; Fraga et al. 1995
Erithridiol	Gonzalez et al. 1979
Glutinol	Fraga et al. 1995; Gonzalez et al. 1979
Oleanolic acid (and derivs)	Fraga et al. 1995; Gonzalez et al. 1979; Sattar et al. 1995[sy,sc,m]
Rhoiptelenol and rhoiptelenone (pentacyclic triterpenes)	Fraga et al. 2003b
Squalene	Aboutabl et al. 2002; Fraga et al. 1995; Gonzalez et al. 1979
Ursolic acid (**21**)	Gonzalez et al. 1979; Sattar et al. 1995[sy,sc,m]
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Sterols and phytol	Amparo & Rodriguez 1976; Marin et al. 1992; Popa et al. 1976[m]
Campesterol	Aboutabl et al. 2002; Fraga et al. 1995; Gonzalez et al. 1979
β-Sitosterol	Aboutabl et al. 2002; Amparo & Rodriguez 1976; Fraga et al. 1995; Gonzalez et al. 1979; Marin et al. 1992; Villar et al. 1984b
Stigmasterol	Aboutabl et al. 2002; Fraga et al. 1995; Gonzalez et al. 1979
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Saponins	Aboutabl et al. 2002; Diaz et al. 1988b; Villar et al. 1984b

Phenolic type	
Flavonoid aglycones	Fefer 1970; Fefer 1971; Fernandez et al. 1988
Acacetin	Gil et al. 1993
Apigenin (derivs.)	Aboutabl et al. 2002; Atoui et al. 2005; Fraga et al. 1995; Tomas-Barberan et al. 1985b[m]; Gonzalez et al. 1978; Gonzalez et al. 1979; Kokkalou & Gabrielli 1997; Tomas-Barberan 1986[m]
Artemetin	Gonzalez et al. 1978; Gonzalez et al. 1979; Tomas-Barberan 1986
Chrysoeriol	Gil et al. 1993a; Palomino et al. 1996a; Tomas-Barberan et al. 1985b[m]; Tomas-Barberan 1986[m]
Cirsiliol	Ferreres et al. 1989; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan et al. 1985aTomas-Barberan et al. 1985b[m]; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989
Cirsilion	Tomas-Barberan 1986
Cirsilineol or anisomelin (and derivs.)	Ferreres et al. 1987; Ferreres et al. 1989; Rodriguez 1977; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986[m]; Tomas-Barberan et al. 1985a; Tomas-Barberan et al. 1985b[m]; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989
Cirsimaritin (derivs.)	Ferreres et al. 1987; Ferreres et al. 1989; Gil et al. 1993; Gonzalez et al. 1978**;** Gonzalez et al. 1979; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986[m]; Tomas-Barberan et al. 1985a; Tomas-Barberan et al. 1985b[m]; Tomas-Barberan et al. 1993; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989
5-Desmethylnobiletin	Palomino et al. 1996a; Rodriguez 1977; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986**;** Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Villar et al. 1985c
Diosmetin	Menkovic et al. 1993[m]
Eupatorin and derivs.	Gil et al. 1993; Gonzalez et al. 1978, Gonzalez et al. 1979; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986, Tomas-Lorente et al. 1989
Gardenin (B or D)	Palomino et al. 1996a; Rodriguez 1977; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Villar et al. 1985c; Villar et al. 1985d
Hispidulin	Gil et al. 1993
5-Hydroxyflavone	Fernandez et al. 1988; Fraga et al. 1995; Gonzalez et al. 1978; Rodriguez 1977; Tomas et al. 1979; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986[m]; Tomas-Barberan et al. 1985b[m]; Tomas-Lorente et al. 1983; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989
8-Hydroxyflavone	Gil et al. 1993; Tomas-Lorente et al. 1988

Hypolaetin	Tomas-Barberan et al. 1985c
Isoscutellarein	Tomas-Barberan et al. 1985c
Kaempferol derivs.	Fraga et al. 1995; Gonzalez et al. 1978; Gonzalez et al. 1979
Leucanthin	Tomas 1979
Luteolin (derivs.)	Gil et al. 1993; Kokkalou & Gabrielli 1997; Palomino et al. 1996a; Pulatova & Aminov 1969[m]; Tomas-Barberan 1986[m]; Tomas-Barberan et al. 1985b[m]; Tomas-Barberan et al. 1985c
8-O-Methylcirsilineol	Ferreres et al. 1987; Palomino et al. 1996a; Tomas-Barberan 1986; Tomas-Barberan et al. 1985a; Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989; Villar et al. 1985c; Villar et al. 1985d
Nepetin	Gil et al. 1993
Nodifloretin	Tomas-Barberan et al. 1985c
Pectolinarigenin	Gil et al. 1993; Gonzalez et al. 1978; Gonzalez et al. 1979; Tomas-Barberan 1986
Primetin	Tomas-Barberan et al. 1985
Salvigenin	Gil et al. 1993; Gonzalez et al. 1978; Gonzalez et al. 1979; Tomas-Barberan 1986; Tomas-Barberan & Wollenweber 1990
Sideritoflavone (and derivs.)	Ferreres et al. 1989; Palomino et al. 1996a; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986[m]; Tomas-Barberan et al. 1985a; Tomas-Barberan et al. 1985b[m]; Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989; Villar et al. 1985c;Villar et al. 1985d
Xanthomicrol	Ferreres et al. 1987; Ferreres et al. 1989; Gonzalez et al. 1978; Palomino et al. 1996a; Tomas-Barberan & Wollenweber 1990; Tomas-Barberan 1986; Tomas-Barberan et al. 1985a; Tomas-Barberan et al. 1985b[m]; Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989; Villar et al. 1985c; Villar et al. 1985d
Flavonoid glycosides	Akcos et al. 1999; Diaz et al. 1988b; Evstatieva 2002[sc], Fefer 1970; Fefer 1971; Ivanova et al. 2005; Pakaln et al. 1976; Papanicolaou & Kokkini 1984[sy,sc]; Pulatova & Aminov 1969[m]; Taskova et al. 1997; Tomas-Barberan et al. 1988b; Tunalier et al. 2004[sc]; Villar et al. 1984b
Apigenin derivs.	Ezer et al. 1992; Gabrielli & Kokkalou 1990; Gil et al. 1993; Tomas-Barberan et al. 1992; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a[m]; Venturella et al. 1995[sy]
Chrysoeriol derivs.	Aboutabl et al. 2002; Ferreres et al. 1987; Ferreres et al. 1989; Gil et al. 1993; Rabanal et al. 1982; Sahin et al. 2005; Tomas-Barberan & Tomas 1985; Tomas-Barberan 1986; Tomas-Barberan et al. 1992; Tomas-Barberan et al. 1988a[m]; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989; Venturella et al. 1977

p-Coumaroylglucosides	Gil et al. 1993; Tomas-Barberan et al. 1992[sy,sc,m]
5-Hydroxyflavone glycosides	Tomas & Ferreres 1980; Venturella et al. 1995[sy]
6-Hydroxyflavone glycosides	Martin-Lomas et al. 1983
8-Hydroxyflavone glycosides	Gil et al. 1993; Harborne et al. 1986; Tomas-Barberan et al. 1984; Tomas-Barberan et al. 1992[sy,sc,m]; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988b[sy,sc,m]
Hypolaetin derivs.: (**30**), (**31**), (**32**)	Aboutabl et al. 2002; Akcos et al. 1999; Ferreres et al. 1987; Ferreres et al. 1989; Gabrielli et al. 2004; Palomino et al. 1996a; Rodriguez-Lyon et al. 2000; Sattar et al. 1993[sy,sc,m]; Tomas et al. 1985; Tomas-Barberan 1986; Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1992; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a[m]; Tomas-Lorente et al. 1989
Isoscutellarein derives.	Aboutabl et al. 2002; Adzet et al. 1990; Ferreres et al. 1987; Ferreres et al. 1989; Gabrielli et al. 2004; Gil et al. 1993; Pallomino et al. 1996b; Rodriguez-Lyon et al. 2000; Sattar et al. 1995a[sc]; Tomas-Barberan 1986; Tomas-Barberan et al. 1985d; Tomas-Barberan et al. 1990; Tomas-Barberan et al. 1992; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a; Tomas-Lorente et al. 1988; Tomas-Lorente et al. 1989
Kaempferide	Tomas-Barberan 1986[m]
Leucanthogenin derives.	Tomas-Barberan 1986
Luteolin derivs.	Gil et al. 1993; Tomas-Barberan 1986[m]; Tomas-Barberan et al. 1984; Tomas-Barberan et al. 1993; Tomas-Barberan et al. 1988a[m]; Tomas-Lorente et al. 1988
4'-Methyl-isoscutellarein derivs.	Ezer et al. 1992; Ferreres et al. 1987; Ferreres et al. 1989; Rodriguez-Lyon et al. 2000; Tomas-Lorente et al. 1989
Nodifloretin derivs.	Tomas-Barberan 1986
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Phenolic acids	Lamaison et al. 1991; Pulatova & Aminov 1970
Caffeic acid (**9**) (and esters)	Atoui et al. 2005; Fefer & Gritsenko 1977; Pulatova & Aminov 1969[m]; Rios et al. 1992; Tunalier et al. 2004[sc]
Chlorogenic acid (**12**)	Atoui et al. 2005; Fefer & Gritsenko 1977; Pulatova & Aminov 1969[m]; Rios et al. 1992
trans-Cinnamic acid (**10**)	Fiamegos et al. 2004
Ester of coumaric acid	Atoui et al. 2005
Ferulic acid (**33**) (and esters)	Fiamegos et al. 2004; Atoui et al. 2005
Homovanillic acid	Fiamegos et al. 2004
p-Hydroxybenzoic acid	Fiamegos et al. 2004
4-Hydroxycinnamic acid	Fiamegos et al. 2004
Isochlorogenic acid	Rios et al. 1992
Neochlorogenic acid	Fefer & Gritsenko 1977
Syringic acid	Fiamegos et al. 2004

Vanillic acid	Fiamegos et al. 2004
Phenylpropanoid glycosides	
Acteoside (Verbascoside) (**29**)	Akcos et al. 1999; Ezer et al. 1992; Sahin et al. 2005
Lavandulifolioside	Akcos et al. 1999
Leucoseptoside A	Akcos et al. 1999; Sahin et al. 2005
Martynoside	Akcos et al. 1999; Sahin et al. 2005
Other phenylpropanoid glycosides	Kokkalou & Gabrielli 1997
Catechuic tannins	Aboutabl et al. 2002; Diaz et al. 1988b; Evstatieva 2002[sc]; Villar et al. 1984b
Coumarins	Pulatova & Aminov 1969[m]
Siderin (**28**) (and derivs.)	Darias et al. 1990; Fraga et al. 1995; Gonzalez et al. 1979; Venturella et al. 1974
Xanthotoxin (furocoumarin)	Aboutabl et al. 2002
Lignans ((+)-sesamin) (**27**)	Darias et al. 1990; Fraga et al. 1995; Gonzalez et al. 1979
Polyhydroxynaphtoquinone	Aboutabl et al. 2002
Miscellaneous	
Fatty acids	Aboutabl et al. 2002; Amparo & Rodriguez 1976; Ertan et al. 2001[l]; Marin et al. 1992[m]
Alkaloids (betain type)	Pulatova & Aminov 1969[m]; Pulatova & Aminov 1970
Minerals	Djordjevic et al. 1993; Ozcan 2004; Zarkovic et al. 1993
Hydrocarbons	Aboutabl et al. 2002
Carbohydrates	Aboutabl et al. 2002

[a] - Superscripts mean that the marked constituents have been found in the species distributed in Bulgaria: sy – in *S. syriaca*; sc – in *S. scardica*; m – in S. *montana;* l – in *S. lanata*

Table 1.4 Biological properties of pure compounds occurring in *Sideritis* (references in this Table are arranged according to year of publication for the same type of activity).

Activity	Pure compounds	References[a]
Antimicrobial and antiviral	4 sp. of *Sideritis*; against G(+), viruses; siderol (**20**) showed moderate activity against G(+) while ursolic (**21**) / oleanolic ac. showed marked activity; the same compounds exerted antiviral activity.	Sattar et al. 1995b[sy,sc,m,l]
	Studied natural diterpenoids were active against G(+), not active against G(−). Galdosol was the most active.	Darias et al. 1990
	Natural and semi-synthetic diterpenoids (against G(−), G(+), acid-fast, yeast). 1-Acetyljativatriol and related terpenoids were active against G(+) and acid-fast bacteria, not active against G(−).	Diaz et al. 1988a
	S. lycia; ajugol (**22**)	Akcos et al. 1998
	Ajugoside (**23**) showed high activity against G(−) & G(+), antifungal activity against yeast.	Ezer et al. 1995a
	Natural and semi-synthetic diterpenoids (against G(−), G(+), acid-fast, yeast). Only foliol and isopusillatriol derivs. were active against G(+) and acid-fast bacteria.	Rodriguez-Linde et al. 1994
	Diterpenoids (linearol, foliol, 7-epicandicandiol, siderol (**20**), etc.); against G(−), G(+), yeast; the most active for both G(−) & G(+) was 7-epicandicandiol.	Kilic et al. 2003; Kilic et al. 2006
	1-Acetyl jativatriol was the most potent from all studied diterpenoids.	Alcaraz & Rios 1991
	From 10 kaurane diterpenoids from *S. stricta* neither of them did not show good activity against G(−) & G(+) nor antifungal activity against yeast.	Kilic 2006
Antiinflammatory (antiulcer, antiarthritic, antirheumatic, antigastric, anticataract, analgesic)	<u>Diterpenes:</u> Borjatriol	Villar et al. 1983; Villar & Alcaraz 1984; Villar et al. 1984a,d; Alcaraz & Villar 1987; Tomas-Barberan et al. 1987
	Linearol	Manez et al. 1989
	Ent-16-hydroxy-13-*epi*-manoyl oxide; a mixture of esters of tyrosol with palmitic, stearic, behenic and lignoceric acids.	Alcaraz et al. 1989a; Alcaraz et al. 1989b

	Ent-13-epi-12α-acetoxymanoyl oxide (= "manoyl oxide F1") & *ent*-8α-hydroxy-13- labda-13(16),14-diene (= "labdane F2") – weak inhibitors.	de las Heras & Hoult 1994
	Tetracyclic kaurene diterpenes (foliol, linearol)	Castrillo et al. 2001
	Andalusol	Gomes-Serranillos et al. 2004
	Labdane-type terpenoids	de las Heras et al. 1994b
	Flavonoids:	
	Flavonoid glyc. (hypolaetin 8-glucoside (**30**): antiinflammatory, antiulcer	Villar et al. 1984c
	Hypolaetin 8-glucoside (**30**): anti-inflammatory.	Villar et al. 1985b,f; Pathak et al. 1991
	Sideritoflavone & hypolaetin 8-glucoside (**30**): antiinflammatory.	Alcaraz & Hoult 1985a
	Flavonoid aglycone (apigenin derivs.) showed a marked activity.	Gabrielli et al. 1990
	Flavonoid glycs. (hypolaetin derivs.) > phenylpropanoids (lavandulifolioside, marthynoside, verbascoside (**29**), leucosceptoside A).	Akcos et al. 1999
	Flavonoid aglyc. (sideritoflavone) >> flavonoid glyc. (hypolaetin 8-glucoside).	Tomas-Barberan et al. 1987
	Acylflavone glucosides	Jimenez et al. 1990
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Enzyme inhibiting	Flavonoid aglycs. (8-methoxy cirsilineol, 5-desmethylnobiletin, cirsiliol, xanthomicrol & others) on soybean lipoxygenase.	Alcaraz et al. 1986
	Flavonoid glyc. (Hypolaetin 8-glucoside (**30**)) & flavonoid aglycs. (hypolaetin, isoscutellarein); Flavonoid aglycs. dose-dependently acted against soybean lipoxygenase and snake venom phospholipase but hypolaetin 8-glucoside was inactive.	Alcaraz & Hoult 1985b
	Flavonoid aglycs. (sideritoflavone & 5-hydroxy aglyc.) & glyc. on aldose reductase, sideritoflavone was among the most active from the others tested.	Villar, Gasco et al. 1984
	Flavonoid aglyc. (sideritoflavone) on aldose reductase.	Pathak et al. 1991
	Flavonoid aglyc. (sideritoflavone) on lens aldose reductase.	Tomas-Barberan et al. 1986
	Flavonoid aglycs. (sideritoflavone, xanthomicrol and cirsiliol)	Mora et al. 1990
Antioxidant	Flavonoid aglycs. (gardenin D >> cirsimaritin > 8-methoxy cirsilineol > 5-desmethylnobiletin > cirsiliol ≈ xanthomicrol ≈ sideritoflavone).	Mora et al. 1990
	Flavonoid glycs. (hypolaetin 8-glucoside (**30**) was the most active; 4 other glyc. studied possessed moderate activity).	Rios et al. 1992

	S. raeseri MeOH ext.; flavonoid glycs. (all 9 compounds show moderate activity)	Gabrieli et al. 2005
	Flavonoids (gardenin D >> hypolaetin 8-glucoside (**30**)) on CCl_4-induced toxicity.	Alcaraz et al. 1990
	S. euboea BuOH fr. → kaempferol shows high activity.	Tsaknis & Lalas 2005
Miscellaneous		
Anti-HIV	Semisynthetic derivs. of linearol	Bruno et al. 2002
Antispasmodic	Flavonoid aglycs. (8-methoxy cirsilineol, cirsilineol),	Tomas-Barberan et al. 1985a
Diuretic	Flavonoid glycs., phenylpropanoids	Akcos et al. 1999
Anti-leishmanical	Flavonoid glycs., phenylpropanoids	Akcos et al. 1999
Cytotoxic	*Ent*-manoyl oxides against *Leishmania donovani* (protozoa distributed by mosquitoes)	Garcia-Granados et al. 1997
Cytostatic	Galdosol >> epigomeric ac. ≈ gomeric ac. ≈ salviol ≈ candicandiol ≈ candidiol ≈ sesamin showed cytostatic activity.	Darias et al. 1990
Immunomodulating	*Ent*-13-epi-manoyl oxides derivs.	Demetzos et al. 1994
	Andalusol	Navarro et al. 2000

Table 1.5 Pharmacological properties of extracts and fractions from *Sideritis* (references in this Table are arranged according to year of publication for the same type of activity).

Activity	Extracts and fractions	References[a]
Antimicrobial and antiviral	Essential oils:	
	S. syriaca & 4 other sp.; against G(−), G(+), yeast; carvacrol was responsible for the activity.	Aligiannis et al. 2001[sy]
	S. lanata & 1 other sp.; against G(−), G(+), yeast.	Ugur et al. 2005[l]
	3 sp. of *Sideritis*; against G(−), G(+), yeast; antifungal was more potent than antimicrobial activity.	Ezer & Abbasoglu 1996
	4 sp. of *Sideritis*; against G(−), G(+), yeast; *S. sipylea* was the most active.	Gergis et al. 1990
	6 sp. of *Sideritis*; against G(−), G(+), acid-fast, yeast; all sp. were active against G(+), acid-fast, yeast, not active against G(−).	Villar et al. 1986a
	S. sipylea; against G(−), G(+), yeast; active against G(+) than G(−) & fungi. Alcohols were responsible for the activity.	Gergis et al. 1991
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	Extracts:	
	11 sp. of *Sideritis*; against G(−), G(+), acid-fast, yeast; EtOAc ≈ MeOH; $CHCl_3$ > C_6H_{14} exts.	Villar et al. 1985e
	4 sp. of *Sideritis*; against G(+), viruses; leaf exudates showed moderate activity against G(+), *S. syriaca & S. scardica* showed minimum activity.	Sattar et al. 1995b[sy,sc,m,l]
	6 sp. of *Sideritis*; against G(−), G(+), acid-fast, yeast; PE ≈ $CHCl_3$ exts. showed higher activity than other exts.; $(CH_3)_2CO$ was more active against G(+); EtOAc was active against G(+); EtOH exts. were active against G(−).	Ezer et al. 1994
	S. clandestina; against G(−) & G(+); CH_2Cl_2 exts. showed marked activity against G(+), not active against G(−).	Schneider et al. 1991
	6 sp. of *Sideritis*; against G(−), G(+); EtOH > Water > EE exts. against G(+), not active against G(−).	Diaz et al. 1988b
	S. montana; against yeast; aqueous exts. against *Yarrowia lipolytica* showed relatively high activity.	Karanika et al. 2001[m]

	7 sp. of *Sideritis*; anticandidal activity of MeOH exts. The most active plants were found to be *S. trojana* and *S. bilgerana.* *S. stricta;* crude acetone ext. against G(−), G(+), yeast – did not show any activity.	Dulger et al. 2006 Kilic 2006
Antiinflammatory (antiarthritic, antirheumatic, antiulcer, antigastric, anticataract)	*S. javalambrensis*; C_6H_{14} > H_2O exts. *S. raeseri;* EtOAc > MeOH exts.(moderate) *S. foetens;* $(CH_3)_2CO$ ext. - Sterol fr. 12 sp. of *Sideritis*; C_6H_{14}, MeOH exts. 8 sp. of *Sideritis*; C_6H_{14}, MeOH exts. *S. candicans;* EtOH exts., $CHCl_3$ fr., H_2O fr. *S. lotsyi;* EtOH exts., $CHCl_3$ fr., H_2O fr. *S. canariensis;* EtOH exts., $CHCl_3$ fr. > H_2O fr. 5 sp. of *Sideritis*; MeOH > C_6H_{14} exts. *S. javalambrensis*; C_6H_{14} ≈ MeOH > CH_2Cl_2, EtOAc, exts. *S. javalambrensis*; C_6H_{14} exts. - lipid fr. *S. taurica;* C_6H_{14}, PE, MeOH, EtOH exts.; CH_2Cl_2 fr. − the highest antiinflammatory activity, BuOH fr. − the highest antiulcer activity. *S. javalambrensis*; C_6H_{14} ≈ MeOH exts. Tea from *S. scardica* 6 sp. of *Sideritis*; EtOH exts. 5 sp. of *Sideritis*; $CHCl_3$, MeOH exts. (dose-dependent way). 4 sp. of *Sideritis*; 20% decoctions; *S. hirsuta* showed the highest activity. *S. javalambrensis*; C_6H_{14} ext. *S. lycia;* PE, $CHCl_3$, EtOAc, H_2O exts., the latter two showed high antiinflammatory activity. Aqueous ext. from *S. caesarea* shows high antiulcerative activity C_6H_{14}, MeOH exts from *S. syriaca*; C_6H_{14} ext. & fr. − antiinflammatory activity; all exts. and fr. – no gastric ulcerogenic activity. Plants exts including 4 *Sideritis sp.* from the flowering shoots all showed antiinflammatory activity against *Mycobacterium butyricum*/carrageenan-induced arthritis in rats.	Villena et al. 2000 Gabrieli et al. 1990 Navarroet al. 2001 Gomez-Serranilos et al. 1998; Gomes-Serranillos et al. 2004 Hernandez-Perez et al. 2004 Hernandez-Perez & Rabanal 2002a Hernandez-Perez & Rabanal 2002b Villar et al. 1986b de las Heras et al. 1994a Godoy et al. 2000 Aboutabl et al. 2002 de las Heras et al. 1990 Alikovski 1999[sc] Yesilada & Ezer 1989 Manez et al. 1990 Zarzuelo et al. 1993 Alcaraz et al. 1989 Akos et al. 1999 Gurbuz et al. 2005 Menghini et al. 2005[sy] Alvarez et al. 1990
Antioxidant	MeOH exts. from *S. scardica* & 26 other sp.; *S. scardica* shows	Tunalier et al. 2004

	remarkable activity.	
	Aqueous exts. from 18 sp. including *S. syriaca; S. syriaca* shows little to moderate activity.	Guvenc et al. 2005
	S. raeseri, aqueous exts.	Triantaphyllou et al. 2001
	S. euboea C_6H_{14}, MeOH, DEE, EtOAc, BuOH exts.; BuOH fr. shows the least oxidation.	Tsaknis & Lalas 2005
	Aqueous exts. of 25 Bulgarian and other medicinal plants from several genera including *S. scardica,* the latter shows moderate activity.	Ivanova et al. 2005 [sc]
	Herbal infusions of 9 plants from several genera including *S. syriaca,* the latter has the weakest activity.	Atoui et al. 2005
Miscellaneous		
Astringent, Vulnerary, Febrifuge, Emmenagogue	Tea from S. *canariensis*, *S. dasygnaphala*	Darias et al. 1990
Antianemia	Tea from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Djordjevic et al. 1993[sc]; Alikovski 1999[sc]
Diuretic	Infusions from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Alikovski 1999[sc]
Asthma	Tea from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Alikovski 1999[sc]
Stenocardia	Tea from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Stojanov 1973 [sc]; Petkov 1982 [sc]; Alikovski 1999[sc]
Digestive	Tea from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Stojanov 1973 [sc]; Font-Quer 1976; Petkov 1982 [sc]; Lubenov 1984 [sc]; Alikovski 1999[sc]
Hepatoprotective	Tea from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Stojanov 1973 [sc]; Petkov 1982 [sc]; Alikovski 1999[sc]
Immunostimulating	Tea from *S. scardica* ("Pirin mountain" or "Mursalski" tea)	Alikovski 1999[sc]
Immunomodulating	*S. foetens;* $(CH_3)_2CO$ ext. - Sterol fr.	Navarro et al. 2001
Against haemorrhoids	Tea from *S. montana*	Lubenov 1984[m]
Analgesic	*S. candicans;* EtOH exts., $CHCl_3$ fr., H_2O fr.	Hernandez-Perez et al. 2004
	S. canariensis; EtOH exts., $CHCl_3$ fr. > H_2O fr.	Hernandez-Perez & Rabanal 2002b
	S. lotsyi; EtOH exts., $CHCl_3$ fr., H_2O fr.	Hernandez-Perez & Rabanal 2002a
	S. taurica; PE exts. > EtOH exts. > CH_2Cl_2 fr. ≈ BuOH fr.	Aboutabl et al. 2002
	C_6H_{14}, MeOH exts from *S. syriaca*; all exts. & fr. → peripheral & central	Menghini et al. 2005[sy]

	analgesic activity.	
Antiestrogenic	2 sp. of *Sideritis;* Water exts.	Kassi et al. 2004
Cytostatic	2 sp. of *Sideritis;* Water exts.	Kassi et al. 2004
Coccidiostat (antiparasitic)	Olympus tea (*S. scardica*)	Floroupaneri et al. 2004 [sc]
Antihyperglycaemic	*S. taurica;* PE exts., EtOH exts., CH_2Cl_2 fr., BuOH fr. (dose-dependent)	Aboutabl et al. 2002
Stomachic	Infusions	Tabanca et al. 2001
Antispasmodic	Infusions	Tabanca et al. 2001
Carminative	Infusions	Tabanca et al. 2001
Antidepressant and stimulant	*S. lanata* & 3 other sp.; aqueous exts. possessed activities on the central nervous system.	Ozturk et al. 1996 [l]
Loosening agent in bronchitis, cough, against cold, asthma	"Pirin mountain" or "Mursalski" tea from *S. scardica*	Stojanov 1973 [sc]; Petkov 1982 [sc]; Alikovski 1999 [sc]

[a] Superscripts mean that the marked constituents have been found in the species distributed in Bulgaria: **sy** - in *S. syriaca*; **sc** - in *S. scardica*; **m** - in *S. montana;* **l** - in *S. lanata;* G(−) - gram-negative; G(+) - gram-positive; > - more; ≈ - equal.
PE - petroleum ether; C_6H_{14} - hexane; EE - Et_2O; CH_2Cl_2 - dichloromethane, $CHCl_3$ - chloroform; EtOAc - ethyl acetate; BuOH - butanol; EtOH -ethanol; MeOH - methanol; H_2O - water extracts.

1.5 References

1.5.1 References on lipid oxidation and antioxidants

Acker S.A.B.E. van, van den Berg D.-J., Tromp M.N.J.L., Griffioen D.H., van Bennekom W.P., van der Vijgh W.J.F., Bast A. (1996). "Structural aspects of antioxidant activity of flavonoids." Free Rad. Biol. Med. **20:** 331-342.
Akoh C.C., Min D.B. (Eds.) (1998). Part III**:** Oxidation. in Food Lipids. Chemistry, Nutrition, & Biotechnology. Marcel Dekker Inc., New York, pp 283-422.
Alcaraz M.J., Ferrandiz M.L. (1987). "Modification of arachidonic metabolism by flavonoids." J. Ethnopharmacol. **21:** 209-229.
Altarejos J., Salido S., Perez-Bonilla M., Linares-Palomino P.J., van Beek T.A., Nogueras M., Sanchez A. (2005). "Preliminary assay on the radical scavenging activity of the olive wood extracts." Fitoterapia **76:** 348-351.
Andersen M., Lauridsen R.K., Skibsted L.H. (2003). "Optimizing the use of phenolic compounds in foods." in Phytochemical functional foods., ch.16 (Eds. Johnson I., Williamson G.), CRC Press, Boca Raton.
Antolovich M., Prenzler P.D., Patsalides E., McDonald S., Robards K. (2002). "Methods for testing antioxidant activity." Analyst **127:** 183-198.
Arnao M.B. (2000). **"**Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case." Trends Food Sci. Technol. **11:** 419-421.
Arnao M.B., Cano A., Hernandez-Ruiz J., Garcia-Canovas F., Acosta M. (1995). "Total antioxidant activity. A method for its estimation in plant material." in Current trends in fruit and vegetables phytochemistry., **ch. 48:** 265-269.
Arnao M.B., Cano A., Hernandez-Ruiz J., Garcia-Canovas F., Acosta M. (1996). "Inhibition by ascorbic acid and other antioxidants of the ABTS oxidation catalyzed by peroxidase: a novel approach for determining total antioxidant status of foods." Anal. Biochem. **236:** 255-261.
Arora A., Strasburg G.M. (1997). "Development and validation of fluorescence spectroscopic assays to evaluate antioxidant efficacy. Application to metal chelators." JAOCS **74:** 1031-1040.
Aruoma O.I. (1998). "Free radicals, oxidative stress, and antioxidants in human health and disease." JAOCS **75:** 199-212.
Aruoma O.I., Halliwell B., Laughton M.J., Quinian G.J., Gutteridge J.M.C. (1989). "The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)-iron(III) complex." Biochem. J. **258:** 617-620.
Bailey M.E., Clarke A.D., Kim Y.S. (1996). "Antioxidant properties of Maillard reaction products as meat flavour compounds." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Baldioli M., Servili M., Perretti G., Montedoro G.F. (1996). "Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil." JAOCS **73:** 1589-1593.
Banias C., Oreopoulou V., Thomopoulos C.D. (1992). "The effect of primary antioxidants and synergists on the activity of plant extracts in lard." JAOCS **69:** 520-524.
Barlow S.M. (1990). "Toxicological aspects of antioxidants used as food additives." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London-New York, ch.7.
Belitz H.-D., Grosch W., Schieberle P. (2004). Food Chemistry. (3rd revised edition), Springer-Verlag, Berlin, ch. 3.
Benavente-Garcia O., Castillo J., Lorente J., Ortuno A., Del Rio J.A. (2000). "Antioxidant activity of phenolics extracted from *Olea europea* L. leaves." Food Chem. **68:** 457-462
Benzie I.F.F., Strain J.J. (1999). "Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous

measurement of total antioxidant power and ascorbic acid concentration." Meth. Enzymol. **299:** 15-27.
Benzie I.F.F., Strain J.J., (1996). "The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay." Anal. Biochem. **239:** 70-76.
Bermond P. (1990). "Biological effects of food antioxidants." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London, ch.6.
Bertelsen G., Christophersen C., Nielsen P.H., Madsen H.L., Stadel P. (1995). "Chromatographic isolation of antioxidants guided by methyl linoleate assay." J. Agric. Food Chem. **43:** 1272-1275.
Beutner St., Bloedorn B., Frixel S., Blanco I.H., Hoffmann Th., Martin H.-D., Mayer B., Noack P., Ruck Ch., Schmidt M., Schulke I., Sell St., Ernst H., Haremza S., Seybold G., Sies H., Stahl W., Walsh R. (2001). "Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of β-carotene in antioxidant functions." J. Sci. Food Agric. **81:** 559-568.
Blois M.S. (1958). "Antioxidant determination by the use of a stable free radical." Nature **181:** 1199-1200.
Bocco A., Cuvelier M.E., Richard H., Berset C. (1998). "The antioxidant activity of various phenolic compounds measured by an accelerated test based on citronellal oxidation." Sci. Alim. **18:** 13-23.
Bondet V., Brand-Williams W., Berset C. (1997). "Kinetics and mechanisms of antioxidant activity using the DPPH free radical method." Lebensm.-Wiss.-Technol. **30:** 609-615.
Bors W., Michel C., Saran M. (1984). "Inhibition of bleaching of the carotenoid crocin, a rapid test for quantifying antioxidant activity." Free Rad. Biol. Med. **796:** 312-319.
Bors W., Michel C., Stettmaier K., Heller W. (1996). "EPR studies of plant polyphenols." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Brand-Williams W., Cuvelier M.E., Berset C. (1995). "Use of a free radical method to evaluate antioxidant activity." Lebensm.-Wiss.-Technol. **28:** 25-30.
Burkow I.C., Moen P., Overbe K. (1992). "Chemiluminescence as a method for oxidative rancidity assessment in autooxidized marine oils." JAOCS **69:** 1108-1111.
Burton G.W., Ingold K.U. (1984). "Beta-carotene: An unusual type of lipid antioxidant." Science **224:** 569-573.
Campos A.M., Lissi E.A. (1996). **"**Kinetcs of the reaction between ABTS derived radical cations and phenols." Int. J. Chem. Kinet. **29:** 219-224.
Cano A., Hernandez-Ruiz J., Garcia-Canovas F., Acosta M., Arnao M.B. (1998). **"**An end-point method for estimation of the total antioxidant activity in plant material." Phytochemistry Anal. **9:** 196-202.
Cao G., Prior R.L. (1998). "Comparison of different analytical methods for assessing total antioxidant capacity of human serum." Clin. Chem. **44:** 1309-1315.
Cao G., Sofic E., Prior R.L. (1996). "Antioxidant capacity of tea and common vegetables." J. Agric. Food Chem. **44:** 3426-3431.
Cao G., Sofic E., Prior R.L. (1997). "Antioxidants and pro-oxidant behaviour of flavonoids : structure-activity relationships." Free Rad. Biol. Med. **22:** 749-760.
Cao G.H., Alessio H.M., Cutler R.G., (1993). "ORAC assay for antioxidants." Free Rad. Biol. Med. **14:** 50-62.
Cash G.A., George G.A., 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. 277-287.
Chan H. W.-S., Prescott F.A.A., Swoboda P.A.T. (1996). "Thermal decomposition of individual positional isomers of methyl linoleate hydroperoxide." JAOCS **53:** 572-576.
Chen J.H., Ho C.-T. (1997). "Antioxidant activity of caffeic acid and its related hydroxycinnamic acid compounds." J. Agric. Food Chem. **45:** 2374-2378.

Chevion S., Berry E.M., Kitrossky N.K., Kohen R. (1997). "Evaluation of plasma low molecular weight antioxidant capacity by cyclic votammetry." Free Rad. Biol. Med. **22:** 411-421.
Christensen T.C., Holmer G. (1996). "Lipid oxidation determination in butter and dairy spreads by HPLC." J. Food Sci. **61:** 486-489.
Cillard J., Cillard P., Cormier M. (1980). "Effect of experimental factors on the pro-oxidant behaviour of α- tocopherol." JAOCS **Aug.:** 254-261.
Cook N.C., Samman S. (1996). "Flavonoids - chemistry, metabolism, cardioprotective effects, and dietary sources." Nutr. Biochem. **7:** 66-76.
Cuvelier M.-E., Richard H., Berset C. (1992). "Comparison of the antioxidant activity of some acid-phenols: structure-activity relationship." Biosci. Biotech. Biochem. **56:** 324-325.
Dalton D.A. (1995). "Antioxidant defenses of plants and fungi." in Oxidative stress and antioxidant defenses in biology. (Ed. Ahmad S.), pp 298-355.
Danchev St. (Ed.) (1997) "Kachestvo na hranite i napitkite." Part IV, NCDO, VIHVP-Plovdiv (in Bulg.).
Dapkevicius A., van Beek T.A., Linssen J.P.H., Venskutonis R. (1998a). 'Rapid spectroscopic screening for antioxidant activity in sage, thyme and oregano isolates with the β-carotene-linoleic acid model system." in Natural Product Analysis. (Eds. Shreier P., Herderich M., Humpf H.-U., Schwab W.), Friedr. Vieweg & Sohn: Braunschweig-Wiesbaden, pp 235-237.
Dapkevicius A., van Beek T.A., Niederländer H.A.G., de Groot A. (1999). "On-line detection of antioxidative activity in HPLC eluates by chemiluminescence." Anal. Chem. **71:** 736-740.
Dapkevicius A., Venskutonis R., van Beek T.A., Linssen J.P.H. (1998b). "Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania." J. Sci. Food Agric. **77:** 140-146.
Decker E.A. (1998a). "Antioxidant mechanisms." in Food Lipids. Chemistry, Nutrition, and Biotechnology. (Eds. Akoh C.C., Min D.B.), Marcel Dekker Inc., New York, pp 397-423.
Decker E.A. (1998b). "Strategies for manipulating the prooxidative/antioxidative balance of foods to maximize oxidative stability." Trends Food Sci. Technol. **9:** 241-248.
Dodd N.J.F. (1995). "Free radicals and food irradiation." in Biochem.Soc.Symp., No.61 - Free radicals and oxidative stress: environment, drugs and food additives. (Eds. Rice-Evans C., Halliwell B. & Lunt G.G.), University of Sussex, Dec.1994, pp 247-258.
Dziedzic S.Z., Hudson B.J.F (1984). "Phenolic acids and related compounds as antioxidants for edible oils." Food Chem. **14:** 45-51.
Dziedzic S.Z., Hudson B.J.F. (1983). "Hydroxy isoflavones as antioxidants for edible oils." Food Chem. **11:** 161-166.
Economou K.D., Oreopoulou V., Thomopoulos C.D. (1991). "Antioxidant activity of some plant extracts of the family Labiatae." JAOCS **68:** 109-11.
Escobar J., Cardenas G., Lissi E. (1997). "Evaluation of the total content of lipid-soluble antioxidants in blood plasma samples employing a simple chemiluminescence quenching procedure." J. Biochem. Biophys. Meth. **35:** 57-60.
Evans R.J., Reynhout G.S. (1992). "Alternates to synthetic antioxidants." in Food Science and Human Nutrition. (Ed. Charalambous G.), pp 27-42.
Finley J.W., Otterburn M.S. (1993). "The consequence of free radicals in foods." in Antioxidants. Chemical, physiological, nutritional and toxicological aspects. (Ed. Williams G.M.), Princeton Scientific Publishing Co. Inc., Princeton, New Jersey, Toxicology and Industrial Health, pp 77-91.
Fogliano V., Verde V., Randazzo G., Ritieni A. (1999). "Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines." J. Agric. Food Chem. **47:** 1035-1040.

Frankel E.N. (1985). "Chemistry of autooxidation: mechanism, products and flavor significance." AOCS Monogr. 15 (Flavor Chem. Fats Oils), pp 1-37.
Frankel E.N. (1993a). "Formation of headspace volatiles by thermal decomposition of oxidized fish oils *vs*. oxidized vegetable oils." JAOCS **70:** 767-772.
Frankel E.N. (1993b). "In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids." Trends Food Sci. Technol. **4:** 220-225.
Frankel E.N. (1996). "Antioxidants in lipid foods and their impact on food quality." Food Chem. **57:** 51-55.
Frankel E.N. (1998). "Lipid oxidation." The Oily Press, Dundee, ch.1a; 3b; 4c; 5d; 6e; 7f; 8g; 11h.
Frankel E.N., Gardner H.W. (1989). "Effect of α-tocopherol on the volatile thermal decomposition products of methyl linoleate hydroperoxides." Lipids **24:** 603-608.
Frankel E.N., Hu M.-L., Tappel A.L. (1989). "Rapid HS-GC of hexanal as a measure of lipid peroxidation in biological samples." Lipids **24:** 976-981.
Frankel E.N., Huang S.-W., Aeschbach R., Prior E. (1996). "Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion." J. Agric. Food Chem. **44:** 131-135.
Frankel E.N., Huang S.-W., Kanner J., German J.B. (1994). "Interfacial phenomena in the evaluation of antioxidants: bulk oils *vs.* emulsions." J. Agric. Food Chem. **42:** 1054-1059.
Frankel E.N., Meyer A.S. (2000). "The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants." J. Sci. Food Agric. **80:** 1925-1941.
Fujimori K., Akutsu K., Horiuchi S. (1994). "Effect of pH on reaction of superoxide." in Frontiers of reactive oxygen species in biology and medicine. (Ed. Asada K., Yoshikawa T.), pp 87-88.
Fukumoto L.R., Mazza G. (2000). "Assessing antioxidant and prooxidant activities of phenolic compounds." J. Agric. Food Chem. **48:** 3597-3604.
Gadow A. von, Joubert E., Hansmann C.F. (1997a). "Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*) with green, oolong and black tea." Food Chem. **60**: 73-77.
Gadow A. von, Joubert E., Hansmann C.F. (1997b). "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.
Gao J.-J., Igalashi K., Nukina M. (1999). "Radical scavenging activity of phenylpropanoid glycosides in *Caryopteris incana*." Biosci. Biotechnol. Biochem. **63:** 983-988.
Garcia-Conesa M.T., Plumb G.W., Waldron K.W., Ralph J., Williamson G. (1997). "Ferulic acid dehydrodimers from wheat bran: isolation, purification, and antioxidant properties of 8-O-4-diferulic acid." Redox Report **3:** 319-323.
Garcia-Plazaola J.I., Becerril J.M. (1999). "A rapid HPLC method to measure lipophilic antioxidants in stressed plants: simultaneous determination of carotenoids and tocopherols." Phytochemistry Anal. **10:** 307-313.
Gebicki J.M., Guille J. (1989). "Spectrophotometric and high-performance chromatographic assays of hydroperoxides by the iodometric technique." Anal. Biochem. **176:** 360-364,
Ghiselli A., Serafini M., Maiani G., Azzini E., Ferro-Luzzi A. (1995). "A fluorescence-based method for measuring total plasma antioxidant capability." Free Rad. Biol. Med. **18:** 29-36.
Ghiselli A., Serafini M., Natella F., Scaccini C. (2000). "Total antioxidant capacity as a tool to assess redox status: critical view and experimental data." Free Rad. Biol. Med. **29:** 1106-1114.
Gil M.I., Tomas-Barberan F.A., Hess-Pierce B., Holcroft D.M., Kader A.A. (2000). "Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing." J. Agric. Food Chem. **48:** 4581-4589.

Glazer A.N. (1990). "Phycoerythrin fluorescence-based assay for reactive oxygen species." Meth. Enzymol. **186:** 161-168.
Gordon M.H. (1990). "The mechanism of antioxidant action *in vitro."* in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London, ch.1, pp 1-18.
Gordon M.H. (2001). "The development of oxidative rancidity in foods." in Antioxidants in food. Practical applications. (Eds. Pokorny J., Yanishlieva N., Gordon M.), ch. 1, pp 7-22, CRC Press, Boca Raton, Woodhead Publishing Ltd., Cambridge, England.
Groot Ae. de (2001). Personal communication, Laboratory of Organic Chemistry, Wageningen University, The Netherlands.
Guo C.J., Cao G.H., Sofic E., Prior R.L. (1997). "HPLC coupled with coulometric array detection of electroactive components in fruit and vegetables – relationship to oxygen radical absorbance capacity." J. Agric. Food Chem. **45:** 1787-1796.
Halliwell B. (1994). "Free radicals and antioxidants: A personal view." Nutr. Rev. **52:** 253-265.
Halliwell B. (1995a). "Antioxidants characterization. Methodology and mechanism." Biochem. Pharmacol. **49:** 1341-1348.
Halliwell B. (1995b). "How to characterize an antioxidant: an update." in Biochem. Soc. Symp., No.61 - Free radicals and oxidative stress: environment, drugs and food additives. (Eds. Rice-Evans C., Halliwell B. & Lunt G.G.), University of Sussex, Dec.1994, pp 73-102.
Halliwell B., Whiteman M. (2004). "Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean?" British J. Pharmacol. **142:** 231-255.
Hamilton R.J., Kalu C., Prisk E., Padley F.B., Pierce H. (1997). "Chemistry of free radicals in lipids." Food Chem. **60:** 193-199.
Hanasaki Y., Ogawa S., Fukui S. (1994). "The correlation between active oxygen scavenging and oxidative effects of flavonoids." Free Rad. Biol. Med. **16:** 845-850.
Heilmann J., Merfort I., Weiss M. (1995). "Radical scavenging activity of different 3',4"-dihydroxyflavonols and 1,5-dicaffeoylquinic acid studied by inhibition of chemiluminescence." Planta Medica **61:** 435-438.
Heinonen M., Rein D., Satue-Gracia M.T., Huang S.W., German J.B. (1998). "Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system." J. Agric. Food Chem. **46:** 917-922.
Hendrich S., Wang G.-J., Lin H.-K., Xi X.,. Tew B.-Y, Wang H.-J., Murphy P.A. (1999). "Isoflavone metabolism and bioavailability." in Antioxidant status, diet, nutrition, and health. (Ed. Papas A.M.), CRC Press, Boca Raton.
Hiramatsu M., Yoshikawa T., Inoue M. (Eds.) (1997). "Food and free radicals. Part 3" Plenum Press, New York.
Ho C.-T., Chen C.-W., Wanasundara U.N., F. Shahidi (1996). "Natural antioxidants from tea." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Hopia A., Huang S.-W., Frankel E.N. (1996). "Effect of α-tocopherol and Trolox on the decomposition of methyl linoleate hydroperoxides." Lipids **31**: 357-365.
Huang D., Ou B., Prior R.L. (2005). "The chemistry behind antioxidant capacity assays." J. Agric. Food Chem. , **53**: 1841-1856.
Huang S.-W., Frankel E.N., Schwarz K., German J.B. (1996). "Effect of pH on antioxidant activity of α-tocopherol and Trolox in oil-in-water emulsions." J. Agric. Food Chem. **44:** 2496-2502.
Hudson B.J.F., Lewis J.I. (1983). "Polyhydroxy flavonoid antioxidant for edible oils. Structural criteria for activity." Food Chem. **10:** 47-55.
Hudson B.J.F., Mahgoub S.E.O. (1980). "Naturally occurring antioxidants for edible oils." J. Agric. Food Chem. **31:** 646-650.

Husain S.R., Cillard J., Cillard P. (1987). "Alfa-tocopherol pro-oxidant effect and malondialdehyde production." JAOCS **64:** 109-111.
Ivanov S., Tanchev S., Obretenov Ts. (1984). "Povishavane kachestvoto na hranitelni producti chrez izpolzvaneto na podobriteli.", Tehnika, Sofia (in Bulg.).
Jacobson G.A. (1993). "Evaluation of oxidized lipids in foods." INFORM **4:** 811-819.
Jorgensen K., Skibsted L.H. (1993). "Carotenoid scavenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity." Z. Lebensm. Unters. Forsch. **196:** 423-429.
Jovanovic S.V., Steenken St., Tosic M., Marjanovic B., Simic M.G. (1994). "Flavonoids as antioxidants." J. Am. Chem. Soc. **116:** 4846-4851.
Kalitchin Zh.D., Boneva M.I., Milkova Ts., Todorova D. (1997). "Study on the antioxidant activity of cholesteryl esters of some phenolic acids by chemiluminescence." J. Photochem. Photobiol. B: Biology **41:** 109-113.
Kanner J., Frankel E., Granit R., German B., Kinsella J.E. (1994). "Natural antioxidants in grapes and wines." J. Agric. Food Chem. **42:** 64-69.
Kansci G., Genot C., Meyner A., Gandemer G. (1997). **"**The antioxidant activity of carnosine and its consequences on the volatile profiles of liposomes during iron/ascorbate induced phopholipid oxidation." Food Chem. **60:** 165-175.
Khal R., Hilderbrant A.G. (1986). "Methodology for studying antioxidant activity and mechanism of action of antioxidants." Food Chem.Toxic. **24:** 1007-1014.
Kim S.Y., Kim S.K., Oh M.J., Jung M.Y. (1994). "Antioxidant activity of selected oriental herb extracts." JAOCS **71:** 633-640.
Kochhar S.P., Rossell J.B. (1990). "Detection, Estimation and Evaluation of Antioxidants in food systems." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Appl. Science, London, ch.2.
Kovatcheva E., Koleva I., Pavlov A., Ilieva M., Michneva M., Konushlieva M. (2001). "Antioxidant activity of extracts from *Lavandula vera* MM cell culture." Food Chem. **72:** 295-300.
Labuza Th.P. (1971). "Kinetics of lipid oxidation in foods." Crit. Rev. Food Technol. **2:** 355-405.
Larson R.A (1997). "Naturally occurring antioxidants", Lewis Publishers, Boca Raton-New York, ch. 1a; 2b; 3c; 4d; 5e; 7f.
Larson R.A. (1995). "Antioxidant mechanisms of secondary natural products." in Oxidative stress and antioxidant defenses in biology. (Ed. Ahmad S.), pp 210-236.
Larson R.A.M. (1988). "The antioxidants of higher plants." Phytochemistry **27:** 969-978.
Laubli M.W., Bruffel P.A. (1986). "Determination of the oxidative stability of fats and oils: Comparison between the active oxygen method and the Rancimat method." JAOCS **63:** 792-795.
Lavelli V., Hippeli S., Peri C., Elstner E.F. (1999). "Evaluation of radical scavenging activity of fresh and air-dried tomatoes by three model reactions." J. Agric. Food Chem. **47:** 3826-3831.
Lien E.J., Ren S., Bui H.-H., Wang R. (1999). "Quantitative structure-activity relationships analysis of phenolic antioxidants." Free Rad. Biol. Med. **26:** 285-294.
Lőliger J. (1991). "Natural antioxidants." Lipid Technol. **April-June:** 58-61
Lucarini M., Pedrieli P., Pedulli G.F., Valgingili L., Gigmes D., Tordo P. (1999). "Bond dissociation energies of the N-H bond and rate constants for the reaction with alkyl, alkoxyl, and peroxyl radicals of phenothiazines and related compounds." J. Am. Chem. Soc. **121:** 11546-11553.
Madhavi D.L., Deshpande S.S., Salunkhe D.K., Eds. (1995). "Food antioxidants. Technological, toxicological and health perspectives.", Marcel Dekker Inc., New York.
Madhavi D.L., Salunkhe D.K. (1995). "Toxicological aspects of food antioxidants." in Food antioxidants. Technological, toxicological and health perspectives. (Eds. Madhavi D.L., Deshpande S.S., Salunkhe D.K.), Marcel Dekker Inc., New York.

Madsen H.L., Bertelsen G. (1995). "Spices as antioxidants." Trends Food Sci. Technol. **6:** 271-277.
Madsen H.L., Bertelsen G., Skibsted L.H. (1997). "Antioxidant activity of spices and spice extracts." in Spices. Flavor, chemistry and antioxidant properties. (Eds. Risch S.J., Ho C.-T.), ACS Symp., Series 660, ch. 14.
Madsen H.L., Sorensen B., Skibsted L.H., Bertelsen G. (1998). "The antioxidative activity of summer savory (*Satureja hortensis* L.) and rosemary (*Rosmarinus officinalis* L.) in dressing stored exposed to light or in darkness." Food Chem. **63:** 173-180.
Mahoney J.R., Graf E. (1986). "Role of α-tocopherol, ascorbic acid, citric acid and EDTA as oxidants in model systems." J. Food Sci. **51:** 1293-1296.
Mahrour A., Lacroix M., Nkesta-Tabiri J., Calderon N., Gagnon M. (1998). "Antioxidant properties of natural substances in irradiated fresh poultry." Radiat. Phys. Chem. **52:** 77-80.
Marco G.J. (1968). "A rapid method for evaluation of antioxidants." JAOCS **45:** 594-598.
Marinova E., Yanishlieva N. (1992). "Inhibited oxidation of lipids II: Comparison of the antioxidative properties of some hydroxy derivatives of benzoic and cinnamic acids." Fat Sci. Technol. **94:** 428-432.
Marinova E.M., Yanishlieva N. (1994). "Effect of lipid unsaturation on the antioxidative activity of some phenolic acids." JAOCS **71:** 427-434.
Mathiesen L., Malterud K.E., Sund R.B. (1997). "Hydrogen bond formation as basis for radical scavenging activity: a structure-activity study of C-methylated dihydrochalcones from *Myrica gale* and structurally related acetophenones." Free Rad. Biol. Med. **22:** 307-311.
Matthaus B.W. (1996). "Determination of the oxidative stability of vegetable oils by Rancimat and conductivity and chemiluminescence measurements." JAOCS **73:** 1039-1043.
Mendez E., Sanhueza J., Speisky H., Valenzuela A. (1996). "Validation of the Rancimat test for the assessment of the relative stability of fish oils." JAOCS **73:** 1033-1037.
Mikkelsen A., Skibsted L.H. (1992). "Kinetics of enymatic reduction of metmyoglobin in relation to oxygen activation in meat products." Z. Lebensm. Unters. Forsch. **194:** 9-16.
Mikova K. (2001). "The regulation of antioxidants in food." in Antioxidants in food. Practical applications. (Eds. Pokorny J., Yanishlieva N., Gordon M.), ch. 11, pp 266-284, CRC Press, Boca Raton, Woodhead Publishing Ltd., Cambridge, England
Milic B.Lj., Djilas S.M., Candanovic-Brunet J.M. (1998). "Antioxidative activity of phenolic compounds on the metal-ion breakdown of lipid peroxidation system." Food Chem. **61:** 443-447.
Miller H.E. (1971). "A simplified method for the evaluation of antioxidants." JAOCS **48:** 91.
Miller N.J., Castelluccio C., Tijburg L., Rice-Evans C. (1996). "The antioxidant properties of theaflavins and their gallate esters - radical scavengers or metal chelators?" FEBS Lett. **392:** 40-44.
Miller N.J., Rice-Evans C., Davies M.J., Gopinathan V., Milner A.A (1993). "Novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates." Clin. Sci. **84:** 407-412.
Miller N.J., Rice-Evans C.A. (1997). "Factors influencing the antioxidant activity determined by the ABTS radical cation assay." Free Rad. Res. **26:** 195-199.
Min D.B. (1998). "Lipid oxidation." in Food Lipids. (Eds. Akoh C.C., Min D.B.), Marcel Dekker Inc., New York, pp 283-297.
Miura T., Muraoka S., Ogiso T. (1998). "Antioxidant activity of adrenergic agents derived from catechol." Biochem. Pharmacol. **55:** 2001-2006.
Miyagawa K., Hirai K., Takezoe R. (1991). "Tocopherol and fluorescence levels in deep-frying oil and their measurement for oil assessment." JAOCS **68:** 163-166.
Moller J.K.S., Madsen H.L., Aaltonen T., Skibsted L.H. (1999). "Dittany (*Origanum dictamnus*) as a source of water-extractable antioxidants." Food Chem. **64:** 215-219.

Moure A., Cruz J.M., Franco D., Dominguez J.M., Sineiro J., Dominguez H., Nunez M.J., Parajo J.C. (2001). "Natural antioxidants from residual sources." Food Chem. **72:** 145-171.
Munday R., Winterbourn C.C. (1989). "Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence system." Biochem. Pharmacol. **38:** 4349-4352.
Nakano M. (1994). "Detection of active oxygen species generated *in vitro* and *in vivo* by chemiluminescence." in Frontiers of reactive oxygen species in biology and medicine. (Eds. Asada K., Yoshikawa T.), pp 73-76.
Nakatani N. (1996). "Antioxidants from spices and herbs." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Namiki M. (1990). "Antioxidants / Antimutagens in Food." Crit. Rev. Food Sci. Nutr. **29:** 273-300.
Nau W.M. (1998). "A fluorescent probe for antioxidants." J. Am. Chem. Soc. **120:** 12614-12618.
Navas M.J., Jimenez A.M. (1996). "Review of chemiluminescent methods in food analysis." Food Chem. **55:** 7-15.
Niederländer H.A.G. (1994). "Chemiluminescence detection in liquid chromatography based on photochemical on-line production of singlet oxygen." Ph.D. Thesis.
Nieto S., Garrido A., Sanhueza J., Loyola L.A., Morales G., Leighton F., Valenzuela A. (1993). "Flavonoids as stabilizers of fish oil: an alternative to synthetic antioxidants." JAOCS **70:** 773-778.
Noguchi N., Niki E. (1999). "Chemistry of active oxygen species and antioxidants." in Antioxidant status, diet, nutrition, and health. (Ed. Papas A.M.), CRC Press, Boca Raton, ch. 1.
Obretenov T., Ivanov S., Peeva D. (1986). "Antioxidative activity of Maillard reaction products obtained from hydrolysates." "Amino-carbonyl reactions in food & biological systems" - Proc. of the 3rd Int. Symp. on the Maillard Reaction, Kodansha Ltd., Tokyo, Japan.
Okuda T., Yoshida T., Hatano T. (1994). "Chemistry and antioxidative effects of phenolic compounds from licorice, tea, and Compositae and Labiatae herbs." in Food Phytochemicals. II: Teas, Spices, & Herbs, ch. 15., pp 133-143.
Osawa T., Katsuzaki H., Kumon H., Kawakishi S., Yamashita K., Nakayama T., Hara Y. (1994). "Protective role of phenolic antioxidants in dietary plants against oxidative damage." in Frontiers of reactive oxygen species in biology and medicine. (Eds. Asada K. & Yoshikawa T.), pp 333-336.
Oxiresearch, Inc. (2002). "Colorimetric, quantitative assay for total antioxidant potential (aqueous samples)."
Pannala A.S., Chan T.S., O'Brien P.J., Rice-Evans C. (2001). "Flavonoid B-ring chemistry and antioxidant activities: fast reaction kinetics." Biochem. Biophys. Res. Commun. **282:** 1161-1168.
Papas, A.M. (Ed.) (1999). "Antioxidant status, diet, nutrition, and health.", CRC Press, Boca Raton.
Pazola Z., Korczak J., Gogolewski M. (1990). "Studies on the antioxidative properties of spices from the Labiatae family. II. Attempt at identification of antioxidative components of rosemary and sage." Roczniki Akademii Rolniczej w Poznaniu **CCXVIII:** 93-107.
Pellegrini N., Simonetti P., Gardana C., Brenna O., Brighenti F., Pietta P. (2000). "Polyphenol content and total antioxidant activity of *Vini Novelli* (Young red wines), J. Agric. Food Chem. **48:** 732-735.
Perez-Bonilla M., Salido S., van Beek T.A., Linares-Palomino P.J., Altarejos J., Nogueras M., Sanchez A. (2006). "Isolation and identification of radical scavengers in olive (*Olea europaea*) wood." J. Chromatogr. A **1112:** 311-318.

Pieri C., Marra M., Moroni F., Recchioni R., Marcheselli F. (1994). "Melatonin: a peroxyl radical scavenger more effective than vitamin E." Life Sci. **55:** 271-276.
Pietta P.-G. (2000). "Flavonoids as antioxidants." J. Nat. Prod. **63:**1035-1042.
Plumb G.W., Chambers S.J., Lambert N., Wanigatunga S., Williamson G. (1997). "Influence of fruit and vegetable extracts on lipid peroxidation in microsomes containing specific cytochrome P450s." Food Chem. **60:** 161-164.
Pokorny J., Nguen H.T.T., Korczak J. (1997). "Antioxidant activities of rosemary and sage extracts in sunflower oil." Nahrung **41:** 176-11.
Pokorny J., Yanishlieva N., Gordon M. (Eds.) (2001). "Antioxidants in food. Practical applications.", part 2, pp 87-147, CRC Press, Boca Raton, Woodhead Publishing Ltd., Cambridge, England
Popov I.N., Lewin G. (1994). "Photochemiluminescent detection of antiradical activity: II. Testing of non-enzymic water-soluble antioxidants." Free Rad. Biol. Med. **17:** 267-271.
Porter W.L. (1993). "Paradoxical behavior of antioxidants in food and biological systems." in Antioxidants. Chemical, physiological, nutritional and toxicological aspects. (Ed. Williams G.M.), pp 93-121, Princeton Scientific Publishing Co., Inc., Princeton, New Jersey, USA.
Porter W.L., Black E.D., Drolet A.M. (1989). "Use of polyamide oxidative fluorescence test on lipid emulsions: contrast in relative effectiveness of antioxidants in bulk *vs*. dispersed systems. J. Agric. Food Chem. **37:** 615-624.
Pratt D.E. (1980). "Natural antioxidants of soybean and other oil seeds." in Autoxidation in Food & Biological Systems. (Eds. Simic M.G., Karel M.), pp 283-293, Plenum Press, New York.
Pratt D.E., Hudson B.J.F (1990). "Natural Antioxidants not exploited commercially." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London, ch.5.
Prior R.L., Cao G., (1999). "*In vivo* total antioxidant capacity: comparison of different analytical methods." Free Rad. Biol. Med. **27:** 1173-1181.
Pryor W.A., Cornicelli J.A., Devall L.J., Tait B., Trivedi B.K., Witiak D.T., Wu M. (1993). "A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants." J. Org. Chem. **58:** 3521-3532.
Pryor W.A., Strickland Th., Church D.F. (1988). "Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous SDS micelle solutions." J. Am. Chem. Soc. **110:** 2224-2229.
Quick K.L., Hardt J.I.M, Dugan L.L. (2000). "Rapid microplate assay for superoxide scavenging efficiency." J. Neurosci. Meth. **97:** 138-144.
Ragnarsson J.O., Labuza T.P. (1977). "Accelerated shelf-life testing for oxidative rancidity in foods - a review." Food Chem. **2:** 291-308.
Ragnarsson J.O., Leick D., Labuza T.P. (1977). "Accelerated temperature study of antioxidants." J. Food Sci. **42:** 1536-1544.
Rajalakshmi D., Narasimhan S. (1995). "Food antioxidants: Sources and methods of evaluation." in Food antioxidants. Technological, toxicological and health perspectives. (Eds. Madhavi D.L., Deshpande S.S., Salunkhe D.K.), Marcel Dekker Inc., New York.
Ramarathnam N., Osawa T., Ochi H., Kawakishi S. (1995). "The contribution of plant food antioxidants to human health." Trends Food Sci. Technol. **6:** 75-82.
Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C. (1999). "Antioxidant activity applying an improved ABTS radical cation decolorization assay." Free Rad. Biol. Med. **26:** 1231-1237.
Reische D.W., Lillard D.A., Eitenmiller R.R. (1998). "Antioxidants." in Food Lipids. Chemistry, Nutrition, and Biotechnology. (Eds. Akoh C.C., Min D.B.), Marcel Dekker, Inc., New York, pp 423-449.
Renaud S., De Lorgeril J.M. (1992). "Wine, alcohol, platelets, and the French paradox for coronary heart disease." Lancet **339:** 1523-1526**.**

Rice-Evans C. (1995). "Plant polyphenols: free radical scavenger or chain breaking antioxidants?" in Biochem. Soc. Symp., No.61 - Free radicals and oxidative stress: environment, drugs and food additives. (Eds. Rice-Evans C., Halliwell B. & Lunt G.G.), University of Sussex, Dec.1994, pp 103-116.
Rice-Evans C., Miller N.J. (1994). "Total antioxidant status in plasma and body fluids." Meth. Enzymol. **234:** 279-293.
Rice-Evans C.A., Miller N.J., Bolwel P.G., Bramley P.M., Pridham J.B. (1995). "The relative antioxidant activities of plant-derived polyphenolic flavonoids." Free Rad. Res. **22:** 375-383.
Rice-Evans C.A., Miller N.J., Paganga G. (1996). "Structure-antioxidant activity relationships of flavonoids and phenolic acids." Free Rad. Biol. Med. **20:** 933-956.
Robards K., Prenzler P.D., Tucker G., Swatsitang P., Glover W. (1999). "Phenolic compounds and their role in oxidative processes in fruits." Food Chem. **66:** 401-436.
Roginsky V.A., Barsukova T.K., Remorova A.A., Bors W. (1996). **"**Moderate antioxidative efficiencies of flavonoids during peroxidation of methyl linoleate in homogeneous and micellar solutions." JAOCS **73:** 777-786.
Roozen J.P. (1987). "Effects of types I, II and III antioxidants on phospholipid oxidation in a meat model for warmed over flavour." Food Chem. **24:** 167-185.
Roozen J.P., Frankel E.N., Kinsella J.E. (1994). "Enzymic and autoxidation of lipids in low fat foods: model of linoleic acid in emulsified triolein and vegetable oils." Food Chem. **50:** 39-43.
Ruiz-Larrea M.B., Mohan A.R., Paganga G., Miller N.J., Bolwell G.B., Rice-Evans C.A. (1997). "Antioxidant activity of phytoestrogenic isoflavones." Free Rad. Res. **26:** 63-70.
Sahu S.C., Gray G.C. (1993). "Interactions of flavonoids, trace metals, and oxygen: Nuclear DNA damage and lipid peroxidation induced by myricetin." Cancer Lett. **70:** 73-79.
Sahu S.C., Gray G.C. (1994). "Kaempferol-induced nuclear DNA damage and lipid peroxidation." Cancer Lett. **85:** 159-164.
Sanchez-Moreno C. (2002). "Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems." Food Sci. Tech. Int. **8:** 121-137.
Sanchez-Moreno C., Larrauri J.A., Saura-Calixto F. (1998). "A procedure to measure the antiradical efficiency of polyphenols." J. Sci. Food Agric. **76:** 270-276.
Sanchez-Moreno C., Larrauri J.A., Saura-Calixto F. (1999). "Free radical scavenging capacity of selected red, rose and white wines." J. Sci. Food Agric. **79:** 1301-1304.
Sawa T., Nakao M., Akaike T., Ono K., Maeda H. (1999). "Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: implication for the anti-tumor-promoter effect of vegetables." J. Agric. Food Chem. **45:** 397-402.
Sawai Y., Sakata K. (1998). "NMR analytical approach to clarify the antioxidative molecular mechanism of catechins using DPPH." J. Agric. Food Chem. **46**:111-114.
Schuller P. (1990). "Natural Antioxidants exploited commercially." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London,, ch.4.
Schwarz K., Bertelsen G., Nissen L.R., Gardner P.T., Heinonen M.I., Hopia A., Huynh-Ba T., Lambelet P., McPhail D., Skibsted L.H., Tijburg L. (2001). "Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assay based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds." Eur. Food Res. Technol. **212:** 319-328.
Sedman J., Ismail A.A., Nicodemo A., Kubow S., van de Voort F.R. (1996). "Application of FTIR/ATR differential spectroscopy for monitoring oil oxidation and antioxidant efficacy." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Shahidi F., Wanasundara P.D. (1992). "Phenolic antioxidants." Crit. Rev. Food Sci. Nutr., **32:** 67-103.

Shahidi F., Wanasundara U.N. (1996). "Application of ^{1}H NMR spectroscopy assessment of oxidative stability of fats and oils." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Shahidi F., Wanasundara U.N. (1998). "Methods of measuring oxidative rancidity in fats and oils." in Food Lipids. Chemistry, Nutrition, and Biotechnology. (Eds. Akoh C.C., Min D.B.), Marcel Dekker, Inc., New York, pp 377-448.
Sherwin E.R. (1968). "Methods for stability and antioxidant measurement." JAOCS **45:** 632A-634A; 646A-648A.
Shukla V.K.S., Wanasundara P.K.J.P.D., Shahidi F. (1996). "Antioxidants from oilseeds." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Singleton V.L., Orthofer R., Lamuela-Raventos R.M. (1999). "Analysis of total phenols and other oxidation substrates and antioxidants by means of FCR." Meth. Enzymol. **299:** 152-178.
Sluis A.A. van der, Decker M., Verkerk R., Jongen W.M.F. (2000). "An improved rapid *in vitro* method to measure antioxidant activity. Application on selected flavonoids and apple juice." J. Agric. Food Chem. **48:** 4116-4122.
Strube M., Haenen G.R.M.M., van der Berg H., Bast A. (1997). "Pitfalls in a method for assessment of total antioxidant capacity." Free Rad. Res. **26:** 515-521.
Taga M.S., Miller E.E., Pratt D.E. (1984). "Chia seeds as a source of natural lipid antioxidants." JAOCS **61:** 928-931.
Takacsova M., Pribela A., Faktorova M. (1995). "Study of the antioxidative effects of thyme, sage, juniper and oregano." Nahrung **39:** 241-243.
Takao T., Kitatani F., Watanabe N., Yagi A., 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.
Thornalley P.J., Bannister J.V. (1985). **"**The spin trapping of superoxide radicals." in CRC Handbook of methods for oxygen radical research. (Ed. Greenwald R.A.), pp 133-207.
Tian K., Dasgupta P.K. (1999). "Determination of oxidative stability of oils and fats." Anal. Chem. **72:**1692-1698.
Torre Boronat de la M.C., Lopez-Tamames E. (1997). "El papel de los antioxidants." Alimentaria **6:** 19-27.
Toyo'oka T., Kashiwazaki T., Kato M. (2003). "On-line screening methods for antioxidants scavenging superoxide anion radical and H_2O_2 by LC with indirect CL detection." Talanta **60:** 467-475.
Tyrrel R.M. (1995). "Ultraviolet radiation and free radical damage to skin." in Biochem. Soc. Symp., No.61 - Free radicals and oxidative stress: environment, drugs and food additives. (Eds. Rice-Evans C., Halliwell B. & Lunt G.G.), University of Sussex, Dec.1994, pp 47-53.
Vekiari S.A., Oreopoulou V., Tzia C., Thomopoulos C.D. (1993). "Oregano flavonoids as lipid antioxidants." JAOCS **70:** 483-487.
Velioglu Y.S., Mazza G., Gao L., Oomach B.D. (1998). "Antioxidant activity and total phenolics of selected fruits, vegetables, and grain products." J. Agric. Food Chem. **46:** 4113-4117.
Vinson J.A., Hontz B.A. (1995). "Phenol antioxidant index: comparative antioxidant effectiveness of red and white wines." J. Agric. Food Chem. **43:** 401-403.
Wada Sh., Fang X. (1992). "The synergistic antioxidant effect of rosemary extract and α-tocopherol in sardine oil, model system and frozen-crushed fish meat." J. Food Process Preserv. **16:** 263-274.
Wanasundara U.N., Shahidi F. (1994). "Stabilization of canola oil with flavonoids." Food Chem. **50:** 393-396.
Wang H., Cao G., Prior R.L. (1997). "The oxygen radical absorbing capacity of anthocyanins." J. Agric. Food Chem. **45:** 304-309.

Wang H., Cao G., Prior R.L., (1996). **"**Total antioxidant capacity of fruits." J. Agric. Food Chem. **44:** 710-705.
Wang H., Nair M.G., Strasburg G.M., Chang Y.-C., Booren A.M. (1999). "Antioxidant and anti-inflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries." J. Nat. Prod. **62:** 294-296.
Wayner D.D.M., Burton G.W., Ingold K.U., Locke S. (1985). "Quantitative measurement of the total peroxyl radical-trapping antioxidant capacity of human blood plasma by controlled peroxidation." FEBS Lett. **187:** 33-37.
Wettasinghe M., Shahidi F. (1999). "Evening primrose: a source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals." J. Agric. Food Chem. **47:** 1801-1812.
White P.J., Xing Y. (1996). "Antioxidants from cereals and legumes." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Whitehead T.P., Thorpe G.H.G., Maxwell S.R.J. (1992). "Enhanced chemiluminescenct assay for antioxidant capacity in biological fluids." Analytica Chimica Acta **266:** 265-277.
Williams R.I., Elliot M.S. (1996). "Antioxidants from grapes and wine: chemistry and health effects." in Natural Antioxidants. Chemistry, health effects & applications. (Ed. Shahidi F.), AOCS Press, Champaign, Illinois, USA.
Yamaguchi F., Yoshimura Y., Nakazawa H., Ariga T. (1999). "Free radical scavenging activity of grape seed extract and antioxidant by ESR spectrometry in an H_2O_2/NaOH/DMSO system." J. Agric. Food Chem. **47:** 2544-2548.
Yamaguchi T., Takamura H., Matoba T., Terao J. (1998). "HPLC method for evaluation of the free radical-scavenging activity of foods by using DPPH." Biosci. Biotechnol. Biochem. **62:** 1201-1204.
Yamamoto Y., Niki E., Tanimura R., Kamiya Y. (1985). "Study of oxidation by chemiluminescence. IV. Detection of low levels of lipid hydroperoxides by chemiluminescence." JAOCS **62:** 1248-1250.
Yamauchi R., Kato K., Ueno Y. (1988). "Formation of trimers of α-tocopherol and its model compound, 2,2,5,7,8-pentamethylchroman-6-ol, in autoxidizing methyl linoleate." Lipids **23:** 779-783.
Yang G.C., Qiang W., Morehouse K.M., Rosenthal I., Ku Y., Yurawecz P. (1991). "Determination of hydroperoxides in edible oils by ESR, TBA assay, and LC-CL techniques." J. Agric. Food Chem. **38:** 896-898.
Yanishlieva-Maslarova N. (2001). "Inhibiting oxidation." in Antioxidants in food. Practical applications. (Eds. Pokorny J., Yanishlieva N., Gordon M.), ch. 3, pp 22-70, CRC Press, Boca Raton, Woodhead Publishing Ltd., Cambridge, England
Yen G.-C., Chen H.-Y. (1995). "Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. **43:** 27-32.
Yoshida T., Mori K., Hatano T., Okumura T., Uehara I., Komagoe K., Fujita Y., Okuda T. (1989). "Studies on inhibition mechanism of autoxidation by tannins and flavonoids. V. Radical-scavenging effects of tannins and related polyphenols on DPPH radical." Chem. Pharm. Bull. **37:** 1919-1921.
Zandi P., Gordon M.H. (1999). "Antioxidant activity of extracts from old tea leaves." Food Chem. **64:** 285-288.
Zhang H.L., Nagatsu A., Sakakibara J. (1996). "Novel antioxidants from safflower (*Carthamus tinctorius* L.) oil cake." Chem. Pharm. Bull. **44:** 874-876.

1.5.2 References on Sideritis

Aboutabl E.A., Elsakhawy F.M., Maklad Y.A., Osman A.F., El-Khrisy E.A.M. (2002). "Phytochemical and pharmacological studies on *Sideritis taurica* Stephan ex Wild." J. Ethnopharmacol. **82:** 177-184.

Adzet T., Canigueral S., Ibanez C. (1989a). "Essential oil of *Sideritis granatensis* (Pau) Rivas-Goday (Lamiaceae)." Flav. Fragr. J. **4:** 129-132.
Adzet T., Conigueral S., Ibanez C., Vila R. (1989b). "Essential oil of *Sideritis reverchonii*." Herba Hungarica **28:** 59-62.
Adzet T., Conigueral S., Monasterio I., Vila R., Ibanez C. (1990). "The essential oil and polyphenols of *Sideritis hyssopifolia* var. *pyreniaca*." J. Ess. Oil Res. **2:** 151-152.
Akcos Y., Ezer N., Calis I., Demirdamar R.B.C. (1999). "Polyphenolic compounds of *Sideritis lycia* and their anti-inflammatory activity." Pharm. Biol. **37:** 118-122.
Akcos Y., Ezer N., Ozcelik B., Abbasoglu U. (1998). "Iridoid glucosides from *Sideritis lycia* Boiss. & Heldr. and its antimicrobial activities." FABAD Farm. Bilimler Derg. **23:** 99-103.
Alcaraz M.J., Anon M.T., Ubeda A. (1990). "Flavonoids as inhibitors of CCl_4-induced cytotoxicity in isoalted hepatocytes." Planta Medica **56:** 679.
Alcaraz M.J., Ferrandiz M.L. (1990). "Diterpenoids from *Sideritis javalambrensis* Pau." Plantes Med. Phytother. **24:** 82-86.
Alcaraz M.J., Ferrandiz M.L., Villar A. (1986). "Flavonoid inhibition of soybean lipoxygenase." Pharmazie **41:** 299-300.
Alcaraz M.J., Garcia-Ochoa S., Jimenez M.J., Valverde S., Villar A. (1989a). "A derivative of ent-13-epi-manoyl oxide isolated from *Sideritis javalambrensis*." Phytochemistry **28:** 1267-1268.
Alcaraz M.J., Hoult J.R.S. (1985). "Effect of hypolaetin-8-glucoside and related flavonoids on soybean lipoxygenase and snake venom phospholipase A2." Arch. Int. Pharmacodyn. **278:** 4-12.
Alcaraz M.J., Jimenez M.J. (1988). "Flavonoids as anti-inflammatory agents." Fitoterapia **59:** 25-38.
Alcaraz M.J., Jimenez M.J., Valverde S., Sanz J., Rabanal R.M., Villar A. (1989). "Anti-inflammatory compounds from *Sideritis javalambrensis* n-hexane extract." J. Nat. Prod. **52:** 1088-1091.
Alcaraz M.J., Rios J.L. (1991). "Pharmacology of diterpenoids." in Ecological Chemistry and Biochemistry of Plant Terpenoids. (Eds. Harborne J.B.; Tomas-Barberan F.A.), 230-263
Alcaraz M.J., Villar A. (1987). "Effect of bojatriol on the synthesis of prostaglandins." Pharmazie **42:** 278
Algarra J., Garcia-Granados A., De Buruaga A.S., De Buruaga J.M. (1983). "Diterpenoids from *Sideritis varoi* Algarra J." Phytochemistry **22:** 1779-1782.
Aligiannis N., Kalpoutzakis E., Chinou I.B., Mitakou S. (2001). "Composition and antimicrobial activity of the essential oils of five taxa of *Sideritis* from Greece." J. Agric. Food Chem. **49:** 811-815.
Alikovski A.S. (1999). "Mursalski chai.", Anri-64 Ltd., Rozhen Ltd., Smolian (in Bulg.).
Alvarez P., Benedi J., Coma I., Iglesias I., Rebuelta M., Villar A. (1990). "Anti-inflammatory activity of various plant extracts." Plantes Med. Phytother. **24:** 92-97.
Amparo L.M., Rodriguez B. (1976). An. Quim. **72:** 578.
Atoui A.K., Mansouri A., Boskou G., Kefalas P. (2005). "Tea and herbal infusions: Their antioxdant activity and phenolic profile." Food Chem. **89:** 27-36.
Ayer W.A., Ball J.-A. H., Rodriguez B., Valverde S. (1974). "The structure of sideritol, a diterpenoid of the ent-atisane class." Can. J. Chem. **52:** 2792-2797.
Barber J.C., Francisco-Ortega J., Santos-Guerra A., Turner K.G., Jansen R.K. (2002). "Origin of Macaronesian *Sideritis* L. (Lamioideae, Lamiaceae) inferred from nuclear and chloroplast sequence datasets." Mol. Phylogenetics Evol. **23:** 293-306.
Baser K.H.C., Bondi M.L., Bruno M., Kirimer N., Piozzi F., Tumen G., Vassallo N. (1996b). "An ent-kaurane from *Sideritis* Huber-Morathii." Phytochemistry **43:** 1293-1295.
Baser K.H.C., Kirimer N., Ozek T., Tumen G., Karaer F. (1996a). "Essential oil composition of three Labiatae endemic to Turkey (*Micromeria fruticosa* (L.) Druce subsp.

giresunica P.H. Davis, *Sideritis lycia* Boiss. & Heldr. and *Sideritis arguta* Boiss. & Heldr.)." J. Essent. Oil Res. **8:** 699-701.
Baser K.H.C., Kirimer N., Tumrn G. (1997). "Essential oil of *Sideritis scardica* Griseb. subsp. *scardica*." J. Essent. Oil Res. **9:** 205-207.
Breton J.L., Gonzalez A.G., Rocha J.M., Panizo P.M., Gonzalez B.R., Valverde S. (1969). "Candicandiol, a new diterpene from *Sideritis candicans* Ait. var. *eriocephala* Webb. (Labiatae)." Tetrahedron Lett. **7:** 599-602.
Bruno M., Piozzi F., Arnold N.A., Baser K.H.C., Tabanca N., Kirimer N. (2005). "Kaurene diterpenoids from three *Sideritis* species." Turk. J. Chem. **29:** 61-64.
Bruno M., Rosselli S., Pibiri I., Kilgore N., Lee K.H. (2002). "Anti-HIV agents derived from the ent- kaurane diterpenoid linearol." J. Nat. Prod. **65:** 1594-1597.
Burzaco A., Velasco-Negueruela A., Perez-Alonso M.J. (1992). "Essential oil analysis of *Sideritis pauli* Pau." Flav. Fragr. J. **7:** 47-48.
Cabrera E., Garcia-Granados A., De Buruaga A.S., De Buruaga J.M. (1983). "Diterpenoids from *Sideritis hirsuta* subsp. *nivalis*." Phytochemistry **22:** 2779-2781.
Cabrera E., Garcia-Granados A., Quecuty M.A. (1988). "Terpenoids from *Sideritis varoi*." Phytochemistry **27:** 183-185.
Carstenn-Lichterfelde C. von, Valverde S., Rodriguez B. (1974). "Studies on diterpenes from genus *Sideritis*. XIII Two new stachene derivatives from *Sideritis angustifolia* (Labiatae)." Aust. J. Chem. **27:** 517-529.
Castrillo A., De Las Heraz B., Hortelano S., Rodriguez B., Villar A., Bosca J L.. (2001). "Inhibition of the nuclear factor kB (NF-kB) pathway by tetracyclic kaurene diterpenes in macrophages." Biol. Chem. **276:** 15854-15860.
Darias V., Bravo L., Rabanal R., Sanchez-Mateo C.C., Martin-Herrera D.A (1990). "Cytostatic and antibacterial activity of some compounds isolated from several Lamiaceae species from the Canary islands." Planta Medica **56:** 70-72.
Demetzos C., Mitaku S., Couladis M., Harvala C., Kokkinopoulos D. (1994). "Natural metabolites of ent-13-epi-manoyl oxide and other cytotoxic diterpenes from the resin "LADANO"of *Cistus creticus*." Planta Medica **60:** 590-591.
Demo A., Petrakis Ch., Kefalas P., Boskou D. (1998). "Nutrient antioxidant in some herbs and Mediterranean plant leaves." Food Res. Int. **31:** 351-354.
Diaz R., Quevedo-Sarmiento J., Ramos-Cormenzana A., Cabo P., Cabo J. (1988b). "Phytochemical and antibacterial screening of some species of Spanish Lamiaceae." Fitoterapia **56:** 329-333.
Diaz R.M., Garcia-Granados A., Moreno E., Parra A., Quevedo-Sarmiento J., De Buruaga A.S. (1988a). "Studies on the relationship of structure to antimicrobial properties of diterpenoid compounds from *Sideritis*." Planta Medica **54:** 301-304.
Disli A., Yildirir Y., Yasar A. (2002). "Galactat, a new diterpene from *Sideritis galactica*." Ankara Univ. Eczacilik Fak. Dergisi **31:** 83-89.
Djordjevic S., Blagojevic S., Sekulovic D., Sekesan V., Runjajic-Antic D. (1993). "Karakterizacija mineralnog sastava ulaznuh komponenti i formulacija fitopreparata za prevenciju i lečenje nutritivnih anemia." in Izvodi Radova 74-75; Arch. Farm. **43:** 225.
Dulger B., Gonuz A., Aysel V. (2006). "Inhibition of clotrimazole-resistant *Candida albicans* by some endemic *Sideritis* sp. from Turkey." Fitoterapia **77:** 404-405.
Ertan A., Azcan N., Demirci B., Baser K.H.C. (2001). "Fatty acid composition of *Sideritis* sp." Chem. Nat. Comp. **37:** 310-303.
Escamilla E.M., Rodriguez B. (1980). "Two new diterpenoid acetates from *Sideritis serrata*." Phytochemistry **19:** 463-464.
Evstatieva L. (2002). "Variation in the flavonoids and tannins content of *Sideritis scardica* Griseb." in Godishnik na Sofiiskiya Universitet "Sv. Kl. Ohridski", Biologicheski facultet, Botanika: v.2, 92: 99-105, Universitetsko izdatelstvo "Sv. Kl. Ohridski" (in Bulg.).
Evstatieva L., Koleva I. (2000). "Cultivation of *Sideritis scadica*." Arandelovac, Yugoslavia, **May 29-June 03:** 189-195.

Evstatieva L., Vasilev P. (1995). "Varhu razpostranenieto, ekologichnite osobenosti i fitocenologichnata rolja na siriiskija chai (*Sideritis syriaca* L.) v Bulgaria." in Sbornik 100 godini ot rozhdenieto na akad. B. Stefanov., **t.2:** 187-192 (in Bulg.).
Ezer N. et al. (1995a). Haccettepe Univ. Eczacilik Fak. Derg. **15:** 15.
Ezer N., Abbasoglu U. (1996). "Antimicrobial activity of essential oils of some *Sideritis* species growing in Turkey." Fitoterapia **67:** 474-475.
Ezer N., Sakar M.K., Rodriguez B., De la Torre M.C. (1992). "Flavonoid glycosides and a phenylpropanoid glycoside from *Sideritis perfoliata*." Int. J. Pharmacognosy **30:** 61-65.
Ezer N., Usluer G., Gunes I., Erol K. (1994). "Antibacterial activity of some *Sideritis* species." Fitoterapia **65:** 549-551.
Ezer N., Vila R., Conigueral S., Adzet T. (1995b). "Essential oil of *Sideritis lycia* Boiss.& Heldr." J. Ess. Oil Res. **7:** 183-185.
Ezer N., Vila R., Conigueral S., Adzet T. (1996). "Essential oil composition of four Turkish species of *Sideritis*." Phytochemistry **41:** 203-205.
Fefer I.M (1970). "Flavonoids from *Sideritis catillaris* Juz." Farm. Zh. **25:** 86.
Fefer I.M. (1971). "Comparative characterisation of flavonoid composition of *Sideritis taurica* Steph., *S. marschalliana* Juz. and *S. ajpetriana* Klok." Farm. Zh. **26:** 86.
Fefer L.M., Gritsenko O.M. (1977). "Phenolic acids of *Sideritis catillaris* L." Farm. Zh. **34**: 92.
Fernandez C., Fraga B.M., Gonzalez A.G., Hernandez M.G., Hanson J.R., Hitchcock P.B. (1985). "Ent-norambrenolide from *Sideritis nutans*." Phytochemistry **24:** 188-189.
Fernandez C., Fraga B.M., Hernandez M.G. (1986a). "Diterpenes from *Sideritis infernalis*." Phytochemistry **25:** 2573-2576.
Fernandez C., Fraga B.M., Hernandez M.G. (1986b). "Diterpenes from *Sideritis nutans*." Phytochemistry **25:** 2825-2827.
Fernandez C., Fraga B.M., Hernandez M.G. (1988). "Flavonoid aglycones from some Canary islands species of *Sideritis*." J. Nat. Prod. **51:** 591-593.
Ferreres F., Tomas-Lorente F., Barberan F.A.T. (1987). "Estudio quimiotaxonomico de *Sideritis spinulosa, S. linearifolia, S. spinulosa x S. linearifolia* y *S. linearifolia x S. spinulosa*." An. Quim. **83:** 356-357.
Ferreres F., Tomas-Lorente F., Barberan F.A.T. (1989). "Biochemical identification of *Sideritis serrata* X *S. bourgaeana* hybrids by HPLC analyses of flavonoids." Z. Naturforsch. **44c:** 568-572.
Fiamegos Y.C., Nanos C.G., Vervoort J., Stalikas C.D. (2004). "Analytical procedure for the in-vial derivatization-extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by GC with mass-selective detection." J. Chromatogr. A **1041:** 11-18.
Flamini G., Cioni P.L., Morelli I., Maccioni S., Tomei P.E. (1994). "Characterization of the volatile fraction of a *Sideritis romana* population from Montemarcello (Eastern Liguria)." J. Ess. Oil Res. **6:** 239-242.
Floroupaneri P., Christaki E., Giannenas I.A., Papazahariadou M., Botsoglou N.A. (2004). "Effect of dietary Olympus tea (*Sideritis scardica*) supplementation on performance of chickens challenged with *Eimeria tenella*." J. Animal Feed Sci. **13:** 301-311.
Font-Quer P. (1976). "Plantas Medicinales." Labor, Barcelona, p. 662.
Fraga B.M., Guillermo R., Hernandez M.G., Mestres T., Arteaga J.M. (1991a). "Diterpenes from *Sideritis canariensis*." Phytochemistry **30:** 3361-3364.
Fraga B.M., Hernandez M.G., Fernandez C., Arteaga J.M. (1987). "Diterpenes from *Sideritis dendrochahorra* and *S. cystosiphon*." Phytochemistry **26:** 775-777.
Fraga B.M., Hernandez M.G., Melchor G., Diaz C.E. (2003a). "On the ent-kaurene diterpenes from *Sideritis athoa*." Nat. Prod. Res. **17:** 141-144.
Fraga B.M., Hernandez M.G., Santana J.M., Arteaga J.M. (1990). "Diterpenes from *Sideritis sventii* and *S. cystosiphon*." Phytochemistry **29:** 591-593.

Fraga B.M., Hernandez M.G., Santana J.M., Arteaga J.M. (1991b). "Diterpenes from *Sideritis ferrensis*." Phytochemistry **30:** 913-915.
Fraga B.M., Hernandez M.G., Santana J.M.H., Terrero D., Galvan M.F. (1995). "A chemotaxonomical study of *Sideritis massoniana* taxa." Biochem. Syst. Ecol. **23:** 835-842.
Fraga B.M., Reina M., Luis J.G., Rodriguez M.L. (2003b). "Rhoiptelenol and rhoiptelenone, two pentacyclic triterpenes from *Sideritis macrostachia*." Z. Naturforsch. [C] **58:** 621-625.
Gabrieli C.N., Kokkalou E. (1990). "A glucosylated acylflavone from *Sideritis raeseri*." Phytochemistry **29:** 681-683.
Gabrieli C.N., Kefalas P. G., Kokkalou E.L. (2005/2004). "Antioxidant activity of flavonoids from *Sideritis raeseri*." J. Ethnopharm. **96:** 423-428.
Gabrieli C.N., Kokkalou E.L., Niopas I. (1990). "Anti-inflammatory activity of a novel glucosilated acylflavone from *Sideritis raeseri*." Sci. Pharm. **58:** 395-397.
Galati E.M., Germano M.P., Rossitto A., Tsakou O., Skaltsa H., Roussis V. (1996). "Essential oil of *Sideritis raeseri* Boiss.& Heldr. ssp. *raeseri.*" J. Ess. Oil Res. **8:** 303-304.
Garcia-Alvarez M.C., Rodriguez B. (1976). "Epoxy-isosidol: a new diterpenoid from *Sideritis biflora*." Phytochemistry **15:** 1994-1995.
Garcia-Alvarez M.C., Rodriguez B. (1980). "Diterpenoids from *Sideritis foetens*." Phytochemistry **19:** 2405-2407.
Garcia-Granados A., Jimenez B., Martinez A., Parra A., Rivas F., Arias J.M. (1994). "Chemical-microbiological synthesis of ent-13-epi-manoyl oxides with biological activities." Phytochemistry **37:** 741-747.
Garcia-Granados A., Linan E., Martinez A., Rivas F., Mesa-Valle C.M., Castilla-Calvente J.J., Osuna A. (1997). "In vitro action of ent-manoyl oxides against *Leishmania donovani*." J. Nat. Prod. **60:** 13-16.
Garcia-Granados A., Martinez A., Molina A., Onorato M.E. (1986). "Terpenoids from *Sideritis varoi* subsp. *oriensis*." Phytochemistry **25:** 2171-2173.
Garcia-Granados A., Martinez A., Molina A., Onorato M.E., Rico M., De Buruaga A.S., De Buruaga J.M. (1985b). "Diterpenoids from *Sideritis varoi* subsp. *cuatrecasasii*: ^{13}C NMR of ent-13-epi-manoyl oxides fuctionalized at C-12*." Phytochemistry **24:** 1789-1792.
Garcia-Granados A., Martinez A., Onorato M.E. (1985a). "Diterpenoids from *Sideritis arborescens* subsp. *paulii.*" Phytochemistry **24:** 517-521.
Garcia-Granados A., Martinez A., Onorato M.E., Socorro O. (1984). "Diterpenoids from *Sideritis pusilla* subsp. *flavovirens*." Phytochemistry **23:** 607-610.
Garcia-Granados A., Molina A. (1989). "New aromadendranic sesquiterpenes from *Sideritis varoi* ssp. *cuatrecasasii*." Can. J. Chem. **67:** 1288-1292.
Garcia-Granados A., Molina A., De Buruaga A.S., De Buruaga J.M. (1985). "Sesquiterpenes from two subspecies of Sideritis varoi." Phytochemistry **24:** 97-101.
Garcia-Granados A., Parra A., Pena A., De Buruaga A.S., De Buruaga J.M, Valverde S. (1980). "Atisideritol, a new ent-atis-13-ene from a *Sideritis pusilla* (Lag) Pau ssp." Tetrahedron Lett. **21:** 3611-3612.
Garciamaroto F., Guilguerrero J.L. (2004). "Essential oil composition of *Sideritis pusilla.*" J. Ess. Oil Res. **16:** 535-538.
Gergis V., Argyriadou N., Poulos C. (1989). "Composition of the essential oils of *Sideritis clandestina* ssp. *cyllenea* and *S. sipylea*." J. Sci. Food Agric. **47:** 501-507.
Gergis V., Spiliotis V., Argyriadou N., Poulos C. (1991). 'Relation between the antimicrobial activity and the chemical composition of the essential oil of *Sideritis sipylea* Boiss. (Labiatae)." Flav. Fragr. J. **6:** 93-95.
Gergis V., Spiliotis V., Poulos C. (1990). "Antimicrobial activity of essential oils from Greek *Sideritis* species." Pharmazie **45:** 70.
Ghoumari H., Benajiba M.-H., Azmani A., Garcia-Granados A., Martinez A., Parra A., Rivas F., Soccoro O. (2005). "Ent-kauranoid derivatives from *Sideritis moorei*." Phytochemistry **66:** 1492-1498.

Gil M.I., Ferreres F., Marrero A., Tomas-Lorente F., Barberan F.A.T. (1993). "Distribution of flavonoid aglycones and glycosides in *Sideriti*s species from the Canary islands and Madeira." Phytochemistry **34:** 227-232.
Godoy A., De las Heras B., Vivas J.M., Villar A. (2000). "Anti-inflammatory properties of a lipid fraction obtained from *Sideritis javalambrensis*." Biol. Pharm. Bull. **23:** 1193-1197.
Gomez-Serranillos M.P., El-Naggar T., Villar A.M., Carretero M.E. (2004). "Analysis and retention behaviour in HPLC of terpenic plant constituents (*Sideritis* sp.) with pharmacological interest." J. Chromatogr. B **812:** 379-383.
Gomez-Serranillos M.P., Carretero E., Slowing K., Palomino O.M., Villarubia A.I., Villar A. (1998). "HPLC quantitative analysis of diterpenoids in *Sideritis* (Labiatae) species." Phytother. Res. **12(Suppl. 1)**: S101-S103.
Gomez-Serranillos M.P., Palomino O.M., Villarubia A.I., Cases M.A., Carretero E., Villar A. (1997). "Analysis of diterpenoids from *Sideritis* species by HPLC." J. Chromatogr. A **778:** 421-425.
Gonzalez A.G., Breton J.L., Fraga B.M., Luis J.G. (1971). "Constituents of Labiatae. IX. Trachinodiol and trachinol, two new diterpenes from *Sideritis canariensis* Ait." Tetrahedron Lett. **33**: 3097-3100.
Gonzalez A.G., Fraga B.M., Hernandez M.G., Larruga F., Luis J.G. (1975). "Four new labdane diterpene oxides from *Sideritis gomerae*." Phytochemistry **14:** 2655-2656.
Gonzalez A.G., Fraga B.M., Hernandez M.G., Larruga F., Luis J.G., Ravelo A.G. (1978). "Flavones from some Canary species of *Sideritis*." J. Nat. Prod. **41:** 279-280.
Gonzalez A.G., Fraga B.M., Hernandez M.G., Luis J.G. (1973a). "New diterpenes from *Sideritis candicans*." Phytochemistry **12:** 2721-2723.
Gonzalez A.G., Fraga B.M., Hernandez M.G., Luis J.G. (1973b). "New diterpenes from *Sideritis canariensis*." Phytochemistry **12:** 1113-1116.
Gonzalez A.G., Fraga B.M., Hernandez M.G., Luis J.G. (1973c). "Vierol and powerol, two new diterpenes from *Sideritis canariensis*." Tetrahedron **29:** 561-563.
Gonzalez A.G., Fraga B.M., Hernandez M.G., Luis J.G., Larruga F. (1979). "Comparative phytochemistry of the genus *Sideritis* from the Canary islands." Chem. System. Ecol. **7:** 115-120.
Gonzalez A.G. et al. (1974). An. Quim. **70:** 730.
Gurbuz I., Ozkan A.M., Yesilada E., Kutsal O. (2005). "Anti-ulcerogenic activity of some plants used in folk medicine of Pinarbasi (Kayseri, Turkey)." J. Ethnopharmacol. **101:** 313-318.
Guvenc A., Houghton P.J., Duman H., Coskun M., Sahin P. (2005). "Antioxidant activity studies on selected *Sideritis* sp. native to Turkey." Pharm. Biol. **43:** 173-177.
Harborne J.B., Barberan F.A.T., Williams C.A., Gil M.I. (1986). "A chemotaxonomic study of flavonoids from European *Teucrium* species." Phytochemistry **25:** 2811-2816.
Hegnauer R., Kooiman P. (1978). "Die systematishe bedeutung von iridoiden inhaltstoffen im Rahmen von Wettstein's Tubiflorae." Planta Medica **33:** 1-33.
Heras B. de las et al. (1994b). Agents Action **41:** 114.
Heras B. de las, Hoult J.R. (1994). "Non-cytotoxic inhibition of macrophage eicosanoid biosynthesis and effects on leukocyte functions and reactive species of two novel anti-inflammatory plant diterpenoids." Planta Medica **60:** 501-506.
Heras B. de las, Vivas J.M., Villar A. (1990). "Anti-inflammatory activity of *Sideritis javalambrensis* in rats." Planta Medica **56:** 659.
Heras B. de las, Vivas J.M., Villar A. (1994a). "Anti-inflammatory activity of *Sideritis javalambrensis* extracts." J. Ethnopharmacol. **41:** 15-17.
Hernandez-Perez M., Rabanal R.M. (2002a). "Analgesic and anti-inflammatory properties of *Sideritis lotsyi* var. *mascaensis*." Phytother. Res. **16:** 264-266.
Hernandez-Perez M., Rabanal R.M. (2002b). "Evaluation of the anti-inflammatory and analgesic activity of *Sideritis canariensis* var. *pannosa* in mice." J. Ethnopharmacol. **81:** 43-47.

Hernandez-Perez M., Sanchez-Mateo C.C., Montalbetti-Moreno Y., Rabanal R.M. (2004). "Studies on the analgesic and anti-inflammatory effects of *Sideritis candicans* Ait. var. *eriocephala* Webb aerial part." J. Ethnopharmacol. **93:** 279-284.
Ivanova D., Gerova D., Chervenkov T., Yankova T. (2005). "Polyphenols and antioxidant capacity of Bulgarian medicinal plants." J. Ethnopharmacol. **96:** 145-150.
Jimenez A. et al. (1990). Sci. Pharm. **58:** 395.
Karanika M.S., Komaitis M., Aggelis G. (2001). "Effect of aqueous extracts of some plants of Lamiaceae family on the growth of *Yarrowia lipolytica*." Int. J. Food Microbiol. **64:** 175-181.
Kassi E., Papoutsi Z., Fokialakis N., Messari J., Mitakou S., Moutsatsou P. (2004). "Greek plant extracts exhibit selective estrogen receptor modulator (SERM)-like properties." J. Agric. Food Chem. **52:** 6956-6961.
Kilic T. (2006). "Isolation and biological activity of new and known diterpenoids from *S. stricta* Boiss. & Heldr." Molecules **11:** 257-262.
Kilic T., Yildiz Y.K., Goren A.C., Tumen G., Topcu G. (2003). "Phytochemical analysis of some *Sideritis* sp. of Turkey." Chem. Nat. Comp. **39:** 453-456.
Kilic T., Yildiz Y.K., Topcu G., Goren A.C., Ay M., Bodige S.G., Watson W.H. (2005). "X-ray analysis of sideroxol from *Sideritis leptoclada*." J. Chem. Cryst. **35:** 647-650.
Kirimer N., Baser K.H.C., Demirci B., Duman. N. (2004). "Essential oils of *Sideritis* sp. of Turkey belonging to the section Empedoclia." Chem. Nat. Comp. **40:** 19-23.
Kirimer N., Baser K.H.C., Ozek T., Tanriverdi H., Kaya A. (1992a). "Composition of the essential oils of two subspecies of *Sideritis germanicopolitana* Bornm." J. Ess. Oil Res. **4:** 533-534.
Kirimer N., Baser K.H.C., Ozek T., Tumen G. (1994). "Essential oils of *Sideritis hispida* P.H.Davis, an endemic species from Turkey." J. Ess. Oil Res. **6:** 435-436.
Kirimer N., Baser K.H.C., Tumen G., Sezik E. (1992b). "Characterization of the essential oil of *Sideritis dichotoma*." J. Ess. Oil Res. **4:** 641-642.
Kirimer N., Kurkcuoglu M., Ozek T., Baser K.H.C., Tumen G. (1996). "Composition of the essential oil of *Sideritis condensata* Boiss. & Heldr." Flav. Flav. J. **11:** 315-320.
Kirimer N., Tabanca N., Baser K.H.C., Tumen G. (2001b). "Composition of the essential oil of *Sideritis congesta* P. H. Davis & Hub.-Mor." J. Ess. Oil Res. **13:** 132-133.
Kirimer N., Tabanca N., Demirci B., Baser K.H.C., Duman N., Aytac Z. (2001a). "The essential oil of a new *Sideritis* sp.: *S. ozturkii* Aytac & Aksoy." Chem. Nat. Comp. **37:** 234-237.
Kirimer N., Tabanca N., Ozek T., Baser K.H.C., Tumen G. (1999). "Composition of essential oil from two endemic *Sideritis* sp. of Turkey." Chem. Nat. Comp. **35:** 61-64.
Kirimer N., Tabanca N., Ozek T., Baser K.H.C., Tumen G., Duman H. (2003). "Composition of essential oils from five endemic *Sideritis* sp.." J. Ess. Oil Res. **15:** 221-225.
Kirimer N., Tabanca N., Ozek T., Tumen G.,. Baser K.H.C. (2000). "Essential oils of annual *Sideritis* sp. growing in Turkey." Pharm. Biol. **38:** 106-111.
Kocabas Y.Z., Karaman S. (2001). "Essential oils of Lamiaceae family from south-east Mediterranean region (Turkey, Pakistan)." J. Biol. Sci. **4:** 1221-1223.
Koedam A. (1986). "Volatile oil composition of Greek mountain tea (*Sideritis* ssp.)." J. Sci. Food Agric. **36:** 681-684.
Kokkalou E. (1987). "Costituants entrainables a la vapeur d'eau de *Sideritis scardica* Griss. ssp. *scardica*." Plantes Med. Phytother. **21:** 262-266.
Kokkalou E., Gabrielli C. (1997). "A new phenylpropanoid glycoside and two flavones from *Sideritis raeseri* Bois. & Heldr." Pharmazie **52:** 322-324.
Koleva I., Handjieva N. (1997). "A study on the iridoid glycosides in *Sideritis* (Lamiaceae)." Scientific works **v. XLII:** 75-79, HIFFI-Plovdiv.
Komaitis M., Falirea A., Voudouris E. (1985). "Constituents of the essential oil of *Sideritis cretica* Boiss." J. Sci. Food Agric. **36:** 970-972.

Komaitis M.E., Melissari-Panagiotou E., Infanti-Papatragianni N. (1992). "Constituents of the essential oil of *Sideritis scardica*." in Off-flavours in foods and beverages, Developments in Food Science 28. (Ed. Charalambous G.), pp 411-416.
Kooiman P. (1972). "The occurence of iridoid glycosides in the Labiatae." Acta Bot. Neerl. **21:** 417-427.
Laer U., Glombitza K.-W., Neugebauer M. (1996). "The essential oil of *Sideritis syriaca*." Planta Medica **62:** 81-82.
Lamaison J.I., Petitjean-Freytet C., Duband F., Carnat A.P. (1991). "Rosmarinic acid content and antioxidant activity in French Lamiaceae." Fitoterapia **62:** 166-171.
Lopez M.A., von Carstenn-Lichterfelde C., Rodriguez B., Fayos J., Martinez-Ripoll M. (1977). "Andalusol, a new diterpenoid from a *Sideritis arborescens* Salzm. ssp. Chemical and X-ray structure determination." J. Org. Chem. **42:** 2517-2518.
Lubenov J. (1984). "Plevelite - vragove i prijateli na choveka.", Zemizdat, Sofia (in Bulg.).
Manez S., Alcaraz M.J., Paya M., Rios J.L., Hancke J.L. (1990). "Selected extracts from medicinal plants as anti-inflammatory agents." Planta Medica **56:** 656.
Manez S., Jimenez A., Villar A., (1991). "Volatiles of *Sideritis mugronensis* flower and leaf." J. Ess. Oil Res. **3:** 395-397.
Manez S., Rios J.L., Paya M., Alcaraz M.J. Tomas-Barberan F.A. (1989). **"**An anti-inflmmatory diterpenoid from *Sideritis flavovirens* Rouy." Int. Symp. on Ecol. Chem. & Biochem. of Plant Terpenoids, Murcia, Spain.
Marin P.D., Sajdl V., Kapor S., Tatic B., Petkovic B., Duletic S (1992). "Fatty acids of the Stachyoideae." Biochem. Syst. Ecol. **20:** 389-392.
Marquez C., Panizo F.M., Rodriguez B., Valverde S. (1975). "A new diterpenoid acetate from *Sideritis reverchonii*." Phytochemistry **14:** 2713-2714.
Martin-Lomas M., Rabanal R.M., Rodriguez B., Valverde S. (1983). "Glucosido de flavonoide aislado de *Sideritis hirsute*." An. Quim. **79:** 230-231
Mateo C., Calderon J., Sanz J. (1988). "Essential oil of some *Sideritis* species from Central and Southern Spain." Phytochemistry **27:** 151-153.
Mateo C., Sanz J., Calderon J. (1983). "Essential oil of *Sideritis hirsute*." Phytochemistry **22:** 171-173.
Menghini L., Massarelli P., Bruni G., Menghini A. (2005). "Preliminary evaluation on anti-inflammtory and analgesic effects of *Sideritis syriaca* L. herba extracts." J. Med. Food **8:** 227-231.
Menkovic N.R., Ristic M., Samardzic Z., Savin K., Kovacevic N. (1991). "The essential oil of *Sideritis scardica*." Planta Medica **57:** A137.
Menkovic N.R., Savin K., Kovacevic N.N., Ristic M.S., Palevitch D. (1993). "Ivestigation of flavonoid complex of *Sideritis montana* L. from different localities in Serbia." (Ed. Putievsky E.), Int. Symp. on Medicinal & Aromatic plants, Tiberias on the Sea of Galilee, Israel, 22-25 Mar, 1993, Acta Horticult. **344:** 582-584.
Mora A., Paya M., Rios J.L., Alcaraz M.J. (1990). "Structure-activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation." Biochem. Pharmacol. **40:** 793-797.
Moron M., Merle H., Primo J., Blazquez M.A., Boira H. (2005). "Diterpene compounds in the essential oil of *Sideritis linearifolia* Lam. growing in Spain." Flav. Fragr. J. **20:** 205-208.
Navarro A., de las Heras B., Villar A. (2000). "Immunomodulating properties of the diterpene analusol." Planta Medica **66:** 289-291.
Navarro A., de las Heras B., Villar A. (2001). "Anti-inflammatory and immunomodulating properties of a sterol fraction from *Sideritis foetens* Clem." Biol. Pharm. Bull. **24:** 470-473.
Oktemer A., Logoglu E. (2003). "Isolation of a new diterpene from *Sideritis congesta*." Commun. Fac. Sci. Univ. Ank., Series B **49:** 1-3.
Ozcan M. (2004). "Mineral contents of some plants as condiments in Turkey." Food Chem. **84:** 437-440.

Ozcan M. (2005). "Chemical constituents of *Sideritis erythrantha* Boiss. & Heldr. var. *erythrantha* used as mountain tea and growing wild in Turkey." J. Ess. Oil-Bearing Plants **8:** 52-55.
Ozcan M., Chalchat J.C., Akgul A. (2001). "Essential oil composition of Turkish mountain tea (*Sideritis* spp.)." Food Chem. **75:** 459-463.
Ozek T., Baser K.H.C., Tumen G. (1993). "The essential oil of *Sideritis athoa* Papanikolaou & Kokkini." J. Ess. Oil Res. **5:** 669-670.
Ozturk Y., Aydin S., Ozturk N., Baser K.H.C. (1996). "Effects of extracts from certain *Sideritis* species on swimming performance in mice." Phytother. Res. **10:** 70-73.
Pakaln D.A., Zakharov A.M., Zakharova O.I., Pakaln A.E. (1976). "A search for flavonoid and iridoid compounds in the Caucasus flora of the Labiatae family." Farmacia **5:** 36-41.
Palomino O.M., Gomez-Serranillos P., Carretero E., Villar A. (1996a). "HPLC of flavonoids from *Sideritis* species." J. Chromatogr. A **731:** 103-108.
Palomino O.M., Sollhuber M., Carretero E., Villar A. (1996b). "Isoscutellarein 7-glucosyl(1-2) xyloside from sixteen species of *Sideritis*." Phytochemistry **42:** 101-102.
Papanicolaou K., Kokkini S.A (1984). "TLC study of some chemical leaf constituents in *Sideritis* L. sect. Empedoclia (Rafin.) Bentham (Labiatae) in Greece." Feddes Rep. **95:** 359-368.
Pathak D., Pathak K., Singla A.K. (1991). "Flavonoids as medicinal agents - recent advances." Fitoterapia **62:** 371-391.
Petkov V. (1982). "Savremenna fitoterapia.", Medicina i fizkultura, Sofia (in Bulg.).
Piozzi F., Venturella P., Bellino A. (1968). "Diterpenes from *Sideritis sicula ucria*." Tetrahedron **24:** 4073-4081.
Popa D.P., Saley L.A., Orguyan T.M. (1976). "Qualitative composition and quantitative content of terpenoids in some species from the Labiatae family." Rast. Res. **12:** 247-252.
Pulatova T.P., Aminov A. (1969). "Phytochemical investigation of *Sideritis montana* L." Khim. Prir. Soedin. **5:** 62-66.
Pulatova T.P., Aminov A. (1970). Uzb. Biol. Zh., **14:** 33.
Quesada T.G. de, Rodriguez B., Valverde S. (1974). "Diterpenes from *Sideritis lagaskana* and *Sideritis valverdei.*" Phytochemistry **13:** 2008.
Quesada T.G. de, Rodriguez B., Valverde S., Huneck S. (1972). "Six new diterpenes from *Sideritis leucantha* Cav. and *S. linearifolia* Lam." Tetrahedron Lett. **22:** 2187-2190.
Quesada T.G. de, Rodriguez B., Valverde S. (1975). "Four new diterpenes from *Sideritis serrata*." Phytochemistry **14:** 517-519.
Rabanal R.M., Valverde S., Martin-Lomas M., Rodriguez B., Chari V.M. (1982). "Chrysoeriol 7-(2"-O-β-D-allopyranosyl)-β-D-glucopyranoside from *Sideritis grandiflora*." Phytochemistry **21:** 1830-1832.
Rios J.L., Manez S., Paya M., Alcaraz M.J. (1992). "Antioxidant activity of flavonoids from *Sideritis javalambrensis*." Phytochemistry **31:** 1947-1959.
Rodriguez B. (1977). "5,4'-Dihydroxy-6,7,8,3'-tetramethoxyflavone from *Sideritis mugronensis*." Phytochemistry **16:** 800-801.
Rodriguez B., Valverde S. (1973). "Borjatriol, a new diterpenoid from *Sideritis mugronensis* Borja (Labiatae)." Tetrahedron **29:** 2837-2843.
Rodriguez B., Valverde S. (1975). "Diterpenes from three *Sideritis* species." Phytochemistry **14:** 1670-1671.
Rodriguez-Garcia I., Munoz-Dorado M., Gomez-Mercado F., Garcia-Maroto F., Guil-Guerrero J.-L. (2004). "Essential oil composition of *Sideritis pusilla* (Lange) Pau ssp." J. Ess. Oil Res. **6:** 535-538.
Rodriguez-Linde M.E., Diaz R.M., Garcia-Granados A., Quevedo-Sarmiento J., Moreno E., Onorato M.R., Parra A., Ramos-Cormenzana A. (1994). "Antimicrobial activity of natural and semisynthetic diterpenoids from *Sideritis* spp." Microbios **77:** 7-13.

Rodriguez-Lyon M.L., Diaz-Lanza A.M., Barnabe M., Villaescusa-Castillo L. (2000). "Flavone glycosides containing acylated sugars from *Sideritis hyssopifolia*." Magn. Reson. Chem. **38:** 684-687.
Sahin F.P., Tasdemir D., Ruedi P., Ezer N., Calis I. (2005/2004). "Three acylated flavone glycosides from *Sideritis ozturku* Aytac & Aksoy." Phytochemistry **65:** 2095-2099.
Sattar A.A., Bankova V., Kujumgiev A., Galabov A., Ignatova A., Todorova C., Popov S. (1995b). "Chemical composition and biological activity of leaf exudates from some Lamiaceae plants." Pharmazie **50:** 62-65.
Sattar A.A., Bankova V., Popov S. (1995a). "Acylated flavonoid glycosides from *Sideritis scardica.*" Fitoterapia **66:** 190.
Sattar A.A., Bankova V., Spassov S., Duddeck H. (1993). "Flavonoid glycosides from *Sideritis* species." Fitoterapia **64:** 278-279.
Schneider K., Novak A., Kubelka W. (1991). "Antibacterial activity and organoleptic properties of *Sideritis clandestina*." Planta Medica **57:** A45.
Schulz H., Oezkan G., Baranska M., Kruger H., Oezcan M. (2005). "Characterization of essential oil plants from Turkey by IR and Raman spectroscopy." Vibrat. Spectr. **39:** 249-256.
Sezik E., Ezer N., Hueso-Rodriguez J.A., Rodriguez B. (1985). "Ent-2α-hydroxy-13-epi-manoyl oxides from *Sideritis perfoliata*." Phytochemistry **24:** 2739-2740.
Stojanov N. (1973). "Nashite lekarstveni rastenia." p. II., Nauka i izkustvo, Sofia (in Bulg.).
Tabanca N., Kirimer N., Baser K.H.C., Husnu C. (2001). "The composition of essential oils from two varieties of *Sideritis erythrantha* var. *erythrantha* and var. *cedretorum*." Turkish J. Chem. **25:** 201-208.
Taskova R., Mitova M., Evstatieva L., Ancev M., Peev D., Hanjieva N., Bankova V., Popov S. (1997). "Iridoids, flavonoids and terpenoids as taxonomic markers in *Lamiaceae, Scrophulariaceae,* and *Rubiaceae."* Bocconea **5:** 631-636.
Tirillini B., Pellegrino R., Menghini L., Pagiotti, Menghini A. (2001). "Composition of the essential oil of *Sideritis syriaca* L. from Italy." J. Ess. Oil Res. **13:** 444-445.
Todorova M.N., Christov R.C., Evstatieva L. (2000). "Essential oil composition from three *Sideritis* sp. from Bulgaria." J. Ess. Oil Res. **12:** 418-420.
Tomas F. (1979). "Leucantina, nuevo flavonoide de la *Sideritis leucantha*." Rev. Agroquim. Tecnol. Aliment. **19:** 224-228.
Tomas F., Ferreres F. (1980). "Two flavone glucosides from *Sideritis leucantha*." Phytochemistry **19:** 2039-2040.
Tomas F., Ferreres F., Guirado A. (1979). "5,3',4'-Trihydroxy-6,7,8-trimethoxyflavone from *Sideritis leucantha*." Phytochemistry **19:** 185-186.
Tomas F., Voirin B., Barberan F.A.T., Lebreton P. (1985). "Hypolaetin 8-glucoside from *Sideritis leucantha*." Phytochemistry **24:** 1617-1618.
Tomas-Barberan F.A. (1986). "The flavonoid compounds from Labiatae." Fitoterapia **57:** 67-95.
Tomas-Barberan F.A., Ferreres F., Tomas F. (1985c). "TLC, UV and acidic treatment in the differentiation of 5,6- and 5,8-dihydroxyflavones, 3-methoxyflavones and flavonols." Tetrahedron **41:** 5733-5740.
Tomas-Barberan F.A., Ferreres F., Tomas-Lorente F., Rivera-Nunez D., Obon-De Castro C. (1990). "A chemotaxonomical study of some Portugese *Sideritis* species." Biochem. Syst. Ecol. **18:** 245-249.
Tomas-Barberan F.A., Gil M.I., Ferreres F., Rivera D., Obon C., Tomas-Lorente F. (1993). "Distribution of 8-hydroxyflavone glycosides and flavonoid aglycones in some Spanish *Sideritis* species." Biochem. Syst. Ecol. **21:** 487-497.
Tomas-Barberan F.A., Gil M.I., Ferreres F., Tomas-Lorente F. (1992). "Flavonoid *p*-coumaroylglucosides and 8-hydroxyflavone allosylglucosides in some Labiatae." Phytochemistry **31:** 3097-3102.

Tomas-Barberan F.A., Grayer-Barkmeijer R.J., Gil M.I., Harborne J.B. (1988b). "Distribution of 6-hydroxy-, 6-methoxy- and 8-hydroxyflavone glycosides in the Labiatae, the Scrophulariaceae and related families." Phytochemistry **27:** 2631-2645.
Tomas-Barberan F.A., Lopez-Gomez C., Villar A., Tomas-Lorente F. (1986). "Inhibition of lens aldose reductase by Labiatae flavonoids." Planta Medica 239-240.
Tomas-Barberan F.A., Manez S., Villar A. (1987). "Identification of antiinflammatory agents from *Sideritis* species growing in Spain." J. Nat. Prod. **50:** 313-314.
Tomas-Barberan F.A., Nunez J.M., Tomas F. (1985b). "An HPLC study of flavones from Spanish *Sideritis* species." Phytochemistry **24:** 1285-1288.
Tomas-Barberan F.A., Rejdali M., Harborne J.B., Heywood V.H. (1988a). "External and vacuolar flavonoids from Ibero-Norrth African *Sideritis* species. A chemosystematic approach." Phytochemistry **27:** 165-170.
Tomas-Barberan F.A., Tomas F. (1985). "An allose-containing 8-hydroxychrisoeriol diglycoside from *Sideiritis leucantha*." Rev. Latinoamer. Quim. **16:** 47-50.
Tomas-Barberan F.A., Tomas F., Ferreres F. (1984). "Two flavone glycosides from *Sideritis leucantha*." Phytochemistry **23:** 2112-2113.
Tomas-Barberan F.A., Tomas F., Ferreres F. (1985d). "Isoscutellarein-7-O-[allosyl (1-2) glucoside] from *Sideritis leucantha*." J. Nat. Prod. **48:** 28-32.
Tomas-Barberan F.A., Tomas F., Nunez J.M. (1985a). "Seasonal variation of free aglycones from *Sideritis leucantha* (Lamiaceae)." Z. Naturforsch. **40c:** 914-916.
Tomas-Barberan F.A., Wollenweber E. (1990). "Flavonoid aglycones from the leaf surfaces of some Labiatae species." Plant Syst. Evol. **173:** 109-118.
Tomas-Lorente F., Ferreres D., Tomas-Barberan F.A., Rivera D., Obon C. (1988). "Some flavonoids and the diterpene borjatriol from some Spanish *Sideritis* species." Biochem. Syst. Ecol. **16:** 33-42.
Tomas-Lorente F., Ferreres F., Guirado A., Trowitzsch (1983). "Flavonas en *Sideritis leucantha*." Anales de Quimica **79:** 96-98.
Tomas-Lorente F., Ferreres F., Tomas-Barberan F.A., Rivera D., Obon C. (1989). "Verification of *Sideritis incana* x *S. angustifolia* hybrids by flavonoid analysis." Phytochemistry **28:** 2141-2143.
Topcu G., Goren A.C., Kilic T., Kemal Y., Tumen G. (2001). "Diterpenes from *Sideritis argyrea*." Fitoterapia **72:** 1-4.
Topcu G., Goren A.C., Kilic T., Turgut Y., Kemal Y., Tumen G. (2002b). "Diterpenes from *Sideritis sipylea* and *S. dichotoma*." Turkish J. Chem. **26:** 189-194.
Topcu G., Goren A.C., Kilic T., Yildiz Y.K., Tumen G. (2002a). "Diterpenes from *Sideritis trojana*." Nat. Prod. Lett. **16:** 33-37.
Topcu G., Goren A.C., Yildiz Y.K., Tumen G. (1999). "Ent-kaurene diterpenes from *Sideritis athoa*." Nat. Prod. Lett. **14:** 123-129.
Triantaphyllou K., Blekas G., Boskou D. (2001). "Antioxidative properties of water extracts obtained from herbs of the species Lamiaceae." Int. J. Food Sci. Nutr. **52:** 313-317.
Tsaknis J., Lalas S. (2005). "Extraction and identification of natural antioxidants from *Sideritis euboea* (mountain tea)." J. Agric. Food Chem. **53:** 6375-6381.
Tűmen G., Baser K.H.C., Kirimer N., Ermin N. (1995). "Essential oil of *Sideritis amasiaca* Bornm." J. Ess. Oil Res. **7:** 699-700.
Tunalier Z., Kosar M., Ozturk N., Baser K.H.C., Duman H., Kirimer N. (2004). "Antioxidant properties and phenolic composition of *Sideritis* sp." Chem. Nat. Comp. **40:** 206-210.
Tzakou O. **(**2002). **"**The essential oil of *Sideritis raeseri* Boiss. & Heldr. ssp. *attica* (Heldr.) Pap. & Kok." J. Ess. Oil Res. **14:** 376-377.
Ugur A., Varol O., Ceylan O. (2005). "Antibacterial activity of *Sideritis curvidens* and *S. lanata* from Turkey." Pharm. Biol. **43:** 47-52.
Venturella P., Bellino A. (1979). "Isolation and partial synthesis of ent-18-acetoxykaur-16-ene-3β,7α, 15β-triol from *Sideritis scardica*." Phytochemistry **18:** 1571-1572.

Venturella P., Bellino A., Marino M.L. (1978). "New diterpenes from *Sideritis sicula*." Phytochemistry **17:** 811-812.
Venturella P., Bellino A., Marino M.L. (1983a). "Ucriol, an epoxy-diterpene from *Sideritis syriaca*." Phytochemistry **22:** 600-601.
Venturella P., Bellino A., Marino M.L. (1983b). "Siderone, a diterpene from *Sideritis syriaca*." Phytochemistry **22:** 2537-2538.
Venturella P., Bellino A., Marino M.L. (1989). "A diterpene, distanol, from *Sideritis distans*." Phytochemistry **28:** 1976-1977.
Venturella P., Bellino A., Marino M.L. (1995). "Three acylated flavone glycosides from *Sideritis syriaca*." Phytochemistry **38:** 527-530.
Venturella P., Bellino A., Papucci A. (1977). "A new flavone glycoside from *Sideritis romana* L." Heterocycles **6:** 267-272.
Venturella P., Bellino A., Piozzi F. (1974). "Revised structure for siderin." Tetrahedron Lett. **12**: 979-981.
Venturella P., Bellino A., Piozzi F. (1975). "Diterpenes from *Sideritis theezans*." Phytochemistry **14:** 1451-1452.
Villar A, Gasco M.A., Alcaraz M.J. (1985f). "Effect of hypolaetin 8-glucoside on human platelet aggregation induced by ADP." Planta Medica **0:** 455—456.
Villar A, Gasco M.A., Alcaraz M.J., Manez S., Cortes D. (1985b). "Hypolaetin 8-glucoside, an antiinflammatory flavonoid from *Sideritis mugronensis*." Planta Medica **0:** 70.
Villar A, Jimenez A., Manez S. (1985c). "HPLC of *Sideritis* polymethoxyflavones." Pharmazie **40:** 731.
Villar A., Gasco M.A., Alcaraz J. (1984c). "Anti-inflammatory and anti-ulcer properties of hypolaetin-8-glucoside, a novel plant flavonoid." J. Pharm. Pharmacol. **36:** 820-823.
Villar A., Jimenez A., Manez S. (1985d). "Sur la distribution des methoxyflavones dans le genre *Sideritis*." Plantes Med. Phytother. **19:** 255-261.
Villar A., Navarro A., Zafra-Polo M.C., Rios J.L. (1984a). "Constituents of the essential oil of *Sideritis mugronensis*." Plantes Med. Phytother. **18:** 150-153.
Villar A., Recio M.C., Rios J.L., Zafra-Polo M.C. (1986a). "Antimicrobial activity of essential oils from *Sideritis* species." Pharmazie **41:** 298-299.
Villar A., Rios J.L., Zafra-Polo M.C., Mares M., Recio M.C. (1985e). "Activite antimicrobienne d'especes de *Sideritis*." Plantes Med. Phytother. **19:** 248-254.
Villar A., Salom R., Alcaraz M.J. (1983). "Effect of borjatriol on established adjuvant arthritis." Pharmazie **38:** 566.
Villar A., Salom R., Alcaraz M.J. (1984d). "An approach to the anti-inflammatory activity of borjatriol." Planta Medica **50:** 90-92.
Villar A., Simeon S., Jimenez F.J., Rios J.L., Zafra-Polo M.C. (1984b). "Screening phytochimique d'especes de *Sideritis*." Fitoterapia **55:** 366-368.
Villar A., Valverde S., De las Heras B., Vivas J. M. (1993). "A manoyl oxide diterpenoid from *Sideritis javalambrensis*." Phytochemistry **34:** 575.
Villar A., Zafra-Polo M.C., Bonanad M.A., Navarro A., Rios J.L. (1985a). "Essential oil *Sideritis javalambrensis*." Pharm. Acta Helv. **60:** 351-352.
Villar A.M., Jimenez M.J., Alcaraz M.J. (1986b). "The anti-inflammatory activity of the genus *Sideritis*: a new insight." Plantes Med. Phytother. **20:** 31-36.
Villena C., Vivas J.M., Villar A. (2000). "Suppression of croton oil-induced rabbit corneal edema by *Sideritis javalambrensis*." J. Ethnopharmacol. **71:** 301-305.
Yesilada E., Ezer N. (1989). "The antiinflammatory activity of some *Sideritis* species growing in Turkey." Int. J. Crude Drug Res. **27:** 38-40.
Zafra-Polo M.C., Blazquez M.A. (1989). "Chemical constituents of the essential oils from *Sideritis scordioides* L. *cavanillesii* Cav. Herb." Plantes Med. Phytother. **23:** 288-292.

Zarkovic B., Dordevic S., Blagojevic S. (1993). "Upoređivanje mineralnog sastava biljke *Sideritis scardica* Griseb., Lamiaceae i zemlosne podloge." in Izvodi Radova 62-63. Arch. Farm. **43:** 237.
Zarzuelo A., Garcia E., Jimenez J., Ocete M.A., Utrilla P. (1993). "Activity of various species of the genus *Sideritis* from the Alpujarra region of Spain." Fitoterapia **64:** 26-30.
Zinchenko T.V., Kostjuchenko O.I., Fefer I.M., Gritsenko E.N., Kobzar A.Y. (1972). "Iridoids of some genera of the family Labiatae." Mater. III Soezda Farm. BSSR, Minsk, 164-166.

1.6 Aim and outline of the thesis

The work as described in this thesis concerns the finding, isolation and evaluation of natural antioxidants. Working on this item is a true research challenge for several reasons:

- There is not and there cannot be one universal method to assess the antioxidant activity of synthetic or natural compounds. Oxidation-antioxidation should be studied from several different points of view, which requires a suitable combination of assessing methods.
- Natural compounds occur in complex matrixes often in low amounts. Natural antioxidants are especially "sensitive" and susceptible to alterations during isolation and purification procedures. Therefore, rapid, selective, sensitive and "mild" screening methods are needed for finding potential candidates with antioxidative properties.

The **aim** of the work described in this thesis consists of three objectives:

(1) Using a given plant as a research object (here three Bulgarian *Sideritis* species, Labiatae) to develop an "algorithm" for studying natural antioxidants of plant origin. This includes choosing the most suitable extraction scheme(s) giving the highest yield and the content of antioxidative compounds, and from here applying the most appropriate methods and improving existing test methods for a rapid, powerful and convenient approach to screen plant extracts and study them in detail.

(2) Following the first objective, to isolate and identify the active components of the studied plants and evaluate their antioxidant activity (or more precisely their radical scavenging activity).

(3) In real systems, antioxidants may behave differently from the model situation because of many possible interactions or interferences. Therefore, isolated natural antioxidants and their "parent" extract should pass a "final check" for their performance in real systems. That is why objective three is to perform initial studies towards the behaviour of putative antioxidants from *Sideritis* species in real foods/cosmetics.

Following the above aims, the main aspects of lipid oxidation, antioxidants and methods for evaluating lipid oxidation and effectiveness of antioxidants are discussed in **Chapter 1.**

The search for a suitable combination of antioxidant activity screening methods is described in **Chapters 2** and **3** for a number of *Sideritis* extracts. The "pros and cons" of three applied methods are discussed in **Chapter 2.** The detailed investigation on the screening of extracts obtained from different *Sideritis* species and populations and the different extraction routes are presented in **Chapter 3.**

Chapters 4 and **5** describe the development of a novel approach, which combines on-line HPLC and antioxidant activity assays in the screening of plant extracts for radical scavenging components. **Chapter 4** presents the use of a model radical ($DPPH^{\bullet}$) in an on-line HPLC-radical scavenging system. The evolution of the on-line HPLC-DPPH method to an on-line HPLC method employing a different model radical ($ABTS^{\bullet+}$), is discussed in **Chapter 5.** For both techniques an optimized instrumental set-up, physico-chemical factors influencing the analysis as well as application to a range of pure antioxidants and extracts are discussed to determine their applicability in qualitative and quantitative measurements.

Chapter 6 deals with the phytochemical study of the putative antioxidants in *Sideritis*, using an antioxidant activity-guided isolation and identification procedures. The evaluation of the antioxidant (radical scavenging) activity by off- and on-line methods is presented as well.

In **Chapter 7** concluding remarks are given. Conclusions are drawn with regard to the best screening methods for "primary" and "secondary" antioxidants and the novel HPLC-radical scavenging detection for on-line assessment of antioxidants. Hyphenated techniques have gained great popularity owing to their advantages. The results of the phytochemical study of putative antioxidants from *Sideritis* plants by off- and on-line analysis are also discussed. Some initial studies are performed to investigate how the extracts and pure compounds will behave in real systems. Various specialists have to combine their efforts to study the behaviour in food products and after processing. A patent has been filed for applying *S. scardica* extract. Finally, future perspectives are outlined.

Screening of Plant Extracts for Antioxidant Activity: a Comparative Study on Three Testing Methods*

2.1 Introduction

Currently used synthetic antioxidants have been suspected to cause or promote negative health effects (Branen 1975; Barlow 1990), hence stronger restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Besides the well known and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices (Kanner et al. 1994; Madsen & Bertelsen 1995; Cao et al. 1996; Wang et al. 1996; Velioglu et al. 1998; Fogliano et al. 1999), many other plant species have been investigated in the search for novel antioxidants (Economou et al. 1991; Chevolleau et al. 1992; Kim et al. 1994). Some natural antioxidants (e.g. rosemary and sage) are already exploited commercially either as antioxidant additives or as nutritional supplements (Schuler 1990).

Lipid oxidation is a complex chain process involving a variety of radicals. The oxidation is influenced by temperature, light, air, physical and chemical properties of the substrate, and the presence of oxidation catalysts or initiators (Frankel & Meyer 2000).

Antioxidants can exercise their protective properties at different stages of the oxidation process and by different mechanisms. There are two main types of antioxidants, namely, "primary" (chain breaking, free radical scavengers) and "secondary" or "preventive" (see Ch. 1.2).

Oxidizable products contain different bulk lipids or their emulsions and various substrates investigation, model systems or accelerated oxidation are used, respectively. A large number of methods have been developed in order to evaluate antioxidant activity (AOA) (Ragnarsson & Labuza 1977; Khal & Hilderbrant 1986; Frankel 1993a; Robards et al. 1999; Moure et al. 2001).

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This renders a direct comparison of antioxidants tested by different methods and in different substrates somewhat difficult. For the assay-guided screening of large numbers of complex samples, rapid, simple and reliable tests are required.

The aim of the present study was to compare three widely used methods for the assessment of AOA, namely, the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) method, the static headspace gas chromatographic (HS-GC) method, and the β-carotene bleaching test (BCBT), and to determine their usefulness in assay-guided screening of plant extracts. Sensitivity, rapidity, applicability, required equipment, correlation of the results with those obtained by other methods, and relation to real lipid systems are discussed. The DPPH$^{\bullet}$ method is representative of the methods employing model radicals in the evaluation of radical scavengers; such methods have gained high popularity over the last decade because of their rapidity and sensitivity. The HS-GC method provides a sensitive determination of volatiles forming the rancid lipid off-flavour. The BCBT method uses a model lipid substrate (linoleic acid) in an emulsified form, and the method lies in between methods employing only model substrates (e.g. DPPH$^{\bullet}$) and those using real lipids.

As different testing methods provide particular, but limited information about AOA, an evaluation of their strengths and limitations should demonstrate the situations in which they are best applicable. The experimental results presented, together with relevant literature material, will illustrate the pros and cons of the methods and will also form the basis for the presented conclusions and recommendations.

2.2 Experimental

2.2.1 Chemicals

All solvents used were of analytical grade; ultra-pure water (0.05 μS/cm) was obtained from a combined Seradest LFM 20 and Serapur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). Reagents and chemicals were purchased from Sigma-Aldrich [Steinheim, Germany; Tween 40, *trans*-β-carotene, **7** (synthetic, 95%) and DPPH$^{\bullet}$, **34** (95%)], Janssen Chimica [Beerse, Belgium; linoleic acid (90%)], Extrasynthese (Genay, France; rosmarinic acid, **11** (RA)], and Fluka [Buchs, Switzerland; BHT, **1** (p.a.)]. See Chapters 1 and 3 for the compounds numbering. Commercial cold-pressed safflower oil, without added synthetic antioxidants, was obtained from Natufood (Harderwijk, The Netherlands) and contained 562 ppm α-tocopherol and traces of γ-tocopherol as determined by HPLC (de Greyt et al. 1998). The oil was stored under nitrogen in the dark at −20°C. Fatty acid composition, determined by GC of the methyl esters, was as follows: 6.9% C16:0, 2.7% C18:0, 12.6% C18:1, 77.5% C18:2.

2.2.2 Preparation of plant extracts

Extracts of differing polarity (see Fig. 2.1) were prepared from 11 populations of three species of *Sideritis* (Labiatae) grown in Bulgaria. Voucher specimens (SOM codes recorded below) were determined by Dr. L. Evstatieva and are deposited in the herbarium of the Institute of Botany (Bulgarian Academy of Sciences, Sofia, Bulgaria). The species and populations used and their origin were as follows: *S. scardica* 1 (LE 7a; cultivar, seeds collected at Pirin mountain, Lozen, Bulgaria, May 1995; SOM 3154a); *S. scardica* 13 (LE 2; natural population, Rodopi mountain, Trigrad, Bulgaria, May 1997); *S. scardica* 14 (LE 7v; cultivar, seeds collected at Pirin mountain, Lozen, Bulgaria, May 1997; SOM 3154v); *S. montana* 4 (LE 12; natural population, Strandja mountain, Dervishki hills, Bulgaria, May 1995; SOM 3253); *S. montana* 5 (LE 30; natural population, Black Sea coast, Primorsko, Bulgaria, August 1993); *S. montana* 11 (LE 31; Black Sea coast, Varna, Bulgaria, July 1995; SOM 1282); *S. syriaca* 6 (LE 5; cultivar, seeds from Spanish origin, Lozen, Bulgaria, 1995); *S. syriaca* 9 (LE 6a = 6b; natural population, Strandja Mountain, Malko Tarnovo, Bulgaria, July 1995; SOM 3151); *S. syriaca* 12 (LE 10; cultivar, seeds from Spanish origin, Lozen, Bulgaria, 1995). Cultivars were grown in the Botanical Garden of the Institute of Botany, Bulgarian Academy of Sciences, Lozen, Bulgaria.

The plant material was air-dried at room temperature in ventilated premises and ground in a grinder before use. The total methanolic (MeOH) extracts were prepared (see Fig. 2.1) by a two-fold maceration (solvent:plant material = 20:1) for 24 h at room temperature. After evaporation of the extract to dryness under vacuum at temperatures not higher than 50 °C, the residue was suspended in water (1:25, w/w) and successively partitioned between water and *t*-butyl methyl ether (*t*-BuOMe), ethyl acetate (EtOAc) and 1-butanol (BuOH). Each liquid– liquid extraction was carried out three times (water:organic solvent = 1:1, v/v). The solvents of the obtained sub-extracts were evaporated under vacuum to dryness and the aqueous extracts were freeze-dried.

2.2.3 Analytical methods

DPPH radical method

A modification of the method of von Gadow et al. (1997a) was used. Ethanolic solutions of $DPPH^{\bullet}$ (10^{-4} M) and *Sideritis* extracts or BHT / RA solutions were mixed in disposable plastic half-microcuvettes (1 cm path length; EMERGO, Landsmeer, The Netherlands,) so that the final

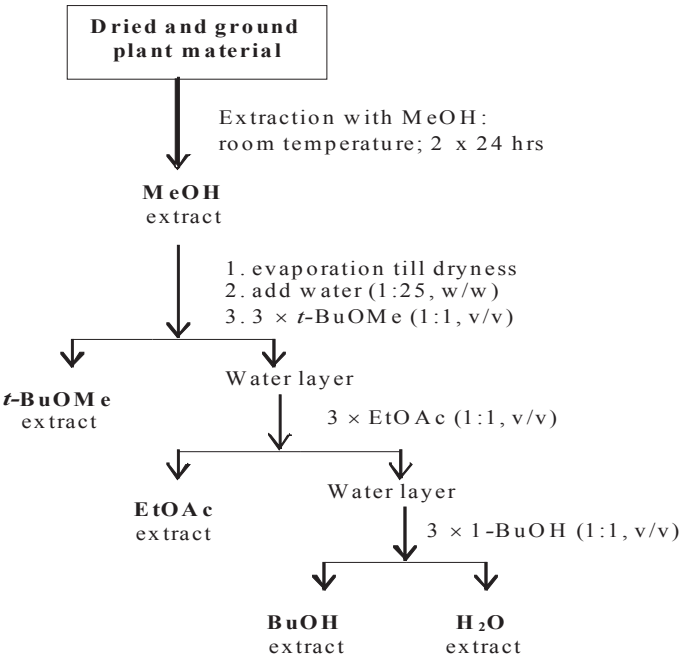

Figure 2.1 Extraction scheme for the preparation of extracts.

mass ratios were extract:DPPH$^{\bullet}$ = 5.5:1, and reference compound:DPPH$^{\bullet}$ = 0.5:1. The samples were incubated for 15 min in the dark at 30 °C and the decrease in absorbance at 517 nm was measured against ethanol using a Specol 11 (Carl Zeiss, Jena, Germany) spectrophotometer. Ethanol was used to zero the spectrophotometer; a blank sample containing the same amount of ethanol and DPPH$^{\bullet}$ was prepared and measured daily. The DPPH$^{\bullet}$ solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4 °C between the measurements. All determinations were performed in triplicate. The radical scavenging activities of the tested samples, expressed as percentage reduction of DPPH$^{\bullet}$, were calculated according to the formula (Yen & Duh 1994):

$$\% \text{ Reduction} = [(A_B - A_A) / A_B] \times 100$$

where A_B and A_A are the absorbance values of the blank and of the test sample, respectively, after 15 min.

Static head space GC (HS-GC)

Safflower oil samples containing methanolic solutions of the extracts (0.1%, w/w) and reference compounds (BHT and RA, each 0.01%, w/w) were prepared. A blank sample was prepared with an equal amount of methanol. Samples (0.20 g) of these solutions were placed into headspace vials (10 mL), sealed with a PTFE/butyl rubber liner, and incubated at 55 °C in a shaker. Sampling was carried out every other day. The amount of hexanal was determined using a Carlo Erba (Milan, Italy) Mega 5300 gas chromatograph equipped with a HS 800 headspace sampler and a wide-bore thick-film capillary DB-WAX (J&W Scientific, Folsom, USA) column (30 m x 0.54 mm i.d.; 1 μm thickness). The temperature programme was 11 min at 40 °C (isothermal), then increased to 170 °C at 5.0 °C/min, then isothermal at 170 °C for 5 min; the helium pressure was 30 kPa; the injection syringe temperature was 70 °C; the injector temperature was 200 °C; and the detector (FID) temperature was 250 °C. Before injection, samples were incubated at 60 °C for 10 min in a 2000 rpm agitator with a 10 s run/10 s stop cycle. The volatiles were cold-trapped using a MFA 815 (Carlo Erba, Milan, Italy) cold trap at −110 °C and injected balistically into the column at 240 °C; the headspace sample volume was 2000 μL. Peak areas for hexanal were processed with Chrom Card (Carlo Erba, Milan, Italy) integration software.

Antioxidant activity was evaluated using the parameters: protection factor $(PF) = IP_A / IP_B$ (Marinova et al. 1991), where IP_A and IP_B are the induction periods for the antioxidant (time in days after which the hexanal formation sharply increased because of the complete consumption of the antioxidant) for the test and blank sample, respectively; and relative rate of hexanal formation until the induction period $(RRH) = V_A / V_B$, where V_A and V_B are the initial rates of hexanal formation of the test and blank sample, respectively, expressed as the slopes of the curves $S_H = f(T)$ [where S_H is the amount of hexanal expressed as hexanal peak area in integration units (IU), and T is the time in days].

β-Carotene bleaching test (BCBT)

A modification of the method described by Dapkevicius et al. (1998a) was employed. β-Carotene (1.0 mg) was dissolved in 5 mL chloroform and to 1 mL of this solution were added linoleic acid (25 μL) and Tween 40 (200 mg). The chloroform was evaporated under vacuum at 40 °C, oxygenated ultra-pure water (50 mL, obtained by bubbling air through the water for 15 min) was added, and the mixture was vigorously shaken. The emulsion obtained was freshly prepared before each experiment. Stock solutions of extracts of *Sideritis* (0.1%) and reference compounds (BHT and RA; 0.01% each) were prepared in ethanol. An aliquot (250 μL) of the β-carotene:linoleic acid emulsion was distributed in each well of 96-well microtitre plates and ethanolic solutions of the test samples (30 μL)

were added. An equal amount of ethanol was used for the blank sample; four replicates were prepared for each of the samples. The microtitre plates were incubated at 55 °C, and the absorbances were measured using a model EAR 400 microtitre reader (SLT Instruments, Grödig, Austria) at 492 nm. Readings of all samples were performed immediately (t = 0 min) and after 105 min of incubation (Pratt & Birac 1979). After this time period the decrease in the absorbance of the blank sample was no longer significant. The AOA coefficient (AAC) was given by the equation (a modified version of the formula of Chevolleau et al. 1992):

$$AAC = [(A_{A,\ 105} - A_{B,\ 105}) / (A_{B,\ 0} - A_{B,\ 105})] \times 1000$$

where $A_{A,\ 105}$ and $A_{B,\ 105}$ are the absorbances of the test and blank sample at t = 105 min, respectively, and $A_{B,\ 0}$ is the absorbance of the blank sample at t = 0 min.

2.3 Results and discussion

The antioxidant activity (AOA) has been found to depend on the hydro-/lipophilicity of the antioxidant. Two well known antioxidants, *tert*-butylated hydroxytoluene, **1** (BHT) and rosmarinic acid, **11** (RA), were chosen as reference compounds since they differ in polarity in a manner similar to the studied plant extracts and have a phenolic group in common with the main active plant constituents.

2.3.1 Antioxidant activity determined by the DPPH method

Free radicals are involved in the propagation of lipid (RH) oxidation, and many radical species of different reactivity are formed (e.g. $^{\bullet}OH$, $O_2^{\bullet-}$, $ROO^{\bullet}$, $RO^{\bullet}$, $R^{\bullet}$, etc.). Relatively stable radicals ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, etc.) are often preferred in the assessment of radical scavenging activity (Miller et al. 1993; Brand-Williams et al. 1995; Fogliano et al. 1999). The stable organic radical $DPPH^{\bullet}$, **34** (see Chapter 3 for the structure) has been widely used in AOA studies of single compounds (Brand-Williams et al. 1995; Sanchez-Moreno et al. 1998), plant extracts (Imai et al. 1994; Yen & Duh 1994; Duh & Yen 1997), foods (Yamaguchi et al. 1998), etc. The method is based on the reduction of alcoholic $DPPH^{\bullet}$ solutions at 517 nm in the presence of an hydrogen donating antioxidant (AH) due to the formation of the non-radical form DPPH-H by the reaction:

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$

The remaining DPPH$^{\bullet}$, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant. The sensitivity of the method is determined by the strong absorbance of DPPH$^{\bullet}$ (Brand-Williams et al. 1995). The method is rapid, a sample analysis takes 15 min in total and little manpower, no expensive reagents or sophisticated instrumentation are required.

Table 2.1 Radical scavenging activity of some *Sideritis* extracts[a] and two reference compounds[b] toward DPPH$^{\bullet}$ expressed as % Reduction.

***Sideritis* species**	**Type of extracts**				
	***t*-BuOMe**[c]	**MeOH**	**EtOAc**	**BuOH**	**H_2O**
S. scardica 14	38.1[d]	94.1	94.4	94.1	49.4
S. syriaca 9	42.1	91.9	94.5	93.8	41.7
S. montana 4	46.5	91.1	94.4	92.4	68.5
S. montana 5	62.7	92.3	95.5	92.7	28.0
S. montana 11	50.2	92.0	93.2	93.3	74.4

[a] - the rest of the studied plant samples gave similar results for radical scavenging activity

[b] - **% Reduction**$_{Rosmarinic\ acid}$ **= 94.5%**; **% Reduction**$_{BHT}$ **= 64.8%**

[c] - ***t*-BuOMe**: *t*-butyl methyl ether extract; **MeOH**: total methanolic extract; **EtOAc**: ethyl acetate extract; **BuOH**: 1-butanol extract; **H_2O**: aqueous extract

[d] - values are mean of three replicates and the relative standard deviations (RSD) for all figures were less than 1%

The method was applied to two known antioxidants (BHT and RA) and to a number of extracts from *Sideritis* species (data in Table 2.1). *t*-Butyl methyl ether, ethyl acetate, 1-butanol, total methanol and aqueous extracts were studied confirming that the DPPH$^{\bullet}$ method is independent of the substrate polarity. Total methanol, ethyl acetate and 1-butanol extracts showed the highest radical scavenging activity, close to that of RA on a weight basis. In preliminary experiments (see Ch. 3), the presence of flavonoids and phenylpropanoid glycosides, was established in these extracts, explaining their high radical scavenging activity. In general, the activity of *t*-butyl methyl ether and aqueous extracts was much lower and was influenced by the origin of the plant sample but not by the polarity. Semi-quantitative TLC tests showed lower amounts of phenolic components in these extracts. Other authors have also reported that hydro-/lipophilicity of a sample does not affect its DPPH$^{\bullet}$ scavenging activity (Yamaguchi et al. 1998; Pekkarinen et al. 1999).

The latter mainly depends on the antioxidant structure, which was also illustrated by the study of RA and BHT on a molecular basis (data in Table 2.2).

Table 2.2 Radical scavenging activity of rosmarinic acid and BHT, expressed as % Reduction, for three molar ratios Antioxidant : DPPH$^{\bullet}$ and their corresponding mass ratios.

mol Antioxidant per mol DPPH	mg Antioxidant per mg DPPH	% Reduction
Rosmarinic acid		
0.2	0.2	93.4
0.5	0.5	94.5
1.0	0.9	95.9
BHT		
0.2	0.1	27.2
0.5	0.3	44.5
1.0	0.5	64.8

The established stronger radical scavenging abilities of RA compared to those of BHT agree with the results of other researchers (Brand-Williams et al. 1995; Chen & Ho 1997). The larger number of electron-donating hydroxyl groups in the RA molecule relative to BHT (Dziedric & Hudson 1984), and the resonance stabilisation of the formed RA radical (Brand-Williams et al. 1995; Sanchez-Moreno et al. 1998) explain the higher radical scavenging activity of RA. Furthermore, a different kinetic behaviour of RA (intermediate) and BHT (slow) has been established. Compounds with rapid or intermediate kinetics have shown a reaction stoichiometry corresponding approximately to the number of electrons available for donation. For slow-reacting compounds (such as BHT) such a correlation did not exist (Bondet et al. 1997) and this was attributed to the complex reaction mechanism, involving one or more secondary reactions.

2.3.2 Antioxidant activity determined by the BCBT method

The method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion (Marco 1968). β-Carotene bleaching, measured by the decrease in the initial absorbance at 470 nm, is slowed down in the presence of antioxidants. The method is widely used in the AOA evaluation of different types of samples such as single compounds (Pratt & Birac 1979; von Gadow et al. 1997a; Abdalla et al. 1999), plant extracts (Chevolleau et al. 1992; von Gadow et al. 1997b;

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Table 2.2 Radical scavenging activity of rosmarinic acid and BHT, expressed as % Reduction, for three molar ratios Antioxidant : $DPPH^{\bullet}$ and their corresponding mass ratios.

mol Antioxidant per mol DPPH	mg Antioxidant per mg DPPH	% Reduction
Rosmarinic acid		
0.2	0.2	93.4
0.5	0.5	94.5
1.0	0.9	95.9
BHT		
0.2	0.1	27.2
0.5	0.3	44.5
1.0	0.5	64.8

The established stronger radical scavenging abilities of RA compared to those of BHT agree with the results of other researchers (Brand-Williams et al. 1995; Chen & Ho 1997). The larger number of electron-donating hydroxyl groups in the RA molecule relative to BHT (Dziedric & Hudson 1984), and the resonance stabilisation of the formed RA radical (Brand-Williams et al. 1995; Sanchez-Moreno et al. 1998) explain the higher radical scavenging activity of RA. Furthermore, a different kinetic behaviour of RA (intermediate) and BHT (slow) has been established. Compounds with rapid or intermediate kinetics have shown a reaction stoichiometry corresponding approximately to the number of electrons available for donation. For slow-reacting compounds (such as BHT) such a correlation did not exist (Bondet et al. 1997) and this was attributed to the complex reaction mechanism, involving one or more secondary reactions.

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Dapkevicius et al. 1998b), grains, fruits, vegetables and foods (Kanner et al. 1994; Velioglu et al. 1998).

The method is sensitive due to the strong absorbance of β-carotene ($\varepsilon^{1\%}_{478}$ = 2280 L/mol.cm) but it is slower (ca. 2 h per assay) than the DPPH$^{\bullet}$ method. Recently, the method has been speeded up using 96-well microtitre plates for sample incubation and an automatic reader for simultaneous absorbance measurements (Dapkevicius et al. 1998a).

Common reagents were used and no special sample treatment was necessary. However, careful solvent evaporation (under vacuum and at temperatures up to 50 °C) and preparation of an emulsion with reproducible composition and droplet-size was essential for achieving reproducible results.

The BCBT method employs an emulsified lipid which introduces an enhanced number of variables influencing oxidation in comparison to bulk lipids. Complex interfacial phenomena which influence the antioxidant behaviour are the result. An interesting phenomenon formulated as the "polar paradox" has been reported (Porter 1993; Frankel et al. 1994): apolar antioxidants exhibit stronger antioxidative properties in emulsions because they concentrate at the lipid:air surface, thus ensuring high protection of the emulsion itself. On the other hand, polar antioxidants remaining in the aqueous phase are more diluted and are thus less effective in protecting the lipid. The opposite is observed in bulk lipids but some solubility is necessary.

The AOA values exhibited by BHT and RA, and by some of the tested *Sideritis* extracts (data in Table 2.3), were in general accordance with the "polar paradox" phenomenon. The more active inhibitors of β-carotene bleaching were mainly among the apolar extracts. The majority of the more polar extracts (methanol, ethyl acetate and 1-butanol) exhibited lower AOA closer to that of RA. Aqueous extracts even showed a prooxidative effect (i.e. a negative AAC).

Some of the extracts did not follow the "polar paradox". Structural features of the antioxidant (e.g. the substitution pattern of the hydroxyl groups) have been found to be more important than the polarity itself in some cases (Pratt & Birac 1979). Furthermore, the complex composition of the extracts could provoke certain interactions (synergistic, additive or antagonistic effects) between their components and/or the medium. It could also affect their partitioning in the medium phases. The antioxidant partitioning into the different phases of an emulsion has been found to affect AOA, although the activity was not proportional to the antioxidant concentration in the lipid phase or the interface (Pekkarinen et al. 1999). Caffeic acid exhibited very poor AOA in a Tween 20 emulsion despite being mainly in the lipid phase. Specific interactions between the acid and

emulsifier such as hydrogen bonding may block the activity-contributing groups (Avila et al. 1995).

Table 2.3 Antioxidant activity (BCBT method) of some *Sideritis* extracts[a] and two reference compounds[b], expressed as AAC.

***Sideritis* species and populations**	**Type of extract**				
	MeOH	***t*-BuOMe**	**EtOAc**	**BuOH**	**H_2O**
S. scardica 13	725	731	562	438	−295
S. scardica 14	701	830	529	632	−123
S. syriaca 9	482	682	524	612	−166
S. syriaca 12	595	468	549	615	−157
S. montana 4	572	467	218	428	−179
S. montana 5	515	229	491	345	−221
S. montana 11	486	586	640	210	−97

[a] - the rest of the studied plant samples gave similar results

[b] - $\mathbf{AAC}_{BHT} = \mathbf{864}$; $\mathbf{AAC}_{Rosmarinic\ acid} = \mathbf{425}$

Linoleic acid can compete with polar compounds (e.g. the water soluble form of tocopherol, namely Trolox) for Tween in the polar region of micelles and at the oil:water interface which results in an increase of Trolox concentration in the water phase. Consequently, Trolox is a less effective antioxidant than α-tocopherol in emulsified linoleic acid. The opposite has been observed for methyl linoleate:water and oil:water emulsions (Huang et al. 1996).

Similar interactions between RA, linoleic acid and Tween may have affected the final AOA of RA in this study. In this way, the low activity observed for RA, a compound recognised as a potent antioxidant, can be explained. Ethyl acetate and 1-butanol extracts of *Sideritis* contain mainly phenolic compounds and are likely to behave similarly to RA as is shown by the data obtained.

Aqueous extracts partitioned predominantly into the water phase, which influenced their activity. Roedig-Penman & Gordon (1998) have shown that the presence of certain

metal salts can result in pro-oxidant behaviour of flavonoids. If such metal salts were present in the plant they would have dissolved in the aqueous phase and thus could have inhibited the activity of antioxidants or even converted them into pro-oxidants.

2.3.3 Antioxidant activity determined by the HS-GC method

The HS-GC method measures the volatile products formed during lipid hydroperoxide decomposition. These compounds are responsible for the rancid off-flavour of the lipid-containing products and are an important factor for their quality. The method has been applied to samples of different nature in various substrates, such as single compounds (Frankel et al. 1996a,b; Roedig-Penman & Gordon 1998; Abdalla et al. 1999), plant extracts (Abdalla & Roozen 1999; Wettasinghe & Shahidi 1999), edible oils (Frankel 1993b) and biological samples (Frankel et al. 1989).

The method is sensitive because the formed volatiles are mainly present in the substrate headspace (Frankel 1998). Incubation of oil samples for at least 10 days is necessary in order to produce sufficient volatiles. Sampling needs to be carried out at least every other day; manpower input can be reduced by using automated sample injection. The method was used to evaluate the "secondary" antioxidant properties of BHT and RA, and some *Sideritis* extracts in commercial bulk safflower oil (data in Table 2.4). It was applied to ethyl acetate and 1-butanol extracts which are rich in phenolic compounds but exhibit low AOA in the BCBT method.

As expected from the "polar paradox", RA inhibited hexanal formation better than BHT in the bulk oil. A similar AOA of the extracts was expected because of their similar composition, observed in chromatographic studies. Two *S. montana* extracts showed a pro-oxidant effect (PF < 1); the different composition of *S. montana* extracts might explain this different behaviour. Discrepancies from the "polar paradox" have been reported by other authors, employing different substrates (oils stripped of endogenous antioxidants, commercial oils, oil:water emulsions) in studies of single compounds and of plant extracts (Frankel et al. 1996a,b; Wettasinghe & Shahidi 1999).

The efficiency of an antioxidant can be estimated either by its effect on the extent of oxidation (here expressed by IP and PF) or on the rate of oxidation (here expressed by the RRH during the induction period). RA and some of the studied extracts retarded hexanal formation (PF > 1.0) but also showed an increased rate of hexanal formation (RRH $\geq$ 1.0). A possible explanation may be sought in the reactions occurring during the lipid (RH) oxidation in the presence of antioxidants. The alkoxyl radicals ($RO^{\bullet}$) produced by the breakdown of lipid hydroperoxides (ROOH) can undergo further reactions to produce various secondary products (carbonyl compounds and short-chain hydrocarbons).

Antioxidants can exercise "secondary" AOA by inhibiting these reactions via different pathways (Frankel 1998). The higher RRH values at a longer IP observed for some of the studied samples might be due to a specific balance between the rates of reactions consuming antioxidant and those regenerating it (Kasaikina et al. 1999). The longer IP might also be due to synergistic effects between oil tocopherols and the other antioxidants present in the samples (Roginsky 1988).

Table 2.4 Antioxidant activity (HS-GC method) of EtOAc- and BuOH extracts from some *Sideritis* species[a], expressed as Protection Factor (PF) and Relative Rate of Hexanal formation (RRH) during the induction period (IP).

Tested sample	EtOAc		BuOH	
	PF	***RRH***	***PF***	***RRH***
S. scardica 13	1.3	2.6	0.7	1.6
S. scardica 14	1.3	1.7	1.0	1.0
S. syriaca 9	1.3	1.4	1.3	1.9
S. montana 4	0.3	1.6	0.6	1.7
S. montana 5	0.3	1.1	0.3	3.7
S. montana 11	1.0	0.9	1.3	1.9

	PF	***RRH***
Blank[b]	1.0	1.0
BHT	1.3	0.7
Rosmarinic acid	1.6	1.0

[a] - the rest of the studied plant samples gave similar results

[b] - IP for the blank oil sample was 7 days

2.4 Comparison of the methods

2.4.1 Necessity of appropriate reference substances

As it is impossible to express the AOA as an absolute value, suitable antioxidants should be used as standards for comparative purposes no matter which method is chosen. The best approach is to choose compounds with similar chemical nature and physicochemical properties as those of the studied samples. The necessity of such standards is well

illustrated in this study: if RA had been tested only by the BCBT method it would have been considered to be a weak antioxidant. However, the strong antioxidant potential of RA has been proven many times by other testing methods. Consequently, the *Sideritis* extracts that show poor antioxidant properties in comparison to BHT but close to RA in the BCBT method should not be "discarded" as poor sources of antioxidants. Similarly, BHT should not be regarded as a weak antioxidant only based on the results of the DPPH$^{\bullet}$ method.

2.4.2 Choice of oxidizable substrate and oxidation conditions

In order to obtain useful data, antioxidants should be studied in an environment similar to the real-life situation. Many studies have shown that the AOA depends on the lipid substrate, which is associated with the oxidation mechanism, antioxidant partitioning, and interactions between antioxidant and medium components. My results also showed different AOA values for the same samples in different substrates.

The independence of the substrate polarity in methods employing model radicals such as DPPH$^{\bullet}$ was confirmed in this study. A drawback, common for all methods using model radicals, is the lack of a direct correlation between the established AOA and its protective properties in real lipid systems. However, radical-trap methods are not devoid of practical value. All powerful natural antioxidants known so far have shown up in the DPPH$^{\bullet}$ assay. A good correlation between DPPH$^{\bullet}$ scavenging activity of some phenolic acids and their inhibitory properties on hydroperoxide formation in bulk oils has been reported. The correlation was very poor in emulsions suggesting that radical scavenging activity of phenolic acids should not be evaluated in emulsions (Pekkarinen et al. 1999). In this study RA did exhibit much stronger activity in the DPPH$^{\bullet}$ system than in the emulsion system (BCBT). These data indicate that in emulsions partitioning effects may be more important than the antioxidant structure. The surface-to-volume ratio has been found to be important for the antioxidative properties in emulsions, hence, reproducible droplet-size formation is crucial. Conflicting results for the same compounds in the BCBT method have been reported by von Gadow et al. (1997a) and Pratt (1980), who have applied different techniques for emulsion preparation (with and without an ultrasonic bath).

The chemical properties of the emulsion components are also important. Linoleic acid emulsions possess other properties than oil-in-water emulsions (Frankel 1998). Furthermore, as a free acid, linoleic acid may influence the dissociation degree of acidic antioxidants (Roginsky 1988) and in this way it is not representative for edible oils/fats as they mainly consist of triglycerides (Frankel 1993a). As a result, an antioxidant may behave differently depending on the emulsion type, as reported in many studies (Huang et al. 1996;

Abdalla et al. 1999). Consequently, results from the BCBT cannot be simply extrapolated to oil-in-water emulsions.

The unusual antioxidative properties of β-carotene itself further complicate the picture. In the BCBT method, β-carotene is a reaction indicator and is oxidised itself, i.e. it can be considered as an antioxidant. However, its protective mechanism is completely different from that of RA and BHT. Burton & Ingold (1984) reported that the AOA of β-carotene is exhibited neither by chain breaking nor by peroxide decomposition. Hence, the exact role of an antioxidant in the β-carotene:linoleic acid system is difficult to explain, especially when complex matrixes such as plant extracts are considered.

Accelerated tests employing high temperatures and/or oxygen supply bring the risk of undesirable alterations (decomposition, evaporation, polymerisation) of the studied antioxidants. The $DPPH^{\bullet}$ method uses very mild experimental conditions, which is an advantage. The linoleic acid oxidation in the BCBT is conducted at relatively low temperature. The HS-GC method is the most flexible of the three methods to variations in substrate type and oxidation conditions.

2.4.3 Importance of the measured parameter

The exhibited AOA depends on the monitored parameter, namely, formation or decomposition of hydroperoxides or radical scavenging activity. A number of reports have shown that a compound ineffective in inhibiting hydroperoxide formation (or even promoting it) may be able to inhibit hydroperoxide breakdown to unwanted volatiles (Frankel. 1996a,b; Huang et al. 1996). I have shown that the same samples can exhibit different AOA values depending on the measured parameter (see Tables 2.1 – 2.4). A possible reason may be the different antioxidant affinity towards $ROO^{\bullet}$ and $RO^{\bullet}$, i.e. it may better exert either chain-breaking (by $ROO^{\bullet}$ deactivation) or preventive (by $RO^{\bullet}$ deactivation) antioxidant properties. The data obtained for RA and BHT by the $DPPH^{\bullet}$ and BCBT methods indicate that the BCBT method is not very appropriate for the evaluation of "primary" antioxidant, i.e. radical scavenging properties.

The HS-GC method monitors an important quality parameter, namely secondary volatile products, as they are found to be the most closely related to the lipid flavour deterioration. A good correlation between the obtained "objective" chromatographic data and the "subjective" sensory panel test results has been established (Frankel 1998); however, the antioxidant effect at a later stage of lipid oxidation is evaluated. Also, antioxidants are not effective if added to the product after the onset of oxidation. Hence, evaluation of only the retardation effect on the decomposition of already formed lipid hydroperoxides is not sufficient to build up the activity profile of an antioxidant.

The generated radical intermediates are responsible for oxidation propagation. The majority of antioxidants exercise antioxidant properties by competing with lipid molecules for the formed radicals. Therefore, it is important to evaluate potential antioxidants by their radical scavenging properties. An effective radical scavenger will favourably influence the inhibition of the oxidation process. Moreover, all known powerful natural radical scavengers are also good antioxidants.

2.4.4 Rapidity, sensitivity, applicability, and equipment

In assay-guided screening of plant samples, simple, sensitive and rapid methods that are applicable to different sample types, have a high throughput and give reproducible results, are preferable. The BCBT method is simple, sensitive and relatively rapid, especially if microtitre plates and a reader device are available. The reproducibility of the results is dependent on many variables. The method should be only used as a preliminary testing procedure for AOA evaluation as it is limited to less polar samples. Otherwise incorrect conclusions (e.g. false negative results) could be drawn. Static HS-GC is a sensitive and reproducible method but requires sophisticated instrumentation. The method is flexible with respect to the type of sample and substrate and the flavour profile of a product can be obtained. It has been ranked as the second most suitable method (after sensory assays) in predicting the stability, shelf life and consumer acceptability of a product (Frankel 1993b). The relatively long required sample oxidation prior to chromatographic measurements significantly slows down the analysis.

The DPPH$^{\bullet}$ method is very rapid, simple, sensitive, reproducible and does not require special instrumentation. In my study, it was very convenient for the screening of large numbers of samples of different polarity because of its high throughput. Microtiter plates can also be used to speed up the analysis (Matsukawa et al. 1997). The known chemical properties of DPPH$^{\bullet}$ enable structure-activity studies (Cotelle et al. 1996; Mathiesen et al. 1997; Yokozawa et al. 1998). An alcoholic DPPH$^{\bullet}$ solution is a convenient TLC spray reagent for a quick qualitative detection of radical scavengers prior to spectrophotometric measurements (Takao et al. 1994). The DPPH$^{\bullet}$ method is not discriminative with respect to the radical species but gives a general idea about the radical quenching ability.

The chemical complexity of plant extracts hampers the explanation and interpretation of their AOA. Recently developed on-line HPLC methods, employing model radicals facilitate the evaluation and elucidation of the antioxidative properties of complex samples (Ogawa et al. 1999; Dapkevicius et al. 1999, 2001; Koleva et al. 2000, 2001).

Owing to the complexity of the oxidation-antioxidation processes, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant

profile of a studied sample. The strengths and limitations of the discussed methods are summarised in Table 2.5. The presented study confirms that a multimethod approach is necessary in AOA assessment (Frankel 1993a; Frankel & Meyer 2000).

Table 2.5 The DPPH, HS-GC and BCBT methods – "pros and cons".

Method	Strengths	Limitations
DPPH	▪ Very rapid ▪ Simple ▪ Sensitive ▪ Reproducible ▪ Sample-polarity independent ▪ No special equipment required ▪ Common reagents ▪ No preliminary sample treatment ▪ Low manpower input ▪ High throughput ▪ Mild experimental conditions → no sample alteration ▪ Easily incorporated in on-line systems ▪ Enables structure-activity studies ▪ Provides preliminary information for lead-finding of novel antioxidants	▪ Not informative about the type of protected lipid substrare ▪ Not specific with respect to the scavenged radical species ▪ Partial correlation with results from other testing methods
HS-GC	▪ Sensitive ▪ Specific → reveals the flavour profile of the lipid degradation products ▪ Reproducible ▪ The best correlation with sensory assays data → provides direct practical information ▪ Flexible with respect to the type of substrate, sample and oxidation conditions ▪ Stability of real lipid systems can be studied → very useful in prediction of stability and shelf-life of lipid products ▪ Complex samples can be measured directly without preliminary extraction or other	▪ Slow ▪ Low throughput ▪ Requires sophisticated instrumentation ▪ Preliminary sample oxidation (e.g. heating at higher temperature) is necessary prior to chromatographic measurements ▪ Relatively high manpower input ▪ Partition phenomena have to be borne in mind in the interpretation of the results

	manipulation	
BCBT	▪ Relatively rapid ▪ Sensitive ▪ No special equipment required ▪ Common reagents ▪ No preliminary sample treatment required ▪ Low to medium manpower input ▪ Acceptable throughput (if microtiter plates and a reader device are available) ▪ Relatively mild oxidation conditions	▪ Sample-polarity dependent → limited applicability for less polar samples ▪ Reproducibility highly dependent on experimental variables ▪ Not specific ▪ The chemical and physical properties of emulsion components may affect the ultimate AOA ▪ Partitioning effects influence the AOA ▪ Does not provide unambiguous information about the type of AOA ▪ Results cannot be extrapolated to real oil-in-water emulsions ▪ Substrate is still somewhat artificial

Independently of the chosen method, suitable reference antioxidants should be tested for comparison. A combination of rapid, sensitive and reproducible methods, preferably requiring small sample amounts, that enable complementary results for "primary" and "secondary" antioxidant properties, should be used whenever an AOA screening is designed. For determination of "primary" AOA, the BCBT method is not the best choice and other methods should be chosen (conjugated dienes, peroxide value, etc.). For evaluation of "secondary" antioxidant properties the HS-GC is preferable. A rapid estimation of radical scavenging abilities by using $DPPH^{\bullet}$ (or another model radical), especially in the on-line variants, could save much laboratory work, furnish preliminary information about the AOA of the screened samples and provide a basis for further isolation procedures. Despite some limitations, the $DPPH^{\bullet}$ method can be really helpful in lead-finding of novel antioxidants in phytochemical screening procedures.

2.5 References

Abdalla A.E., Roozen J.P. (1999). "Effect of plant extracts on the oxidative stability of sunflower oil and emulsion." Food Chem. **64:** 323-329.
Abdalla A.E., Tirzite D., Tirzitis G., Roozen J.P. (1999). "Antioxidant activity of 1,4-dihydropyridine derivatives in β-carotene-methyl linoleate, sunflower oil and emulsions." Food Chem. **66:** 189-195.
Avila D.V., Ingold K.U., Lusztyk J., Green W.H., Procopio D.R.(1995). "Dramatic solvent effects on the absolute rate constant for abstraction of the hydroxylic hydrogen atom from t-butyl hydroperoxide and phenol by the cumyloxyl radical. The role of hydrogen bonding." JAOCS **117:** 2929-2930.
Barlow S.M. (1990). "Toxicological aspects of antioxidants used as food additives." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Sciences, London, pp 253-307.

Bondet V., Brand-Williams W., Berset C. (1997). "Kinetics and mechanisms of antioxidant activity using the DPPH free radical method." Lebensm-Wiss. Technol. **30:** 609-615.
Brand-Williams W., Cuvelier M.E., Berset C. (1995). "Use of a free radical method to evaluate antioxidant activity." Lebensm-Wiss.-Technol. **28:** 25-30.
Branen A.L. (1975). "Toxicology and biochemistry of butylated hydroxy anisole and butylated hydroxy toluene." JAOCS **52:** 59-63.
Burton G.W., Ingold K.U. (1984). "β-Carotene: an unusual type of lipid antioxidant." Science **224:** 569-573.
Cao G., Sofic E., Prior R.L. (1996). "Antioxidant capacity of tea and common vegetables." J. Agric. Food Chem. **44:** 3426-3431.
Chen J.H., Ho C.-T. (1997). "Antioxidant activity of caffeic acid and its related hydroxycinnamic acid compounds." J. Agric. Food Chem. **45:** 2374-2378.
Chevolleau S., Mallet J.F., Ucciani E., Gamisans J., Gruber M. (1992). "Antioxidant activity in leaves of some mediterranean plants." JAOCS **69:** 1269-1271.
Cotelle N., Bernier J.-L., Catteau J.-P., Pommery J., Wallet J.-C., Gaydou E.M. (1996). "Antioxidant properties of hydroxy-flavones." Free Rad. Biol. Med. **20:** 35-43**.**
Dapkevicius A., van Beek T.A., Linssen J.P.H., Venskutonis R. (1998a). "Rapid spectroscopic screening for antioxidant activity in sage, thyme and oregano isolates with the β-carotene-linoleic acid model system." in Natural Product Analysis. (Eds. Schreier P., Herderich M., Humpf H.-U., Schwab W.), Friedr. Vieweg & Sohn, Braunschweig, pp 235-237.
Dapkevicius A., van Beek T.A., Niederländer H.A.G. (2001). "Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in HPLC eluates." J. Chromatogr. A **912:** 73-82.
Dapkevicius A., van Beek T.A., Niederländer H.A.G., de Groot Ae. (1999). "On-line detection of antioxidative activity in HPLC eluates by chemiluminescence." Anal. Chem. **71:** 736-740.
Dapkevicius A., Venskutonis R., van Beek T.A., Linssen J.P.H. (1998b). "Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania." J. Sci. Food Agric. **77:** 140-146.
Duh P.-D., Yen G.-C. (1997). "Antioxidative activity of three herbal water extracts." Food Chem. **60:** 639-645.
Dziedric S.Z., Hudson B.J.F. (1984). "Phenolic acids and related compounds as antioxidants." Food Chem. **14:** 45-51.
Economou K.D., Oreopoulou V., Thomopoulos C.D. (1991). "Antioxidant activity of some plant extracts of the family Labiatae." JAOCS **68:** 109-111.
Fogliano V., Verde V., Randazzo G., Ritieni A. (1999). "Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines." J. Agric. Food Chem. **47:** 1035-1040.
Frankel E.N. (1993a). "In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids." Trends Food Sci. Technol. **4:** 220-225.
Frankel E.N. (1993b). "Formation of headspace volatiles by thermal decomposition of oxidized fish oils *vs* oxidized vegetable oils." JAOCS **70:** 767-772.
Frankel E.N. (1998). "Lipid Oxidation." The Oily Press: Dundee.
Frankel E.N., Hu M.-L., Tappel A.L. (1989). "Rapid HS-GC of hexanal as a measure of lipid peroxidation in biological samples." Lipids **24:** 976-981.
Frankel E.N., Huang S.-W., Aeschbach R., Prior E. (1996a). "Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion." J. Agric. Food Chem. **44:** 131-135.
Frankel E.N., Huang S.-W., Kanner J., German .B. (1994). "Interfacial phenomena in the evaluation of antioxidants: bulk oils *vs* emulsions." J. Agric. Food Chem. **42:** 1054-1059.

Frankel E.N., Huang S.-W., Prior E., Aeschbach R. (1996b). "Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions." J. Sci. Food Agric. **72:** 201-208.
Frankel E.N., Meyer A.S. (2000). "The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants." J. Sci. Food Agric. **80:** 1925-1941.
Gadow A. von, Joubert E, Hansmann CF. (1997a). "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.
Gadow A. von, Joubert E., Hansmann C.F. (1997b). "Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*) with green, oolong and black tea." Food Chem**. 60:** 73-77.
Greyt W.F. de, Petrauskaite V., Kellens M.J., Huyghebaert A.D. (1998). "Analysis of tocopherols by GC and HPLC: a comparative study." Fett/Lipid **100:** 503-507.
Huang S.-W., Hopia A., Schwarz K., Frankel E.N., German J.B. (1996). "Antioxidant activity of α-tocopherol and Trolox in different lipid substrates: bulk oils *vs* oil-in-water emulsions." J. Agric. Food Chem. **44:** 444-452.
Imai J., Ide N., Nagae S., Moriguchi T., Matsuura H., Itakura Y. (1994). "Antioxidant and radical scavenging effects of aged garlic extract and its constituents." Planta Medica **60:** 417-420.
Kanner J., Frankel E.N., Granit R., German J.B., Kinsella J.E. (1994). "Natural antioxidants in grapes and wines." J. Agric. Food Chem. **42:** 64-69.
Kasaikina O.T., Kortenska V.D., Yanishlieva N.V. (1999). "Role of chain transfer and recombination/disproportion reaction of the inhibitor's radical in the inhibited lipid oxidation." Russ. Chem. Bull. **48:** 1891-1896 (translated from Russ).
Khal R., Hilderbrandt A.G. (1986). "Methodology for studying antioxidant activity and mechanism of action of antioxidants." Food Chem. Toxicol. **24:** 1007-1014.
Kim S.Y., Kim S.K., Oh M.J., Jung MY. (1994). "Antioxidant activity of selected oriental herb extracts." JAOCS **71:** 633-640.
Koleva I.I., Niederländer H.A.G., van Beek T.A. (2000). "An on-line HPLC method for detection of radical scavenging compounds in complex mixtures." Anal. Chem. **72:** 2323-2328.
Koleva I.I., Niederländer H.A.G., van Beek T.A. (2001). "Application of ABTS radical cation for selective on-line detection of radical scavengers in HPLC eluates." Anal. Chem. **73:** 3373-3381.
Madsen H.L., Bertelsen G. (1995). "Spices as antioxidants." Trends Food Sci. Technol. **6:** 271-277.
Marco G.J. (1968). "A rapid method for evaluation of antioxidants." JAOCS **45:** 594-598.
Marinova E., Yanishlieva N., Ganeva I. (1991). "Antioxidative effect of Bulgarian rosemary and inhibiting activity of its carnosol." Oxidation Commun. **14:** 125-131.
Mathiesen L., Malterud K.E., Sund R.B. (1997). "Hydrogen bond formation as basis for radical scavenging activity: a structure-activity study of C-methylated dihydrochalcones from *Myrica gale* and structurally related acetophenones." Free Rad. Biol. Med. **22:** 307-311.
Matsukawa R., Dubinsky Z., Kishimoto E., Masaki K., Masuda Y., Takeuchi T., Chihara M., Yamamoto Y., Niki E., Karube I. (1997). "A comparison of screening methods for antioxidant activity in seaweeds." J. Applied Phycol. **9:** 29-35.
Miller N.J., Rice-Evans C., Davies M.J., Gopinathan V., Milner A. (1993). "A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates." Clin. Sci. **84:** 407-412.
Moure A., Cruz J.M., Franco D., Dominguez J.M., Sineiro ., Dominguez H., Nunez M.J., Parajo J.C. (2001). "Natural antioxidants from residual sources." Food Chem. **72:** 145-171.

Ogawa A., Arai H., Tanizawa H., Miyahara T., Toyo'oka T. (1999). "On-line screening method for antioxidants by LC with chemiluminescence detection." Anal. Chim. Acta **383:** 221-230.
Pekkarinen S.S., Stockmann H., Schwarz K., Heinonen M., Hopia A.I. (1999). "Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate." J. Agric. Food Chem. **47:** 3036-3043.
Porter W.L. (1993). "Paradoxical behaviour of antioxidants in food and biological systems." in Antioxidants. Chemical, physiological, nutritional and toxicological aspects. (Ed. Williams G.M.), Princeton Scientific Publ. Co. Inc., Princeton, New Jersey, pp 93-121.
Pratt D.E. (1980). "Natural antioxidants of soybean and other oil seeds." in Autoxidation in Food and Biological Systems. (Eds. Simic M.G., Karel M.), Plenum Press, New York, pp 283-293.
Pratt D.E., Birac P.M. (1979). "Source of antioxidant activity of soybeans and soy products." J. Food Sci. **44:** 1720-1722.
Ragnarsson J.O., Labuza T.P. (1977). "Accelerated shelf-life testing for oxidative rancidity in foods - a review." Food Chem. **2:** 291-308.
Robards K., Prenzler P.D., Tucker G., Swatsitang P., Glover W. (1999). "Phenolic compounds and their role in oxidative processes in fruits." Food Chem. **66:** 401-436.
Roedig-Penman A., Gordon M.H. (1998). "Antioxidant properties of myricetin and quercetin in oil and emulsions." JAOCS **75:** 169-180.
Roginsky V.A. (1988). "Phenol Antioxidants. Efficiency and Reactivity.", Nauka, Moscow (in Russ).
Sanchez-Moreno C., Larrauri J.A., Saura-Calixto F.A. (1998). "Procedure to measure the antiradical efficiency of polyphenols." J. Agric. Food Chem. **76:** 270-276.
Schuler P. (1990). "Natural antioxidants exploited commercially." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Sciences, London, pp 99-170.
Takao T., Kitatani F., Watanabe N., Yagi A., 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.
Velioglu Y.S., Mazza G., Gao L., Oomach B.D. (1998). "Antioxidant activity and total phenolics of selected fruits, vegetables, and grain products." J. Agric. Food Chem. **46:** 4113-4117.
Wang H., Cao G., Prior R.L. (1996). "Total antioxidant capacity of fruits." J. Agric. Food Chem. **44:** 710-705.
Wettasinghe M., Shahidi F. (1999). "Evening primrose: a source of natural antioxidants and scavengers of hydrogen peroxide and oxygen-derived free radicals." J. Agric. Food Chem. **47:** 1801-1812.
Yamaguchi T., Takamura H., Matoba T., Terao J. (1998). "HPLC method for evaluation of the free radical-scavenging activity of foods by using DPPH." Biosci. Biotechnol. Biochem. **62:** 1201-1204.
Yen G.-C., Duh P.-D. (1994). "Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species." J. Agric. Food Chem. **42:** 629-632.
Yokozawa T., Chen C.P., Dong E., Tanaka T., Nonaka G.-I., Nishioka I. (1998). "Study on the inhibitory effect of tannins and flavonoids against the 1,1'-diphenyl-2-picrylhydrazyl radical." Biochem. Pharmacol. **56:** 213-222.

Antioxidant Activity Screening of Extracts from *Sideritis* species (Labiatae) Grown in Bulgaria*

3.1 Introduction

Lipid oxidation is a highly deteriorative process in foods, as it leads to unacceptable properties for the customer and to loss in nutritional value. As a serious consequence health disorders like atherosclerosis, cancerogenesis, etc. can arise (Papas 1999). Hence, the presence of antioxidants in foods is essential for their quality and safety. The importance of natural antioxidants for use as food additives or nutritional supplements has already been established. The commonly used synthetic antioxidants possess negative side effects (Barlow 1990). This has resulted in stronger restrictions on their use and in shifting the consumer and scientific interests toward natural alternatives. Although natural compounds, including antioxidants, can be toxic under some conditions, more often they possess additional useful properties (antibacterial, antimutagenic, immuno-stimulating, etc.) and their different protective mechanisms rank them high in the choice for use in food products. Medicinal plants and herbs are a promising and diverse source of natural antioxidants (Kim et al. 1994; Madsen & Bertelsen 1995). Many plant species have been studied for antioxidant activity, and some of them have even become commercial products. Tea (Yen & Chen 1995), rosemary, sage and other herbs, and the products obtained from them, are well known examples (Lőliger 1991; Marinova et al. 1991). Plants belonging to the genus *Sideritis* (Labiatae) are well known in Bulgarian and other folk medicines for treating various infections, inflammations, etc. Many reports are devoted to their chemical composition and pharmacological activities. The presence of terpenoids (Rodriguez & Valverde 1975), essential oils (Mateo et al. 1988) and flavonoids (Ezer et al. 1992; Gil et al. 1993) has been established. From some species, a coumarin (siderin) (Gonzalez et al. 1972), a lignan ((+)-sesamin) (Fraga et al. 1995) and phenylpropanoid glycosides (Ezer et al. 1992; Kokkalou & Gabrieli 1997) have been isolated.

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Although the antiinflammatory principles of *Sideritis* plants have been well studied (Tomas-Barberan et al. 1987), as have the antimicrobial effects of its diterpenoids (Diaz et al. 1988) and essential oils (Villar et al. 1986), little has so far been done on their possible antioxidant activity. The antioxidant and superoxide scavenging activities of several flavonoids and polymethoxyflavones, some isolated from Spanish *Sideritis* species, using $FeSO_4$/cysteine-induced microsomal lipid peroxidation and the nitroblue tetrazolium test, have been reported (Mora et al. 1990; Rios et al. 1992).

The *Sideritis* species grown in Bulgaria have not been studied in detail. Among the compounds so far detected are the diterpenoid siderol and ursolic/oleanolic acids (Sattar et al. 1995a) and flavonoids (Sattar et al. 1993; Sattar et al. 1995b). The antibacterial and antiviral properties of some Bulgarian *Sideritis* species have been reported (Sattar et al. 1995a). The presence of flavonoids and phenylpropanoid glycosides in *Sideritis* plants, as well as their established antiinflammatory properties, suggest a possible antioxidant activity of *Sideritis* extracts.

Antioxidants affect the process of lipid oxidation at different stages due to differences in their mode of action (Larson 1997). Because of the complexity of the oxidation process itself, the diversity of the substrates and the active species involved, the application of different test methods is necessary in the evaluation of antioxidants (Frankel & Meyer 2000; Koleva et al. 2002).

The present study is aimed at the evaluation of the antioxidant activity of different extracts from several *Sideritis* species and populations, grown in Bulgaria, by using three testing methods: the *β*-carotene bleaching test (BCBT), 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging method and static headspace gas chromatography (HS-GC). As the extracts studied have been produced by three extraction routes, the results obtained will show the most suitable route with regard to the preparation of extracts rich in antioxidative active components, as well as the most promising *Sideritis* species/population(s). Additionally, this initial screening will reveal at which stage of lipid oxidation a particular extract would be most effective as an antioxidant with respect to its possible practical implementation in real food systems.

3.2 Experimental

3.2.1 Chemicals

Reagents and compounds were purchased from: Sigma-Aldrich Chemie GmbH, Steinheim, Germany [Tween40, *trans*-*β*-carotene, **7** (synthetic, 95%) and 2,2-diphenyl-1-picrylhydrazyl, **34** ($DPPH^{\bullet}$, 95%)]; Janssen Chimica, Beerse, Belgium [linoleic acid

(90%)]; Extrasynthese, Genay, France [rosmarinic acid, **11**]; Fluka, Buchs, Switzerland [butylated hydroxytoluene, **1** (BHT, p.a.)]. Authentic samples of flavonoids **31** and **32**, purified from natural sources, were a kind gift from Dr. V. Bankova (Bulgarian Academy of Sciences, Institute of Organic Chemistry, Sofia, Bulgaria). See Figure 3.2 and Chapter 1 for the structures of the compounds. Commercial cold-pressed safflower oil without added synthetic antioxidants was obtained from Natufood BV, Harderwijk, The Netherlands. It contained 562 ppm α-tocopherol and traces of γ-tocopherol as determined by high-performance liquid chromatography (HPLC) (de Greyt et al. 1998). The oil was stored under nitrogen in the dark at −20 °C. Fatty acid composition determined by GC as methyl esters was as follows: 6.9% C16:0, 2.7% C18:0, 12.6% C18:1, 77.5% C18:2. All solvents used were of analytical grade or HPLC grade (for HPLC analyses). Ultra-pure water (0.05 μScm^{-1}) was obtained from a combined Seradest LFM 20 and Serapur Pro 90C apparatus (Seral, Ransbach-Baumbach, Germany).

3.2.2 Preparation of plant extracts

Aerial parts of *Sideritis* plants were collected at the flowering stage, air dried at ambient temperature with forced ventilation, and ground in a grinder before use. Voucher specimens were determined by Dr. L. Evstatieva and deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Sofia (SOM). The species and populations used and their origins were as follows: *S. scardica* 1 (LE 7a; cultivar, seeds collected at Pirin mountain, Lozen, Bulgaria, May 1995; SOM 3154a); *S. scardica* 13 (LE 2; natural population, Rodopi mountain, Trigrad, Bulgaria, May 1997); *S. scardica* 14 (LE 7v; cultivar, seeds collected at Pirin mountain, Lozen, Bulgaria, May 1997; SOM 3154v); *S. montana* 4 (LE 12; natural population, Strandja mountain, Dervishki hills, Bulgaria, May 1995; SOM 3253); *S. montana* 5 (LE 30; natural population, Black Sea coast, Primorsko, Bulgaria, August 1993); *S. montana* 11 (LE 31; Black Sea coast, Varna, Bulgaria, July 1995; SOM 1282); *S. syriaca* 6 (LE 5; cultivar, seeds from Spanish origin, Lozen, Bulgaria, 1995); *S. syriaca* 9 (LE 6a = 6b; natural population, Strandja mountain, Malko Tarnovo, Bulgaria, July 1995; SOM 3151); *S. syriaca* 12 (LE 10; cultivar, seeds from Spanish origin, Lozen, Bulgaria, 1995). Cultivars were grown in the Botanical Garden of the Institute of Botany, Bulgarian Academy of Sciences, Lozen, Bulgaria. Dried and ground plant material was extracted according to the extraction schemes presented in Figure 3.1. All extracts were evaporated under vacuum till dryness at temperatures not higher than 50 °C. Water and hot water extracts were freeze dried.

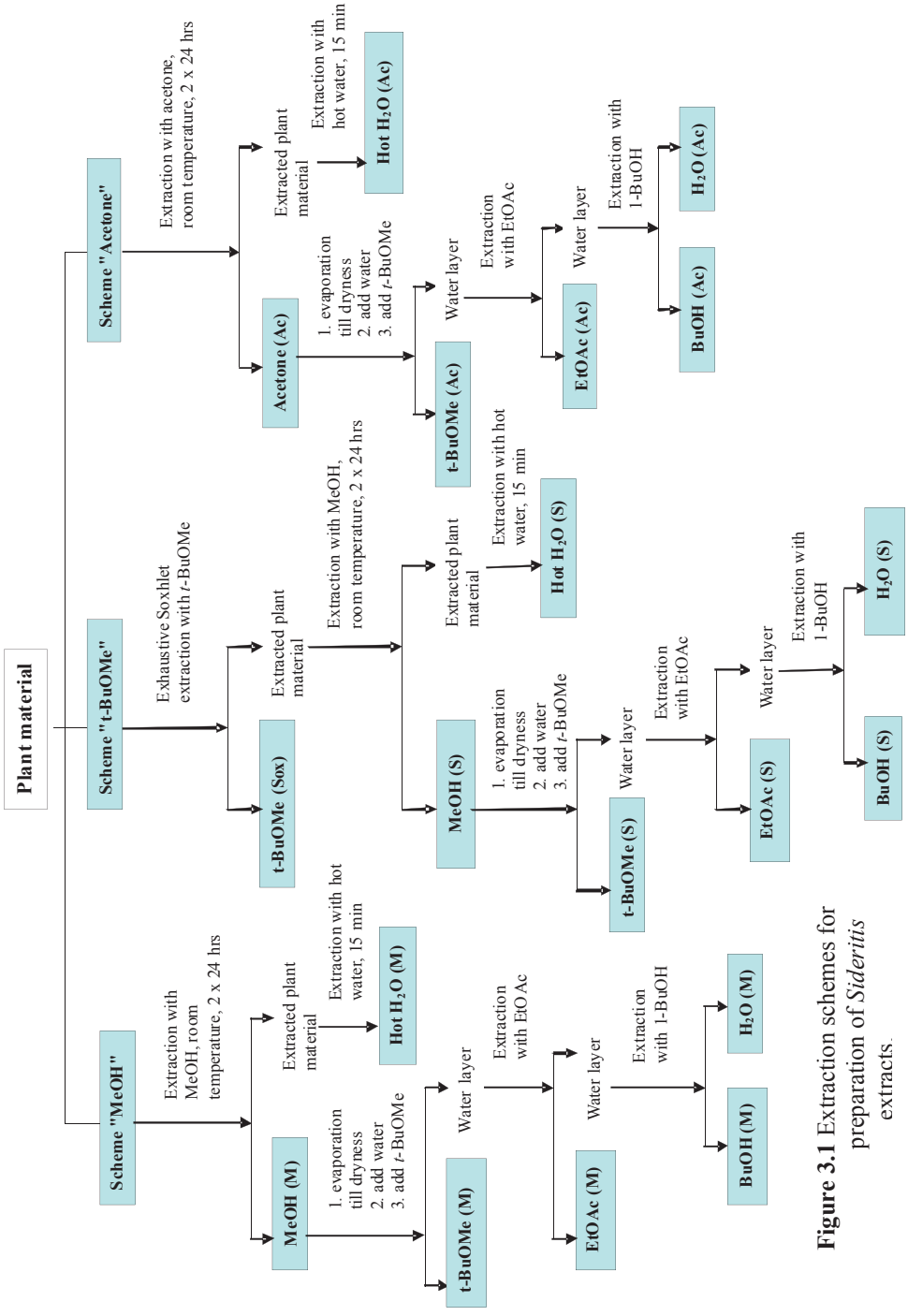

Figure 3.1 Extraction schemes for preparation of *Sideritis* extracts.

[*Abbreviations*: **MeOH** and **M** - methanol (extract); ***t*-BuOMe** and **S** - *tert*-butyl methyl ether (extract); **Ac**- acetone (extract); **EtOAc** – ethyl acetate (extract); **BuOH** – 1-butanol (extract); **Sox** – Soxhlet (extract)]

3.2.3 Chromatographic and spectral analyses

Thin-layer chromatography (TLC)

Pre-coated silica gel 60 F_{254} thin-layer chromatography (TLC) plates (0.2 mm; Merck, Darmstadt, Germany) and two solvent systems ($CHCl_3$:MeOH:H_2O = 60:22:4 and 6:4:1) were used for the TLC studies of extracts. Detection was carried out under UV light. For qualitative detection of radical scavenging compounds, the TLC plates were sprayed with 1 mM methanolic solution of $DPPH^{\bullet}$ radical. Radical scavengers produce yellow spots on a purple background.

High-pressure liquid chromatography (HPLC)

The HPLC system used consisted of the following: an HPLC eluent pump (Waters 600E System Controller, Milford, MA); a programmable photodiode array detector (Waters 996) and an autosampler (Waters 717plus). Separations were carried out with an Alltima C18 HPLC column (250 × 4.6 mm i.d., 5 μm; Alltech Associates Inc., Deerfield, IL) with a guard column. Stock solutions of *Sideritis* extracts (5 $mg.cm^{-3}$) were prepared in MeCN:H_2O (1:1, v/v) or in water (for aqueous extracts). Further dilution with water to a concentration of 1 $mg.cm^{-3}$ was carried out before injection (20 μL) onto the HPLC column. The separation was performed by a step gradient with 5% MeCN in water (solvent **A**) and MeCN (solvent **B**) at a flow rate of 0.8 $mL.min^{-1}$ as follows: linear gradient from 100 to 80% **A** for 5 min; 5 min 80% **A**, isocratic; 20 min linear gradient from 80 to 65% **A**; 10 min isocratic hold at 65% **A**; 10 min linear gradient from 65 to 0% **A**; 5 min 0% **A**, isocratic; 5 min linear gradient from 0 to 100% **A** and a 10 min isocratic hold at 100% **A** for re-equilibration of the column before the next injection. UV detection was carried out at 280 and 330 nm.

Spectroscopic methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-E 200 and a DPX 400 spectrometer. The ^{13}C NMR spectra and distortionless enhancement by polarization transfer (DEPT) spectra were recorded on a Bruker DPX 400 spectrometer operating at 100 MHz. Samples were dissolved in DMSO-d_6 (for flavonoids) and CD_3OD (for verbascoside).

3.2.4 Methods for determination of antioxidant activity

The antioxidant activity of *Sideritis* extracts, BHT (**1,** Fig. 3.2) and rosmarinic acid (**11,** Fig. 3.2) was determined using the methods described by Koleva et al. (2002). Below, a brief explanation of the experimental protocols is given.

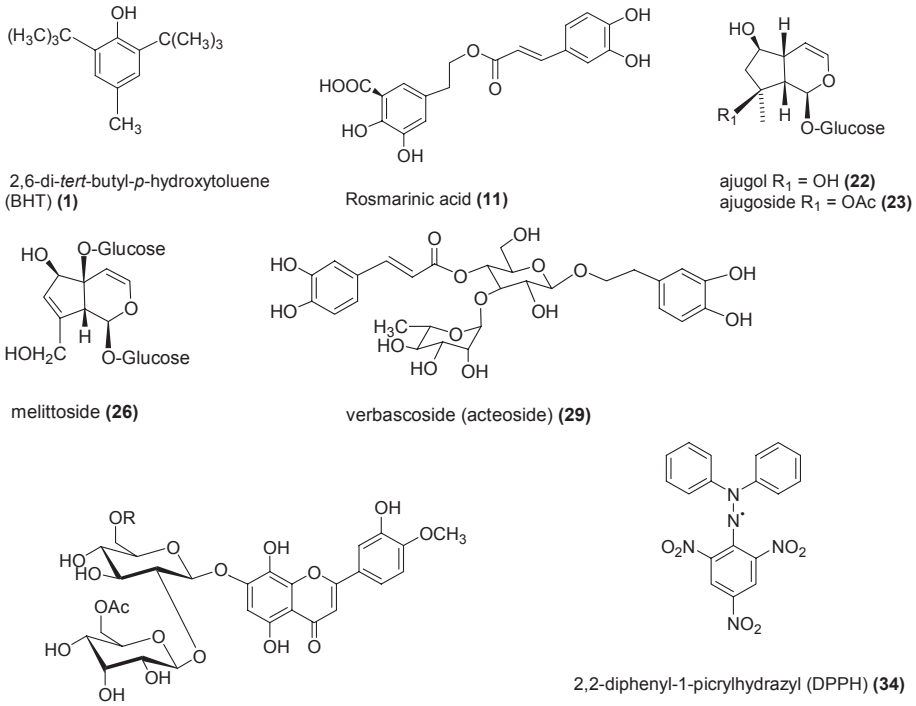

Figure 3.2 Structures of the compounds mentioned in the text (see Ch. 1 for the numbering of the compounds).

Determination of antioxidant activity using β-carotene bleaching test (BCBT)

Trans-β-carotene − linoleic acid emulsion (250 µL) was distributed in each of the wells of 96-well microtitre plates and ethanol solutions (30 µL) of the antioxidants (0.1% of extracts and 0.01% each for BHT and rosmarinic acid) to be tested were added. Four replicates were prepared for each of the samples. The microtitre plates were incubated at 55 ºC. The absorbance was measured in an EAR 400 Microtiter Reader (SLT Instruments, Austria) at

492 nm (Dapkevicius et al. 1998). Readings of all samples were performed immediately (t = 0 min) and at t = 105 min (Pratt & Birac 1979). After 105 min the decrease in the absorbance of the blank sample was no longer significant. The antioxidant activity coefficient (AAC) was calculated from the data using the formula reported by Chevolleau et al. (1992), slightly modified as follows:

$$AAC = [(A_{A,105} - A_{B,105}) / (A_{B,0} - A_{B,105})] \times 100$$

where $A_{A,105}$ and $A_{B,105}$ are the absorbances of the test and blank sample, respectively at t = 105 min and A_B,0 is the absorbance of the blank sample at t = 0 min.

Determination of antioxidant activity by static headspace gas chromatography (HS-GC)
Solutions of safflower oil containing methanolic solution of the extracts to be tested (0.1%, w/w) and reference compounds (BHT and rosmarinic acid, each 0.01%, w/w) were prepared. A blank sample was prepared with an equal amount of methanol. Samples (0.20 g) of these solutions were placed into 10 mL headspace vials. The samples were incubated at 55 °C in a shaker. Sampling was carried out every other day. The hexanal formation was monitored using a Carlo Erba Mega 5300 gas chromatograph with an HS 800 headspace sampler and a wide-bore–thick film capillary DB-WAX column (30 m × 0.54 mm, 1 μm thickness). The temperature program is given in detail by Koleva et al. (2002). The antioxidant activity was evaluated using the protection factor (PF) (Marinova et al. 1991),

$$PF = IP_A / IP_B$$

and the relative rate of hexanal (RRH) formation until the induction period (IP)

$$RRH = V_A / V_B$$

where IP_A and IP_B are the induction periods (time in days after which the hexanal formation increases sharply because of the complete consumption of the antioxidant) for the test and blank sample, respectively; V_A and V_B are the initial rates of hexanal formation of the antioxidant and blank sample, respectively, expressed as the slopes of the curves $S_H = f(T)$ [where S_H is the amount of hexanal, expressed as hexanal peak area in integration units (IU) and T is the time in days].

Antioxidant Activity Screening of Extracts from *Sideritis* species (Labiatae) Grown in Bulgaria

Determination of free radical scavenging activity using DPPH free radical method

An ethanolic solution of DPPH$^{\bullet}$ (10^{-4} M) (Fig. 3.2, **34**) and sample solution (*Sideritis* extracts, BHT and rosmarinic acid) were mixed so that the final mass ratios were extract:DPPH$^{\bullet}$ = 5.5:1 and reference compound:DPPH$^{\bullet}$ = 0.5:1. Triplicate samples were incubated for 15 min in the dark at 30 °C and the decrease in absorbance at 517 nm was measured. The radical scavenging activity of the samples tested, expressed as percentage reduction of DPPH$^{\bullet}$, was calculated according to the following formula (Yen & Duh 1994):

$$Reduction\ (\%) = [(A_B - A_A) / A_B] \times 100$$

where A_B is the absorbance of the blank sample and A_A is the absorbance of the antioxidant tested after 15 min.

3.3 Results and discussion

3.3.1 Antioxidant activity of Sideritis extracts according to the β-carotene bleaching test method (BCBT)

Table 3.1 shows the yields of the different extracts. Depending on the solvent, different yields were obtained. For the extraction scheme 'MeOH', the highest yield was found for MeOH, whereas the yields of the other extracts were much lower. The highest yield was also found for MeOH in the extraction scheme '*t*-BuOMe', whereas in the extraction scheme 'Acetone' the highest yield was found for the hot H_2O extract.

Because of the different polarity of the extracts tested — from apolar (*t*-butyl methyl ether extracts) to very polar (water and hot-water extracts) — BHT (AAC = 864) and rosmarinic acid (AAC = 425), representing apolar and polar antioxidants respectively, were used as reference compounds (Koleva et al. 2002).

The antioxidant activities of all *Sideritis* extracts tested, expressed as AAC, are presented in Table 3.2. *t*-Butylmethyl ether extracts and total methanol extracts from *S. scardica* showed higher activity (AAC between 830 and 700) than the other extracts. The more polar extracts (EtOAc and BuOH) and rosmarinic acid exhibited moderate to weak antioxidant activity (AAC between 600 and 300) according to this method. As a whole, the weakest inhibitors of *β*-carotene loss turned out to be the most polar extracts — hot aqueous and aqueous extracts, the latter even showing pro-oxidant effects (negative AAC). Both were found to contain mainly polar iridoid glycosides (Koleva & Handjieva 1997) (**22**; **23**; **26**, Fig. 3.2). These glycosides do not possess structural features implying antioxidant properties, i.e. functional groups capable of donating electrons or hydrogen atoms.

However, some antioxidant activity was still observed for some of these extracts, which might be due to polar phenolic compounds present at low concentrations.

Table 3.1 Yields of *Sideritis* extracts, obtained from all populations studied (For abbreviations, see Fig. 3.1).

***Sideritis* species and populations**	**Extract yield ($g.kg^{-1}$ dried plant material)**					
***Type of extract according to extraction scheme "MeOH"*[a]**						
	MeOH	***t*-BuOMe**	**EtOAc**	**BuOH**	**H_2O**	**Hot H_2O**
S. scardica 1	120.7	22.4	22.5	31.0	37.4	63.4
S. scardica 13	157.2	27.4	23.5	35.8	65.7	71.5
S. scardica 14	157.0	29.4	19.9	38.6	66.0	58.6
S. syriaca 6	163.5	27.6	19.9	42.7	70.1	69.7
S. syriaca 9	140.4	23.5	21.2	35.3	60.3	66.3
S. syriaca 12	159.6	25.1	23.6	33.1	63.8	64.4
S. montana 4	128.3	30.8	34.5	33.5	24.4	53.3
S. montana 5	161.1	33.9	42.9	38.8	43.6	69.4
S. montana 11	141.3	22.6	26.5	26.5	60.5	76.3
Type of extract according to extraction scheme "t-BuOMe"						
	***t*-BuOMe (Sox)**	**MeOH**	***t*-BuOMe**	**EtOAc**	**BuOH**	**H_2O**	**Hot H_2O**
S. scardica 14	20.8	129.6	14.6	16.7	38.4	52.4	79.4
Type of extract according to extraction scheme "Acetone"						
	Acetone	***t*-BuOMe**	**EtOAc**	**BuOH**	**H_2O**	**Hot H_2O**
S. scardica 14	37.7	13.9	10.4	10.4	00.9	132.5

The similar activities of EtOAc and BuOH extracts are most likely due to the presence of substances being partitioned into both solvents. TLC and HPLC separations of these extracts showed that they have a similar composition (Fig. 3.3). The main components of these extracts were identified as verbascoside (**29,** Fig. 3.2), hypolaetin-4'-methylether-7-*O*-[6'''-*O*-acetyl-*β*-D-allopyranosyl-(1'''-2'')-*β*-D-glucopyranoside] (**31,** Fig. 3.2) and hypolaetin-4'-methylether-7-*O*-[6'''-*O*-acetyl-*β*-D-allopyranosyl-(1'''-2'')-*β*-D-6''-*O*-acetyl-*β*-D glucopyranoside] (**32,** Fig. 3.2). Flavonoids were identified by comparing their HPLC retention times and spectral data (UV, NMR) with those of reference samples and literature data (Sattar et al. 1993; Sattar et al. 1995b). Verbascoside (**29**) was identified by comparison with previously reported spectral data (Gao et al. 1999). Several other phenolic components of the flavonoid and phenylpropanoid types were also isolated and their

structures will be further elucidated. Comparison of the AAC of *S. scardica* 14 extracts obtained by the three extraction routes showed that the initial extraction solvent had little effect on the antioxidant activity of the same types of extract (e.g. *t*-BuOMe extracts obtained by the three extraction routes had AAC values between 760 and 830). Methanol gave approximately four times higher yields than acetone; hence, it was preferred as an initial extraction solvent for all plant samples studied.

Table 3.2 Antioxidant activity (BCBT method) of *Sideritis* extracts, obtained from all populations studied according to extraction scheme "MeOH", expressed as AAC, for the various extract types.

***Sideritis* species and populations**	**AAC**					
	MeOH	***t*-BuOMe**	**EtOAc**	**BuOH**	**H_2O**	**Hot H_2O**
S. scardica 1	709	670	408	551	−266	367
S. scardica 13	725	731	562	438	−295	266
S. scardica 14	701	830	529	632	−123	554
S. syriaca 6	526	355	611	574	−169	411
S. syriaca 9	482	682	524	612	−166	428
S. syriaca 12	595	468	549	615	−157	645
S. montana 4	572	467	218	428	−179	103
S. montana 5	515	229	491	345	−221	197
S. montana 11	486	586	640	210	−97	256

Values are means of four replicates and the RSD is less than 5%

AAC_{BHT} = 864; **$AAC_{Rosmarinic\ acid}$ = 425**

The results obtained are not unusual when considered in the light of the 'polar paradox' phenomenon, in which the polar antioxidants tend to be more active in bulk oil systems and the apolar antioxidants exhibit stronger antioxidant activity in emulsions (Porter 1980; Frankel et al. 1994). However, some of the extracts did not behave according to this phenomenon. For example, *t*-BuOMe(M) extracts from *S. syriaca* 6 and *S. montana* 5 were less active than EtOAc(M) and BuOH(M) extracts from the same plants. A possible reason could be the multicomponent nature of the extracts, which could involve synergistic, additive or antagonistic effects, i.e. a complex mechanism of action. Other factors to

consider include the chemical and physical properties of linoleic acid, *β*-carotene and the emulsion itself (Koleva et al. 2002).

Extracts from *S. scardica* and *S. syriaca* species exhibited similar activity and were, in general, more active than the extracts from *S. montana* species. Each of the *S. montana* populations showed its own specific pattern of activity in their extracts, which could mean that the habitat influence is much stronger for this species than for the other two species. A semi-quantitative TLC screening of the total MeOH extracts showed that all *S. scardica* and *S. syriaca* samples were chemically very similar, whereas *S. montana* samples were clearly different and even showed intraspecies variations.

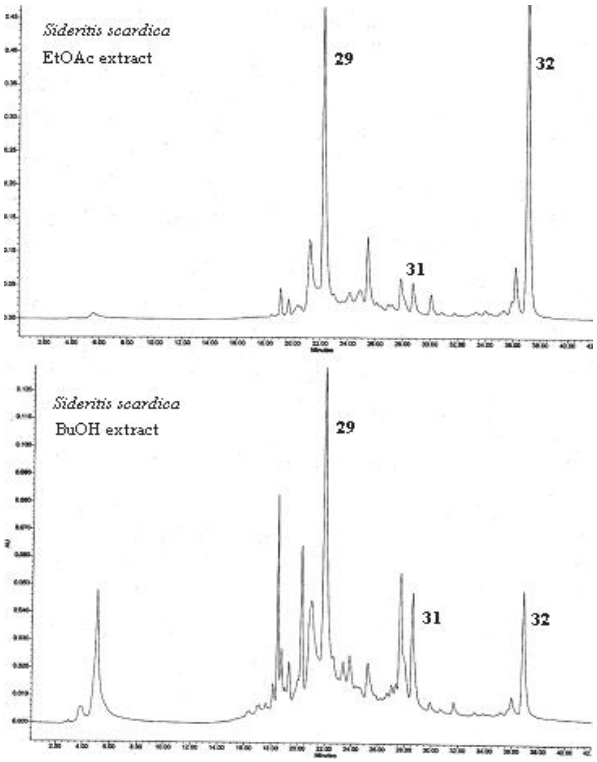

Figure 3.3 HPLC profiles of *S. scardica* EtOAc and BuOH extracts. Chromatogrphic conditions: Alltima RP18 column, 250 × 4.6 mm i.d., 5 μm; flow rate 0.8 mL.min^{-1}; MeCN:H_2O gradient (see Experimental section for details); UV detection was carried out at 330 nm.

3.3.2 Antioxidant activity of Sideritis extracts according to the static headspace gas chromatography method (HS-GC)

Since the lipid hydroperoxides formed rapidly decompose to stable secondary oxidation products after heating (in cooked, fried products, for example), it is useful to follow the lipid oxidation by monitoring the formation of such reaction products. Hexanal is one of the major secondary compounds formed during the lipid oxidation and it is responsible for the rancid off-odour of lipid-containing products (Frankel 1991). The literature data concerning the inhibiting properties of natural antioxidants on the decomposition of lipid hydroperoxides and the formation of secondary products are not very abundant. Static HS-GC has been applied to assess this type of antioxidant activity of rosemary (Frankel et al. 1996), grapes (Meyer et al. 1997), extracts from several Labiatae plants (Abdalla & Roozen 1999) and phenolics in virgin olive oil (Satue et al. 1995).

Table 3.3 Antioxidant activity (HS-GC method) of EtOAc- and BuOH extracts from *Sideritis,* expressed as protection factor (PF) and relative rate of hexanal formation (RRH) until the induction period (IP).

Tested sample	**EtOAc extracts**		**BuOH extracts**	
	PF	***RRH***	***PF***	***RRH***
S. scardica 1[a]	1.3	1.7	1.3	1.1
S. scardica 13	1.3	2.6	0.7	1.6
S. scardica 14 (scheme "MeOH")	1.3	1.7	1.0	1.0
S. scardica 14 (scheme "t-BuOMe")	1.3	1.8	1.3	1.0
S. scardica 14 (scheme "Acetone")	1.3	2.0	0.7	1.2
S. syriaca 6	1.3	1.4	1.3	1.7
S. syriaca 9	1.3	1.4	1.3	1.9
S. syriaca 12	1.3	1.7	1.3	2.3
S. montana 4	0.3	1.6	0.6	1.7
S. montana 5	0.3	1.1	0.3	3.7
S. montana 11	1.0	0.9	1.3	1.9
	PF		***RRH***	
Blank[b]	1.0		1.0	
BHT	1.3		0.7	
Rosmarinic acid	1.6		1.0	

[a] - EtOAc and BuOH extracts from all *Sideritis* species were obtained according to extraction scheme "MeOH". *S. scardica* 14 plant samples were also processed according to the schemes "*t*-BuOMe" and "Acetone".

[b] - IP for the blank oil sample was 7 days

The presence of flavonoid and phenylpropanoid glycosides in EtOAc- and BuOH extracts explains their established antioxidant activity. The antioxidant properties of many flavonoid and phenylethanoid glycosides have been reported (Mora et al. 1990; Xiong et al. 1996; Roedig-Penman & Gordon 1998; Gao et al. 1999). Despite their 'phenolic' nature, the EtOAc and BuOH extracts showed low antioxidant activity in the BCBT assay, close to that of rosmarinic acid. Because a low activity of an antioxidant in emulsion systems is often correlated with a higher activity in bulk oils ('polar paradox'), these extracts were also investigated for their ability to inhibit hexanal formation in bulk safflower oil. The 'secondary' antioxidant properties of the samples tested were estimated on the basis of the following parameters: protection factor (PF) and relative rate of hexanal (RRH) formation until the induction period (IP).

Table 3.3 shows a tendency for the ethyl acetate extracts from *S. syriaca* and *S. scardica* and the butanol extracts from *S. syriaca* to protect the oil from rancidity, in terms of hexanal formation, in the same order as BHT but less than rosmarinic acid. *S. scardica* BuOH extracts, as a whole, were less active than their *S. syriaca* counterparts and *S. scardica* EtOAc extracts. *S. montana* extracts showed lower activity. In fact, two of them (*S. montana* 4 and *S. montana* 5) even showed pro-oxidant properties (PF < 1).

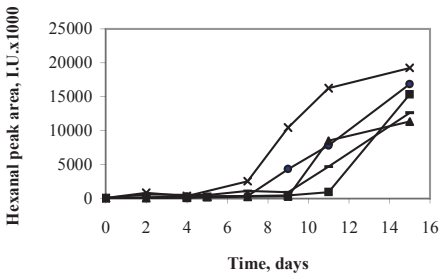

Figure 3.4 Changes in hexanal formation during oxidation of safflower oil in time in the presence of (▲) BHT, (■) rosmarinic acid, (—) *Sideritis syriaca* 12 – BuOH (M) and (×) *Sideritis montana* 4 – BuOH (M) extracts compared to a blank oil sample (●).

Figure 3.4 presents data on the formation of hexanal in bulk safflower oil for some of the extracts studied and the reference compounds. Again, the choice of the initial extraction solvent ("MeOH", "*t*-BuOMe", "Acetone") did not influence the results with this method (see the data for *S. scardica* 14 extracts in Table 3.3).

The results were obtained with commercial oil and showed that most of the above-described *Sideritis* extracts (and/or their components) could, in principle, serve as natural antioxidative additives for retardation of edible oil rancidity provided that they do not possess toxic effects or impart unwanted taste/colour to foods. After these screening experiments, further work will be performed to describe the antioxidative activity in more detail.

3.3.3 Radical scavenging activity of Sideritis extracts determined by the DPPH method

As mentioned earlier in Chapters 1 and 2, various radicals formed during lipid oxidation are among the main causes for oxidative damage to human health (Williams 1993). Antioxidants can exercise their protective function by scavenging free radicals, which are the main propagators of lipid oxidation. $DPPH^{\bullet}$ is a stable organic radical, often used in evaluation of radical scavenging activity of antioxidants — natural and synthetic pure compounds (Brand-Williams et al. 1995; von Gadow et al. 1997), plant extracts (Yen & Duh 1994; Koleva et al. 2002), foods (Yamaguchi et al. 1998). Alcoholic solutions of $DPPH^{\bullet}$ have a characteristic absorption maximum at 517 nm. When an electron- or hydrogen-atom donating antioxidant (AH) is added to $DPPH^{\bullet}$, there is a decrease in absorbance at 517 nm due to the formation of the non-radical form DPPH-H which does not absorb at 517 nm:

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$

All previously described *Sideritis* extracts were screened for radical scavenging activity against $DPPH^{\bullet}$ (Table 3.4). The most active extracts were the EtOAc and BuOH extracts, as well as total MeOH and Acetone extracts. This is due to the presence of flavonoid and phenylpropanoid glycosides — compounds known as potential hydrogen donors and radical scavengers (Rice-Evans et al. 1996; Gao et al. 1999). In some of the extracts (MeOH, EtOAc and BuOH) the percentage of reduction reached nearly 100%. This suggests that these extracts contain higher concentrations of active compounds than those needed in the reaction of $DPPH^{\bullet}$ scavenging. In the follow-up work it will be necessary to apply diluted samples of the extracts to provide better evidence for which extracts are the most efficient ones. Spraying of TLC plates of *Sideritis* extracts with a $DPPH^{\bullet}$ alcoholic solution showed that all spots corresponding to phenolic compounds reacted with the $DPPH^{\bullet}$ reagent.

In general, the radical scavenging activities of *t*-butyl methyl ether extracts and water extracts were much lower and strongly influenced by the plant sample origin. TLC and HPLC analysis of water extracts showed the presence of the same phenolics as found in EtOAc and BuOH extracts, but in much lower concentrations. $DPPH^{\bullet}$ spraying of TLC plates with ether extracts showed that they were poorer in $DPPH^{\bullet}$ scavenging components. No substantial influence of the extraction procedure was observed.

When the same type of extract is considered for all plant samples tested, little difference in relative activity was observed. In contrast to the BCBT method, the apolar *t*-butyl methyl ether extracts were not the most active in the $DPPH^{\bullet}$ test system. This fact

could mean that either their components do not possess good hydrogen donating properties or that some kinetic factors influenced their reaction with the radical, or that there are components interfering with the radical scavenging process. Bondet et al. (1997) have found that the radical scavenging activity of a particular antioxidant depends on its structure as well as on the type of reaction kinetics. The higher antioxidant activity of these extracts according to the BCBT method could mean that their protective properties are highly dependent on the surface phenomena of the methods in emulsion systems, such as BCBT, or on reactions between the functional groups of the compounds and the substrate (Koleva et al. 2002).

Table 3.4 Radical scavenging activity of *Sideritis* extracts and two reference compounds[a] against DPPH$^{\bullet}$ expressed as % Reduction for the various extract types.

***Sideritis* species and populations**	***t*-BuOMe**	**MeOH**	**EtOAc**	**BuOH**	**H_2O**	**Hot H_2O**
S. scardica 1[b]	44[a]	93	95	94	35	85
S. scardica 13	28	94	96	93	58	85
S. scardica 14 (MeOH)	38	94	94	94	49	86
S. scardica 14 (t-BuOMe)	59 67[c]	94	97	97	39	84
S. scardica 14 (Acetone)	45	90	96	94	90	88
S. syriaca 6	38	92	96	95	32	87
S. syriaca 9	42	92	95	94	42	86
S. syriaca 12	27	93	94	95	33	87
S. montana 4	47	91	94	92	69	82
S. montana 5	63	92	96	93	28	79
S. montana 11	50	92	93	93	74	83

% Reduction$_{Rosmarinic\ acid}$ **= 95 %**; **% Reduction**$_{BHT}$ **= 65 %**

[a] - Values are mean of three replicates and the RSD is less than 1%

[b] - Extracts from all *Sideritis* species were obtained according to the extraction scheme "MeOH". *S. scardica* 14 plant samples were also processed by schemes "*t*-BuOMe" and "Acetone".

[c] - Value for *t*-BuOMe(Sox) from *S. scardica* 14 (scheme "*t*-BuOMe")

For comparative purposes, the radical scavenging activities of rosmarinic acid and BHT were also determined. Similar to the BCBT and HS-GC protocols, the pure reference compounds were applied in a ten-times lower concentration than the extracts (here 0.5:1, w/w). Rosmarinic acid showed 95% Reduction of DPPH$^{\bullet}$, whereas BHT exhibited only 65% Reduction. Compared on a molar basis, BHT was also a much weaker radical

scavenger than rosmarinic acid (Koleva et al. 2002). The most active *Sideritis* extracts possessed radical quenching activity comparable to that of rosmarinic acid, and even the less active extracts can be compared in their potency to BHT. The results show that the most active *Sideritis* extracts can serve as a promising source for the isolation of radical scavenging compounds.

3.4 Conclusions

The *Sideritis* species investigated contain components with antioxidant abilities. The methods applied in this study considered the antioxidant properties of the extracts studied as determined by different testing methods. Such an approach is necessary in evaluation of novel antioxidants, since the antioxidant activity is dependent on many factors, such as the type of oxidizable substrates, oxidation conditions, parameters measured, partition phenomena, etc. (Frankel & Meyer 2000; Koleva et al. 2002). The high radical scavenging activity of EtOAc and BuOH extracts can be correlated to the presence of flavonoid and phenylpropanoid glycosides. These types of compounds have also been found to be effective in rancidity retardation of edible oils, with or without natural tocopherols, and may explain the antioxidant activity exhibited in the HS-GC method. Consequently, the active *Sideritis* extracts, their components or their enriched fractions might be useful in rancidity retardation of edible oils. The observed higher activity of the apolar *t*-BuOMe extracts, compared with the other more polar extracts in the BCBT system, can be attributed to favourable interfacial phenomena in the emulsion medium and to the presence of some apolar phenolic compounds. The results from the BCBT assay suggest that some apolar *Sideritis* components or extracts might be inhibitors of lipid oxidation in emulsions. However, additional studies with isolated pure compounds in real oil-in-water emulsions are necessary to prove this (Koleva et al. 2002). The presence of pro-oxidative substances in some of the sub-extracts has been established. Hence, a preliminary fractionation and screening of the fractions obtained may be useful for the removal (partial or complete) of pro-oxidative components. The initial extraction solvent had little effect on the activity of the sub-extracts obtained in this study. Owing to the multicomponent composition of the extracts, further research will be carried out to isolate the individual active compounds, to evaluate their antioxidant properties and to study possible synergistic or inhibitory effects. The different species of *Sideritis* were collected over several years (1993 − 1997). In future studies, attention will be given to the influence of geographical origin, time of harvest, soil and climatological conditions. Such parameters could influence the presence and concentration of secondary metabolites, and thus the antioxidative activity.

3.5 References

Abdalla A.E., Roozen J.P. (1999). "Effect of plant extracts on the oxidative stability of sunflower oil and emulsion." Food Chem. **64:** 323-329.
Barlow S.M. (1990). "Toxicological aspects of antioxidants used as food additives." In Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Sciences, London, pp 253-307.
Bondet V., Brand-Williams W., Berset C. (1997). "Kinetics and mechanisms of antioxidant activity using the DPPH free radical method." Lebensm-Wiss. Technol. **30:** 609-615.
Brand-Williams W., Cuvelier M.E., Berset C. (1995). "Use of a free radical method to evaluate antioxidant activity." Lebensm-Wiss. Technol. **28:** 25-30.
Chevolleau S., Mallet J.F., Ucciani E., Gamisans J., Gruber M. (1992). "Antioxidant activity in leaves of some mediterranean plants." JAOCS **69:** 1269-1271.
Dapkevicius A., van Beek T.A., Linssen J.P.H., Venskutonis R. (1998). "Rapid spectroscopic screening for antioxidant activity in sage, thyme and oregano isolates with the β-carotene-linoleic acid model system." in Natural Product Analysis. (Eds. Schreier P., Herderich M., Humpf H.-U., Schwab W.), Friedr. Vieweg & Sohn, Braunschweig, pp 235-237.
Diaz R.M., Garcia-Granados A., Moreno E., Parra A., Quevedo- Sarmiento J., De Buruaga A.S. (1988). "Studies on the relationship of structure to antimicrobial properties of diterpenoid compounds from *Sideritis*." Planta Medica **54:** 301–304.
Ezer N., Sakar M.K., Rodriguez B., de la Torre M.C. (1992). "Flavonoid glycosides and a phenylpropanoid glycoside from *Sideritis perfoliata*." Int. J. Pharmacol. **30:** 61–65.
Fraga B.M., Hernandez M.G., Santana J.M.H., Terrero D., Galvan M.F. (1995). "A chemotaxonomical study of *Sideritis massoniana* taxa." Biochem. Syst. Ecol. **23:** 835–842.
Frankel E.N. (1991). "Recent advances in lipid oxidation." J. Sci. Food Agric. **54:** 495–511.
Frankel E.N., Huang S.-W., Aeschbach R., Prior E. (1996). "Antioxidant activity of a rosemary extract and its constituents carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion." J. Agric. Food Chem. **44:**131–135.
Frankel E.N., Huang S.-W., Kanner J., German J.B. (1994). "Interfacial phenomena in the evaluation of antioxidants: bulk oils *vs* emulsions." J. Agric. Food Chem. **42:** 1054–1059.
Frankel E.N., Meyer A.S. (2000). "The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants." J. Sci. Food Agric. **80:** 1925-1941.
Gadow A. von, Joubert E., 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.
Gao J.-J., Igalashi K., Nukina M. (1999). "Radical scavenging activity of phenylpropanoid glycosides in *Caryopteris incana*." Biosci. Biotechnol. Biochem. **63:** 983–988.
Gil M.I., Ferreres F., Marrero A., Tomas-Lorente F., Tomas- Barberan F.A. (1993). "Distribution of flavonoid aglycones and glycosides in *Sideritis* species from the Canary islands and Madeira." Phytochemistry **34:** 227–232.
Gonzalez A.G., Fraga B.M., Hernandez M., Gand Luis J.G. (1972). "Siderin, a new coumarin from *Sideritis canariensis*." Phytochemistry **11:** 2115–2116
Greyt de W.F., Petrauskaite V., Kellens M.J., Huyghebaert A.D. (1998). "Analysis of tocopherols by GC and HPLC: a comparative study." Fett/Lipid **100:** 503-507.
Kim S.Y., Kim S.K., Oh M.J., Jung M.Y. (1994). "Antioxidant activity of selected oriental herb extracts." JAOCS **71:** 633-640.
Kokkalou E., Gabrieli C. (1997). "A new phenylpropanoid glycoside and two flavones from *Sideritis raeseri* Bois et Heldr." Pharmazie **52:** 322–324.
Koleva I.I., Handjieva N.V. (1997)."A study on the iridoid composition of *Sideritis*." Sci. Works HIFFI Plovdiv **42:** 75–79 (in Bulg.).
Koleva I.I., van Beek T.A., Linssen J.P.H., de Groot Ae., Evstatieva .N. (2002). "Screening of plant extracts for antioxidant activity — a comparative study on three testing methods." Phytochem. Anal. **13:** 8–17.

Larson R.A. (1997). Naturally Occurring Antioxidants., Lewis Publishers, New York.
Lőliger J. (1991). "Natural antioxidants." Lipid Technol. **3:** 58–61.
Madsen H.L., Bertelsen G. (1995). "Spices as antioxidants." Trends Food Sci. Technol. **6:** 271-277.
Marinova E., Yanishlieva N., Ganeva I. (1991). "Antioxidative effect of Bulgarian rosemary and inhibiting activity of its carnosol." Oxidation Commun. **14:** 125-131.
Mateo C., Calderon J., Sanz J. (1988). "Essential oil of some *Sideritis* species from central and southern Spain." Phytochemistry **27:** 151–153.
Meyer A.S., Yi O.-S., Pearson D.A., Waterhouse A.L., Frankel E.N. (1997). "Reduction of human low density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (*Vitis vinifera*). J. Agric. Food Chem. **45:** 1638–1643.
Mora A., Paya M., Rios J.L., Alcaraz M.J. (1990). "Structure–activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation." Biochem. Pharmacol. **40:** 793–797.
Papas A.M. (1999). Antioxidant Status, Diet, Nutrition, and Health. CRC Press, Boca Raton.
Porter W.L. (1980). "Recent trends in food applications of antioxidants." in Autooxidation in Food and Biological Systems. (Eds. Simic M.G., Karel M.), Plenum Press, New York, pp 295–365.
Pratt D.E., Birac P.M. (1979). "Source of antioxidant activity of soybeans and soy products." J. Food Sci. **44:** 1720-1722.
Rice-Evans C.A., Miller N.J., Paganga G. (1996). "Structure–antioxidant activity relationships of flavonoids and phenolic acids." Free Rad. Biol. Med. **20:** 933–956.
Rios J.L., Manez S., Paya M., Alcaraz M.J. (1992). "Antioxidant activity of flavonoids from *Sideritis javalambrensis*." Phytochemistry **31:** 1947–1959.
Rodriguez B., Valverde S. (1975). "Diterpenes from three *Sideritis* species." Phytochemistry **14:** 1670 1671.
Roedig-Penman A., Gordon M.H. (1998). "Antioxidant properties of myricetin and quercetin in oil and emulsions." JAOCS **75:** 169-180.
Sattar A.A., Bankova V., Kujumgiev A., Galabov A., Ignatova A., Todorova C., Popov S. (1995a). "Chemical composition and biological activity of leaf exudates from some Lamiaceae plants." Pharmazie **50:** 62–65.
Sattar A.A., Bankova V., Popov S. (1995b). "Acylated flavonoid glycosides from *Sideritis scardica*." Fitoterapia **66:** 190.
Sattar A.A., Bankova V., Spassov S., Duddeck H. (1993). "Flavonoid glycosides from *Sideritis* species." Fitoterapia **64:** 278–279.
Satue M.T., Huang S.-W., Frankel E.N. (1995). "Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached, and decolorized olive oil." JAOCS **72:** 1131–1137.
Tomas-Barberan F.A., Manez S., Villar A. (1987). "Identification of antiinflammatory agents from *Sideritis* species growing in Spain." J. Nat. Prod. **50:** 313–314.
Villar A., Recio M.C., Rios J.L., Zafra-Polo M.C. (1986). "Antimicrobial activity of essential oils from *Sideritis* species." Pharmazie **41:** 298–299.
Williams G. (1993). "Antioxidants. Chemical, Physiological, Nutritional and Toxicological Aspects.", Princeton Scientific Publishing Co. Inc., Princeton.
Xiong Q., Kadota Sh., Tani T., Namba Ts. (1996). "Antioxidative effects of phenylethanoids from *Cistanche deserticola*." Biol. Pharm. Bull. **19:** 1580–1585.
Yamaguchi T., Takamura H., Matoba T., Terao J. (1998). "HPLC method for evaluation of the free radical-scavenging activity of foods by using DPPH." Biosci. Biotechnol. Biochem. **62:** 1201-1204.
Yen G.-C., Chen H.-Y. (1995). "Antioxidant activity of various tea extracts in relation to their antimutagenicity." J. Agric. Food Chem. **43:** 27–32.
Yen G.-C., Duh P.-D. (1994). "Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species." J. Agric. Food Chem. **42:** 629-632.

A Rapid On-Line HPLC Method for Detection of Radical Scavenging Compounds in Complex Mixtures*

4.1 Introduction

Free radicals formed during oxidation processes occurring in various products and living organisms are known to be responsible for oxidative product deterioration, health damage, and accelerated aging (Finley & Otterburn 1993; Aruoma 1998). In recent years an increasing interest in naturally occurring antioxidants can be noticed (Evans & Reynhout 1992; Nieto et al. 1993; Madsen & Bertelsen 1995).

In the search for new natural antioxidants, complex mixtures are frequently encountered. Bioassay-guided fractionation of plant samples is time-consuming and labour-intensive. For antioxidants, there is often a loss in activity during the isolation and purification procedures due to decomposition. For this reason, availability of a rapid method for screening and activity evaluation of the samples is essential in order to avoid many of these problems. A method combining separation and activity evaluation would present a major advantage for such investigations. However, reports concerning on-line separation and antioxidant activity assessment are scarce. In 1999 as far to my knowledge, only two reports had been published. Both concerned on-line detection of HPLC antioxidative eluates by means of chemiluminescence (Dapkevicius et al. 1999; Ogawa et al. 1999). Furthermore, a flow system without separation has been described (Niederländer et al. 1994). Although very sensitive, these methods require a special instrumental set-up and expensive and unstable reagents. There are also limitations regarding the HPLC mobile-phase composition.

This chapter presents a simple and rapid method for HPLC-DPPH on-line detection of antioxidative compounds in complex mixtures such as plant extracts/fractions, foods, and drugs without the use of chemiluminescence.

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4.2 Experimental

4.2.1 Instrumental set-up

On-line system. A scheme of the instrumental set-up is given in Figure 4.1. The HPLC system used consistsed of the following: HPLC pump delivery system (Waters 600E System Controller, Milford, MA); a programmable photodiode array detector (Waters 994) connected to a Waters 5200 printer plotter (data were processed using Waters 991 PDA software); a variable- wavelength Gilson 115 UV detector (Gilson Medical Electronics, Inc., Middleton, WI) connected to a recorder (Kipp & Zonen BD40, Delft BV, The Netherlands); a syringe pump for delivery of DPPH$^{\bullet}$ solution (laboratory-made, Free University, Amsterdam, The Netherlands). The separations were carried out on an Alltima C18 HPLC column (5 μm, 250 x 4.6 mm i.d., Alltech Associates, Inc., Deerfield, IL). Samples were injected with a Gilson 231 sample injector equipped with a 20-μL loop. The reaction coil was made of PEEK tubing 15 m x 0.25 mm i.d. (tubing of a larger inner diameter is unsuitable due to undesirable peak broadening). Detection of DPPH$^{\bullet}$ reduction was carried out at 517 nm. The UV detection wavelengths for the tested compounds were chosen according to their characteristic absorption maxima (range 0.01 AU, rise time 0.5 s).

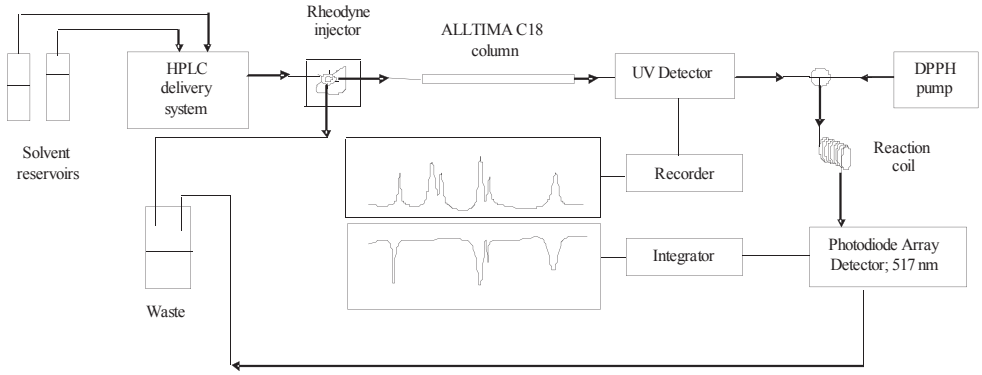


Figure 4.1 Instrumental set-up for the HPLC-DPPH on-line detection of radical scavenging compounds.

Off-line system. The off-line measurements were carried out on a Lambda 18 UV/Visible spectrometer (Perkin-Elmer, Corp., Norwalk, CT) at 517 nm in disposable polystyrene cuvettes (1-cm path length, Greiner Labortechnik, Alphen a/d Rijn, The Netherlands) against methanol as a reference solution. The reagents were mixed at concentrations and in

ratios corresponding to the optimized on-line experiments. To minimize air bubble formation, solvent mixtures were degassed by sonication before use. Measurements of pH were carried out with a Hana Instruments HI 9025 (Portugal) pH meter.

4.2.2 Reagents and tested antioxidants

All solvents used were of HPLC grade (Lab-Scan Analytical Sciences Ltd., Dublin, Ireland). Ultrapure water (0.05 μS/cm) was obtained from a combined Seradest LFM 20 and Serapur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). Before use in the HPLC system, all samples solutions and solvents were membrane filtered (0.45 μm, Type RC 55, Schleicher & Schuell, Dassel, Germany). During the HPLC runs, solvents were continuously degassed by sparging with helium.

The following reagents and compounds were used: 2,2-diphenyl-1-picrylhydrazyl, **34** (DPPH$^{\bullet}$ 95%), eugenol, **35** (99%, GC), isoeugenol, **36** (98%), kaempferol, **37** (90%), quercetin dihydrate, **14** (98%), α-tocopherol, **5** (95%), and Trolox, **38** (97%) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); glacial acetic acid (100%) and Na_2HPO_4 dihydrate (p.a.) from Merck (Darmstadt, Germany); citric acid monohydrate (p.a.) and sodium acetate (anhydrous, p.a.) from Acros Organics (Geel, Belgium); rutin trihydrate, **15** (90%) from Fluka (Buchs, Switzerland); and rosmarinic acid, **11** from Extrasynthese (Genay, France). Carnosic acid, **13** was isolated from dried rosemary leaves in our laboratory (for compounds numbering see Chs. 1 and 3).

DPPH$^{\bullet}$ solutions were freshly prepared in methanol every day and kept at 4 °C, in a volumetric flask protected from light. Eight to ten different dilutions of each antioxidant in methanol were prepared and tested. Plant extracts were prepared by extraction of air-dried, ground plant material from *Sideritis* species (Labiatae) with methanol, evaporation of the solvent to dryness, and successive partitioning between water and *tert*-butyl methyl ether, ethyl acetate, and 1-butanol. The obtained sub-extracts were evaporated to dryness under vacuum at temperatures not higher than 50 °C. The remaining aqueous layer was freeze-dried. Extract solutions were prepared in methanol or water (1 mg/mL).

4.3 Results and discussion

4.3.1 Physicochemical factors influencing the antioxidant-DPPH$^{\bullet}$ reaction rate

The absorbance at 517 nm, corresponding to the "remaining" DPPH$^{\bullet}$ concentration, is influenced by several parameters (apart from the chemical nature and concentration of the antioxidants as will be discussed later): *(1)* concentration of the DPPH$^{\bullet}$ stock solution; *(2)* reaction time between the antioxidant and DPPH$^{\bullet}$; *(3)* composition and pH of the HPLC

mobile phase. The influence of these factors was determined under off- and on-line conditions.

The primary aim was to detect antioxidative components at low concentrations. The system was therefore optimized with respect to the limits of detection. The limit of detection (LOD) was calculated according to

$$LOD = -2t\sigma_{blank}$$

where σ_{blank} is the standard deviation of the blank signal and t is the Student's t statistic.

For method optimization under the on-line conditions, LOD was evaluated in terms of a field blank response (LOD_{noise}, AU) with the coefficient $t = 2.5$ for $n = 24$ measurements of the blank signal with a confidence interval of 99%. For determination of the limits of detection of the tested compounds ($LOD_{compound}$, μg/mL), the coefficient $t = 1.714$ for $n = 24$ measurements of the blank signal with a confidence interval of 90% was used. The negative peak resulting from the reduction of DPPH$^{\bullet}$ by a radical scavenging compound was considered detectable if its height (depth) exceeded the calculated $LOD_{compound}$.

4.3.2 Influence of DPPH$^{\bullet}$ stock solution concentration

Five different concentrations of DPPH$^{\bullet}$ stock solution in methanol were prepared: 10^{-3}, 10^{-4}, 5 x 10^{-5}, 10^{-5}, and 5 x 10^{-6} M. Tests were carried out at a flow rate of 0.7 mL/min for both the DPPH$^{\bullet}$ solution and the HPLC mobile phase (methanol), which proved to be the most stable flow rate for the DPPH delivery pump. In Figure 4.2(a) it is shown that the noise level increased rapidly with increasing DPPH$^{\bullet}$ concentration, at concentrations higher than 10^{-5} M. However, a decrease in DPPH$^{\bullet}$ concentration below 10^{-5} M did not contribute to a further decrease in noise. The influence of DPPH$^{\bullet}$ stock solution concentration on the $LOD_{compound}$ values for several of the tested antioxidants, isoeugenol, α-tocopherol, and eugenol, was checked with the two DPPH$^{\bullet}$ concentrations showing the lowest noise levels − 10^{-5} and 5 x 10^{-6} M. The three compounds chosen cover the three main types of kinetic behaviour with respect to their reaction with DPPH$^{\bullet}$ − rapid, intermediate, and slow, respectively (Brand-Williams et al. 1995; Sanchez-Moreno et al. 1998). In all cases, the $LOD_{compound}$ values shifted to higher antioxidant concentrations when 5 x 10^{-6} M DPPH$^{\bullet}$ was used. On the basis of these results, 10^{-5}M DPPH$^{\bullet}$ was chosen as the optimum DPPH$^{\bullet}$ stock solution concentration.

4.3.3 Influence of reaction time

Some additional experiments with two of the antioxidants tested, eugenol and isoeugenol, were carried out to establish that the variables (concentration of DPPH$^{\bullet}$ solution and reaction time) were largely independent. Signal-to-noise (S/N) ratios were determined at two different DPPH$^{\bullet}$ concentrations (10^{-5} and 5 x 10^{-6} M) and reaction times (9 and 30 s, corresponding to reaction coils of 4.4 m and 15 m x 0.25 mm i.d.). The results for isoeugenol are given in Table 4.1 (similar results were also obtained for eugenol). The highest S/N ratio was determined at 10^{-5} M DPPH$^{\bullet}$ solution and 30-s reaction time. On the basis of these data, the reaction coil of 15 m x 0.25 mm i.d. (30-s reaction time) was chosen for further experiments.

Table 4.1 S/N ratios determined for isoeugenol (5.10^{-5} mol/L) at two reaction times and two DPPH$^{\bullet}$ stock solution concentrations.

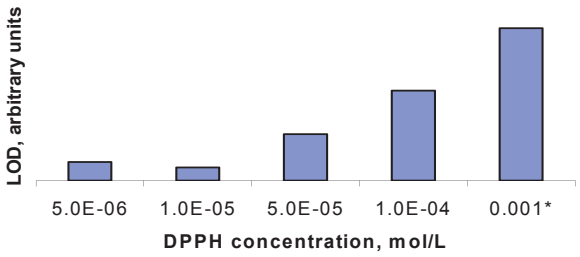
Reaction time, s	**DPPH$^{\bullet}$ stock solution concentrations, mol/L**	
	5.10^{-6}	***10^{-5}***
9	4.5	10.9
30	10	21.8

4.3.4 Influence of the mobile-phase composition and pH

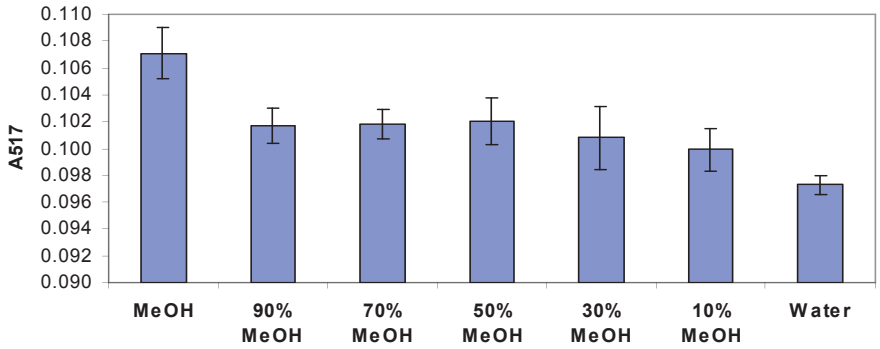
The suitability of the method for analyses involving acidic mobile phases was checked by using buffers of different pH and composition. According to Blois (1958), DPPH$^{\bullet}$ is moderately stable with respect to changes in pH values and its absorbance at 517 nm can be considered as independent of pH from 5.0 to 6.5. Methanol, acetonitrile, and mixtures of these with water and various buffers (pH 2.2-6.6) were studied under off- and on-line conditions.

In the off-line experiments solvent mixtures of 90, 70, 50, 30, and 10% methanol and acetonitrile in water and acetate buffer as well as pure methanol, acetonitrile, water, and acetate buffer were mixed with 10^{-5} M DPPH$^{\bullet}$ and the absorbance at 517 nm (A^{517}) was recorded after 30 s. The changes in A^{517} with changes in the solvent mixture composition are presented in Figure 4.2(b) (for methanol-water mixtures). Replacing methanol by acetonitrile gave similar changes in A^{517}. Addition of either water or buffer to the organic solvent decreased A^{517} values.

(a)



(b)



(c)

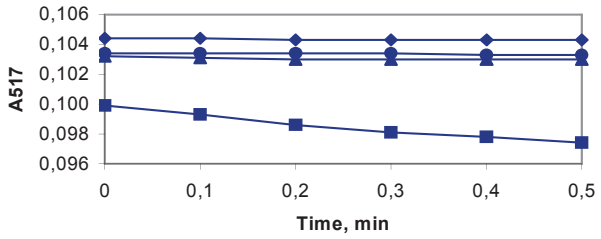


Figure 4.2 (a) Changes in LOD in terms of blank response signal with DPPH$^{\bullet}$ stock solution concentration, mol/L (* means: LOD/10 is plotted). **(b)** Changes in A^{517} with changes in the solvent mixture composition for methanol-water mixtures (mean values for four replicates ± SD). **(c)** Time course of the absorbance at 517 nm (A^{517}) for several mobile phase mixtures of different pH (♦methanol, • citric acid-phosphate buffer (CPB) pH 5.6, ▲ CPB pH 3, ■ CPB pH 2.2).

This effect was more distinct when changing from 100% organic solvent to organic solvent-water or buffer solutions and from 10% organic solvent to pure water or buffer. Thus, HPLC gradients with mobile-phase composition from 10% to 90% organic solvent can be applied with this method without significant changes in DPPH$^{\bullet}$ absorbance. Pure organic solvents can be used in isocratic runs. The use of pure water caused the strongest decrease in the DPPH$^{\bullet}$ absorbance. Water has been reported to promote radical recombination (Karel 1980). Although the systems described by Karel (1980) are different from the DPPH on-line set-up, the results obtained here are in general accordance with these earlier observations.

In the on-line experiments, two solvent mixtures, 100% organic solvent (methanol or acetonitrile) and their 10% solutions in both water and acetate buffer, were used. No significant changes in LOD_{noise} were observed for any of the mobile phase combinations tested.

To establish the influence of mobile-phase pH, citric acid-Na_2HPO_4 buffers (CPB) of seven different pH values (from 2.2 to 6.6) were tested under off-line conditions. Ultrapure water, 0.1 M citric acid (pH 2.05), and acetate buffer (pH 5.6) were also included in this experiment.

The absorbance at 517 nm was monitored during 30 s. Results are presented in Figure 4.2(c). "Highly acidic" systems (pH 2.2) caused a drastic decrease in DPPH$^{\bullet}$ absorbance over 30 s. Consequently, low-pH buffers should not be used as mobile phases in this method as they contribute to lower S/N ratios under on-line conditions. Although DPPH$^{\bullet}$ solutions have been reported to be stable only in the range of pH 5-6.5, the results obtained show that buffers with pH down to 3 can still be used with this method. Most probably, the reason for the "broadening" of the suitable pH range is the short reaction time (30 s) during which there is no significant decrease in absorbance. The use of buffer at pH 6.6 resulted in formation of a cloudy suspension when mixed with DPPH$^{\bullet}$ solution. Thus, this buffer is not suitable for on-line application as it would cause a high noise level. Using buffers with the same pH but of different composition (citric acid-phosphate buffer and acetate buffer) did not cause changes in the absorbance values. On the basis of both off- and on-line experiments, it can be concluded that mobile phases containing down to 10% methanol or acetonitrile can be used in the proposed method. Buffers with pH 3-6 can also be used when necessary for improvement of the HPLC separation.

4.3.5 Influence of the chemical nature of the antioxidants

The method was applied to compounds of different chemical nature, belonging to the three main types of kinetic behaviour toward DPPH$^{\bullet}$ (slow, intermediate and rapid). Their limits

of detection, $LOD_{compound}$ (μg/mL), and the minimum detectable amounts (ng) were determined. The data obtained are presented in Table 4.2. The structures of the compounds studied are presented in Figure 4.3. A combined plot of the UV and $DPPH^{\bullet}$ quenching profiles of nine dilutions of Trolox (**38**) is presented in Figure 4.4.

Table 4.2 Limits of Detection and Minimum amounts detectable of some antioxidants in the HPLC-DPPH on-line system under isocratic conditions.

Tested compound	Kinetic behaviour	Limit of Detection* LOD (μg/mL)	Minimum amount detectable (ng)
Quercetin, **14**	slow	0.33	6.6
Carnosic acid, **13**	no data	0.41	8.2
Rosmarinic acid, **11**	intermediate	0.83	17
Trolox, **38**	no data	1.5	30
Isoeugenol, **36**	rapid	1.8	36
Rutin, **15**	slow	2.2	44
Kaempferol, **37**	no data	2.3	46
α-Tocopherol, **5**	intermediate	17	340
Eugenol, **35**	slow	94	1900

* - determined with 20-μL loop

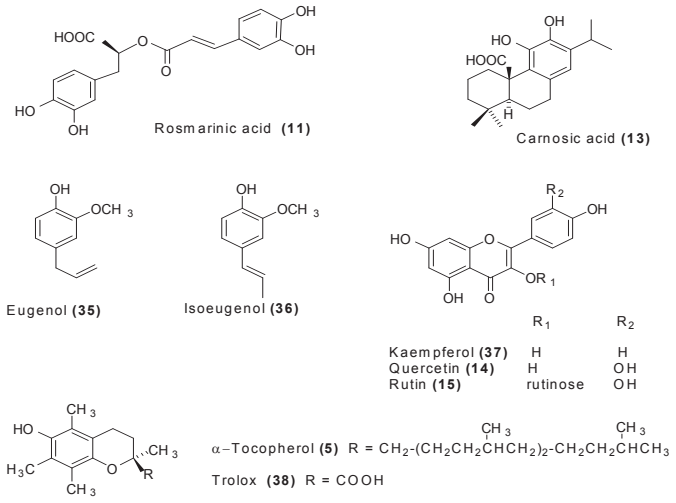


Figure 4.3 Structures of the tested antioxidants
(see Ch. 1 for the numbering of the compounds).

Carnosic acid (**13**) and rosmarinic acid (**11**) were among the most potent radical scavengers of DPPH$^{\bullet}$, the former being more active. Carnosic acid was also found to be a stronger antioxidant than rosmarinic acid by other testing methods (Frankel et al. 1996). Comparing the three flavonoids tested (kaempferol (**37**), quercetin (**14**), rutin (**15**)), quercetin was more active than both its glycoside rutin and kaempferol. Other authors (van Gadow et al. 1997; Sanchez-Moreno et al. 1998) have reported similar results for the pair rutin-quercetin. Glycosylation has been found to diminish the radical scavenging abilities of flavonoids (Pratt 1980). The weaker radical scavenging activity of kaempferol could be explained by the fact that it possesses one phenolic OH group less than quercetin and rutin.

Trolox (**38**) has been found to be a potent antioxidant by many different methods (Miller 1993; Hopia 1996). In this method, it showed stronger antioxidant activity than its fat-soluble form α-tocopherol (**5**). Similar results have been reported by Yamaguchi et al. (1998) also using DPPH$^{\bullet}$. Possibly, steric factors strongly influence the reaction of these compounds with DPPH$^{\bullet}$ since their lipo-/hydrophilicity is not likely to have any effect in the presented method.

Eugenol (**35**) and its isomer isoeugenol (**36**) greatly differed in their scavenging activity toward DPPH$^{\bullet}$. These results are in accordance with those obtained by the group of Berset (Brand-Williams et al. 1995; Bondet et al. 1997), who have proposed reaction mechanisms for these compounds with DPPH$^{\bullet}$.

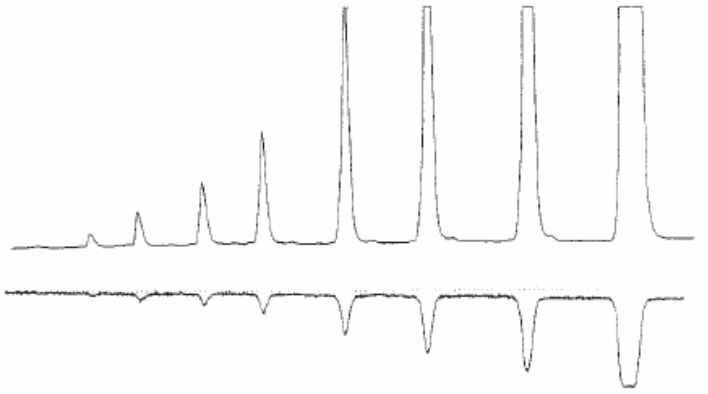


Figure 4.4 Combined plot of the UV and DPPH$^{\bullet}$ reduction profiles of different concentrations of Trolox (**38**) sequentially injected (left to right: 0.25, 2.5, 6.3, 12.5, 25, 63, 125, 250, 1250 μg/mL) under isocratic conditions.

Comparison of the $LOD_{compound}$ and minimum detectable amounts for some of the studied pure compounds as determined by this method and the on-line HPLC-chemiluminescence method proposed by Dapkevicius et al. (1999) shows that the latter is more sensitive. This may be due to the inherently higher sensitivity of chemiluminescence compared to UV/Visible spectroscopy (Poole & Poole 1991) and/or to the difference in the stability between $DPPH^{\bullet}$ and the luminol radical. Another reason for the lower sensitivity observed might be due to the electronic noise at 517 nm of the diode array detector used. A dedicated visible wavelength detector with a tungsten lamp, which was not available to us, is likely to lead to lower limits of detection.

To elucidate antioxidant kinetic behaviour with this method, additional studies are necessary. Some preliminary experiments showing the suitability of my system to perform such studies have been carried out. Several concentrations of eugenol and isoeugenol (compounds having slow and rapid kinetics, respectively) were tested at two different concentrations of $DPPH^{\bullet}$ stock solutions and two reaction times. A good fit of the experimental data with the kinetic models developed was obtained.

4.3.6 Application of the method to complex mixtures (plant extracts/fractions)

The method described can be used for a rapid detection of antioxidative (radical scavenging) components in complex mixtures such as plant extracts/fractions, foods, etc. It was applied to ethyl acetate, 1-butanol, and aqueous extracts from *Sideritis* plants. Preliminary studies (unpublished) have shown that these extracts possess components with radical scavenging activity. Combined UV and $DPPH^{\bullet}$ quenching chromatograms under gradient conditions of a crude aqueous extract from *Sideritis scardica* are presented in Figure 4.5. No preliminary sample preparation was necessary, thus confirming that the method can save time, labour input, and chemicals. Injecting three different concentrations of the same extract resulted in a proportional change in the negative peak height/area of the reduced $DPPH^{\bullet}$. These results suggest the method can be applied to quantitative determinations of radical scavenging components in complex matrixes after calibration for the compounds to be quantified. In the chromatogram presented, only the component marked with an * does not possess any radical scavenging ability. Although it is one of the major extract constituents, no corresponding negative peak of reduced $DPPH^{\bullet}$ is observed. The application of the method to the other extracts mentioned in the Experimental Section also showed the presence of radical scavenging components. Those extract components that cause strong $DPPH^{\bullet}$ scavenging are of interest for further investigations.

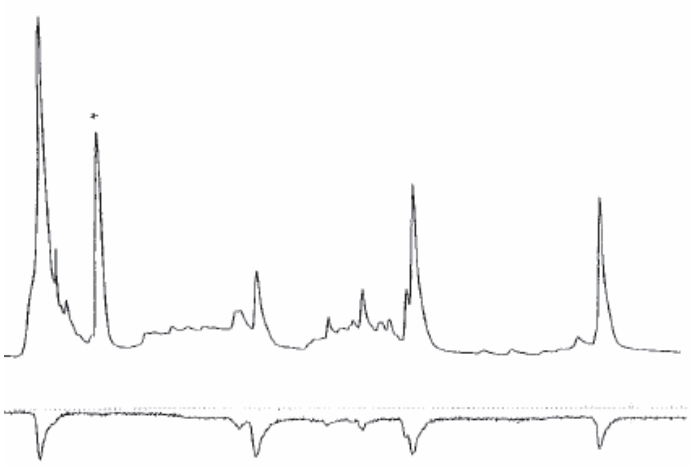


Figure 4.5 Combined UV and DPPH$^{\bullet}$ reduction chromatograms of *S. scardica* crude aqueous extract (1 mg/mL in water). Chromatographic conditions: RP-18 column (see Experimental Section), gradient elution at 0.7 mL/min (methanol-water (44:56, v/v) to methanol (100%) over 50 min), UV detection at 254 nm.

4.4 Conclusion

A rapid HPLC method for on-line detection of antioxidative components in complex mixtures was developed. It is characterized by the following: **(1)** a broad range of applicability. *(a)* compounds of different kinetic behaviour toward DPPH$^{\bullet}$ can be determined; *(b)* isocratic and gradient runs with mobile phases of different composition and pH can be carried out whereas other methods published (Dapkevicius 1999) have limitations with respect to some mobile phase modifiers; *(c)* compounds over a broad range of polarity, and p*K*a can be evaluated since HPLC mobile phases, ranging from 10 to 90% organic solvent in water or buffers (pH 3-6), can be used; *(d)* use of a readily available and non-expensive instrumental set-up; **(2)** use of cheap, stable and commonly available chemicals; **(3)** the method is easy to perform. The method can be applied for a quick screening of antioxidants or more precisely radical scavenging activity of complex samples such as plant extracts, foods, and drugs as well as for quantitative analysis. The greatest benefit of the method is that it is immediately clear which constituent possesses radical scavenging activity. Thus, it is no longer necessary to purify every single constituent for off-line assays, leading to very significant reductions of costs and faster results.

4.5 References

Aruoma O.I. (1998). "Free radicals, oxidative stress, and antioxidants in human health and disease." JAOCS **75:** 199-212.
Blois M.S. (1958). "Antioxidant determination by the use of a stable free radical." Nature **181:** 1199-1200.
Bondet V., Brand-Williams W., Berset C. (1997). "Kinetics and mechanisms of antioxidant activity using the DPPH free radical method." Lebensm-Wiss.-Technol. **30:** 609-615.
Brand-Williams W., Cuvelier M.E., Berset C. (1995). "Use of a free radical method to evaluate antioxidant activity." Lebensm-Wiss.-Technol. **28:** 25-30.
Dapkevicius A., van Beek T.A., Niederländer H.A.G., de Groot A. (1999). "On-line detection of antioxidative activity in HPLC eluates by chemiluminescence." Anal. Chem. **71:** 736-740.
Evans R.J., Reynhout G.S. (1992). "Alternates to synthetic antioxidants." in Food science and human nutrition. (Ed. Charalambous G.), Elsevier, Amsterdam, pp 27-42.
Finley J.W., Otterburn M.S. (1993). "The consequence of free radicals in foods." in Antioxidants. Chemical, physiological, nutritional and toxicological aspects. (Ed. Williams G.M.), Princeton Scientific Publishing Co., Inc., Princeton, NJ, pp 77-91.
Frankel E.N., Huang S.-W., Aeschbach R., Prior E. (1996). "Antioxidant activity of a rosemary extract and its constituents carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion." J. Agric. Food Chem. **44:**131–135.
Gadow A. von, Joubert E., 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.
Hopia A., Huang S.-W., Frankel E.N. (1996). "Effect of alpha-tocopherol and trolox on the decomposition of methyl linoleate hydroperoxides." Lipids **31:** 357-365.
Karel M. (1980). "Lipid oxidation, secondary reactions, and water activity of foods." in Autooxidation in Food and Biological Systems. (Eds. Simic M.G., Karel M.), Plenum Press, New York, pp 191-206.
Madsen H.L., Bertelsen G. (1995). "Spices as antioxidants." Trends Food Sci. Technol. **6:** 271-277.
Miller N.J., Rice-Evans C., Davies M.J., Gopinathan V., Milner A. (1993). "A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates." Clin. Sci. **84:** 407-412.
Niederländer H.A.G., de Jong M.M., Gooijer C., Velthorst N.H. (1994). "Chemiluminescence detection in liquid chromatography based on photochemical on-line production of singlet oxygen." Anal. Chim. Acta **290:** 201-214.
Nieto S., Garrido A., Sanhueza J., Loyola L.A., Morales G., Leighton F., Valenzuela A. (1993). **"**Flavonoids as stabilizers of fish oil: an alternative to synthetic antioxidants." JAOCS **70:** 773-778.
Ogawa A., Arai H., Tanizawa H., Miyahara T., Toyo'oka T. (1999). "On-line screening method for antioxidants by LC with chemiluminescence detection." Anal. Chim. Acta **383:** 221-230.
Poole C.F., Poole S.K. (1991). "Chromatography today.", Elsevier, Amsterdam, pp 567-586.
Pratt D.E. (1980). "Natural antioxidants of soybean and other oil seeds." in Autoxidation in Food and Bilogical Systems. (Eds. Simic M.G., Karel M.), Plenum Press, New York, pp 283-293.
Sanchez-Moreno C., Larrauri J.A., Saura-Calixto F.A. (1998). "Procedure to measure the antiradical efficiency of polyphenols." J. Agric. Food Chem. **76:** 270-276.
Yamaguchi T., Takamura H., Matoba T., Terao J. (1998). "HPLC method for evaluation of the free radical-scavenging activity of foods by using DPPH." Biosci. Biotechnol. Biochem. **62:** 1201-1204.

Application of ABTS Radical Cation for Selective On-Line Detection of Radical Scavengers in HPLC Eluates*

5.1. Introduction

A great deal of knowledge has accumulated over recent years regarding the role of free radicals in oxidative stress in living organisms (Aruoma 1998) and about their propagative role in lipid oxidation and the subsequent quality deterioration of foods (Finley & Otterburn 1993), cosmetics (Tyrrel 1995), etc. Because of their high reactivity, most free radicals react rapidly with oxidizable substrates. This necessitates discriminative assays for the evaluation of radical-trapping properties. Such methods often utilize stable model free radicals as indicators for radical-scavenging abilities, among which the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) (Brand-Williams et al. 1995) and the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), by some authors also referred to as $ABTS^{\bullet-}$ (Scott et al. 1993; Campos & Lissi 1996; van der Berg et al. 1999; Huang et al. 2005) have gained the highest popularity. These radicals have been useful in the antioxidant activity evaluation of various samples, because the assay protocols are simple and require only a spectrophotometer. However, it has been recognized that the exhibited radical scavenging abilities (in terms of both stoichiometry and reaction rates) depend on the assay method used (Mantle et al. 1998). For example, antioxidants can differ significantly in their reactivity toward $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ (Lissi et al. 1999). In 1993, Miller et al. (1993) proposed a common parameter for antioxidant activity evaluation, called the Trolox equivalent antioxidant capacity value (TEAC), based on the use of the relatively long-lived $ABTS^{\bullet+}$. The TEAC value represents the antioxidant reactivity relative to a standard of 1.0 mmol/L Trolox, a water-soluble synthetic derivative of vitamin E.

The $ABTS^{\bullet+}$ has been used in the evaluation of the antioxidant activity of single compounds (Rice-Evans et al. 1995; Miller et al. 1996) and mixtures of various nature (e.g.

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body fluids (Rice-Evans & Miller 1994), foods and beverages (Miller et al. 1995; Amao et al. 1996) and plant extracts (Mantle et al. 1998; Pietta et al. 1998)).In the search for novel antioxidants, the current focus is towards those of plant origin. This application requires screening of many plant extracts for antioxidant activity. Consequently, rapid methods allowing a high-throughput screening are needed. Whenever mixtures are concerned, the antioxidant capacity has usually been expressed in terms of total antioxidant activity (TAA) (Arnao et al. 1996; Mantle et al. 1998; Cano et al. 1998). In complex mixtures, additive, synergistic, or inhibitory effects among the components have been observed (Benavente-Garcia et al. 2000). To evaluate the contribution of each single sample component, knowledge of its molecular structure is required. In the search of novel active compounds in complex matrixes, this is complicated and difficult.

Recently developed on-line methods combine the advantages of rapid and sensitive activity assay protocols with an HPLC separation for a rapid detection of radical scavenging components in complex mixtures. Two of the reported methods are based on the determination of luminol-chemiluminescence inhibition as an indicator of radical-trapping activity (Dapkevicius et al. 1999; Ogawa et al. 1999). Another one uses the $DPPH^{\bullet}$ stable radical in a simple HPLC on-line system (Koleva et al. 2000), however, this method showed limited sensitivity for compounds having slow reaction kinetics towards $DPPH^{\bullet}$ (e.g. BHT).

This chapter describes the detection of radical-scavenging components in complex matrixes by an on-line HPLC method using $ABTS^{\bullet+}$. The method is a useful and complementary extension of the previously reported HPLC-DPPH on-line method (Koleva et al. 2000) for the screening of complex mixtures for radical scavengers.

5.2 Experimental

5.2.1 Instrumental set-up

The block scheme of the instrumental set-up is presented in Figure 5.1. The HPLC system that was used consisted of the following: an HPLC eluent pump (Waters 600E System Controller; Milford, MA); a programmable photodiode array detector (Waters 994) connected to a Waters 5200 printer plotter (data were processed using Waters 991 PDA software) [For the analyses of the extracts, the photodiode array detector was replaced by a model 757 UV-Vis absorbance detector (Applied Biosystems Inc.; Foster City, CA)]; a UV-Vis absorbance detector (model 759A) equipped with a tungsten lamp (Applied Biosystems Inc.; Foster City, CA) and connected to a recorder (Kipp & Zonen BD40; Delft, The Netherlands); and a syringe pump (maximum volume content, 45 mL) for delivery of

$ABTS^{\bullet+}$ solution (laboratory-made; Free University, Amsterdam, The Netherlands). Separations were carried out on an Alltima C18 HPLC column (5 μm, 250 x 4.6 mm i.d., Alltech Associates Inc.; Deerfield, IL). An additional pulse dampener (SSI, Inc.; State College, PA) was mounted upstream from the injector to further reduce pressure pulsations. Samples were injected using a Gilson 231 sample injector equipped with a 10-μL loop. The reaction coils used were made of PEEK tubing of the following sizes: 4.4 m x 0.25 mm i.d., 13.7 m x 0.25 mm i.d., and 6.5 m x 0.50 mm i.d. Detection of $ABTS^{\bullet+}$ quenching was carried out at 734 nm (range, 0.02 AUFS; rise time, 5 s). At this wavelength, the extinction coefficient of $ABTS^{\bullet+}$ (ε^{734}) is 1.5 x 10^4 $L.mol^{-1}.cm^{-1}$ (Scott et al. 1993). This absorbance maximum was preferred over that at 414 nm (ε^{414} = 3.6 x 10^4 $L.mol^{-1}.cm^{-1}$) because it reduces the risk of interfering absorbance from sample components. The UV detection wavelengths for the test compounds were chosen according to their characteristic absorbance maxima.

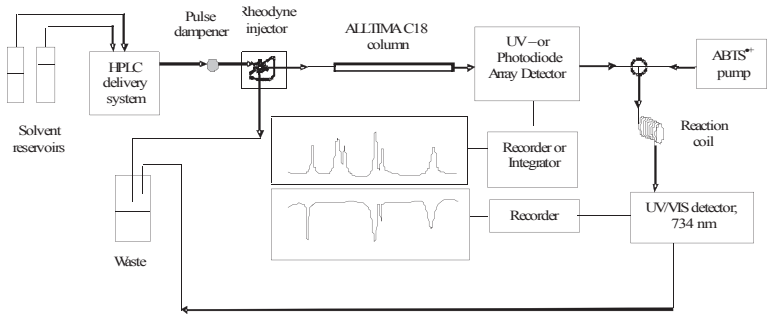


Figure 5.1 Instrumental set-up for the HPLC-$ABTS^{\bullet+}$ on-line detection of radical scavenging compounds.

The off-line UV-Vis measurements were carried out on a Lambda 18 UV-Vis spectrometer (Perkin-Elmer Corp.; Norwalk, CT) at 734 nm in disposable polystyrene cuvettes (1-cm path length; Greiner Labortechnik; Alphen a/d Rijn, The Netherlands). All spectrometric measurements were carried out at least in triplicate. Measurements of pH were carried out using a Hana Instruments (Portugal) HI 9025 pH meter.

5.2.2 Reagents and tested antioxidants

All solvents used were of HPLC grade (Lab-Scan Analytical Sciences Ltd.; Dublin, Ireland). Ultrapure water (0.05 µS/cm) was obtained from a combined Seradest LFM 20

and Serapur Pro 90 C apparatus (Seral; Ransbach-Baumbach, Germany). Before use in the HPLC system, all sample solutions and solvents were membrane-filtered (0.45 µm, type RC 55; Schleicher & Schuell; Dassel, Germany). During the HPLC runs, solvents were continuously sparged with helium. All solvents used were flushed with nitrogen before use.

The following reagents and compounds were used: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), potassium persulfate ($K_2S_2O_8$, 99%), eugenol, **35** (99%, GC), isoeugenol, **36** (98%), quercetin dihydrate, **14** (98%), α-tocopherol, **5** (95%), and Trolox, **38** (97%) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); KCl (p.a.), NaCl (p.a.), glacial acetic acid (100%), and Na_2HPO_4 dihydrate (p.a.) from Merck (Darmstadt, Germany); ascorbic acid, **6** (for titration) and citric acid monohydrate (p.a.) from Acros Organics (Geel, Belgium); 2,6-di-*tert*-butyl-4-methylphenol, **1** (BHT, p.a.) and rutin trihydrate, **15** (90%) from Fluka (Buchs, Switzerland); rosmarinic acid, **11** from Extrasynthese (Genay, France); glutathione, reduced (GSH, 98%), from Boehringer Mannheim B. V. (Almere, The Netherlands); 3,4-dimethoxybenzoic acid (99%) and KH_2PO_4 monobasic (p.a.) from Janssen Chimica (Beerse, Belgium). For compounds numbering see Chapters 1 and 4.

Preparation of $ABTS^{\bullet+}$ solutions

ABTS (2 mM) was dissolved in 8 mM phosphate-buffered saline (PBS, consisting of 8.2 g NaCl, 0.27 g KH_2PO_4, 1.4 g Na_2HPO_4, and 0.15 g KCl per L) and adjusted to pH 7.4 with NaOH, and potassium persulfate solution in ultrapure water was added (0.3 mM final concentration) to produce the $ABTS^{\bullet+}$ radical cation. At this ratio of the reagents, no unreacted $K_2S_2O_8$ is left because ABTS and persulfate react stoichiometrically at a ratio of 2:1. To obtain a maximum conversion of ABTS to $ABTS^{\bullet+}$ the mixture was stored in the dark at room temperature for 16-17 h before use (Re et al. 1999). This $ABTS^{\bullet+}$ stock solution was stable for more than 2 days when kept at room temperature during measurements (protected from light) and at 4 °C during the night. Fresh working solutions (kept in a volumetric flask protected from light) of appropriate concentration were prepared in 10% methanol in PBS for each refill of the $ABTS^{\bullet+}$ syringe pump. Before use, the concentration of the $ABTS^{\bullet+}$ working solution was determined by measuring its absorbance at 734 nm.

Preparation of sample solutions

Stock solutions of the tested antioxidants (1 or 10 mM) were prepared in methanol and stored at −20 °C (exceptions: stock solutions of ascorbic acid and GSH were freshly prepared in ultrapure water). For the determination of the minimum detectable

concentration (MDC), 8-10 dilutions of each compound were prepared and sequentially injected under the following isocratic conditions: 100% MeOH (BHT and α-tocopherol); MeOH-H_2O (80:20, v:v) (eugenol, isoeugenol, rutin, and quercetin); MeOH-H_2O-HOAc (85:13:2, v:v) (Trolox, 3,4-dimethoxybenzoic acid and rosmarinic acid); MeOH-H_2O-HOAc (15:84:1, v:v) (ascorbic acid); MeOH-H_2O (15:85, v:v) (glutathione).

Plant extracts were prepared by extraction of air-dried, ground plant material from *Sideritis* species (Labiatae) with methanol, evaporation of the solvent to dryness, and successive partitioning between water and *tert*-butyl methyl ether, ethyl acetate, and 1-butanol. The obtained sub-extracts were evaporated to dryness under vacuum at temperatures not higher than 50 °C. The remaining aqueous layer was freeze-dried. Stock solutions from ethyl acetate (or butanol) extracts were prepared in methanol and from aqueous extracts in water (1 mg/mL), and stored at −20 °C. Working dilutions of appropriate concentration were freshly prepared in 50% methanol or pure water and 10 µL was injected for HPLC analysis. Ethyl acetate extracts were separated by a step gradient using 10% MeOH acidified with 0.1% HOAc (Solvent **A**) and MeOH acidified with 0.1% HOAc (Solvent **B**) at a flow rate of 0.8 mL/min as follows: 3 min, 70% **A**, isocratic; 3 min, linear gradient from 70 to 65% **A**; 15 min, isocratic hold at 65% **A**; 15 min, linear gradient from 65 to 45% **A**; 20 min, 45% **A**, isocratic; 3 min, linear gradient from 45 to 20% **A**; 16 min, isocratic hold at 20% **A**; 3 min, linear gradient from 20 to 70% **A**; and a 7 min isocratic hold at 70% **A** for re-equilibration of the column before the next injection. UV detection was carried out at 254 nm.

Rosemary extract was prepared from dried rosemary leaves (100 mg) by extraction using 2 mL MeCN-H_2O-HOAc (70:30:1, v:v) for 15 min in an ultrasonic bath. The supernatant was pipetted off and filtered through a 0.45-µm filter. Suitable working solutions were prepared in MeCN-H_2O-HOAc (70:30:1, v:v) and 10 μL was injected onto the HPLC column. Step gradients with 10% MeCN acidified with 0.1% HOAc (Solvent **A**) and MeCN acidified with 0.1% HOAc (Solvent **B**) at a flow rate of 0.8 mL/min were used for separation as follows: 5 min, 85% **A**, isocratic; 12 min, linear gradient from 85 to 50% **A**; 10 min, isocratic hold at 50% **A**; 3 min, linear gradient from 50 to 35% **A**; 20 min, 35% **A**, isocratic; 3 min, linear gradient from 35 to 0% **A**; 5 min, isocratic hold at 0% **A**; 5 min, linear gradient from 0 to 85% **A**; and an 8 min isocratic hold at 85% **A** for re-equilibration of the column before the next injection. UV detection was carried out at 280 nm.

Measurement of antioxidant capacity expressed as TEAC

To avoid peak broadening and an associated dilution during chromatography (i.e. to ensure uniform experimental conditions for all tested compounds), the HPLC column was

mounted before the injection port, and the analyses were carried out in flow-injection mode. The HPLC mobile phase (MeOH:H_2O = 85:15, v:v) was run at 0.8 mL/min and $ABTS^{\bullet+}$ solution (5.5 µM, in 10% MeOH in PBS), at 0.5 mL/min. Four to five different concentrations (triplicate injections) of the studied antioxidants were used to construct calibration curves $h = a \; x \; c + b$, where h represents the height (depth) of the negative peak resulting from the $ABTS^{\bullet+}$ quenching in mm; c is the compound concentration in μM, a is the slope, and b is the y-intercept of the obtained curve ($r^2 \geq 0.99$). The TEAC value was derived using the following formula:

$$TEAC = a_{sample} / a_{Trolox}$$

Each compound was tested at the above-mentioned concentrations in triplicate on at least two separate days. For Trolox, a concentration range was analyzed and a calibration curve calculated daily to ensure accurate TEAC determination of the compounds tested on the same day. TEAC values were determined at three reaction times: 10, 30 and 60 s (see part 5.3.2).

5.3 Results and discussion

5.3.1 Stability of $ABTS^{\bullet+}$ solutions

Different methods have been used for generation of $ABTS^{\bullet+}$, either before or after the addition of the antioxidant [metmyoglobin, H_2O_2 (Miller et al. 1993); horseradish peroxidase, H_2O_2 (Arnao et al. 1996); using a thermally decomposing azo compound 2,2'-azobis-(2-amidopropane) hydrochloride (ABAP) (van den Berg et al. 1999); by oxidation with strong oxidizers such as MnO_2 (Miller et al. 1996), Br_2 (Scott et al. 1993), and $K_2S_2O_8$ (Re et al. 1999); or by pulse radiolysis (Wolfenden & Willson 1982)].

Within my investigations, $ABTS^{\bullet+}$ was produced by oxidation of ABTS (in excess) with potassium persulfate prior to the addition of antioxidants. The presence of unreacted ABTS has been found to be important for a stable production of $ABTS^{\bullet+}$ (Wolfenden & Willson 1982; Cano et al. 1998). The prior generation of the radical in a stable form prevents possible interactions between the primary oxidant ($K_2S_2O_8$) and sample components (Re et al. 1999). It also avoids over estimation of activity as a result of compounds that have an inhibitory effect on the radical formation. Only the radical scavenging properties are assessed (Strube et al. 1997).

Time stability tests of $ABTS^{\bullet+}$ working solutions obtained with diluting solvents of different composition were performed over a period of 1 min. Before measuring, the

solvent mixture to be studied (0.8 mL) was mixed with 5 μM $ABTS^{\bullet+}$ solution in PBS (0.5 mL). Solvent mixtures containing varying concentrations (0 − 100%) of organic modifier (methanol or acetonitrile) and with varying pH (2-9) were considered. The effect of the diluting solvent pH was studied with citric acid-Na_2HPO_4 buffers (CPB) (pH from 3 to 7.4) as well as with H_2O, PBS (pH 7.4), 0.1 M citric acid (pH 2), and 0.2 M Na_2HPO_4 (pH 9). Neither the buffered solvents nor water had any influence on the absorbance over a period of 1 min. Acidic (pH 2) and basic (pH 9) solutions showed slightly increased and decreased initial absorbance values, respectively. In later on-line HPLC experiments, it was found that the HPLC solvents should not be acidified with strong acids, such as trifluoroacetic acid (TFA), because it caused strong $ABTS^{\bullet+}$ quenching. It has been assumed that the TFA anion masks the positive charge on an analyte molecule (within this context, it should be noted that TFA has been observed to suppress the signals of radical cations in LC/MS experiments (Niessen 1999). Changing the organic modifier or its percentage caused no significant absorbance instability over 1 min. In Figure 5.2 the time stability of $ABTS^{\bullet+}$ solutions diluted with some of the studied solvents is presented. A trend of decrease in the initial absorbance value with increasing organic solvent content was observed, but the difference between the two extreme initial absorbance values was sufficiently small to be neglected. The obtained results suggest that HPLC mobile phases containing up to 100% organic solvent (methanol or acetonitrile) and with pH down to 3 can be used in both isocratic and gradient runs with this method.

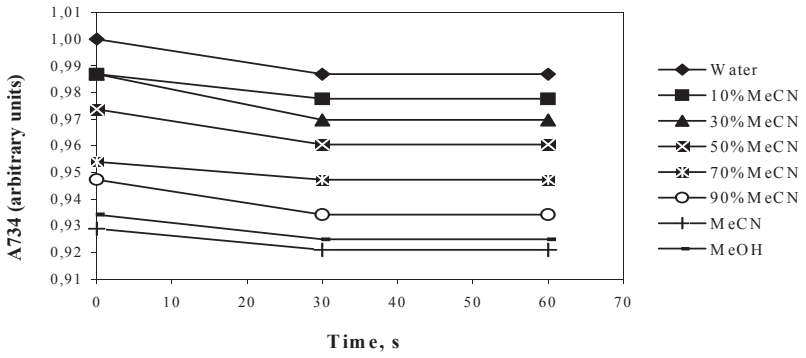


Figure 5.2 Stability of $ABTS^{\bullet+}$-diluted solutions during 1 min, depending on the diluting-solvent composition.

In previously reported experimental protocols, $ABTS^{\bullet+}$ working solutions were prepared in PBS (Miller et al. 1993; van den Berg et al. 1999). The dependence of the long-

term stability of $ABTS^{\bullet+}$ working solutions (prepared from the stock solution after a 10-times dilution with the studied solvent) on the content of organic solvent (methanol) in PBS (0, 10, 30, 50, 70, 90, and 100% methanol) was checked over a period of 4 h. Increasing the methanol content to more than 10% decreased the long-term stability of the $ABTS^{\bullet+}$ solutions. For example, the decrease in asorbance was about 30% of its initial value at the end of the 4-h period with 50% methanol as a diluting solvent. The working $ABTS^{\bullet+}$ solutions were sufficiently stable over the total analysis time not to significantly affect the S/N ratios or the obtained detection limits. Consequently, fresh $ABTS^{\bullet+}$ working solutions should be prepared before each single use in either PBS or PBS containing no more than 10% methanol.

5.3.2 Optimization of the on-line experimental set-up

When $ABTS^{\bullet+}$ was applied as a post-column reagent solution in the on-line system, a similar effect of the content of organic modifier was observed. With three compositions of the $ABTS^{\bullet+}$-diluting solvents (PBS, 10% MeOH in PBS, and 50% MeOH in PBS), the signal-to-noise (S/N) ratio for 10-µL injections of a 1 µM Trolox solution was studied ($ABTS^{\bullet+}$ reagent flow concentration, 5.5 and 11 µM). Using 10% MeOH in PBS resulted in a slight increase of the S/N ratio as compared to that obtained with PBS, whereas using 50% MeOH in PBS led to a lower S/N ratio. Hence, 10% methanol in PBS was chosen as a diluting solvent for the $ABTS^{\bullet+}$ stock solution for all further experiments.

Reagent concentration and reaction time influence the S/N ratio. Both of these parameters were optimized with respect to the S/N ratio for Trolox. As established prevoiusly (Koleva et al. 2000), the sensitivity of the method depends on the reactor concentration of the model free radical. The $ABTS^{\bullet+}$ reactor concentration was, therefore, optimized under on-line conditions by monitoring the S/N ratio for 10-µL injections of a 1 µM Trolox solution (measurements in triplicate). The $ABTS^{\bullet+}$ reactor concentrations were changed by changing the concentration of the working solution (3, 5.5, 11, 25, and 50 μM) and the flow rate (0.1, 0.3, 0.5, 0.7, and 0.9 mL/ min) of the syringe pump delivering the $ABTS^{\bullet+}$ solution. For these experiments, $ABTS^{\bullet+}$ working solutions were prepared with PBS. The HPLC mobile phase ($MeOH:H_2O:HOAc$ = 85:13:2, v:v:v) was run at 0.8 mL/min. As can be seen from Figure 5.3(a), the S/N ratio is essentially inversely proportional to the $ABTS^{\bullet+}$ reactor concentration. However, at very low $ABTS^{\bullet+}$ reactor concentrations (< 1 µM), the noise generated by system components (e.g. detector noise and short-term flow instabilities) becomes limiting, which leads to an optimum S/N ratio at ~2.5 µM $ABTS^{\bullet+}$ reactor concentration. Furthermore, the reagent-pump flow rate becomes an important parameter at these low $ABTS^{\bullet+}$ reactor concentrations.

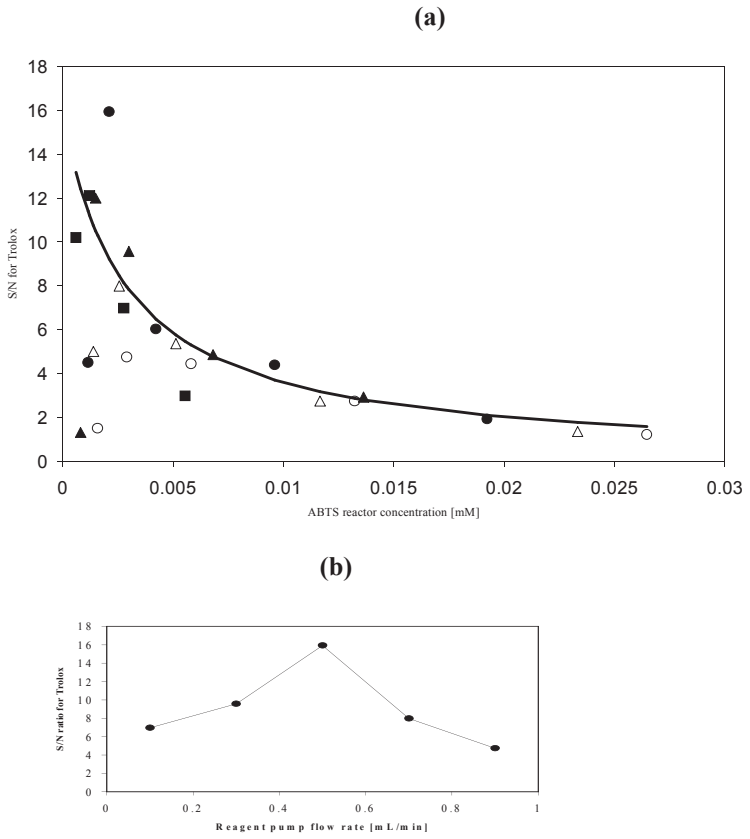


Figure 5.3 (a) Effect of $ABTS^{\bullet+}$ reactor concentration on the signal-to-noise ratio (S/N) for 10-µL injections of a 1 µM Trolox solution. ABTS reactor concentration is changed as a function of reagent flow concentration and flow speed (■ = 0.1 $mL.min^{-1}$, ▲ = 0.3 $mL.min^{-1}$, ● = 0.5 $mL.min^{-1}$, Δ = 0.7 $mL.min^{-1}$, ○ = 0.9 $ml.min^{-1}$). Inverse proportionality is shown from a least-squares fit (solid line), excluding the data at very low $ABTS^{\bullet+}$ reactor concentration. **(b)** Effect of $ABTS^{\bullet+}$ pump flow rate on the S/N ratio at an $ABTS^{\bullet+}$ reactor concentration of ca. ~2.5 µM.

This flow rate influences the S/N ratio via its effect on the column effluent dilution and analyte residence time in the reactor coil. At an $ABTS^{\bullet+}$ reactor concentration of ~2.5 µM, it can be seen from Figure 5.3(b) that a reagent-pump flow rate of 0.5 mL/min gives an optimum S/N ratio. The same findings were obtained using eugenol (0.5 µM injections) as

a model compound. As a result, a 5.5 μM $ABTS^{\bullet+}$ solution at a flow rate of 0.5 mL/min (providing an $ABTS^{\bullet+}$ reactor concentration of 2.1 μM) was used in all further experiments.

For optimization of the reaction time, S/N ratios for 10-μL injections of a 1 μM Trolox solution were determined for three reaction coils providing reaction times (at 1.3 mL/min total flow rate) of 10 s (coil dimensions, 4.4 m x 0.25 mm i.d.), 30 s (coil dimensions: 13.7 m x 0.25 mm i.d.), and 60 s (coil dimensions: 6.5 m x 0.5 mm i.d.).

As expected, as a result of peak broadening, relatively low S/N ratios were obtained with the wide-bore reaction coil. However, mounting a 0.25-mm i.d. reaction coil providing 1 min reaction time required a length of ~30 m, resulting in high back pressure, which was undesirable. Neither narrow-bore coil differed significantly with respect to S/N ratios obtained for Trolox. To ensure a sufficiently long reaction time for compounds with slower reaction kinetics toward $ABTS^{\bullet+}$ and considering the UV absorbance stability of $ABTS^{\bullet+}$ solutions over 1 min (see Fig. 5.2), the 30-s reaction coil was selected for all further experiments.

5.3.3 Determination of Minimum Detectable Concentration (MDC) and Minimum Detectable Amount (MDA)

For determination of MDC (mol/L) and MDA (ng), the coefficient t = 1.725 for n = 21 measurements of the blank signal with a confidence interval of 90% was used, and MDC was calculated as

$$MDC = -2t\sigma_{blank}$$

where σ_{blank} is the standard deviation of the blank signal and t is the Student's t statistic.
The blank sample consisted of HPLC mobile phase with the composition used for the tested compound mixed with the $ABTS^{\bullet+}$ working solution at its optimum concentration and flow rate. The negative peak resulting from the bleaching of $ABTS^{\bullet+}$ by a radical-scavenging compound was considered detectable if its height (depth) exceeded the calculated MDC value.

A number of known reference compounds were studied, and their MDC and MDA were determined under the optimized on-line conditions (data in Table 5.1). A combined plot of the UV and $ABTS^{\bullet+}$-quenching profiles of seven dilutions of BHT (**1**) sequentially injected under isocratic conditions is presented in Figure 5.4. The MDA values obtained with the HPLC-ABTS on-line system are lower and significantly different from those obtained with the HPLC-DPPH and HPLC-CL methods for most of the tested compounds.

These observations could be explained in the light of the differences in reaction kinetics involving different radical species.

Table 5.1 Minimum Detectable Concentrations (MDC) and Minimum Detectable Amount (MDA) of some antioxidants in the HPLC-ABTS, HPLC-DPPH and HPLC-CL on-line systems under isocratic conditions.

Tested Compound	**MDC[a], μM**	**MDA, ng** (HPLC-ABTS)	**MDA[b], ng** (HPLC-DPPH)	**MDA[c], ng** (HPLC-CL)
Trolox (**38**)[d]	0.09	0.21	1.0	5.2
Eugenol (**35**)	0.13	0.21	60	0.23
Isoeugenol (**36**)	0.16	0.27	2.2	3.9
Rosmarinic acid (**11**)	0.12	0.43	0.07	0.43
Ascorbic acid (**6**)	0.24	0.43	3.9	0.18
Rutin (**15**)	0.09	0.55	4.0	15
Quercetin (**14**)	0.19	0.58	0.93	4.5
Glutathione	0.19	0.60	ND	ND
BHT (**1**)	0.53	1.2	360	20
α-Tocopherol (**5**)	0.29	1.3	5.5	10

[a] - determined with a 10-μL loop; 30 s reaction time

[b] - obtained with the HPLC-DPPH method (Koleva et al. 2000; Dapkevicius et al. 2001)

[c] - obtained with the HPLC-Chemiluminescence method (Dapkevicius et al. 1999)

[d] - see Chapters 1 and 4 for the numbering of the compounds

ND - not done

For example, the reaction kinetics between phenols (PhOH) and $ABTS^{\bullet+}$ has been found to differ from that between phenols and $DPPH^{\bullet}$ over a similar range of concentrations. Campos & Lissi (1996) have suggested that this difference can be partly a result of different equilibrium displacements in reaction 1 as a result of the fact that the reactions of $DPPH^{\bullet}$ are carried out in the absence of added DPPH-H (i.e. the reduced form), but the reduced form ABTS is always present in the systems containing $ABTS^{\bullet+}$ (see *Stability of* $ABTS^{\bullet+}$ *solutions*).

$$\text{Radical}^{\bullet} + \text{PhOH} \rightleftharpoons \text{Reduced form} + \text{PhO}^{\bullet} \qquad (1)$$

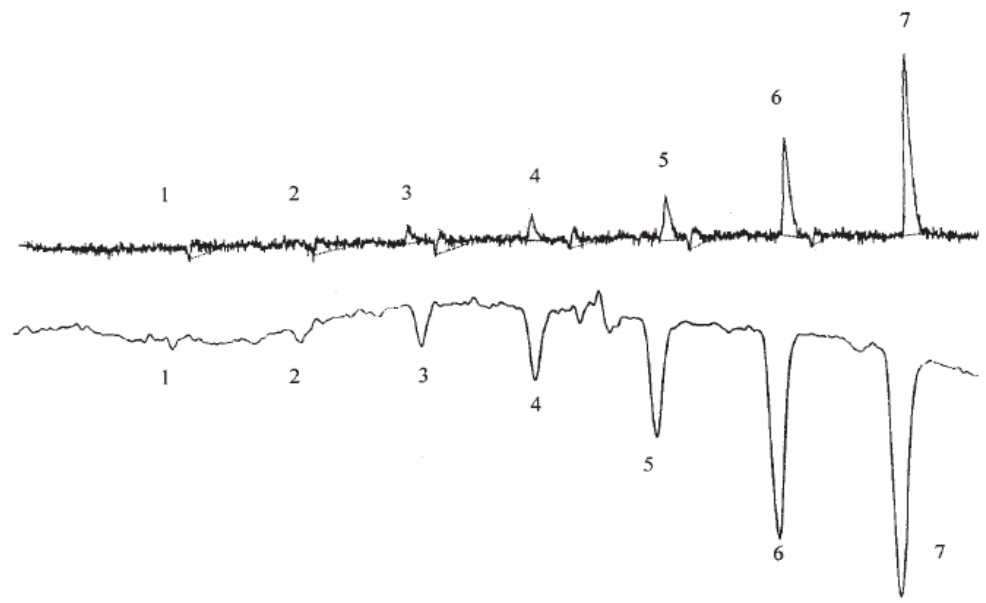


Figure 5.4 Combined plot of the UV and $ABTS^{\bullet+}$-reduction profiles of different concentrations of BHT (**1**) sequentially injected (left to right: 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50 μM) under isocratic conditions. Chromatographic conditions: Alltima C18 HPLC column (5 μm, 250 × 4.6 mm i.d.); mobile phase 100% MeOH; flow rate 0.8 mL/min; sample size 10 μL; detection at 280 nm; peak widths of peak 7: UV trace, 2.8 mm; $ABTS^{\bullet+}$-reduction trace, 6.2 mm.

Furthermore, different mechanisms for the reactions of phenols with $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ have been reported (Brand-Williams et al. 1995; Campos & Lissi 1996; Bondet et al. 1997; Lissi et al. 1999). Reactions of phenols with $ABTS^{\bullet+}$ are usually rapid, but the reactions with $DPPH^{\bullet}$ differ from compound to compound. Three types of kinetics, rapid, intermediate and slow, have been distinguished for the reactions between $DPPH^{\bullet}$ and phenols (Brand-Williams et al. 1995; Campos & Lissi 1996). Lissi et al. (1999) have stated that "the reaction of $DPPH^{\bullet}$ with phenols is complex and involves several reversible reactions rendering difficult an interpretation of reaction rates in these systems". Additionally, the reaction mechanism between phenols and $ABTS^{\bullet+}$ has been suggested to involve secondary reactions of the phenoxyl radicals ($PhO^{\bullet}$) formed in reaction 1. The MDA value depends on both the reaction stoichiometry and the initial rate between the

antioxidant and $ABTS^{\bullet+}$. Differences in MDAs for various antioxidants can be the result of changes in either or both of these factors.

It should be noted that, as expected, compounds without hydrogen-donating groups (e.g. 3,4-dimethoxybenzoic acid) do not exhibit any radical-scavenging activity towards $ABTS^{\bullet+}$ in the on-line system, even at high concentrations (100 µM). It can therefore be concluded that $ABTS^{\bullet+}$ quenching is, indeed, a result of radical scavenging activity. Hence, false positive results for radical trapping abilities are unlikely.

5.3.4 Suitability of the method for quantitative determinations

Some linearity assessments have been carried out to prove the suitability of the method for quantitative determinations. Different concentrations of test compounds resulted in a proportional change in the corresponding negative peak heights, that is, the method was fully linear over the concentration range that was tested. The data obtained are presented in Table 5.2.

Table 5.2 Calibration curve ($h = a \times c + b$; r^2) data for several known compounds (obtained under the optimized experimental conditions).

Test compound[a]	a	b	linear range [µM]	data points in linear range [n]	r^2
α-Tocopherol (**5**)	7.608	0.469	0.5 - 5	4	0.9994
Rosmarinic acid (**11**)	10.376	2.521	0.25 - 5	5	0.9985
Quercetin (**14**)	16.594	–1.621	0.25 - 5	5	0.9994
Eugenol (**35**)	18.044	1.249	0.25 - 5	5	0.9988

[a] - see Chapters 1 and 4 for the numbering of the compounds

5.3.5 On-line HPLC-ABTS system and TEAC determination

In assays using $ABTS^{\bullet+}$, a common parameter, TEAC, is used to assess the antioxidant activity. It has been recognized that the reaction time is crucial for the obtained TEAC value, which is a logical consequence of a difference in reaction rate constants between the antioxidant being evaluated and the standard (i.e., Trolox). Consequently, different TEAC values for the same compounds have been reported (van den Berg et al. 1999; Re et al. 1999). Measuring the TEAC of an antioxidant at different reaction times provides information with respect to both the extent and the rate of the radical-scavenging reaction (Re et al. 1999). The on-line HPLC-ABTS system was used in flow injection mode to

determine the TEAC values for a number of known antioxidants at three reaction times: 10, 30, and 60 s. For some of the studied compounds, literature data are available, and they were compared with the values obtained using the on-line method (data in Table 5.3). Correlations with literature data are expected to hold when a chromatographic separation is included, assuming that reactor antioxidant concentrations are corrected for dilution (band broadening) accompanying chromatographic separation. Additionally, the separation has to be carried out under isocratic conditions, and the solvent composition should be identical to that used under off-line conditions.

Table 5.3 TEAC of various antioxidants at three different reaction times obtained under optimized conditions in a flow-injection mode.

Tested compound[a]	**Reaction time, s**				
	10 (*n*)	*10* [b]	**30** (*n*)	**60** (*n*)	*60* [c]
Trolox (**38**)[d]	1.00	*1.00*	1.00	1.00	*1.00*
Eugenol (**35**)	1.46 ± 0.06 (48)	*ND*	1.52 ± 0.09 (48)	1.48 ± 0.08 (45)	*ND*
Isoeugenol (**36**)	0.72 ± 0.06 (45)	*ND*	0.81 ± 0.09 (45)	1.06 ± 0.01 (45)	*ND*
Rosmarinic acid (**11**)	2.11 ± 0.03 (30)	*ND*	2.55 ± 0.20 (42)	2.79 ± 0.03 (30)	*ND*
Ascorbic acid (**6**)	0.52 ± 0.04 (21)	*0.86 ± 0.02*	0.63 ± 0.11 (15)	1.01 ± 0.01 (21)	*1.05 ± 0.02*
Rutin (**15**)	1.76 ± 0.07 (42)	*ND*	2.16 ± 0.02 (39)	2.40 ± 0.17 (42)	*ND*
Quercetin (**14**)	2.13 ± 0.09 (36)	*3.49 ± 0.33*	2.54 ± 0.05 (42)	2.76 ± 0.20 (42)	*2.77 ± 0.02*
BHT (**1**)	0.10 ± 0.02 (24)	*ND*	0.27 ± 0.01 (24)	0.39 ± 0.06 (24)	*ND*
α-Tocopherol (**5**)	0.98 ± 0.02 (30)	*0.95 ± 0.02*	0.93 ± 0.04 (30)	0.96 ± 0.02 (30)	*0.89 ± 0.05*

[a] - TEACs are means ± SD (*n* = the total number of injections done for all tested concentrations of a compound). The listed compounds were dissolved in methanol, except ascorbic acid, which was dissolved in water.

[b] - Data from van den Berg et al. (1999) − $ABTS^{\bullet+}$ generated with ABAP; ascorbic acid dissolved in PBS, Trolox, quercetin and α-tocopherol – in ethanol.

[c] - Data from Re et al. (1999) − $ABTS^{\bullet+}$ generated with $K_2S_2O_8$; ascorbic acid dissolved in water, Trolox, quercetin and α-tocopherol – in ethanol.

[d] - see Chapters 1 and 4 for the numbering of the compounds

ND - not done

At a reaction time of 1 min, a good agreement was observed between the TEAC values determined and those reported by Re et al. (1999). The authors used $ABTS^{\bullet+}$ pregenerated by oxidation of ABTS with $K_2S_2O_8$. Furthermore, there is a good correlation with the literature data for TEAC values of α-tocopherol obtained at 10 s and 1 min (van den Berg et al. 1999; Re et al. 1999). However, the TEAC values calculated for ascorbic acid and quercetin at 10 s, that is, during the first stage of the reaction, differ from those presented by van den Berg et al. (1999). This discrepancy is possibly due to the different experimental protocols (different ways of producing $ABTS^{\bullet+}$) used. Influence of the assay protocol has been demonstrated in several cases (Romay et al. 1996; Miller & Rice-Evans 1997; van den Berg et al. 1999; Re et al. 1999), where different TEAC values at the same reaction time have been reported for some of the tested compounds. Below, the implications of the measured and cited TEAC values for mechanistic classification are discussed in more detail.

For most of the studied compounds, a TEAC value changing with the end-point time was observed. Such a result is expected on the basis of a difference in reaction rate constant between the antioxidant being evaluated and the standard (i.e., Trolox). However, in addition to these kinetic differences, secondary reactions involving reaction products and $ABTS^{\bullet+}$ may influence the TEAC values. Similar results reported in other works (Romay et al. 1996; van den Berg et al. 1999; Re et al. 1999) have been partially attributed to such a "biphasic pattern" of the reaction of some compounds with $ABTS^{\bullet+}$. Van den Berg et al. (1999) demonstrated this hypothesis with quercetin and ascorbic acid. As a possible explanation, the authors suggested the formation of reaction products reacting more slowly with $ABTS^{\bullet+}$ or intramolecular rearrangements of antioxidants leading to slowing of the reaction. Romay et al. (1996) also reported a biphasic reaction pattern for ascorbic acid and bilirubin with $ABTS^{\bullet+}$. Furthermore, such behaviour has been demonstrated for some flavonoids (Re et al. 1999). Although a reaction time dependence for most of the studied compounds (including ascorbic acid), is also observed with the on-line method, differences in kinetic behaviour seem to suffice to explain these data. Such a method is preferable, because the importance of secondary processes is highly questionable when considering real biological systems. Differences with literature may well originate from the fact that with previous assays, it has been impossible to monitor the initial time period (about 10 s) after the initiation of the reaction.

Trolox, the compound used for standardization purposes, reacts very fast (less than 10 s) with $ABTS^{\bullet+}$ (Campos & Lissi 1996; Romay et al. 1996; van den Berg et al. 1999). Given the fact that the reaction between Trolox and $ABTS^{\bullet+}$ is completed within 10 s, the TEAC values obtained for α-tocopherol at 10, 30, and 60 s indicate that its reaction with $ABTS^{\bullet+}$

has actually finished after 10 s, as well. Similar behaviour has been reported for α-tocopherol (Campos & Lissi 1996; Romay et al. 1996; van den Berg et al. 1999) and can be attributed to its structural similarity with Trolox.

Because glycosylation has been found to diminish the radical scavenging activity of flavonoids in other studies (Pratt 1980), the higher activity for quercetin as compared to its glycoside rutin is expected. The importance of small changes in antioxidant structure in determining radical scavenging activity is illustrated by the difference in TEAC values observed for eugenol and isoeugenol.

Finally, the TEAC of an antioxidant has been found to be solvent-dependent. For example, *β*-carotene exhibits high radical-scavenging activity when dissolved in THF, twice less activity in EtOH, and no activity in acetone (van den Berg et al. 1999). Solvent dependence was confirmed by some of my own results. For example, a TEAC value about 1.5 times lower was obtained for ascorbic acid dissolved in methanol, as compared to its TEAC in water.

5.3.6 Application of the HPLC-ABTS method to complex samples

In Figures 5.5(a) and 5.5(b), the application of the proposed method for the screening of two plant extracts is illustrated. Preliminary studies, as well as the data obtained with the HPLC-DPPH system (Koleva et al. 2000), showed the presence of radical-scavenging compounds in *Sideritis* extracts, mainly flavonoids and phenylpropanoid glycosides, that is, compounds with known radical scavenging properties. Rosemary extracts are currently commercially used as natural antioxidants. The main contributors to their high antioxidant activity are carnosol, carnosic acid, and rosmarinic acid (Frankel et al. 1996). No preliminary sample preparation was necessary, indicating that a considerable amount of time, labour, and reagents can be spared by applying the method. The peaks in the chromatograms marked with an asterisk (*) do not have a counterpart negative peak of reduced $ABTS^{\bullet+}$. This means that the corresponding components do not possess radical scavenging properties. Similarly to the pure compounds (see Table 5.2), injecting different concentrations of the extracts resulted in a proportional change in the corresponding negative peak heights, which confirms the suitability of the method for quantitative determinations.

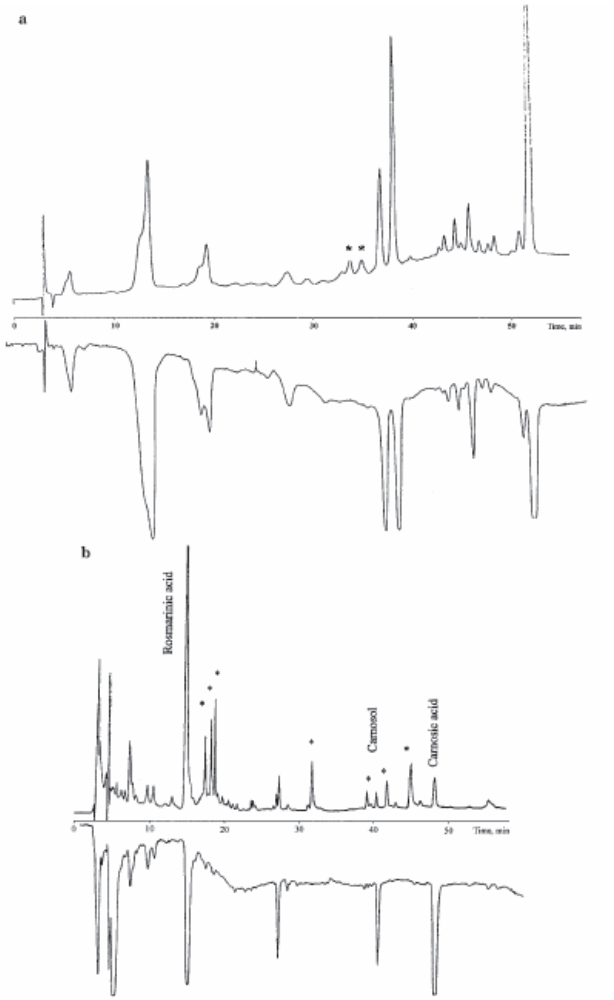


Figure 5.5 (a) Combined plot of the UV and $ABTS^{\bullet+}$-quenching chromatograms of *Sideritis syriaca* ethyl acetate extract. Chromatographic conditions: Alltima C18 HPLC column (5 μm, 250 × 4.6 mm i.d.); step gradient with acidified MeOH – H_2O mixtures; flow rate 0.8 mL/min; sample size 10 μL; detection, 254 nm; **(b)** Combined plot of the UV and $ABTS^{\bullet+}$-quenching chromatograms of a rosemary leaf extract. Chromatographic conditions: Alltima C18 HPLC column (5 μm, 250 × 4.6 mm i.d.); step gradient with acidified MeCN – H_2O mixtures; flow rate 0.8 mL/min; sample size 10 μL; detection, 280 nm. Detailed chromatographic conditions for both extracts are given in the Experimental.

5.4 Conclusion

A rapid and sensitive HPLC method for on-line detection of radical-scavenging compounds in complex matrixes using $ABTS^{\bullet+}$ was developed. The method proves to be a useful and complementary extension of the previously developed HPLC-DPPH on-line method. The method combines some of the characteristics of the HPLC-DPPH system (Koleva et al. 2000) like simple set-up, selectivity, and on-line detection with several additional advantages: **(1)** higher sensitivity as compared to the on-line methods developed previously (HPLC-DPPH and HPLC-CL); **(2)** the ability to perform isocratic and gradient HPLC runs over a broader range of solvent compositions [from 0 to 100% organic component in water, buffers, or weak acids (pH 3−7.4)]; consequently, a broad range of compounds can be analyzed with this method; **(3)** TEAC values can be determined in flow-injection mode at different reaction times. In the screening of complex samples, compounds with different kinetics toward $ABTS^{\bullet+}$ will be encountered. The rate constants of rapidly reacting compounds are generally difficult to determine if special equipment (e.g. a stop-flow device) is not available. As a result, only the stoichiometry can be determined for such rapidly reacting compounds (Lissi et al. 1999). The presented system provides an alternative tool for performing kinetic studies on both fast- and slow-reacting compounds, because reaction coils of different lengths can be easily installed. The method can be applied for a quick screening of complex mixtures of different origins (plant extracts, foods and beverages, biological samples) for radical-trapping components.

For reactive antioxidants, detection by $ABTS^{\bullet+}$ reduction sometimes proves to be more sensitive than normal UV detection (see Figs. 5.4 and 5.5). Hence, the method may be useful in product quality evaluation with regard to antioxidative constituents as well as for monitoring modification processes in studies aimed at the production of materials with an increased antioxidant content. Because of its high reactivity, radical-scavenging activity toward $ABTS^{\bullet+}$ can serve as a reason to expect similar activity toward active radical species encountered in food or biological systems although antioxidant activity determined with model systems cannot be simply extrapolated to an *in vivo* efficiency or to complex real systems such as foods. Nevertheless, model systems represent a rapid, simple, and inexpensive way for screening compounds in search of new antioxidants. A combined use of HPLC-radical on-line methods is useful in the evaluation of radical-scavenging properties because it provides a more comprehensive picture of the radical-scavenging profile of the studied antioxidant.

5.5 References

Arnao M.B., Cano A., Hernandez-Ruiz J., Garcia-Canovas F., Acosta M. (1996). "Inhibition by ascorbic acid and other antioxidants of the ABTS oxidation catalyzed by peroxidase: a novel approach for determining total antioxidant status of foods." Anal. Biochem. **236:** 255-261.
Aruoma O.I. (1998). "Free radicals, oxidative stress, and antioxidants in human health and disease." JAOCS **75:** 199-212.
Benavente-Garcia O., Castillo J., Lorente J., Ortuno A., del Rio J.A. (2000). "Antioxidant activity of phenolics extracted from *Olea europea* L. leaves." Food Chem. **68:** 457-462.
Berg R. van der, Haenen G.R.M.M., van den Berg H., Bast A. (1999). "Applicability of an improved Trolox equvalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures." Food Chem. **66:** 511-517.
Bondet V., Brand-Williams W., Berset C. (1997). "Kinetics and mechanisms of antioxidant activity using the DPPH free radical method." Lebensm-Wiss.-Technol. **30:** 609-615.
Brand-Williams W., Cuvelier M.E., Berset C. (1995). "Use of a free radical method to evaluate antioxidant activity." Lebensm-Wiss.-Technol. **28:** 25-30.
Campos A.M., Lissi E.A. (1996). "Kinetcs of the reaction between ABTS derived radical cations and phenols." Int. J. Chem. Kinetics **29:** 219-224.
Cano A., Hernandez-Ruiz J., Garcia-Canovas F., Acosta M., Arnao M.B. (1998). "An end-point method for estimation of the total antioxidant activity in plant material." Phytochem. Anal. **9:** 196-202.
Dapkevicius A., van Beek T.A., Niederländer H.A.G. (2001). "Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in HPLC eluates." J. Chromatogr. A **912:** 73-82.
Dapkevicius A., van Beek T.A., Niederländer H.A.G., de Groot A. (1999). "On-line detection of antioxidative activity in HPLC eluates by chemiluminescence." Anal. Chem. **71:** 736-740.
Finley J.W., Otterburn M.S. (1993). "The consequence of free radicals in foods." in Antioxidants. Chemical, physiological, nutritional and toxicological aspects. (Ed. Williams G.M.), Princeton Scientific Publishing Co., Inc., Princeton, NJ, pp 77-91.
Frankel E.N., Huang S.-W., Aeschbach R., Prior E. (1996). "Antioxidant activity of a rosemary extract and its constituents carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion." J. Agric. Food Chem. **44:**131–135.
Huang D., Ou B., Prior R.L. (2005). "The chemistry behind antioxidant capacity assays." J. Agric. Food Chem. **53:** 1841-1856.
Koleva I.I., Niederländer H.A.G., van Beek T.A. (2000). "An on-line HPLC method for detection of radical scavenging compounds in complex mixtures." Anal. Chem. **72:** 2323-2328.
Lissi E.A., Modak B., Torres R. Escobar J., Urzua A. (1999). "Total antioxidant potential of resinous exudates from *Heliotropium* species, and a comparison of the ABTS and DPPH methods." Free Rad. Res. **30:** 471-477.
Mantle D., Anderton J.G., Falkous G., Barnes M., Jones P., Perry, E.K. (1998). "Comparison of methods for determination of total antioxidant status: application to analysis of medicinal plant essential oils." Comp. Biochem. Physiology, Part B **121:** 385-391.
Miller N.J., Diplock A.T., Rice-Evans C.A. (1995). "Evaluation of the total antioxidant activities as a marker of the deterioration of apple juice on storage." J. Agric. Food Chem. **43:** 1794-1801.
Miller N.J., Rice-Evans C., Davies M.J., Gopinathan V., Milner A. (1993). "A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates." Clin. Sci. **84:** 407-412.

Miller N.J., Rice-Evans C.A. (1997). "Factors influencing the antioxidant activity determined by the ABTS radical cation assay." Free Rad. Res. **26:** 195-199.
Miller N.J., Sampson J., Candeias L.P., Bramley P.M., Rice-Evans C.A. (1996). "Antioxidant activities of carotenes and xanthophylls." FEBS Lett. **384:** 240-242.
Niessen W.M.A. (1999). "Liquid Chromatography - Mass Spectrometry.", 2nd ed., Marcel Dekker, New York, p 320.
Ogawa A., Arai H., Tanizawa H., Miyahara T., Toyo'oka T. (1999). "On-line screening method for antioxidants by LC with chemiluminescence detection." Anal. Chim. Acta **383:** 221-230.
Pietta P., Simonetti P., Mauri P. (1998). "Antioxidant activity of selected medicinal plants." J. Agric. Food Chem. **46:** 4487-4490.
Pratt D.E. (1980). "Natural antioxidants of soybean and other oil seeds." in Autoxidation in Food and Bilogical Systems. (Eds. Simic M.G., Karel M.), Plenum Press, New York, pp 283-293.
Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C. (1999). "Antioxidant activity applying an improved ABTS radical cation decolorization assay." Free Rad. Biol. Med. **26:** 1231-1237.
Rice-Evans C., Miller N.J. (1994). "Total antioxidant status in plasma and body fluids." Meth. Enzymol. **234:** 279-293.
Rice-Evans C.A, Miller N.J., Bolwel P.G, Bramley P.M., Pridham J.B. (1995). "The relative antioxidant activities of plant-derived polyphenolic flavonoids." Free Rad. Res. **22:** 375-383.
Romay C., Pascual C., Lissi E.A. (1996). "The reaction between ABTS radical cation and antioxidants and its use to evaluate the antioxidant status of serum samples." Braz. J. Med. Biol. Res. **29:** 175-183.
Scott S.L., Chen W.-J., Bakac A., Espenson J.H. (1993). "Spectroscopic parameters, electrode potentials, acid ionization constants, and electron exchange rates of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) radicals and ions." J. Phys. Chem. **97:** 6710-6714.
Strube M., Haenen G.R.M.M., van den Berg H., Bast A. (1997). "Pitfalls in a method for assessment of total antioxidant capacity." Free Rad. Res. **26:** 515-521.
Tyrrel R.M. (1995). "Ultraviolet radiation and free radical damage to skin." in Biochem. Soc. Symp., No.61 - Free radicals and oxidative stress: environment, drugs and food additives. (Eds. Rice-Evans C., Halliwell B. & Lunt G.G.), University of Sussex, Dec.1994, pp 47-53.
Wolfenden B.S., Willson R.L. (1982). "Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions: pulse radiolysis studies of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)." J. Chem. Soc., Perkin Trans. II: 805-812.

Antioxidative Components of *Sideritis syriaca* and *Sideritis scardica* Grown in Bulgaria

6.1 Introduction

Natural antioxidants of plant origin have attracted lot of attention in recent years. This is due to the suspected toxic effects of synthetic antioxidants and the evidence that the intake of natural compounds can contribute to a healthier life (Madhavi & Salunkhe 1995; Barlow 1990; Evans & Reynhout 1992; Nieto et al. 1993; Schuller 1990; Madsen & Bertelsen 1995). Many plant species have been investigated in the search for active antioxidative components among them spices, teas, seeds and wine (Madsen & Bertelsen 1995; Schuller 1990; Altarejos et al. 2005; Cao et al. 1996; Pellegrini et al. 2000). The chemical nature of the isolated compounds greatly varies but substances of phenolic nature such as flavonoids, phenolic acids, isoflavones, phenylpropanoids, anthocyanidins, chalcones and catechins are most often responsible for the antioxidant activity (see Introduction, 1.2.2 – Figs. 1.13 & 1.14).

Plants from the genus *Sideritis* (Labiatae) are well known in Bulgarian and other folk medicines for treating various infections and inflammations (see Introduction, 1.4.3). Different pharmacological activities have been established, mainly antiinflammatory and antimicrobial (see Introduction, 1.4.3 - Table 1.4 & 1.5). Diterpenoids, essential oils and flavonoids have been found to be the main types of chemical constituents. From some species a coumarin (siderin), a lignan ((+)-sesamin) and a few phenylpropanoid glycosides have been isolated. Some minor constituents such as triterpenoids, iridoids, sterols, phenolic acids and fatty acids, occur in some plants of the genus (see Introduction, 1.4.2 - Table 1.3).

Four *Sideritis* species are distributed in Bulgaria – *S. scardica, S. syriaca, S. montana* and *S. lanata* (see Introduction, 1.4.1 - Fig. 1.16). Their chemical composition has not been studied in detail. Among the compounds so far detected are the diterpenoid siderol and ursolic/oleanolic acids, essential oil, iridoids, flavonoids and the phenylpropanoid glycoside verbascoside (acteoside) (see Introduction, 1.4.2 - Table 1.3). The antioxidant activity screening of a number of extracts from *S. syriaca*, *S. scardica* and *S. montana,* which differ

in polarity, has shown that only the extracts from the first two species may serve as a promising source of antioxidative compounds (Koleva et al. 2002; Koleva et al. 2003). *S. syriaca* and *S. scardica* species have a similar chemical composition and antioxidant activity in on-line methods (Koleva et al. 2003). The previous studies on the radical scavenging activity of *Sideritis* extracts and their components using a novel HPLC-DAD-DPPH (Koleva et al. 2000) and HPLC-DAD-ABTS (Koleva et al. 2001) on-line approach have enabled their selective assay-guided isolation. Recently, Ivanova et al. (2005) have evaluated herbal teas from 21 Bulgarian medicinal plants from several genera including *S. scardica* using ABTS method and TEAC.

This chapter describes the isolation and identification of the putative radical scavenging components of Bulgarian populations of *S. syriaca* and *S. scardica*. Structures were confirmed by spectroscopic methods. The radical scavenging activity of the isolated components have been evaluated off-line with the stable radical $DPPH^{\bullet}$, expressed as % Reduction, and compared to that of rutin and rosmarinic acid, caffeic acid and chlorogenic acid. On-line assays have also been performed.

6.2 Experimental

6.2.1 Chemicals

All solvents used for extraction, chromatographic separation and isolation of the compounds ($CHCl_3$, *t*-butyl methyl ether (*t*-BuOMe), petroleum ether (PE), dichloromethane (DCM), diethyl ether (Et_2O), acetone, MeOH, EtOAc, *n*-BuOH) were redistilled or of analytical grade. Methanol used for radical scavenging activity was of HPLC grade. Deuterated methanol, $CDCl_3$ and DMSO-d_6 were used to prepare solutions of purified compounds for NMR analysis and were purchased Sigma-Aldrich Chemie (Steinheim, Germany). The following reagents were used: 2,2-diphenyl-1-picrylhydrazyl radical, **34** ($DPPH^{\bullet}$) and caffeic acid, **9** from Sigma-Aldrich Chemie (Steinheim, Germany); rosmarinic acid, **11** from Extrasynthése (Genay, France); rutin trihydrate, **15** (90%) from Fluka (Buchs, Switzerland); chlorogenic acid, **12** from Carl Roth (Karlsruhe, Germany). For compounds numbering see Chapters 1 and 3. Ultra pure water (0.05 μS/cm) was obtained from a combined Seradest LFM 20 and Serapur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). Before use in the HPLC system, all samples solutions and solvents were membrane filtered (0.45 μm, Type RC 55, Schleicher & Schuell, Dassel, Germany). During the HPLC runs, solvents were continuously degassed by sparging with helium.

6.2.2 Plant material and extraction

Above-ground parts of *S. syriaca* or *S. scardica* were collected at flowering stage at the experimental field of the Botanical Garden of the Institute of Botany, Bulgarian Academy of Sciences, Lozen, Bulgaria. Cultivars from *S. syriaca* 16 (Co 1124) and *S. scardica* 14 (SOM 3154v) (see also Chs. 2 and 3) were dried at room temperature under forced ventilation in closed premises and ground before use. Voucher specimens were determined by Dr. L. Evstatieva and deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Sofia.

The finely ground, dried plant material (aerial parts) of *S. syriaca* (0.57 kg) was macerated with MeOH (2 × 12 L, each extraction 48 hrs, room temperature, under N_2 bubbling, shaking) = methanolic extract (ME). After filtration, the extracts were combined and evaporated *in vacuo* at 50 °C till dryness. The yield was 93.3 g. The plant material, after the extraction with MeOH, was macerated with hot water (5.5 L, 90 ºC) under N_2 bubbling for 15 min and then freeze-dried = hot water extract (hWE, yield 27.5 g). The methanolic extract (93.3 g) was dispersed in warm water (0.3 L, 50 ºC). The obtained aqueous dispersion was successively extracted with *t*-butyl methyl ether (*t*-BuOMe) (6 × 0.25 L) = BuOMe (16 g), EtOAc (14 × 0.3 L) = EAE (12.2 g) and 1-BuOH (10 × 0.3 L) = BE (34 g). The solvents of the obtained sub-extracts were evaporated *in vacuo* to dryness. The remaining water layer was freeze-dried = WE-1 (22.7 g). Fractions were monitored and screened for the presence of radical scavenging compounds by qualitative TLC under UV light (254 nm) and spraying with a 1 mM methanolic solution of $DPPH^{\bullet}$. Active components give yellow spots on a purple background. The ME, EAE and BE sub-extracts were the most active. BE was partitioned between warm water and EtOAc:BuOH = 1:4. As result, a Bu/EA solution (~31 g) (see Fig. 6.1) and a water-soluble fraction = WE-2 (~2.5 g) were obtained. The Bu/EA solution was subjected to VLC on Si gel (SiO_2) as stationary phase and EtOAc–MeOH mixtures (0 to 100% MeOH) as eluents. Fifty five fractions of ca. 0.4 L were collected. Fraction 8 (218 mg) (EtOAc-MeOH = 80:20) was rechromatographed by column chromatography (CC) on Sephadex LH-20 (Seph-1) with 40 → 60% MeOH to give **1** (100 mg). The most active TLC-DPPH-screened fractions 13-15 (6.30 g) were pooled and 5 g were rechromatographed by CC on Si gel (SiO_2-1) using $CHCl_3$:MeOH:H_2O = 60:22:4 → 6:4:1. 249 fractions were collected among them frs. 15-20 (**2**, 170 mg); frs. 21-32; frs. 33-48 (**3**, 1110 mg); frs. 49-63; frs. 64-69; frs. 84-145; frs. 176-195 and frs. 196-247. Frs. 1-14 and 248-249 were discarded as they did not contain any compounds. Frs. 21-32 were rechromatographed by CC on Si gel (SiO_2-2a) to give **2**, **3**, and a mixture. The latter was rechromatographed by CC on Sephadex LH-20 (Seph-3) with 50 → 100%

MeOH to give **4** (14 mg), a mixture and **2**. To separate the mixture, it was twice rechromatographed by CC on Sephadex LH-20 (Seph-5) with 40 → 60% MeOH to give **5** (9 mg) and **6** (9.5 mg), respectively. Frs. 49-63 were separated by CC on Si gel (SiO_2-1a) using $CHCl_3$:MeOH:H_2O = 60:22:4 → 100% MeOH to give **3** (46 mg), **7** (112 mg) and a mixture. To separate the latter they were rechromatographed by CC on Si gel (SiO_2-1b) using the same solvent system to give some more **3** and the unknown **8** (43 mg). Frs. 64-69 were rechromatographed by CC on Sephadex LH-20 (Seph-2) with 50 → 100% MeOH to give more **8** (22 mg) and a mixture. To purify the mixture, it was rechromatographed on Sephadex LH-20 (Seph-2a) with 30% MeOH to give **9** (30 mg). Frs. 70-175 did not yield any pure compounds. Frs. 176-195 contained several compounds including derivatives of verbascoside. They were further separated by MPLC and LC-NMR. Detailed spectroscopic studies to elucidate them are on-going and will be reported elsewhere. The WE-1 (22.7 g) was subjected to $MPLC_{RP\text{-}18}$ with 3% MeCN to give melittoside (**10**, 15 mg). In Figure 6.1 a schematic fractionation of *S. syriaca* is presented.

The finely ground, dried plant material (aerial parts) of *S. scardica* (0.29 kg) was subjected to exhaustive Soxhlet extraction with *t*-BuOMe (1.5 L) for 5 hours = BuOMe-Sox extract (0.607 g). The residual plant material (0.29 kg) was extracted with MeOH (2 x 0.8 L, each extraction 48 hrs, room temperature, under N_2 bubbling, shaking) = methanolic extract (ME, 3.78 g). Finally, the residual plant material was extracted with hot water (350 mL, 15 min, 90 ºC) = hWE (2.13 g). To an aliquot of sample (2.77 g) from ME, warm water (80 mL, 50 ºC) was added and subsequently it was extracted with *t*-BuOMe (160 mL) = BuOMe (0.31 g), EtOAc (260 mL) = EAE (0.35 g), 1-BuOH (260 mL) = BE (0.82 g). The remaining aqueous phase was freeze-dried = WE (1.13 g). The BuOMe-Sox extract (0.607 g) was separated by CC on Si gel with eluents of increasing polarity PE-EtOAc (100% PE → PE-EtOAc = 1:1 → 100% EtOAc). In total 107 frs. were collected. Fractions 81-82, which were eluted with PE-EtOAc = 1:1, gave almost pure **11** (siderol). Siderol was purified by means of preparative TLC using PE:EtOAc = 30:70. Four frs. were collected and fr. S1 gave pure siderol (**11**, 17.6 mg). In Figure 6.2 a schematic presentation of the fractionation of *S. scardica* is given. From *S. scardica*-BE compounds **2** (29 mg), **3** (104 mg), **3** + **7** (22 mg), **1** (148 mg) and **12** were isolated. Compound **12** needed further purification. It was separated by CC on Polyamide 6S (PA-B) using 30 → 100% MeOH and finally 10 mg were obtained from **12**. *S. syriaca* and *S. scardica* showed similar patterns of radical scavenging compounds in the four extracts studied: ME, EAE, BE and hWE extracts (see also Chs. 2 & 3).

6.2.3 Thin-layer chromatography

Analytical TLC-I of extracts and purified compounds was performed on Si gel pre-coated (0.2 mm) 60 F_{254} aluminum- or plastic backed TLC plates (Merck, Darmstadt, Germany). For the development several mobile phases were used: $CHCl_3$:MeOH:H_2O (60:22:4; 6:4:1); PE:Et_2O = 1:1; PE:EtOAc = 80:20; 30:70. The compounds were visualized by UV (254 nm), by iodine vapours or by heating at 105 ºC. For qualitative detection of radical scavenging compounds, the TLC plates were sprayed with a 1 mM methanolic solution of $DPPH^{\bullet}$. Radical scavengers produced yellow spots on a purple background.

Analytical TLC-II was performed on the same plates as analytical TLC-I. In 2004 the purity of the compounds **1** − **9**, **12** were checked to see whether any decomposition had occurred since their isolation in 2000. All compounds were still pure except for compound **4** where two spots were present. Compound **4** was not subjected further to HPLC analysis.

Preparative TLC**:** PSC Kieselgel 60 F_{254S}; 20 x 20 cm; 0.5 mm with conc. zone 4 x 20 cm; PE:EtOAc = 30:70.

6.2.4 Chromatographic separation and isolation

- ***Open column chromatography (CC):*** Si gel (SiO_2), Kieselgel 60; Pharmacia Sephadex LH-20; Polyamide 6S (Riedel-de Haën AG, Seelze, Germany) (see Figs. 6.1 & 6.2, and the text from Plant material & extraction Section).
- ***Vacuum liquid chromatography (VLC)*****:** Si gel, Kieselgel 60.
- ***Medium Pressure Liquid Chromatography* (*MPLC*)** of:
  - *S. syriaca*-WE: Gilson 303 pump, manometric module 804C, Gilson fraction collector 202, Jobin Yvon 50 x 4 cm axial compression column, sample 0.6 g; RP-18 (Baker Bond Phase C18, J.T. Baker Mallinckrodt Baker B.V., Deventer, Holland), 40 μm particle size; mobile phase 3% MeCN in H_2O; flow rate 5 mL/min, 10 bar, 115 frs. of 20 mL.
  - *S. syriaca*-Bu/EA solution, RP-18 (Baker Bond Phase C18, J.T. Baker Mallinckrodt Baker B.V., Deventer, Holland), 15 → 50% MeOH in H_2O.
- ***Semi-preparative HPLC:*** the separation of MPLC of *S. syriaca*-WE was followed by semi-preparative HPLC with UV detector (215 nm) with mobile phase 3% MeCN in H_2O, Alltima C18, 5 μm, 250 x 4.6 mm with a guard column and filter, injection volume 225 μL, Alltech Associates (Breda, Holland), flow rate 1 mL/min.
- ***Analytical HPLC-I:*** Alltima C18, 5 μm, 250 x 4.6 mm with a guard column and filter; 92% **A** (10% MeCN in H_2O) and 8% **B** (MeCN); flow rate 0.8 mL/min; injection volume 20 μL; UV detection 254 nm.

- ***Analytical HPLC-II:*** Lichrosorb C18, 5 μm, 250 x 4.6 mm with a guard column and filter; solvent system, flow rate and injection volume were the same as for HPLC-I; UV detection with a fixed wavelength of 280 nm.
- ***LC-NMR:*** Alltima C18, 5 μm, 150 x 4.6 mm; flow rate 0.8 mL/min; **A:** H_2O, **B:** MeCN, **C:** D_2O; from 0 to 5 min linear gradient from 95% **A** + 5% **B** to 80% **A** + 20% **B**; from 5 min to 10 min 80% **A** + 20% **B** isocratic; from 10 to 11 min linear gradient from 80% **A** + 20% **B** to 80% **C** + 20% **B**; from 11 to 30 min linear gradient from 80% **C** + 20% **B** to 65% **C** + 35% **B**; from 30 to 35 min linear gradient from 65% **C** + 35% **B** to 100% **B**; from 35 min to 42 min 100% **B** isocratic; from 42 to 47 min linear gradient from 100% **B** to 95% **A** + 5% **B**; from 47 min to 55 min 95% **A** + 5% **B** isocratic for re-equilibration of the column before the next injection; oven temperature 20 ºC, injection volume 60 μL, Bruker LC22 pump, Bruker DAD.
- ***HPLC-DPPH:*** The column was the same as for analytical HPLC-I. Flow rate 0.8 mL/min; **A**: 5% MeCN in water, **B**: MeCN; Linear gradient from 100 to 80% **A** for 5 min; 5 min 80% **A**, isocratic; 20 min linear gradient from 80 to 65% **A**; 10 min isocratic at 65% **A**; 10 min linear gradient from 65 to 0% **A**; 5 min 0% **A**, isocratic; 5 min linear gradient from 0 to 100% **A** and 10 min isocratic at 100% **A** for re-equilibration of the column before the next injection (Gradient 9). Flow 2.10^{-5} M $DPPH^{\bullet}$, 0.8 mL/min; detection at 330 nm (UV) and 515 nm ($DPPH^{\bullet}$).

6.2.5 Radical scavenging activity (RSA) assays

The RSA towards $DPPH^{\bullet}$ of the isolated substances and reference compounds – rutin, rosmarinic acid, caffeic acid and chlorogenic acid, was determined. The experimental protocol used was as follows:

Off-line DPPH

One mL 10^{-4} M methanolic solution of $DPPH^{\bullet}$ and 200 μL of putative antioxidant (1 mg/mL; mass ratio AO:$DPPH^{\bullet}$ = 5:1), 200 μL of reference (0.5 mg/mL; mass ratio AO:$DPPH^{\bullet}$ = 2.5:1) or 10 μL of fractions (1 mg/mL) + 190 μL MeOH, were shaken and allowed to stand for 10 min in the dark at room temperature and the decrease of absorbance at λ = 515 nm was measured against methanol using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The initial absorbance at 515 nm was 0.870 ± 0.030. The absorbance of a blank sample containing the same amount of MeOH and $DPPH^{\bullet}$ solution was prepared and measured daily. $DPPH^{\bullet}$ solution was freshly prepared prior to each experiment and kept in the dark at 4 °C between the measurements. All determinations were performed in

triplicate. The RSA of the tested samples, expressed as % Reduction of DPPH$^{\bullet}$, was calculated according to the formula:

$$\% \text{ Reduction} = [(A_B - A_A) / A_B] \times 100$$

where A_B is the absorbance of the blank sample (t = 0), and A_A is the absorbance of the tested sample after 10 min (Cotelle et al. 1996; Pekkarinen et al. 1999; Schwarz et al. 2001).

On-line detection of radical scavengers

On-line HPLC detection (see Section 6.2.4 and Ch. 3) was performed using the methods of Koleva et al. (2000) and Dapkevicius et al. (2001). The profiles of the BE sub-extracts of *S. syriaca* and *S. scardica* are presented in Figures 6.7 & 6.8. The chromatographic and DPPH conditions are presented in Section 6.2.4 and Chapters 3 & 4.

6.2.6 Structure elucidation of isolated compounds

The following spectroscopic techniques were used for the elucidation of the purified compounds: UV, FT-IR, FAB-MS, ESI(negative)-MS, ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, TOCSY, ROESY, HMQC, HMBC) in addition to comparison with reference compounds and literature data. Ultraviolet (UV) spectra were recorded in methanol on a Perkin-Elmer UV/Vis spectrophotometer Lambda 15 (Perkin-Elmer Instruments, Norwalk, CT, USA) using quartz cells of 1-cm path length. Infrared (IR) spectra were performed on a FT-IR Perkin-Elmer 1750 spectrometer (Perkin-Elmer Instruments, Norwalk, CT, USA) using a capillary layer (c.l.) in MeOH or KBr plates. Only characteristic absorptions (ν, cm^{-1}) are reported. One-dimensional off-line NMR spectra were recorded on a Bruker DPX 300 or Bruker Avance 400 NMR spectrometer (Bruker Daltonik GmbH, Rheinstetten, Germany) using tetramethylsilane (TMS) and CD_3OD, DMSO-d_6 or $CDCl_3$. LC-NMR was carried out with a Bruker LC22 pump, Bruker DAD detector, Bruker loop storage, and Bruker Avance 400 MHz NMR spectrometer equipped with an LC-NMR probe 60 μL measuring volume. The system was controlled by HystarNT^{TM} software. Off-line fast atom bombardment (FAB-MS) was done on a Finnigan Matt 95 and spectra were recorded dissolved in a glycerol matrix (scan range from *m/z* 30 to *m/z* 900). Infusion ESI-Mass spectra (MS) were recorded on a Thermo Finnigan LCQ. LC/ESI-MS was done on an LCQ iontrap from Thermoquest with electrospray ionization (ESI-negative mode).

The following compounds were isolated:

Verbascoside (syn. acteoside, kusaginin) (1) ($C_{29}H_{36}O_{15}$, MW 624): Light-brown amorphous powder; *UV* λ_{max}^{MeOH} (nm): 326, 287s, 246s; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3349 (OH), 2937 (C-H), 1699 (conj. ester), 1631 (C=C), 1604, 1525 (aromatic ring); R_t* (see p. 156 for explanation) = 21.5 min; *m/z (rel. int. %) FAB-MS*: 115 (100), 133 (73), 137 (45), 163 (82), 186 (51), 207 (68), 302 (19), 325 (95), 471 (22), 625 (29), 647 $[M+Na]^+$ (15); *ESI-MS (−):* 623 $[M-H]^-$ (100). For NMR see Tables 6.3 & 6.4. It was compared to literature data (Miyase et al. 1982; Ezer et al. 1992; Gao et al. 1999; de Santos-Galindez et al. 2000).

Hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside (2) ($C_{32}H_{36}O_{19}$, MW 724): Yellow amorphous powder; *UV* λ_{max}^{MeOH} (nm): 339, 297s, 276, 254; *IR* ν_{max}^{KBr} (cm^{-1}): 3410 (OH), 2920 (C-H), 1739, 1661 (C=C), 1615 (-O-, aromatic ring), 1587 (aromatic ring), 839 (trisubstituted aromatic ring); R_t = 36.5 min; *m/z (rel. int. %) FAB-MS*: 301 (8), 315 (5), 316 (38), 317 (100), 725 $[M-H]^-$ (75); *ESI-MS (−):* 723 $[M+H]^+$ (100). For NMR see Tables 6.1 & 6.2. It was compared with authentic **2** and also to literature data (Lenherr & Mabry 1987; Sattar et al. 1993).

Hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside (3) ($C_{30}H_{34}O_{18}$, MW 682): Yellow amorphous powder; *UV* λ_{max}^{MeOH} (nm): 341, 297s, 275, 254; *IR* ν_{max}^{KBr} (cm^{-1}): 3417 (OH), 2922 (C-H), 1744, 1662 (C=C), 1618 (-O-, aromatic ring), 1589 (aromatic ring), 1272 (OMe), 839 (trisubstituted aromatic ring); R_t = 28 min; *m/z (rel. int. %) FAB-MS*: 282 (8), 316 (27), 317 (56), 318 (13), 338 (6), 683 $[M-H]^-$ (100); *ESI-MS (−):* 315 (10), 681 $[M+H]^+$ (100). For NMR see Tables 6.1 & 6.2. It was compared to authentic **3** and also to literature data (Sattar et al. 1993; Venturella et al. 1995; Marin et al. 2004; Albach et al. 2005).

Isoscutellarein-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside (4) ($C_{30}H_{34}O_{17}$, MW 666): Deep-brown jelly-like solid; *UV* λ_{max}^{MeOH} (nm): 321s, 303, 276; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3367 (OH), 2917 (C-H), 1733, 1654 (C=C), 1607 (-O-, aromatic ring), 1219 (OMe), 833 (*p*-disubstituted aromatic ring); R_t = 35.7 min; *m/z (rel. int. %) FAB-MS*: 43 (9), 115 (8), 285 (10), 300 (43), 301 (100), 302 (19), 316 (8), 317 (16), 667 $[M+H]^+$ (56); *ESI-MS (−):* 299 (16), 665 $[M-H]^-$ (100). For NMR see Tables 6.1 & 6.2. It was compared to literature data (Ezer et al. 1992; Albach et al. 2003; Marin et al. 2004; Meremeti et al. 2004; Saracoglu et al. 2004; Gabrieli et al. 2005).

Luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside (5) ($C_{30}H_{34}O_{17}$, MW 666): Off-white amorphous powder; *UV* λ_{max}^{MeOH} (nm): 344, 265s, 252; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3339 (OH), 2918 (C-H), 1729, 1658 (C=C), 1609 (-O-, aromatic ring), 1260 (OMe), 823 (trisubstituted aromatic ring); R_t = 27.5 min; *m/z (rel. int. %) FAB-MS*: 85 (15), 115 (100), 171 (16), 207 (91), 263 (15), 299 (14), 300 (28), 301(72), 302 (15),

667 $[M+H]^+$ (95); *ESI-MS (−):* 299 (45), 665 $[M-H]^-$ (100). For NMR see Tables 6.1 & 6.2 and Figure 6.6(a).

Hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside (6) ($C_{30}H_{34}O_{18}$, MW 682): Deep-brown jelly-like solid; *UV* λ_{max}^{MeOH} (nm): 337, 297s, 278, 254; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3392 (OH), 1733, 1657 (C=C), 826 (trisubstituted aromatic ring); R_t = 31.2 min; *m/z (rel. int. %) FAB-MS*: 84 (17), 85 (93), 115 (100), 169 (46), 177 (27), 207 (34), 316 (28), 317 (63), 318 (13), 683 $[M+H]^+$ (16); *ESI-MS (−):* 315 (10), 681 $[M-H]^-$ (100). For NMR see Tables 6.1 & 6.2 and Figure 6.6(b).

Isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside (7) ($C_{29}H_{32}O_{17}$, MW 652): Yellow amorphous powder; *UV* λ_{max}^{MeOH} (nm): 326, 306, 275, 226; *IR* ν_{max}^{KBr} (cm^{-1}): 3435 (OH), 2924 (C-H), 1736 (ester C=O), 1658 (C=C), 1609 (-O-, aromatic ring), 1588 (aromatic ring), 835 (*p*-disubstituted aromatic ring); R_t = 27.2 min; *m/z (rel. int. %) FAB-MS*: 85 (16), 115 (31), 133 (27), 151 (32), 163 (18), 207 (15), 286 (55), 287 (100), 653 $[M+H]^+$ (98); *ESI-MS (−):* 651 $[M-H]^-$ (100). For NMR see Tables 6.1 & 6.2. It was compared to literature data (Lenherr & Mabry 1987; El-Ansari et al. 1991; Sattar et al. 1995; Rodriguez-Lyon et al. 2000; Albach et al. 2003; Marin et al. 2004; Meremeti et al. 2004; Saracoglu et al. 2004; Albach et al. 2005; Gabrieli et al. 2005; Sahin et al. 2006).

3''-O-*p*-Coumaroyl-6'''-O-acetyl melittoside (8) ($C_{32}H_{40}O_{18}$, MW 712): Deep-brown jelly-like solid; *UV* λ_{max}^{MeOH} (nm): 310, 226s; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3393 (OH), 2943 (C-H); R_t = 18.2 min; *m/z (rel. int. %) FAB-MS*: 91 (24), 106 (20), 107 (24), 115 (85), 131 (23), 147 (100), 165 (30), 177 (21), 351 (15), 735 $[M+Na]^+$ (20); *ESI-MS (−):* 623 (20), 637 (40), 711 $[M-H]^-$ (100). For NMR see Tables 6.3 & 6.4 and Figure 6.6(c).

Hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside (9) ($C_{29}H_{32}O_{18}$, MW 668): Yellow-brown amorphous powder; *UV* λ_{max}^{MeOH} (nm): 345, 329s, 274, 254s; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3391 (OH), 2918 (C-H), 1723, 1659 (C=C), 1611 (-O-, aromatic ring), 1587 (aromatic ring), ~1272 (OMe), 839 (trisubstituted aromatic ring); R_t = 23.9 min; *m/z (rel. int. %) FAB-MS*: 115 (100), 171 (11), 207 (71), 263 (8), 299 (9), 302 (17), 303 (36), 669 $[M+H]^+$ (27); *ESI-MS (−):* 667 $[M-H]^-$ (100). For NMR see Tables 6.1 & 6.2. It was compared to literature data (Lenherr & Mabry 1987; Rodriguez-Lyon et al. 2000; Marin et al. 2004).

Melittoside (10) ($C_{21}H_{32}O_{15}$, MW 524): Off-white amorphous powder; *UV* λ_{max}^{H2O} (nm): 208; *IR* ν_{max}^{KBr} (cm^{-1}): 3401 (OH), 2906 (C-H), 1653 (C=C), 1073 (-O-); *^{1}H NMR* (DMSO-d_6, 250 MHz) δ (ppm): 5.40 (1H, d, J = 3.5 Hz, H-1), 6.32 (1H, d, J = 7 Hz, H-3), 5.07 (1H, d, J = 7 Hz, H-4), 4.22 (1H, bs, H-6), 5.64 (1H, bs, H-7), 3.36 (1H, bs, H-9), 4.53, 4.40 (2H, dd, J = 2 x 7.6, H_2-10), 3.01-3.68 (sugars); *^{13}C NMR* (CD_3OD, 250 MHz) δ (ppm): 93.2 (C-

1), 142.5 (C-3), 104.5 (C-4), 79.1 (C-5), 78.9 (C-6), 127.2 (C-7), 146.3 (C-8), 50.5 (C-9), 60.0 (C-10), 98.7 (C-1'), 74.8 (C-2'), 77.1 (C-3'), 69.8 (C-4'), 76.2 (C-5'), 61.1 (C-6'), 97.1 (C-1''), 74.1 (C-2''), 77.5 (C-3''), 70.7 (C-4''), 77.2 (C-5''), 61.7 (C-6''); *m/z (rel. int. %) FAB-MS*: 547 $[M+Na]^+$ (100). It was compared to authentic melittoside and also to literature data (Swiatek et al. 1981; Bianco et al. 1983; Matsumoto et al. 1989).

Siderol (11) ($C_{22}H_{34}O_3$, MW 346): White amorphous powder; *UV* λ_{max}^{hexane} (nm): 211; *IR* ν_{max}^{KBr} (cm^{-1}): 3471 (OH), 2993 (C-H), 1708 and 1268 (AcO), 1644 (C=C), 3059 and 817 (trisubstituted C=C); *^{1}H NMR* ($CDCl_3$, 250 MHz) δ (ppm): 0.71 (s, Me), 1.09 (s, Me), 1.72 (d, J = 1.7 Hz, allylic Me), 2.09 (s, AcO), 3.00 and 3.34 (q, J = 11 Hz, −C−C<u>H</u>$_2$OH), 4.71 (t, 6 Hz, −C<u>H</u>OAc), 5.27 (bs, allylic −C<u>H</u>=C−); *^{13}C NMR* ($CDCl_3$, 250 MHz) δ (ppm): 15.38 (C-17), 17.35 (C-19), 17.75 (C-20), 17.90 (C-11), 18.27 (C-2), 21.47 (Ac-Me), 23.46 (C-6), 24.75 (C-12), 35.18 (C-3), 36.96 (C-4), 39.06 (C-10), 39.81 (C-13, C-14), 41.96 (C-1), 44.81 (C-5, C-9), 51.82 (C-8), 71.35 (C-18), 78.26 (C-7), 129.76 (C-15), 143.79 (C-16), 170.81 (CO). It was compared to authentic siderol and also to literature data (Piozzi et al. 1968).

Leucosceptoside A, 3'''-O-methyl-verbascoside (12) ($C_{30}H_{38}O_{15}$, MW 638): Off-white amorphous powder; *UV* λ_{max}^{MeOH} (nm): 326, 292s, 229s; *IR* $\nu_{max}^{c.l.}$(cm^{-1}): 3300 (OH), 2927 (C-H), 1695 (conj. ester), 1635 (C=C), 1600, 1516 (aromatic ring); R_t = 24.7 min; *m/z (rel. int. %) FAB-MS*: 85 (41), 113 (15), 114 (29), 115 (100), 137 (21), 171 (13), 177 (48), 207 (33), 339 (20), 661 $[M+Na]^+$ (19); *ESI-MS (−):* 637 $[M-H]^-$ (30). For NMR see Tables 6.3 & 6.4. It was compared to literature data (Miyase et al. 1982; Ismail et al. 1995; Kirmizibekmez et al. 2005).

(* − R_t (min) for all isolated compounds were obtained with 5% MeCN in water (A) → MeCN (B), Gradient 9 (see Experimental section, item 6.2.4).

6.3 Results and discussion

6.3.1 Isolation and structure elucidation of compounds from S. syriaca and S. scardica extracts

The extraction-fractionation trees of *S. syriaca* and *S. scardica* are presented in Figures 6.1 and 6.2, respectively. The structures of the isolated flavonoids (**2** − **7**, **9**) and phenylpropanoids (**1**, **12**) are given in Figures 6.3 and 6.4, respectively. At the very beginning my studies were focussed on the chemotaxonomy of the genus *Sideritis*. For this reason it was isolated siderol and melittoside. Later my subject changed to "New methods for the screening of antioxidants in three *Sideritis* species" (see 1.6). Although siderol (**11**) and melittoside (**10**) are not antioxidants, their structures were presented in Figure 6.5.

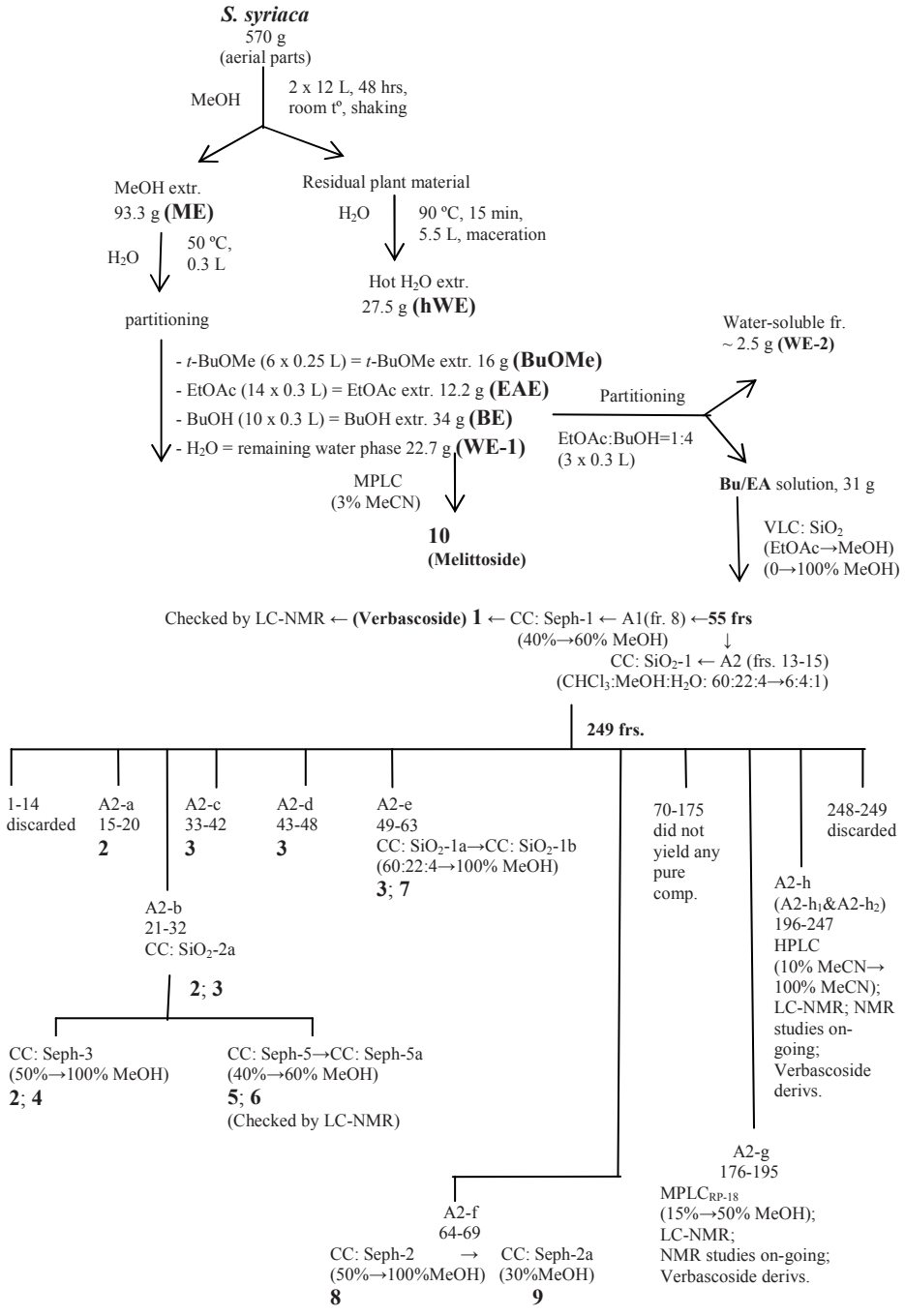

S. syriaca
570 g
(aerial parts)
MeOH
2 x 12 L, 48 hrs,
room tº, shaking
MeOH extr.
93.3 g (ME)
Residual plant material
H2O
90 ºC, 15 min,
5.5 L, maceration
Hot H2O extr.
27.5 g (hWE)
H2O
50 ºC,
0.3 L
partitioning
- t-BuOMe (6 x 0.25 L) = t-BuOMe extr. 16 g (BuOMe)
- EtOAc (14 x 0.3 L) = EtOAc extr. 12.2 g (EAE)
- BuOH (10 x 0.3 L) = BuOH extr. 34 g (BE)
- H2O = remaining water phase 22.7 g (WE-1)
Water-soluble fr.
~ 2.5 g (WE-2)
Partitioning
EtOAc:BuOH=1:4
(3 x 0.3 L)
Bu/EA solution, 31 g
MPLC
(3% MeCN)
10
(Melittoside)
VLC: SiO2
(EtOAc→MeOH)
(0→100% MeOH)
Checked by LC-NMR ← (Verbascoside) 1 ← CC: Seph-1 ← A1(fr. 8) ←55 frs
(40%→60% MeOH)
CC: SiO2-1 ← A2 (frs. 13-15)
(CHCl3:MeOH:H2O: 60:22:4→6:4:1)
249 frs.
1-14
discarded
A2-a
15-20
2
A2-b
21-32
CC: SiO2-2a
2; 3
CC: Seph-3
(50%→100% MeOH)
2; 4
CC: Seph-5→CC: Seph-5a
(40%→60% MeOH)
5; 6
(Checked by LC-NMR)
A2-c
33-42
3
A2-d
43-48
3
A2-e
49-63
CC: SiO2-1a→CC: SiO2-1b
(60:22:4→100% MeOH)
3; 7
A2-f
64-69
CC: Seph-2 → CC: Seph-2a
(50%→100%MeOH) (30%MeOH)
8 9
70-175
did not
yield any
pure
comp.
A2-g
176-195
MPLCRP-18
(15%→50% MeOH);
LC-NMR;
NMR studies on-going;
Verbascoside derivs.
A2-h
(A2-h1&A2-h2)
196-247
HPLC
(10% MeCN→
100% MeCN);
LC-NMR; NMR
studies on-
going;
Verbascoside
derivs.
248-249
discarded

Figure 6.1 (previous page) Schematic presentation of fractionation of *S. syriaca.* [All column chromatography (CC) on Si gel (SiO_2) was performed with $CHCl_3$:MeOH:H_2O = 60:22:4 → 6:4:1; Polyamide (PA); Sephadex LH-20 (Seph); Vacuum liquid chromatography on Si gel (VLC); HPLC; $MPLC_{RP-18}$].

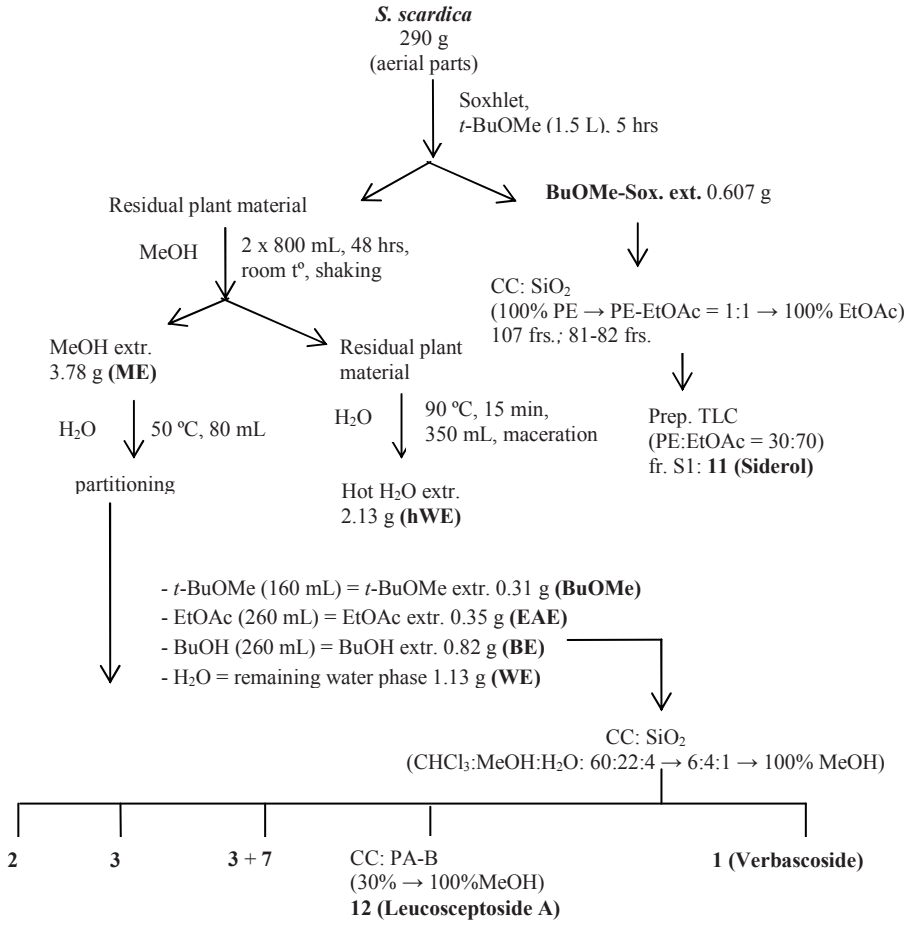
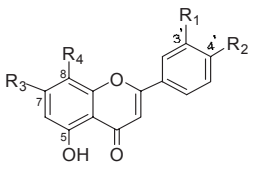


Figure 6.2 Schematic presentation of fractionation of *S. scardica* [Column chromatography (CC) on: Si gel (SiO_2); Polyamide (PA); Thin Layer Chromatography (TLC)].

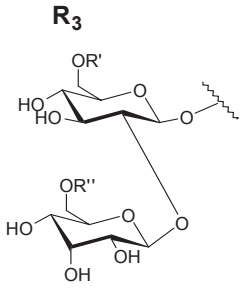
Proton (^1H) and carbon (^{13}C) NMR spectra of flavonoids (**2** − **7**, **9**) are given in Tables 6.1 and 6.2 and ^1H- and ^{13}C NMR spectra of phenylpropanoid (**1**, **12**) and iridoid (**8**)

glycosides are presented in Tables 6.3 and 6.4. Relevant parts of the HMBC spectrum of flavonoids **5**, **6** and iridoid glycoside **8** are presented in Figure 6.6 (a-c), respectively.

For *S. syriaca* it was decided to follow the fractionation scheme "MeOH" depicted in Figure 3.1. It worked fine and it led to some poorly active and very active radical scavenging fractions (sub-extracts) (Table 3.4). Most of the activity was concentrated in the ethyl acetate (EAE), butanol (BE) and hot water fractions (hWE) with % DPPH reduction values of around 90%. Preliminary research described in Chapter 3 has already shown that there is considerable overlap in the constituents present in the EAE, BE and hWE. Of these 3 active fractions the BE was the largest one (34 g) and therefore initially I focused my attention on the BE fraction. As this fraction still contained some troublesome polar constituents (sugars) that interfered with chromatography, one more partitioning step was carried out to remove the interfering "polars". On a weight basis more than 90% of the constituents remained in the organic layer. This fraction was separated by vacuum liquid chromatography (VLC) on silica gel and fractions were



	R_1	R_2	R_3	R_4
2	OH	OCH_3	sugars (see R_3)	OH
3	OH	OCH_3	sugars (see R_3)	OH
4	H	OCH_3	sugars (see R_3)	OH
5	OH	OCH_3	sugars (see R_3)	H
6	OH	OCH_3	sugars (see R_3)	OH
7	H	OH	sugars (see R_3)	OH
9	OH	OH	sugars (see R_3)	OH



	R'	R''
2	Ac	Ac

hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside]

3	H	Ac

hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside]

4	H	Ac

isoscutellarein-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside]

5	H	Ac

luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside]

6	Ac	H

hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside]

7	H	Ac

isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside]

9	H	Ac

hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside]

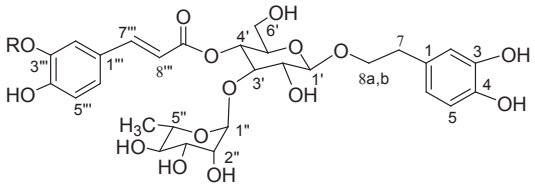
Figure 6.3 Structures of the isolated flavonoids.

Table 6.1 ^{1}H NMR Data for **2**, **3**, **4**, **5**, **6**, **7**, and **9** (measured in DMSO).

	2	**3**	**4**	**5**	**6**	**7**	**9**
Position	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
3	6.79, s	6.79, s	6.92, s	6.83, s	6.81, s	6.83, s	6.70, s
6	6.63, s	6.68, s	6.69, s	6.43, d (2.0)	6.61, s	6.69, s	6.67, s
8	-	-	-	6.77, d (2.0)	-	-	-
2'	7.50, d (2.2)	7.50, d (2.2)	8.10, d (9.1)	7.44, d (2.2)	7.52, d (2.2)	7.98, d (8.9)	7.46, bs
3'	-	-	7.14, d (9.1)	-	-	6.95, d (8.9)	-
4' O-CH_3	3.88, s	3.87, s	3.87, s	3.87, s	3.87, s	-	-
5'	7.11, d (8.6)	7.11, d (8.6)	7.14, d (9.1)	7.11, d (8.6)	7.12, d (8.6	6.95, d (8.9)	6.89, d (8.3)
6'	7.61, dd (8.6, 2.2)	7.60, dd (8.6, 2.2)	8.10, d (9.1)	7.57, dd (8.6, 2.2)	7.61, dd (8.5, 2.1)	7.98, d (8.9)	7.47, dd (8.3, 2.2)
1"	5.17, d (7.6)	5.09, d (7.6)	5.08, d (7.6)	5.24, d (7.3)	5.19, d (7.6)	5.06, d (7.6)	5.08, d (7.6)
2"	3.64, dd (8.6, 7.6)	3.60, dd (8.4, 8.1)	3.59, dd (9.0, 7.8)	3.46, m	3.67, dd (8.6, 7.9)	3.59, dd (9.1, 7.9)	3.60, dd (8.8, 7.6)
3"	3.53, dd (9.1, 8.6)	3.51, m	3.51, dd (9.0, 9.0)	3.52, m	3.53, dd (9.4, 8.6)	3.51, dd (9.1, 8.9)	3.51, dd (9.3, 8.8)
4"	3.28, dd (9.6, 9.1)	3.25, m	3.26, m	3.26, m	3.27, dd (9.4, 9.1)	3.25, m	3.25, m
5"	3.76, m	3.46, m	3.45, m	3.50, m	3.77, ddd (9.3, 7.4, 1.9)	3.45, m	3.44, m
6a"	4.32, dd (11.9, 1.9)	3.73, d (11.1)	3.74, d (10.5)	3.73, m	4.32, dd (12.0, 1.5)	3.73, d (11.3)	3.73, d (10.5)
6b"	4.12, dd (12.1, 7.1)	3.49, m	3.49, m	3.49, m	4.11, dd (11.9, 6.9)	3.49, m	3.49, m
6"acetyl	2.04, s	-	-	-	2.04, s	-	-
1"'	4.92, d (8.1)	4.93, d (8.1)	4.92, d (8.1)	4.79, d (7.8)	4.90, d (7.9)	4.92, d (8.1)	4.92, d (8.1)
2"'	3.25, m	3.25, m	3.26, m	3.19, m	3.21, dd (8.0, 2.6)	3.25, m	3.25, m
3"'	3.91, m	3.92, m	3.91, m	3.88, m	3.88, m	3.92, m	3.91, m
4"'	3.39, dd (9.8, 2.7)	3.39, m	3.40, m	3.31, m	3.49, m	3.41, dd (10.0, 1.9)	3.40, dd (9.9, 2.6)
5"'	3.85, ddd (10.1, 3.6, 3.6)	3.85, ddd (9.8, 3.8, 3.6)	3.87, m	3.76, m	3.61, ddd (9.8, 5.7, 2.3)	3.87, ddd (9.8, 4.1, 2.7)	3.84, ddd (9.8, 3.6, 3.6)
6a"'	3.99, m	4.00, m	4.02, m	4.07, dd (11.7, 1.3)	3.40, m	4.03, m	3.99, m
6b"'	3.99, m	4.00, m	4.02, m	3.97, dd (11.7, 5.4)	3.40, m	4.03, m	3.99, m
6"'acetyl	1.86, s	1.86, s	1.87, s	1.93, s	-	1.87, s	1.86, s
Spectr. freq.	500	500	400	400	500	500	400

Table 6.2 13C NMR Data for 2, 3, 4, 5, 6, 7, and 9 (measured in DMSO, spectral frequency 100 MHz).

Position	**2**	**3**	**4**	**5**	**6**	**7**	**9**
2	164.0	164.1	163.7	164.1	163.9	164.1	164.4
3	103.4	103.4	103.4	103.9	103.4	102.6	102.6
4	182.4	182.5	182.5	182.0	182.4	182.4	182.3
5	152.2	152.3	152.2	161.1	152.4	152.2	152.2
6	99.1	99.4	99.5	99.6	98.6	99.4	99.3
7	150.4	150.8	150.7	162.9	151.0	150.6	150.6
8	127.4	127.6	127.6	94.8	127.1	127.6	127.5
9	144.0	144.0	143.9	157.0	144.5	143.8	143.9
10	105.5	105.6	105.6	105.5	105.3	105.6	105.5
1'	123.1	123.1	122.9	122.9	123.1	121.3	121.3
2'	113.2	113.2	128.6	113.1	113.2	128.7	113.5
3'	146.8	146.8	114.7	146.8	146.8	116.0	145.9
4'	151.3	151.4	162.5	151.4	151.3	161.4	150.4
4' O-CH_3	55.8	55.9	55.7	55.8	55.8	-	-
5'	112.1	112.2	114.7	112.2	112.2	116.0	116.0
6'	119.0	119.1	128.6	118.9	119.0	128.7	119.3
1"	99.4	100.0	100.0	98.9	99.1	100.0	100.0
2"	82.0	82.3	82.6	82.7	80.9	82.6	82.3
3"	75.2	75.6	75.6	75.8	75.4	75.6	75.6
4"	69.4	69.3	69.3	69.1	69.5	69.3	69.3
5"	73.6	77.1	77.2	77.0	73.6	77.1	77.1
6"	63.2	60.6	60.6	60.5	63.2	60.6	60.5
6"acetyl C=O	170.2	-	-	-	170.3	-	-
6"acetyl CH_3	20.6	-	-	-	20.6	-	-
1'''	102.2	102.3	102.6	102.5	101.6	102.7	102.3
2'''	71.4	71.6	71.6	71.7	71.4	71.5	71.6
3'''	70.8	70.9	70.8	70.9	71.0	70.8	70.9
4'''	66.9	67.0	66.9	67.1	67.1	66.8	66.9
5'''	71.4	71.5	71.6	71.5	74.6	71.5	71.6
6'''	63.5	63.5	63.6	64.0	60.9	63.8	63.5
6'''acetyl C=O	170.3	170.4	170.4	170.4	-	170.4	170.4
6''' acetyl CH_3	20.5	20.5	20.5	20.6	-	20.5	20.5

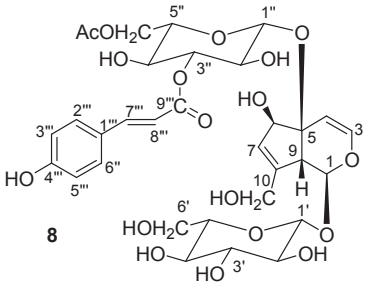


R: H verbascoside, acteoside (**1**)
CH_3 leucosceptoside A, 3'''-O-methyl verbascoside (**12**)

Figure 6.4 Structures of verbascoside (**1**) and leucosceptoside A (**12**).

Table 6.3 ^{1}H NMR Data for **1**, **12**, and **8** (spectral frequency 400 MHz).

Position	**1** δ_H (*J* in Hz)	**12** δ_H (*J* in Hz)	Position	**8** δ_H (*J* in Hz)
Caffeoyl (Feruloyl) moiety			**Iridiod moiety**	
2'''	7.21, d (2.0)	7.21, bs	1	5.46, d (4.9)
3''' O-CH_3	-	3.75, s	3	6.39, d (6.4)
5'''	6.95, d (8.3)	6.75, d (8.1)	4	5.09, d (6.1)
6'''	7.15, dd (8.4, 2.1)	7.04, d (8.3)	6	4.41, bs
7'''	7.71, d (15.9)	7.49, d (15.9)	7	5.75, dd (2.0, 1.5)
8'''	6.41, d (15.9)	6.35, d (15.9)	9	3.27, m
Aglycone moiety			10a	4.23, m
2	6.85, d (2.2)	6.58, m	10b	4.11, m
5	6.85, d (8.1)	6.59, m	**Glucoses**	
6	6.75, dd (8.4, 2.1)	6.45, m	1'	4.58, d (7.8)
7	2.84, m	2.64, m	2'	3.20, dd (9.0, 7.8)
8a	4.08, m,	3.83, m	3'	3.34, dd (9.1, 8.9)
8b	3.86, m	3.55, m	4'	3.24, m
Glucose			5'	3.25, m
1'	4.52, d (8.1)	4.30, d (7.8)	6a'	3.84, m
2'	3.46, dd (9.0, 8.3)	3.16, m	6b'	3.62, m
3'	3.87, dd (9.0, 9.0)	3.64, m	1"	4.77, d (7.8)
4'	4.95, dd (9.5, 9.3)	4.67, dd (9.8, 9.3)	2"	3.44, dd (9.4, 7.9)
5'	3.69, m	3.42, m	3"	5.06, dd (9.3, 9.3)
6a'	3.69, m	3.35, m	4"	3.48, dd (9.8, 9.3)
6b'	3.57, m	3.29, m	5"	3.62, m
Rhamnose			6a"	4.34, dd (11.9, 1.8)
1"	5.10, d (1.7)	4.97, bs	6b"	4.14, dd (12.0, 6.6)
2"	3.99, dd (3.3, 1.8),	3.65, m	6" acetyl	2.07, s
3"	3.63, dd (9.8, 3.4)	3.29, m	**Coumaroyl moiety**	
4"	3.31, dd (9.5, 9.5)	3.06, dd (9.3, 9.3)	2''' + 6'''.	44, d (8.6)
5"	3.49, m	3.34, m	3''' + 5'''	6.77, d (8.6)
6"	1.05, d (6.4)	0.92, d (6.1)	7"	7.63, d (15.9)
			8'''	6.36, d (15.9)
Solvent	MeCN/H_2O	DMSO		CD_3OD



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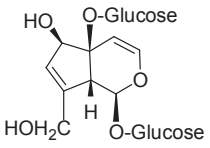
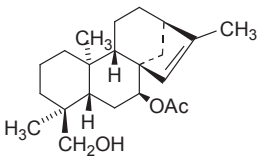


melittoside (**10**)



siderol (**11**)

Figure 6.5 Structures of the isolated 3"-O-*p*-coumaroyl-6"´-O-acetyl melittoside (**8**), melittoside (**10**) and siderol (**11**).

Table 6.4 ^{13}C NMR Data for **1**, **12**, and **8** (spectral frequency 100 MHz).

Position	**1**	**12**	**12**	Position	**8**
Caffeoyl (Feruloyl) moiety				**Iridiod moiety**	
1'''	127.6	127.7	125.8	1	95.3
2'''	114.6	111.8	111.3	3	144.1
3'''	148.0	149.4	148.1	4	105.6
3''' O-CH_3	-	56.5	55.7	5	81.2
4'''	149.8	150.8	149.5	6	81.0
5'''	116.5	116.3	115.7	7	128.0
6'''	123.2	124.4	123.4	8	147.2
7'''	146.8	147.9	145.8	9	52.1
8'''	115.1	115.1	114.3	10	61.1
9'''	168.3	168.3	166.1	**Glucoses**	
Aglycone moiety				1'	99.1
1	131.4	131.5	129.4	2'	74.8
2	117.1	117.1	116.5	3'	77.6
3	146.1	146.1	145.1	4'	71.5
4	144.6	144.7	143.7	5'	78.4
5	116.3	116.5	115.7	6'	62.7
6	121.2	121.3	119.8	1''	99.6
7	36.5	36.5	35.2	2''	73.4
8	72.3	72.3	70.5	3''	78.7
Glucose				4''	69.9
1'	104.1	104.2	102.5	5''	75.1
2'	76.2[a]	76.2[c]	74.7	6''	64.6
3'	81.6	81.5	79.4	6'' acetyl C=O	173.3
4'	70.4	70.6[d]	69.3	6'' acetyl CH_3	21.1
5'	76.0[a]	76.0[c]	74.7	**Coumaroyl moiety**	
6'	62.3	62.4	60.9	1'''	127.2
Rhamnose				2'''	131.2
1''	103.0	103.0	101.5	3'''	116.8
2''	72.3[b]	72.3[e]	70.7	4'''	161.3
3''	72.1[b]	72.1[e]	70.5	5'''	116.8
4''	73.7	72.8	71.8	6'''	131.2
5''	70.4	70.4[d]	69.0	7'''	146.7
6''	18.5	18.4	18.3	8'''	115.4
				9'''	169.0
Solvent	CD_3OD	CD_3OD	DMSO		CD_3OD

[a,b,c,d,e] values may have to be interchanged

monitored for composition, purity and activity by TLC-DPPH. One fraction yielded after further purification verbascoside (**1**), a very potent antioxidant. Verbascoside and all other isolated compounds were identified by spectroscopic means (UV, IR, MS, NMR) and, if possible, by comparison with authentic reference materials.

The most active fractions from the VLC separation were pooled and separated by column chromatography on silica gel. In total 249 fractions were collected and these were further separated by various techniques and media. The known hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside]

(**2**), hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**3**), isoscutellarein-4'-methyl-ether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**4**), isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**7**) and, hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**9**) could be isolated. Two new flavonoid glycosides were isolated, namely luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**5**) and hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] (**6**). Additionally a new esterified iridoid was isolated: 3''-O-*p*-coumaroyl-6''-O-acetyl melittoside (**8**). These three compounds have not been reported before as natural products. Their structure elucidation is described below.

Compound **5** was isolated as an off-white amorphous powder. Its UV spectrum in MeOH showed three bands at 252, 265 (shoulder) and 344 nm. It was reminiscent of the UV spectrum of luteolin 7-[6'''-acetylallosyl-(1-2)-glucoside] except for the high wavelength band (344 versus 335 nm) (El-Ansari et al. 1991). The proton NMR spectrum confirmed a luteolin moiety with 2 meta-coupled protons in the A-ring, an H-3 in the C-ring, and 3 protons in the B-ring.

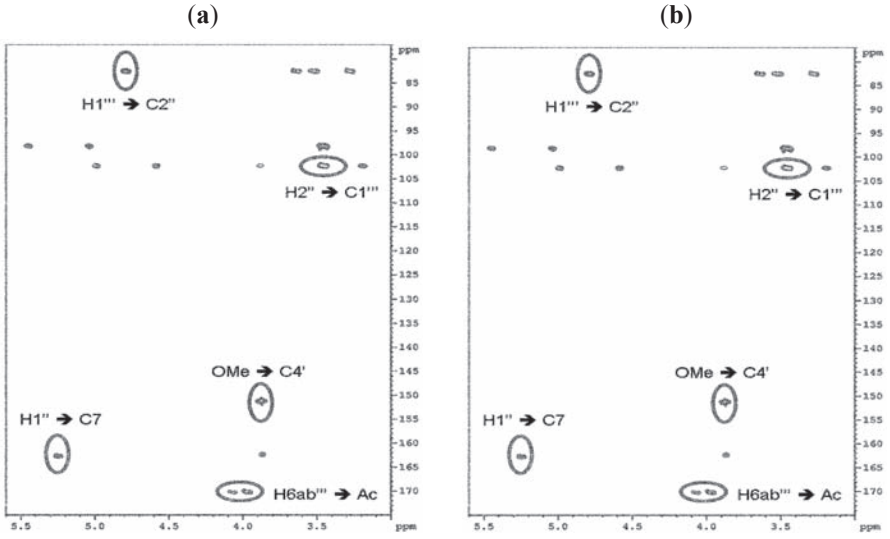


Figure 6.6 Close-ups of HMBC spectra of: (**a**) Luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside (**5**) and (**b**) Hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside (**6**)**.**

Two singlets at 1.93 and 3.87 ppm suggested methoxy and acetyl groups. The molecular weight was determined at 666 Da ($C_{30}H_{34}O_{17}$). This molecular weight corresponds with a methoxylated luteolin, two hexose units and an acetyl group. The fragment at *m/z* 299 in the negative mode ESI-MS confirmed the presence of a methoxylated luteolin moiety (El-Ansari et al. 1991). To determine the nature of the sugar units, and the connection and location of the methoxy, acetyl and sugar units, a complete set of 2D NMR spectra was recorded (COSY, TOCSY, ROESY, HMQC, HMBC). By means of these spectra a full interpretation was possible and all shifts and couplings constants could be assigned (see Tables 6.1 and 6.2 and Fig. 6.6(a) for a close-up of the HMBC spectrum). With the help of the HMBC spectrum all connections could be unequivocally determined. Thus compound **5** was deduced to be luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1'''-2'')-β-D-glucopyranoside]. A comparison with the spectral data of the closely related 6-hydroxy-luteolin-4'-methylether-7-O-α-rhamnopyranosyl-(1'''-2'')[6''-O-acetyl-β-glucopyranoside] (Albach et al. 2003) and 4H-1-benzopyran-4-one, 7-[[2''-O-(6'''-O-acetyl-β-D-allopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4'-hydroxy-3'-methoxyphenyl) (syn. stachyspinoside) (Calis et al. 2002) confirmed this further.

Compound **6** was isolated as a deep-brown jelly-like solid. Its UV spectrum was almost identical to that of **3** and is typical of a hypolaetin-type flavone. The pseudomolecular ions in the (+)-ESI-MS and (–)-ESI-MS were found at 683 $[M-H]^-$, and 681 $[M+H]^+$, respectively. This corresponded to a molecular formula of $C_{30}H_{34}O_{18}$ (MW = 682), i.e. the same as for **3**. Also the fragmentation was similar to that of **3** and highly suggestive of a methoxylated hypolaetin moiety (El-Ansari et al. 1991). At first glance the NMR spectra of **3** and **6** looked similar and confirmed the presence of a hypolaetin type flavone (Lenherr et al. 1984 and Lenherr & Mabry 1987). There were only minor differences in the sugar part, especially around the C-4, C-5, and C-6 atoms. To determine the exact location of sugars, a HMBC spectrum was recorded and all shifts and couplings constants could be assigned (see Tables 6.1 and 6.2 and Fig. 6.6(b) for a close-up of the HMBC spectrum). Thus compound **6** was deduced to be hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside].

Compound **8** was isolated as an amorphous solid. Its UV showed a maximum at 310 nm indicating an extended chromophore like a cinnamoyl unit. This was confirmed by the ^{1}H NMR spectrum that showed two characteristic 1H doublets at 6.36 and 7.63 ppm with a 16 Hz coupling constant. Additionally signals characteristic of a 1,4-disubstituted benzene nucleus, a melittoside aglycone, two sugar units and an acetyl group were observed. The presence of these groups was confirmed by mass spectrometry. The pseudomolecular ions in the (+)-ESI-MS and (–)-ESI-MS were found at 735 $[M+Na]^+$ and 711 $[M-H]^-$,

respectively. This corresponded to a molecular formula of $C_{32}H_{40}O_{18}$ (MW = 712). A closer study of the ^{1}H and ^{13}C NMR spectra showed great similarities with the spectra of melittoside (Swiatek 1981) and sammangaoside (= melittoside-3"-β-glucoside) (Kanchanapoom 2001). Relative to melittoside an extra *p*-coumaroyl and an acetyl unit were present. The acetyl unit had to be present at a 6-position of one of the two glucose moieties because of two double doublets at 4.34 ppm (J =11.9 and 1.8 Hz) and 4.14 ppm (J = 12.0 and 6.6 Hz). To determine the exact location of both the *p*-coumaroyl and acetyl unit, a complete set of 2D NMR spectra was recorded (COSY, TOCSY, ROESY, HMQC, HMBC). By means of these spectra a full interpretation was possible and all

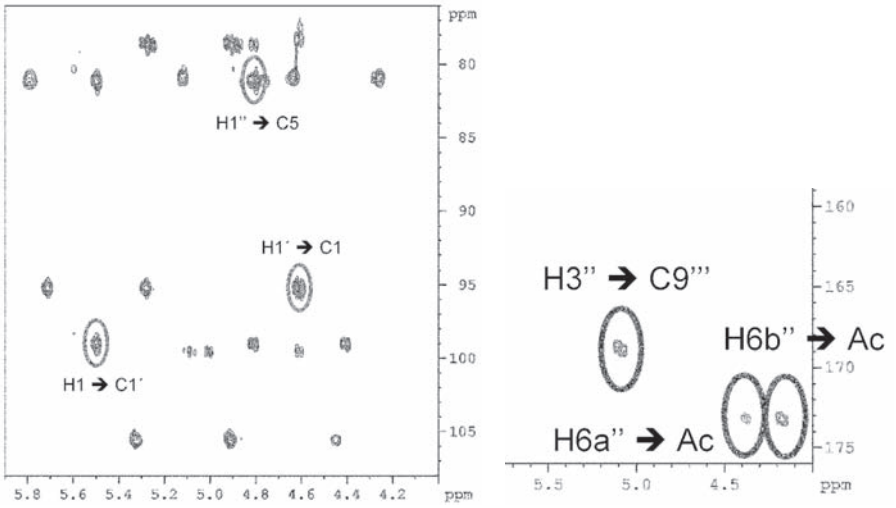


Figure 6.6(c) Close-ups of the HMBC of 3"-O-*p*-coumaroyl-6"-O-acetyl melittoside (**8**).

shifts and couplings constants could be assigned (see Tables 6.3 and 6.4). Most valuable for determining the points of connection of the different units was the HMBC spectrum with clear cross peaks between the carbonyl of the acetyl and both H-6" of the glucose, the carbonyl of the *p*-coumaroyl unit and H-3", the anomeric H-1" and C-5, C-1' and H-1 and C-1 and H-1' (see Fig. 6.6c). Thus compound **8** was deduced to be 3"-O-*p*-coumaroyl-6"-O-acetyl melittoside.

Some minor active constituents from fractions 176-247 could not be isolated in sufficient quantities in pure form and were therefore studied by LC-NMR. They appear to be new derivatives of verbascoside (**1**) with an additional sugar unit. Similar to verbascoside they show strong to medium radical scavenging activity. Spectroscopic

studies are still on-going to elucidate their structures. Due to a lack of time the active fractions EAE and hWE were not studied or fractionated. The complete fractionation scheme of the *S. syriaca*-BE is given in Figure 6.1 and the on-line UV / DPPH chromatograms are given in Figure 6.7. Apart from the new **5**, **6**, and **8** compounds **1**, **4**, **7**, **9** and **12** have not been identified before in *S. syriaca.*

For *S. scardica* a slightly different fractionation scheme was followed. When I started my research in 1998, I extracted all *Sideritis* sp. in order to choose the best extraction scheme. *S. scardica* 14 was extracted by Soxhlet extraction with *t*-BuOMe given the fact that only from this species I had enough material for extraction. The goal of performing a Soxhlet extraction with *t*-BuOMe before the extraction with methanol was to remove many interfering non-polar constituents with marginal activity. Later on, in 2001, I extracted only *S. syriaca* because the results presented in Chapter 3 showed that almost no antioxidant compounds were present in the *t*-BuOMe (Soxhlet) fraction. Moreover, I no longer had sufficient amounts of *S. scardica*. Preliminary research described in Chapter 3 showed considerable overlap in the constituents present in the EAE and BE fractions (Fig. 3.3) and similar to *S. syriaca* I decided to work initially on the BE fraction. By column chromatography the known verbascoside (**1**), hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopy-ranoside] (**2**), hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**3**), isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**7**) and leuco-sceptoside A (**12**) were purified. The full fractionation scheme of the *S. scardica*-BE is given in Figure 6.2 and the on-line UV / DPPH chromatograms are given in Figure 6.8. Due to a lack of time the active fractions EAE and hWE were not studied or fractionated. Apart from the new **5, 6** and **8**, compounds **1**, **4**, **9** and **12** have not been identified before in *S. scardica.*

6.3.2 Radical scavenging activity according to the DPPH method

The radical scavenging activity of the compounds isolated from *S. syriaca* and *S. scardica* has been determined on-line and off-line against DPPH$^{\bullet}$ radical. The on-line results are shown in Figures 6.7 and 6.8 while the off-line results can be found in Table 6.5.

Verbascoside **1** (76% Red.) was the most active antioxidant from all studied samples. It was also a more active radical scavenger than caffeic acid (CA) and rosmarinic acid (RA), two well known, potent natural antioxidants. It was found to be a potent radical scavenger by other authors, too although not the most active among all the phenylpropanoids studied (Xiong et al. 1996; Gao et al. 1999; Takeda et al. 1999).

Fraction A2-a and fraction A2-b gave **2**. As **2** contains a methoxy group in the B-ring and is glycosylated at C7, it shows low radical scavenging activity. However in the on-line HPLC-DPPH method **2** manifested itself among the strongest antioxidants. This might be a matter of concentration.

Table 6.5 Percent Reduction (%) of DPPH$^{\bullet}$ by compounds isolated from *Sideritis* and reference compounds.

Number	mM Antioxidant per mM DPPH$^{\bullet}$	mg Antioxidant per mg DPPH$^{\bullet}$	Reduction (%) of DPPH$^{\bullet}$[a]
1	3.4	5.0	76.3
2	2.9	5.0	15.3
3	3.1	5.0	28.0
5	3.1	5.0	no reduction
6	3.1	5.0	no reduction
7	3.1	5.0	26.4
8	2.9	5.0	18.6
9	3.1	5.0	50.3
12	3.4	5.0	12.4
Reference compounds			
CA	5.9	2.5	70.6
RA	2.9	2.5	65.1
Rutin.3H$_2$O	1.6	2.5	40.3
ChA	2.9	2.5	38.0

RA - rosmarinic acid; *CA* - caffeic acid; *ChA* - chlorogenic acid

[a] - Reduction % values are means of three replicates and the RSD is less than 1%.

The other pure compounds showed low or no activity in the off-line DPPH assay. All of these compounds were less potent radical scavengers than the reference substances. Compound **7** showed some activity against DPPH$^{\bullet}$ (26% Red.). In the on-line HPLC-DPPH method fraction A2-e (see Fig. 6.1) exhibited strong activity mainly due to the presence of **7**. Gabrieli et al. (2005) investigated nine glucosides isolated from *S. raeseri*. Compounds **3** (28% Red.), **4** and **7** (26% Red.) were among those tested and they all showed moderate

activity against DPPH•. They explained the rather low activity for 5,8-dihydroxy flavones by intermolecular hydrogen bond formation between the OH at C-5 and the carbonyl group, and/or by the enhanced inductive effect of the 7-O-glycoside substituent as opposed to a free phenolic group. From all the compounds isolated, compounds **7** and **9** are the only ones that are not O-methylated at C-4' (=R_2; see Fig. 6.3) and it is known that O-methylation reduces antioxidant activity (Robards et al. 1999; Fukumoto & Mazza 2000; Pietta 2000).

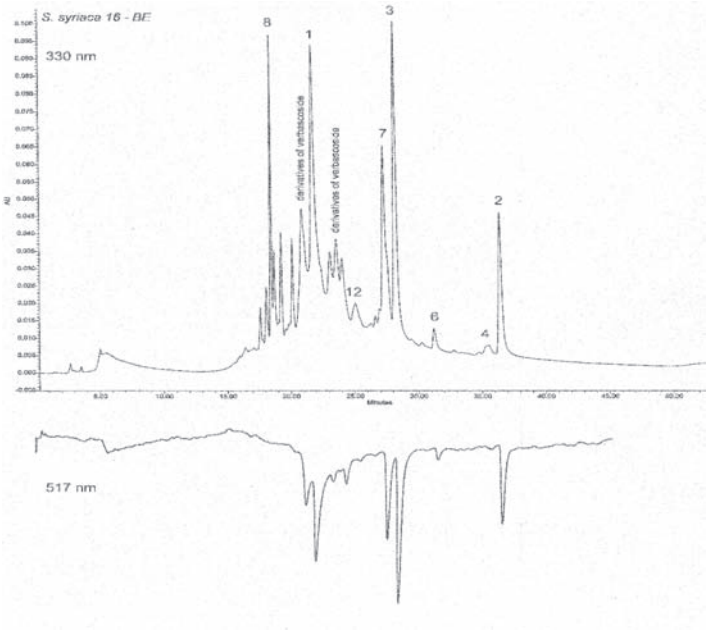


Figure 6.7 Combined plot of the UV and 2.5 x 10^{-5} M DPPH•-reduction chromatograms of *S. syriaca*-BE extract. 30 µL of a 1 mg/mL solution were injected. Chromatographic conditions: see Experimental. Detection at 330 nm and 517 nm.

Compound **5**, which had no hydroxyl group at C-8 (=R_4; see Fig. 6.3) in the A-ring but was otherwise similar to the other compounds showed no reduction in the off-line DPPH method and a low activity in the on-line HPLC-DPPH assay. Compound **6** showed no reduction in the off-line DPPH assay but moderate activity in the on-line version. This must be due to concentration effects.

Fraction A2-f yielded **9** (50% Red.) as main constituent. With its 50% Reduction, compound **9** was less active than CA and RA but more active than rutin and ChA even when expressed in molar ratios. Fraction A2-f also contained **8** but this compound showed

no activity in the on-line HPLC-DPPH assay and weak activity in the off-line DPPH assay. The low radical scavenging activity can be explained by the presence of only a single phenolic group.

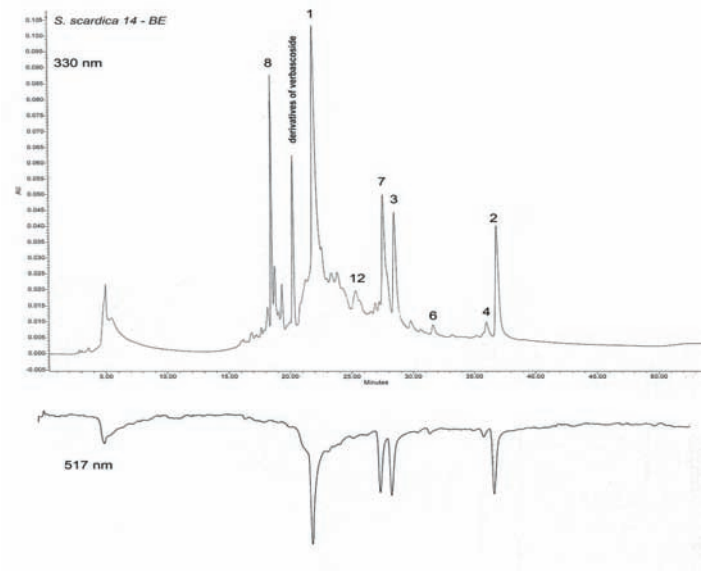


Figure 6.8 Combined plot of the UV and 2.5 x 10^{-5} M DPPH$^{\bullet}$-reduction chromatograms of *S. scardica*-BE extract. 30 µL of a 1 mg/mL solution were injected. Chromatographic conditions: see Experimental. Detection at 330 nm and 517 nm.

For leucosceptoside A (**12**) the discrepancies between off- (12% Red.) and on-line methods (relatively moderate activity, especially in *S. syriaca*-BE) are obviously a matter of concentration. Xiong et al. (1996) studied antioxidant activity of several phenylethanoids isolated from *Cistanche deserticola* against DPPH$^{\bullet}$. Strong activity for verbascoside (**1**) was reported. Additionally cistanoside A, a compound that contains one more glucose unit than leucosceptoside A (**12**) was shown to possess moderate RSA. This lower activity relative to **1** is due to the presence of feruloyl moiety instead of a caffeoyl moiety (Robards et al. 1999; Fukumoto & Mazza 2000; Pietta 2000).

For all fractions and compounds with a lower activity in the off-line assay than in the on-line assay, most likely this is due to concentration differences (e.g. **2**, **6**, **7** and **12**) and/or alteration/degradation during purification procedures (e.g. **4**). The on-line HPLC-DPPH experiments were performed in 2000 while the off-line DPPH experiments were done in 2005.

6.4 Conclusions

The substances present in *S. syriaca* and *S. scardica* possessed very different radical scavenging activities (RSA) − from high through moderate to no RSA based on the on-line and off-line DPPH methods. In the off-line DPPH method the most active radical scavengers were verbascoside (**1**) and hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**9**) (Table 6.5) relative reference compounds. Three novel compounds were isolated from both *Sideritis* species. They were identified as luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**5**), hypolaetin-4'-methylether-7-O-[β-D-allopyrano-syl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] (**6**) and 3''-O-*p*-coumaroyl-6''-O-acetyl melittoside (**8**). The peak of **5** (R_t ~ 27.5 min) appeared as a shoulder of peak **7** in the HPLC-DPPH chromatograms of both *Sideritis* species. Although it was one of the main peaks in the UV-based chromatogram, **8** showed no RSA in the on-line HPLC-DPPH method and low RSA (19% Red.) in the off-line DPPH method.

The discrepancies between off- and on-line experiments might be due to alteration or degradation of active antioxidative constituents during fractionation procedures (see e.g. **4**) or too low concentration in off-line assays (see e.g. **2**, **6**, **7** and **12**) or both. It is notable that in the on-line HPLC-DPPH chromatograms, *S. syriaca*-BE and *S. scardica*-BE showed almost the same profile. *S. syriaca* contained a few more constituents. The antioxidants (radical scavengers) in *S. syriaca*-BE were in order of decreasing activity: hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**3**) > verbascoside (**1**) > isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**7**) > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] (**2**). In *S. scardica*-BE this order was: verbascoside (**1**) > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**3**) ≈ isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**7**) ≈ hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] (**2**). The major peaks as well as some of the minor ones are indicated in the chromatograms in Figures 6.7 and 6.8. Hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] (**9**) (50% Red.) was not visible in either of the chromatograms. Most probably it co-eluted with verbascoside (**1**) as its R_t was 22-23 min. From the NMR experiments carried out so far, other antioxidants (also indicated in Figures 6.7 and 6.8) seem to be derivatives of verbascoside with an extra sugar.

Methanol (ME), ethyl acetate (EAE) and hot water extracts (hWE) from both species were also studied by on-line HPLC-DPPH. ME, EAE and BE showed a similar profile of

constituents. The active constituents in the hWE were not studied in detail due to a lack of time. The compounds responsible for the radical scavenging are possibly very polar, highly glycosylated flavonoids or phenylpropanoids. Overall **1**, **2**, **3**, and **7** were the most active radical scavengers present like in the BE, however their concentrations varied in the different extracts.

Further experiments have to be carried out to see what is more appropriate for application in real systems − an extract/fraction or pure compound(s), and for what type of real systems (foods, cosmetics) they are more suitable i.e. bulk oils, emulsions or both.

6.5 References

Albach D.C., Grayer R.J., Jensen S.R., Ozgokce F., Veitch N.C. (2003). "Acylated flavone glycosides from *Veronica*." Phytochemistry **64:** 1295-1301.
Albach D.C., Grayer R.J., Kite G.C., Jensen S.R. (2005). "*Veronica*: acylated flavone glycosides as chemotaxonomic markers." Biochem. System. Ecol. **33:** 1167-1177.
Altarejos J., Salido S., Perez-Bonilla M., Linares-Palomino P.J., van Beek T.A., Nogueras M., Sanchez A. (2005). "Preliminary assay on the radical scavenging activity of olive wood extracts." Fitoterapia **76**: 348-351.
Barlow S.M. (1990). "Toxicological aspects of antioxidants used as food additives." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London, ch.7.
Bianco A., Passacantilli P., Polidori G., Nicoletti M. (1983). "^{1}H and ^{13}C NMR data of C-6 epimeric iridoids." Org. Magn. Reson. **21:** 460-461.
Calis I., Kirmizibekmez H., Ersoz T., Saracoglu I., Donmez A. A., Mitova M., Handjieva N., Popov S. (2002). "Iridoid, phenylethanoid and flavonoid glycosides from *Phlomis sintensii*." Acta Pharm. Turcica **44:** 195-203.
Cao G., Sofic E., Prior R.L. (1996). "Antioxidant capacity of tea and common vegetables." J. Agric. Food Chem. **44**: 3426-3431.
Chari V.M., Grayer-Barkmeijer R.J., Harborne J.B., Osterdahl B.-G. (1981). "An acylated allose-containing 8-hydroxyflavone glycoside from *Veronica filiformis*." Phytochemistry **20:** 1977-1979.
Cotelle N., Bernier J.-L., Catteau J.-P., Pommery J., Wallet J.-C., Gaydou E.M. (1996). "Antioxidant properties of hydroxy-flavones." Free Rad. Biol. Med. **20**: 35-43.
Dapkevicius A., van Beek T.A., Niederländer H.A.G. (2001). "Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in HPLC eluates." J. Chromatogr. A **912:** 73-82.
El-Ansari M.A., Barron D., Abdalla M.F., Saleh N.A.M., Le Quere J.L. (1991). "Flavonoid constituents of *Stachys aegyptiaca*." Phytochemistry **30:** 1169-1173.
Evans R.J., Reynhout G.S. (1992). "Alternates to synthetic antioxidants." in Food Science and Human Nutrition. (Ed. Charalambous G.), pp 27-42.
Ezer N., Sakar M.K., Rodriguez B., de la Torre M.C. (1992). "Flavonoid glycosides and a phenylpropanoid glycoside from *Sideritis perfoliata*." Int. J. Pharmacognosy **30:** 61-65.
Fukumoto L.R., Mazza G. (2000). "Assessing antioxidant and prooxidant activities of phenolic compounds." J. Agric. Food Chem. **48:** 3597-3604.

Gabrieli C.N., Kefalas P. G., Kokkalou E.L. (2005). "Antioxidant activity of flavonoids from *Sideritis raeseri*." J. Ethnopharmacol. **96:** 423-428.
Gao J.-J., Igalashi K., Nukina M. (1999). "Radical scavenging activity of phenylpropanoid glycosides in *Caryopteris incana*." Biosci. Biotechnol. Biochem. **63**: 983–988.
Ismail L.D., El-Azizi M.M., Khalifa T.I., Stermitz F.R. (1995). **"**Verbascoside derivatives and iridoid glycosides from *Penstemon crandallii.*" Phytochemistry **39:** 1391-1393.
Ivanova D., Gerova D., Chervenkov T., Yankova T. (2005). "Polyphenols and antioxidant capacity of Bulgarian medicinal plants." J. Ethnopharmacol. **96:** 145-150.
Kanchanapoom T., Kasai R., Chumsri Ph., Hiraga Y., Yamasaki K. (2001). "Megastigmane and iridoid glucosides from *Clerodendrum inerme*." Phytochemistry **58:** 333–336.
Kirmizibekmez H., Montoro P., Piacente S., Pizza C., Donmer A., Calis I. (2005). "Identification by HPLC-PAD-MS and quantification by HPLC-PAD of phenylethanoid glycosides of five Phlomis sp." Phytochemical Anal. **16**: 1-6.
Koleva I.I., Linssen J.P.H., van Beek T.A., Evstatieva L., Kortenska V., Handjieva N. (2003). "Antioxidant activity screening of extracts from *Sideritis* species (Labiatae) grown in Bulgaria." J. Sci. Food Agric. **83***:* 809–819.
Koleva I.I., Niederländer H.A.G., van Beek T.A. (2000). "An on-line HPLC method for detection of radical scavenging compounds in complex mixtures." Anal. Chem. **72:** 2323-2328.
Koleva I.I., Niederländer H.A.G., van Beek T.A. (2001). "Application of ABTS radical cation for selective on-line detection of radical scavengers in HPLC eluates." Anal. Chem. **73:** 3373-3381.
Koleva I.I., van Beek T.A., Linssen J.P.H., de Groot Ae., Evstatieva L. (2002). "Screening of plant extracts for antioxidant activity — a comparative study on three testing methods." Phytochemical Anal. **13**: 8–17.
Lenherr A., Lahloub M. F., Sticher O. (1984). "Three flavonoid glycosides containing acetylated allose from *Stachys recta*." Phytochemistry **23:** 2343-2345.
Lenherr A., Mabry T.J. (1987). "Acetylated allose-containing flavonoid glucosides containing from *Stachys anisochila*." Phytochemistry **26:** 1185-1188.
Madhavi D.L., Salunkhe D.K. (1995). "Toxicological aspects of food antioxidants." in Food antioxidants. Technological, toxicological and health perspectives. (Eds. Madhavi D.L., Deshpande S.S., Salunkhe D.K.), Marcel Dekker Inc., New York.
Madsen H.L., Bertelsen G. (1995). "Spices as antioxidants." Trends Food Sci. Technol. **6**: 271-277.
Marin P. D., Grayer R.J., Grujic-Jovanovic S., Kite G.C., Grayer R.J., Kite G.C., Veitch N.C. (2004). "Glycosides of tricetin methyl ethers as chemotaxonomic markers in *Stachys* subgenus *Betonica*." Phytochemistry **65:** 1247-1253.
Matsumoto M., Shoyama Y., Nishioka I., Irino N. (1989). "Constituents of regenerated shoot and cultured root tissue of *Rehmannia glutinosa*." Phytochemistry **28:** 2331-2332.
Meremeti A., Karioti A., Skaltsa H., Heilmann J., Sticher O. (2005). "Secondary metabolites from *Stachys ionica*." Biochem. Syst.. Ecol. **33:** 139-151.
Miyase T., Koizumi A., Ueno A., Noro T., Kuroyanagi M., Fukushima S., Akiyama Y., Takemoto T. (1982). "Studies on the acyl glycosides from *Leucoseptrum japonicum* (Miq.) Kitamura et Murata." Chem. Pharm. Bull. **30:** 2732-2737,

Nieto S., Garrido A., Sanhueza J., Loyola L.A., Morales G., Leighton F., Valenzuela A. (1993). "Flavonoids as stabilizers of fish oil: an alternative to synthetic antioxidants." JAOCS **70**: 773-778.
Pekkarinen S.S., Stockmann H., Schwarz K., Heinonen M., Hopia A.I. (1999). "Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate." J. Agric. Food Chem. **47:** 3036-3043.
Pellegrini N., Simonetti P., Gardana C., Brenna O., Brighenti F., Pietta P. (2000). "Polyphenol content and total antioxidant activity of *Vini Novelli* (Young red wines), J. Agric. Food Chem. **48**: 732-735.
Pietta P.-G. (2000). "Flavonoids as antioxidants." J. Nat. Prod. **63:**1035-1042.
Piozzi F., Venturella P., Bellino A., Mondelli R. (1968). "Diterpenes from *Sideritis sicula ucria.*" Tetrahedron **24**: 4073-4081.
Robards K., Prenzler P.D., Tucker G., Swatsitang P., Glover W. (1999). "Phenolic compounds and their role in oxidative processes in fruits." Food Chem. **66:** 401-436.
Rodriguez-Lyon M.L., Diaz-Lanza A.M., Barnabe M., Villaescusa-Castillo L. (2000). "Flavone glycosides containing acylated sugars from *Sideritis hyssopifolia*." Magn. Reson. Chem. **38**: 684-687.
Sahin F.P., Ezer N., Calis I. (2006). "Terpenic and phenolic compounds from *Sideritis stricta*." Turk. J. Chem. **30:** 495-504.
Santos-Galindez J. de, Diaz-Lanza A.M., Matellano L.F., Sanchez A.R. (2000). "A new phenylpropanoid glycoside isolated from *Scrophularia scordonia* L." Magn. Reson. Chem. **38**: 688-691.
Saracoglu I., Harput U.S., Ogihara Y. (2004). "Acylated flavone glycosides from *Veronica pectinata* var. *glandulosa* and *V. persica*." Turk. J. Chem. **28:** 751-759.
Sattar A.A., Bankova V., Popov S. (1995). "Acylated flavonoid glycosides from *Sideritis scardica*." Fitoterapia **66**: 190.
Sattar A.A., Bankova V., Spassov S., Duddeck H. (1993). "Flavonoid glycosides from *Sideritis* species." Fitoterapia **64**: 278–279.
Schuller P. (1990). "Natural Antioxidants exploited commercially." in Food Antioxidants. (Ed. Hudson B.J.F.), Elsevier Applied Science, London, ch.4.
Schwarz K., Bertelsen G., Nissen L.R., Gardner P.T., Heinonen M.I., Hopia A., Huynh-Ba T., Lambelet P., McPhail D., Skibsted L.H., Tijburg L. (2001). "Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assay based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds." Eur. Food Res. Technol. **212**: 319-328.
Swiatek L., D. Lehmann, Chaudhuri R. K., Sticher O. (1981). "Occurrence of melittoside in the seeds of *Plantago media*." Phytochemistry **20:** 2023-2024..
Takeda T., Narukawa Y., Hada N. (1999). "Studies on the constituents of *Leonotis nepetaefolia*." Chem. Pharm. Bull. **47:** 284-286.
Venturella P., Bellino A., Marino M.L. (1995). "Three acylated flavone glycosides from *Sideritis syriaca*." Phytochemistry **38:** 527-530.
Xiong Q., Kadota Sh., Tani T., Namba Ts. (1996). "Antioxidative effects of phenylethanoids from *Cistanche deserticola*." Biol. Pharm. Bull. **19**: 1580–1585.

Conclusions

The research in this thesis was centred on the rapid and robust evaluation of antioxidant activity of complex mixtures such as plant extracts. In section 1.6 three aims were formulated and at the end of this study it is good to look at them retrospectively to see what has been achieved.

Concerning the first aim, the development of an "algorithm" for studying natural antioxidants, this study was directed to establish the best screening and isolation procedures for antioxidative components. Three different screening methods (the β-carotene bleaching test = BCBT, the headspace GC method = HS-GC and the off-line radical scavenging assay with the artificial DPPH radical = DPPH method) and various extraction schemes were used. Three plants from the genus *Sideritis* grown in Bulgaria served as test species. Methanol turned out to be a better extraction solvent than acetone or *t*-BuOMe in a Soxhlet apparatus. The BCBT turned out to be the most suitable preliminary assay. HS-GC was preferred for "secondary" oxidative products and the DPPH method was best when radical scavenging properties had to be assessed. As the BCBT can serve only as a preliminary procedure, one should not use the BCBT for evaluation of "primary" i.e. "chain-breaking" antioxidant properties, as the outcome is influenced by many variables. As the antioxidants found in *Sideritis* are representative of many antioxidants, the procedures can be applied to other species as well, i.e. the procedure can serve as a general algorithm.

The good results obtained from the off-line DPPH method led to the idea to develop a novel HPLC-DPPH method to rapidly and sensitively pinpoint individual antioxidants in complex mixtures with as few as possible fractionation procedures. The instrumental set-up and physico-chemical parameters were studied. The method was successful for qualitative and semi-quantitative measurements of pure antioxidants and extracts. The use of the alternative $ABTS^{\bullet+}$ radical cation made the method in most cases even more sensitive. However, the simplicity of the HPLC-DPPH method makes it preferable and many researchers have copied it. The two papers on the on-line methodology have been cited seventy times.

Concerning the second aim, a range of flavonoid-, phenylpropanoid- and iridoid glycosides were isolated from *S. syriaca* and *S. scardica* partially using the earlier developed on-line methodology and their radical scavenging properties were evaluated. A distinction has to be made between radical scavenging activity and antioxidant activity

(capacity). All known powerful natural radical scavengers are also good antioxidants. The study showed moderate to low or even no radical scavenging activity based on the off-line DPPH method. In contrast, the respective antioxidants showed strong to moderate radical scavenging activity in the on-line DPPH method. This suggested alteration or degradation of fractions or active antioxidative compounds during isolation procedures. In my opinion, although extraction and fractionation was carried out under mild conditions (maceration, shaking, bubbling with N_2, heating not above 50 ºC), even milder conditions would be necessary to preserve the original activity of the parent extract or fractions. Another plausible explanation might be concentration effects i.e. a too low concentration in the assays. Three novel compounds were isolated: the flavonoid glycosides luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **5** and hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **6** and the iridoid glycoside 3''-O-*p*-coumaroyl-6''-O-acetyl melittoside **8** from both *Sideritis* species. Their structures were confirmed by spectroscopic methods. Studies on compounds of phenylpropanoid nature with an extra sugar are still on-going. The radical scavenging activity of the new compounds was rather low (e.g. **8**) or even negligible (e.g. **5**, **6**) in the off-line DPPH method. The low activity of the first compound was explicable given the fact that there are no ortho or para groups. For flavonoid (**6**) there are discrepancies between off- and on-line DPPH methods. After the elucidation of the still unknown verbascoside derivatives, structure-activity studies might be performed to determine the influence of glycosylation and the effect of the various substituents present in the A- and B-rings.

Concerning the third aim for a "final check", the HS-GC method was applied to investigate how *Sideritis* extracts and pure components would behave in real systems: bulk oils and emulsions. Conjugated dienes and hexanal formation were monitored. Results for a range of pure compounds and extracts using rosmarinic acid and BHT as standards, showed that *Sideritis* extracts could serve as antioxidants. As for the pure compounds (the most active flavonoid glycosides and verbascoside in the off-line DPPH method), they all showed better inhibitory effects with regard to dienes and hexanal formation in both test systems than the extracts. However, the costs of the isolation and purification procedures are too high even when they are applied at the 0.01 to 0.03% level that is normal in foods and cosmetics. Also the costs of the toxicity studies of pure compounds would be prohibitive. Thus the use of extracts appears more feasible. The extracts should not have any odour, taste or colour, be compatible with food/cosmetic products and be safe. In future there should be teamwork of nutritionists, chemists and economists to evaluate the application of extracts in food/cosmetic products.

Summary

Oxygen is an element of dual character – on the one hand it is the first life-essential element, needed for normal functioning of all aerobic systems, on the other hand it is involved in a number of oxidation processes that produce radicals with potentially harmful consequences. Lipids are among the major components of many foods. They are susceptible to oxidative reactions. The use of added substances (antioxidants) able to delay oxidative processes is indispensable in food/cosmetic preservation.

Several synthetic (BHT, BHA, TBHQ) and a few natural antioxidative compounds (tocopherol, L-ascorbic acid, β-carotene) are officially permitted for use in foods/cosmetics. Because of toxicological concerns of some synthetic antioxidants, a preference for "all-natural" antioxidants has intensified the search for novel antioxidants in aromatic and medicinal plants. General background information about lipid oxidation, antioxidant mechanisms, chemical nature of antioxidants, methods for evaluating of antioxidant activity and determination of the level of lipid oxidation is given in **Chapter 1**. In this thesis Bulgarian *Sideritis* plants are screened for antioxidants. For this purpose several off- and on-line methods are discussed. A literature survey of the botanical, chemical and pharmacological profile of the genus *Sideritis* (Labiatae) is presented.

Three widely employed methods for the evaluation of antioxidant activity, namely the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging method, the static headspace gas chromatography assay (HS-GC) and the β-carotene bleaching test (BCBT), are compared with regard to their application in the screening of plant extracts (**Chapter 2**). The strengths and limitations of each method are illustrated by testing a number of extracts of different polarity from plants of the genus *Sideritis*, and two known antioxidants − BHT and rosmarinic acid. The sample polarity turns out to be important for the exhibited activity in the BCBT and HS-GC methods but not for the DPPH method. The value of the BCBT appears to be limited to less polar samples. β-Carotene is a reaction indicator and is oxidised itself. It can be considered as an antioxidant, too, therefore the exact role of an antioxidant in the β-carotene:linoleic acid system is difficult to explain. However, the BCBT is simple, sensitive and relatively rapid. Although slower, the HS-GC method is used for assessing the antioxidant inhibitory properties on the formation of secondary

volatile products. It is sensitive and reproducible. With this test, the antioxidant effect at a later stage of lipid oxidation is evaluated.

Being rapid, simple, sensitive, reproducible and independent of sample polarity, the DPPH method is very convenient for the screening of many samples for radical scavenging activity. A drawback, common to all methods using model radicals, is the lack of a direct correlation between the established antioxidant activity and its protective properties in real lipid systems. The known chemical properties of $DPPH^{\bullet}$ enable structure-activity studies. The DPPH method is not discriminative with respect to the radical species but gives a general idea about the radical scavenging ability.

Plant samples from several species and populations of the genus *Sideritis* grown in Bulgaria (*S. scardica*, *S. syriaca* and *S. montana*) are extracted with different solvents (MeOH, acetone, *t*-BuOMe followed by extraction with MeOH) and the extracts are tested on their antioxidative capacity using the methods mentioned in Chapter 2. The results are compared with the antioxidant capacity of two reference compounds of different polarity, BHT and rosmarinic acid (**Chapter 3**). The highest antioxidant capacity in the BCBT is close to that of BHT and is observed for the more apolar extracts. The inhibitory effect on *β*-carotene discoloration of the polar extracts and rosmarinic acid is much lower than that of BHT. The reduction of hexanal formation in the HS-GC method in bulk safflower oil by most of *S. syriaca* and *S. scardica* extracts is as effective as BHT but less so than rosmarinic acid. *S. montana* extracts show weak antioxidant or even pro-oxidant properties. Extracts from all *Sideritis* plants studied exhibit strong radical scavenging activities against $DPPH^{\bullet}$, close to that of rosmarinic acid. *S. montana* extracts contain slightly weaker radical inhibitors than the extracts from the other two species. The antioxidant activity of *Sideritis* extracts is due to the presence of flavonoid and phenylpropanoid glycosides, as shown in Chapter 6.

Following the good results obtained by the DPPH off-line method, a rapid on-line method for screening of complex mixtures for radical scavenging components is developed using a methanolic solution of $DPPH^{\bullet}$ (**Chapter 4**). The HPLC-separated analytes react post-column with the $DPPH^{\bullet}$ solution, and any reduction is detected as a negative peak. An optimized instrumental and experimental set-up is presented. The method proves suitable for both isocratic and gradient HPLC runs with mobile-phase compositions ranging from 10 to 90% organic solvent in water or buffer (pH 3-6). The method is simple, is broadly applicable, uses common instruments and inexpensive and stable reagents, is time-saving and follows a non-laborious experimental protocol. It can be used for quantitative analysis. The method is applied to several pure natural antioxidants and a crude aqueous extract of *S.*

scardica. The limits of detection are 0.33 (for quercetin) to 94 (for eugenol) μg/mL, depending on the compound tested.

As the HPLC-DPPH method has been successful, another radical ($ABTS^{\bullet+}$ radical cation) [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)], is utilized in an on-line HPLC method for the detection of radical scavengers in complex matrixes (**Chapter 5**). The HPLC-separated analytes react post-column with preformed $ABTS^{\bullet+}$ and the induced reduction is detected as a negative peak. Similar to the HPLC-DPPH method, an optimized instrumental and experimental set-up is presented. The method is suitable for both isocratic and gradient HPLC runs using mobile phases containing up to 100% organic solvent or its solution in water, weak acids, or buffers (pH 3-7.4). This method has the same advantages as the HPLC-DPPH method. The method is applied to several pure natural antioxidants and a *S. syriaca* EtOAc extract. The minimum detectable concentration varies from 0.02 (for Trolox and rutin) to 0.13 (for BHT) μg/mL, depending on the compound tested. The method can be applied for kinetic studies because reaction coils of different lengths can be easily installed.

As a result of all these off- and on-line studies, 7 flavonoid glycosides, 2 phenylpropanoid glycosides plus 2 unknown phenylpropanoid glycosides, 2 iridoid glycosides and the diterpene siderol are isolated from Bulgarian populations of *S. scardica* and *S. syriaca* (**Chapter 6**). The structures are elucidated by spectroscopic methods, reference compounds and literature data. Three novel compounds were isolated from both *Sideritis* species, namely luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **5**, hypolaetin-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **6** and 3''-O-*p*-coumaroyl-6''-O-acetyl melittoside **8**. The structures of two unknown phenylpropanoid glycosides with an extra sugar are on-going. The radical scavenging activity of all isolated compounds is evaluated off-line by using the $DPPH^{\bullet}$ radical and is compared to the activities of rosmarinic acid, caffeic acid, chlorogenic acid and rutin. In the off-line DPPH method the most active radical scavengers are verbascoside **1** (76% Red.) and hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **9** (50% Red.). On-line HPLC-DPPH assays are also performed. In the on-line HPLC-DPPH method the most active antioxidants (radical scavengers) in *S. syriaca*-BE are hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **3** > verbascoside **1** > isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **7** > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **2**. In *S. scardica*-BE the most active radical scavengers are verbascoside **1** > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **3** ≈ isoscutellarein-7-O-[6'''-O-acetyl-β-D-

allopyranosyl-(1-2)-β-D-glucopyranoside] **7** ≈ hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **2**. The presence of putative antioxidants, more precisely radical scavengers, in *Sideritis* plants makes them a possible source of natural antioxidants.

Concluding remarks are made in **Chapter 7.** Following the aims stated in Chapter 1.6 an "algorithm" for antioxidant activity in phytochemical studies is presented. The effect of extracts and pure compounds isolated from them in real food systems (diene conjugation and hexanal formation in bulk oils and emulsions) is discussed. Toxicity studies and compatibility with food/cosmetics are necessary. For use in foods such products (extracts/fractions and pure compounds) will, in all probability, not be utilized. For cosmetics, the regulations are not so strict and their use is permitted.

Due to the complexity of the oxidation-antioxidation processes, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a sample. A multimethod approach including on-line assays, is necessary in antioxidative activity assessment. A combination of rapid, sensitive and reproducible methods, preferably requiring small sample amounts, producing complementary results for "primary" and "secondary" antioxidant properties, as well as radical scavenging ones should be used whenever an antioxidative activity screening is designed.

Samenvatting

Zuurstof is een element met een dualistisch karakter – aan de ene kant is het absoluut noodzakelijk voor het normaal functioneren van alle aërobe systemen, aan de andere kant is het betrokken bij oxidatieprocessen waarbij reactieve radicalen vrijkomen met mogelijk schadelijke gevolgen. Lipiden (vetten) zijn één van de belangrijkste componenten in levensmiddelen. Ze zijn gevoelig voor oxidatief bederf en reactieve radicalen spelen hierbij een belangrijke rol. Door het toevoegen van antioxidanten aan levensmiddelen en cosmetische producten worden deze oxidatieve processen vertraagd en de houdbaarheid verlengd.

Verschillende synthetische (BHT, BHA, TBHQ) en enkele natuurlijke antioxidanten (tocopherol (vitamine E), ascorbinezuur (vitamine C), β-caroteen, rozemarijn extract) zijn officieel toegestaan in levensmiddelen en cosmetische producten. Vanwege toxicologische bezwaren tegen sommige synthetische antioxidanten, wordt er momenteel veel onderzoek naar natuurlijke antioxidanten afkomstig uit planten uitgevoerd.

Algemene achtergrond informatie over vetoxidatie, werkingsmechanismen van antioxidanten, structuren van antioxidanten, technieken om antioxidatieve activiteit te bepalen en de bepaling van de mate van vetoxidatie wordt gegeven in **Hoofdstuk 1**. In dit proefschrift wordt een onderzoek beschreven naar de aanwezigheid van antioxidanten in *Sideritis* planten van Bulgaarse afkomst. De resultaten van een literatuuronderzoek naar de botanie, chemie en farmacologie van het genus *Sideritis* behorend tot de familie der Labiatae, wordt gepresenteerd.

Drie veel toegepaste methoden om antioxidatieve activiteit te evalueren, namelijk de 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) methode, de statische headspace bemonsteringstechniek (HS-GC) en de β-caroteen ontkleuringstest (BCBT), worden vergeleken op hun toepasbaarheid in het screenen van plantenextracten (**Hoofdstuk 2**). De voor- en nadelen van elke methode zijn onderzocht door een aantal extracten van verschillende polariteit van het genus Sideritis en twee bekende antioxidanten (BHT en rozemarijnzuur) te testen. De polariteit van een monster is van belang bij het meten van de antioxidatieve activiteit in de BCBT en de HS-GC test, maar niet bij de DPPH methode. De BCBT test is minder geschikt voor polaire monsters. In deze test wordt β-caroteen gebruikt als indicator en ontkleurd door oxidatie. Het kan echter ook als een antioxidant worden

beschouwd en daardoor is de precieze rol van een te testen antioxidant in het β-caroteen/linoleenzuur systeem moeilijk te achterhalen. Echter, de BCBT test is eenvoudig, gevoelig en relatief snel uitvoerbaar. De HS-GC methode kost meer tijd en wordt gebruikt om de remmende eigenschappen van antioxidanten op de vorming van secundaire oxidatieproducten te beoordelen. Deze test is gevoelig en reproduceerbaar.
De DPPH test is snel, eenvoudig, gevoelig, reproduceerbaar, onafhankelijk van de polariteit van het monster en erg geschikt voor het screenen van veel monsters op hun radicaal afvangend vermogen. Een nadeel is dat er geen direct verband bestaat tussen de gemeten antioxidatieve activiteit en de beschermende eigenschappen in een echt levensmiddel. De bekende chemische eigenschappen van DPPH• maken het erg geschikt voor studies naar structuur activiteits relaties (SAR). De DPPH methode geeft een algemene indruk van het radicaal afvangend vermogen van een component.
Plantaardig materiaal van drie Sideritis soorten afkomstig van verschillende populaties in Bulgarije *(Sideritis scardica, S. syriaca en S. montana)* zijn geëxtraheerd met verschillende oplosmiddelen (methanol, aceton, *tert*-butyl-methyl-ether gevolgd door extractie met methanol) en de extracten zijn getest op hun antioxidatieve capaciteit met de in hoofdstuk 2 genoemde testen. De uitkomsten zijn vergeleken met die van twee referentie verbindingen, te weten BHT en rozemarijnzuur (**Hoofdstuk 3**). De meer apolaire extracten waren het meest actief in de BCBT test en hun activiteit was bijna vergelijkbaar met die van BHT. Het remmende effect op de β-caroteen ontkleuring van de polaire extracten en van rozemarijnzuur is veel minder dan die van BHT. De vermindering van de vorming van hexanal in de HS-GC methode in saffloerolie door extracten van *S. syriaca* en *S. scardica* is net zo effectief als bij BHT, maar minder dan bij rozemarijnzuur. Extracten van *S. montana* zijn duidelijk minder actief of versnellen soms zelfs de oxidatie. Van alle drie *Sideritis* soorten laten de extracten een sterk radicaal afvangend vermogen zien in de DPPH• test, vergelijkbaar met dat van rozemarijnzuur. Toch zijn extracten van *S. montana* iets zwakkere radicaal afvangers dan de extracten van de andere twee soorten. De antioxidatieve activiteit van Sideritis extracten wordt veroorzaakt door de aanwezigheid van flavonoid- en fenylpropaan glycosiden, zoals beschreven staat in hoofdstuk 6.
Na de goede resultaten verkregen met de DPPH off-line test, is een snelle on-line methodiek om complexe mengsels van antioxidanten te screenen ontwikkeld (**Hoofdstuk 4**). Met behulp van HPLC worden de componenten gescheiden en actieve componenten (fenolen) reageren post-column met een toegevoegde methanolische DPPH• oplossing. Gereduceerd DPPH• (DPPHH) is kleurloos in plaats van paars en dus is elke reductie door een eluerend fenol zichtbaar als een negatieve piek. Door dit profiel te vergelijken met het normale HPLC-UV profiel wordt direct duidelijk welke componenten in het extract

antioxidatieve activiteit bezitten. De instrumentele en experimentele opzet is geoptimaliseerd. Deze methode is geschikt voor zowel isocratische als gradient HPLC analyses met een mobiele fase van 10-90% organisch oplosmiddel in water of buffer (pH 3-6). De methode is eenvoudig, breed inzetbaar, en gebruikt standaard apparatuur, goedkope en stabiele reagentia en bespaart veel tijd. Zij kan gebruikt worden voor kwantitatieve analyses. De methode is toegepast op verschillende zuivere natuurlijke antioxidanten en een ruw waterig extract van *S. scardica*. De detectielimieten variëren van 0.33 (quercetine) tot 94 (eugenol) µg/L, afhankelijk van welke component er getest wordt.

Door het succes van de DPPH methode, is ook een ander radicaal ($ABTS^{\bullet+}$ radicaal kation; 2,2'- azinobis-(3-ethylbenzothiazoline-6-sulfonaat) toegepast in een on-line HPLC methode om radicaal afvangde componenten te detecteren in complexe mengsels (**Hoofdstuk 5**). Het principe en de voordelen van deze methode zijn dezelfde als die voor $DPPH^{\bullet}$. De methode kan gecombineerd worden met zowel isocratische als gradient HPLC met mobiele fases tot 100% organisch oplosmiddel in waterige zwakke zuren of buffers (pH 3 - 7.4). Ze is toegepast op zuivere natuurlijke antioxidanten en een *S. syriaca* ethylacetaat extract. De detectie limieten variëren van 0.02 (Trolox en rutine) tot 0.13 (BHT) µg/mL. De methode kan worden toegepast voor kinetische studies omdat de reactietijd eenvoudig gevarieerd kan worden door de dimensies van de post-column reactieloop te veranderen.

Het resultaat van deze studies is dat 7 flavonoid glycosiden, 2 fenylpropaan glycosiden, 2 iridoid glycosiden, het diterpeen siderol en 2 niet-geïdentificeerde fenylpropaan glycosiden zijn geïsoleerd uit *S. scardica* en *S. syriaca* (**Hoofdstuk 6**). De chemische structuren zijn opgehelderd door middel van spectroscopische methoden, referentie verbindingen en data uit de literatuur. Drie nieuwe verbindingen zijn geïdentificeerd: luteoline-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **5**, hypolaetine-4'-methylether-7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **6** en 3''-O-*p*-coumaryl-6''-O-acetyl melittoside **8**. Er wordt nog gewerkt aan de structuuropheldering van de twee niet-geïdentificeerde fenylpropaan glycosides. Het radicaal afvangend vermogen van alle geïsoleerde verbindingen is geëvalueerd met behulp van het DPPH radicaal en is vergeleken met de activiteiten van rozemarijnzuur, koffiezuur, chlorogeenzuur en rutine. De meeste actieve radicaal afvangende verbindingen zijn verbascoside **1** (76% red.) and hypolaetine-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **9** (50% red.). In de on-line HPLC-DPPH methode zijn de meest actieve antioxidanten (radicaal afvangers) in het *S. syriaca*-butanol extract: hypolaetine-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **3** > verbascoside **1** > isoscutellareine-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **7** > hypolaetine-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-

2)-6"-O-acetyl-β-D-glucopyranoside] **2**. In het *S. scardica* butanol extract zijn de meest actieve radicaal afvangers: verbascoside **1** > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **3** ≈ isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **7** ≈ hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6"-O-acetyl-β-D-glucopyranoside] **2**. De aanwezigheid van deze antioxidanten in *Sideritis* planten maakt hen tot een mogelijke bron van natuurlijke antioxidanten.

In **hoofdstuk** 7 wordt terug gekeken op de behaalde resultaten en de doelstellingen zoals geformuleerd in hoofdstuk 1.6. Diverse concluderende opmerkingen worden gemaakt. Een algoritme voor het bepalen van antioxidatieve activiteit in fytochemische studies is ontwikkeld. Het effect van extracten en zuivere verbindingen in echte voedselsystemen (diëen conjugatie en hexanal vorming in plantaardige oliën en emulsies) wordt bediscussiëerd. Deze producten zullen waarschijnlijk geen toepassing vinden in voedsel. Voor cosmetica is de wetgeving echter minder streng en kan het gebruik wel worden toegestaan.

Vanwege de complexiciteit van het oxidatie proces en de remming daarvan, is het duidelijk dat niet één enkelvoudige test in staat is om een goed beeld te geven van het antioxidant profiel van een monster. Meerdere methoden moeten uitsluitsel geven. Een combinatie van snelle, gevoelige en reproduceerbare methoden, die uitgevoerd kunnen worden met kleine monsterhoeveelheden en gegevens opleveren over de “primaire”, “secundaire” antioxidatieve en radicaal afvangende eigenschappen, moet gebruikt worden voor de screening van planten op antioxidatieve activiteit.

Резюме

Кислородът е елемент с двойнствен характер – от една страна, той е първият жизнено-важен елемент за нормалното функциониране на всички аеробни системи, от друга страна е отговорен за редица окислителни процеси, в резултат на които се получават радикали с потенциално вредни последици. Липидите са сред основните компоненти на много храни. Те са подложени на окислителни реакции. Използването на добавки като антиоксидантите, способни да предотвратяват или забавят окислителните процеси, е неизменна част от съвременното запазване на хранителните и козметични продукти.

Няколко синтетични (БХТ, БХА, ТБХХ) и определен брой природни антиоксиданти (токоферол, L-аскорбинова киселина, β-каротен) са официално разрешени за употреба в храните и козметиката. Поради опасения за токсикологичното действие на синтетичните антиоксиданти, все повече предпочитания се отдават на т. нар. "напълно натурални" ("all-natural") антиоксиданти. Те са причина да се интензифицира процеса на търсене на нови антиоксиданти в ароматичните и лекарствени растения. В **Глава 1** са представени най-общо липидното окисление, антиоксидантните механизми, химичната природа на антиоксидантите, методите за оценка на антиоксидантната активност и определяне степента на липидно окисление. В тази дисертация като обект на изследване служат български растения от род *Sideritis*, които са изследвани за съдържание на антиоксиданти. За тази цел са дискутирани редица off- и on-line методи. Обширен литературен преглед е представен върху ботаническия, химическия и фармакологичския профил на род *Sideritis* (Labiatae).

За оценка на различни екстракти от род *Sideritis* по отношение на тяхната антиоксидантната активност, са използвани три широко използвани метода, а именно 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) радикалулавящия метод, HS-газова хроматография (HS-GC) и метода с обезцветяване на β-каротен (ВСВТ). Те са сравнени с оглед на тяхното приложение в отсяването (screening) на растителни екстракти (**Глава 2**). Предимствата и недостатъците на всеки метод са илюстрирани чрез тестване на редица екстракти с различна полярност от растения от род *Sideritis*, и на два известни антиоксиданта – БХТ и розмаринова киселина (РК). Стойносттите на антиоксидантния коефициент (при ВСВТ) показват, че по-голяма активност имат

екстрактите с по-ниска полярност в сравнение с тези с по-висока полярност. Полярността се оказва важна за ВСВТ и HS-GC методите, но не и за DPPH метода. Един от недостатъците на ВСВТ е, че самият β-каротен е реакционен индикатор т.е. той също е антиоксидант, и затова точната роля на изследваните антиоксиданти в системата β-каротен : линолова киселина е трудно да бъде обяснена. ВСВТ е лесноприложим, чувствителен и сравнително бърз метод. Въпреки, че е по-бавен от ВСВТ, HS-GC се използва за оценка на инхибиращите свойства на антиоксидантите по отношение на вторични летливи продукти на окисление. Той е чувствителен и възпроизводим. С HS-GC метода се оценява реалния антиоксидантен ефект на един по-късен етап на липидното окисление.

DPPH методът е удобен за screening на много проби за радикалулавяща активност, понеже е бърз, лесноприложим, чувствителен, възпроизводим и не зависи от полярността на пробата. Като недостатък на метода, общ за всички методи, използващи моделни радикали, е липсата на директна връзка между антиоксидантната активност и предпазващите свойства в реални липидни системи. Известните химични свойства на DPPH$^{\bullet}$ позволяват изследвания за връзката структура на съединението−антиоксидант. С DPPH метода не може да се разграничи вида на радикала, но той дава обща представа за радикалулавящата активност.

Растителни проби от различни видове и популации от род *Sideritis*, растящи в България (*S. scardica*, *S. syriaca* и *S. montana*), са екстрахирани с различни разтворители (MeOH, ацетон и *t*-BuOMe, последвана от екстракция с MeOH) и екстрактите са тествани за техния антиокислителен капацитет с използване на методите, разгледани в Глава 2. Резултатите са сравнени с антиокислителния капацитет на избрани два стандарта с различна полярност − БХТ и РК (**Глава 3**). Според ВСВТ най-висок и близък до БХТ е антиокислителния капацитет на по-неполярните екстракти. Обезцветяването на β-каротена в присъствие на полярните екстракти и РК е много по-малко, от това при БХТ. Редукцията на образуването на хексанал в сафлорово масло на екстракти чрез HS-GC показва, че при повечето екстракти от *S. scardica* и *S. syriaca* ефективността в сафлорово масло е еднаква с тази на БХТ, но е по-малка в сравнение с РК. Екстрактите от *S. montana* показват слаби антиоксидантни и дори про-оксидантни свойства. Екстрактите от всички растения от род *Sideritis* демонстрират силна радикалулавяща активност спрямо DPPH$^{\bullet}$, близо до тази на РК. Екстрактите от *S. montana* съдържат по-слаби радикалуловители, отколкото екстрактите от другите два вида. Антиоксидантната активност на екстрактите от *Sideritis* се дължи главно на присъствието на флавоноидни и фенилпропаноидни гликозиди, както е показано в Глава 6.

Въз основа на получените добри резултати, получени при off-line DPPH метода, е разработен бърз on-line метод за screening на сложни смеси от радикалулавящи компоненти чрез HPLC с метанолен разтвор на DPPH$^{\bullet}$ (**Глава 4**). Разделените по този начин компоненти се детектират чрез редукционен процес след колоната с разтвора на DPPH$^{\bullet}$. Всяка редукция се регистрира като отрицателен пик. Представена е оптимизирана инструментална и експериментална схема. Методът е подходящ за изократични и градиентни HPLC режими с подвижни системи от 10 до 90% органичен разтворител във вода или буфер (pH 3-6). Методът е лесноосъществим, широко приложим, използва достъпна апаратура, евтини и стабилни реактиви, спестява време и следва опростен експериментален протокол. Може успешно да бъде използван за количествен анализ. Приложен е към редица чисти природни антиоксиданти и воден екстракт от *S. scardica*. Пределната коцентрация варира от 0.33 (за кверцетин) до 94 (за евгенол) μg/mL.

Тъй като HPLC-DPPH методът се оказа успешен, друг радикал (ABTS$^{\bullet+}$ радикал катион) [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)], е използван във вариант on-line HPLC метод за детекция на радикалуловители в комплексни матрици (**Глава 5**). Разделените чрез HPLC вещества реагират след колоната с предварително получен ABTS$^{\bullet+}$. Предизвиканата редукция на радикала се регистрира като отрицателен пик. Подобно на HPLC-DPPH метода, и тук е представена оптимизирана инструментална и експериментална схема. Методът е подходящ за изократични и градиентни HPLC режими с подвижна система до 100% органичен разтворител или негов разтвор във вода, слаби киселини или буфер (pH 3-7.4). Методът се отличава със същите предимства като HPLC-DPPH метода. Той е приложен към някои чисти природни антиоксиданти и EtOAc екстракт от *S. syriaca*. Пределната коцентрация варира от 0.02 (за Тролокс и рутин) до 0.13 (за БХТ) μg/mL. В много случаи той е по-чувствителен от HPLC-DPPH метода. Методът може да се прилага и за кинетични изследвания, тъй като след колоната лесно може да се монтират реактори с различна дължина на капилярата.

Като резултат от всички тези off- и on-line изследвания, 7 флавоноидни гликозида, 2 фенилпропаноидни гликозида и 2 неизвестни засега фенилпропаноидни гликозида, 2 иридиодни гликозида и дитерпена сидерол са изолирани от български популации на *S. scardica* и *S. syriaca* (**Глава 6**). Структурите на съединенията са доказани чрез спектроскопски методи (UV, IR, едно- и дву-димензионален NMR, FAB-MS и ESI-MS), стандартни вещества и по литературни данни. Три нови съединения от двата вида *Sideritis* са изолирани, а именно luteolin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **5**, hypolaetin-4'-methylether-

7-O-[β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **6** и 3''-O-*p*-coumaroyl-6''-O-acetyl melittoside **8**. Предстои да се докажат структурите на двата неизвестни засега фенилпропаноидни гликозида с допълнителен захарен остатък.

Радикалулавящата активност с DPPH$^{\bullet}$ на всички изолирани съединения е оценена off-line, и сравнена с розмаринова киселина, кафеена киселина, хлорогенова киселина и рутин. Според off-line DPPH метода, най-активни радикалулавящи компоненти са verbascoside **1** (76% Red.) и hypolaetin-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **9** (50% Red.). Бяха проведени също и on-line HPLC-DPPH изследвания. При HPLC-DPPH метода най-активни антиоксиданти (радикалуловители) в *S. syriaca*-ВЕ са hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **3** > verbascoside **1** > isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **7** > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **2**. В *S. scardica*-ВЕ най-активни радикалуловители са verbascoside **1** > hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **3** ≈ isoscutellarein-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-β-D-glucopyranoside] **7** ≈ hypolaetin-4'-methylether-7-O-[6'''-O-acetyl-β-D-allopyranosyl-(1-2)-6''-O-acetyl-β-D-glucopyranoside] **2**. Присъствието на предполагаеми антиоксиданти, по-точно радикалуловители, в растенията от род *Sideritis* ги прави възможен източник на натурални антиоксиданти.

Глава 7 представлява заключение. Следвайки целите, поставени в Глава 1.6, е разработен "алгоритъм" за оценка на антиоксидантна активност във фитохимичните изследвания. Сравнява се ефекта на екстрактите и чисти вещества, изолирани от тях, в реални хранителни системи (спрегнати диени и образуване на хексанал в сафлорово масло и емулсия). За окончателна им оценка като антиоксиданти в хранителни и козметични продукти са необходими допълнителни токсикологични изследвания, както и такива за съвместимост с храните.

Като се има в предвид комплексността на процеса окисление-антиокисление, очевидно не може да бъде намерен един единствен метод, който да осигурява изчерпателна картина на антиоксидантния профил на дадена система субстрат-антиоксидант. Многобройни методи, включващи и on-line методите, са нужни, когато трябва да се оцени реалната антиоксидантна активност. За тази цел трябва да се използва комбинация от бързи, чувствителни и възпроизводими методи, осигуряващи допълващи се резултати за "първични" и "вторични" антиоксидантни свойства, както и радикалулавящи такива, когато се планира тестване за антиоксидантна активност.

List of Abbreviations

$^1O_2$-Q - $^1O_2$ Quencher
$^3O_2$-S - $^3O_2$ Scavenger
ABAP - 2,2'-Azobis-(2-amidopropane) hydrochloride
ABTS - 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate); **$ABTS^{+\bullet}$** - 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation
AH - Phenolic antioxidant
AnV - Anisidine Value
AO - Antioxidant
AOA - Antioxidant Activity
AOM - Active Oxygen Method
AUC - Area-Under-Curve
BCBT - β-Carotene Bleaching Test
BHA - 2-*tert*-Butyl-*p*-hydroxyanisole
BHT - 2,6-Di-*tert*-butyl-*p*-hydroxytoluene
CA - Caffeic acid
CC - Column Chromatography
ChA - Chlorogenic acid
CL - Chemiluminescence
COSY - Correlated Spectroscopy
CPB - Citric-Phosphate Buffer
DAD - Diode Array Detector
DBO - n, π^* Singlet-excited azoalkane 2,3-diazabicyclo[2.2.2.]-oct-2-ene
DEPT - Distortionless Enhancement by Polarization Transfer
DMPD - N,N-Dimethyl-1,4-phenylenediamine
DMSO - Dimethylsulfoxide
DNA - Deoxyribonucleic acid
DPPH - 2,2-Diphenyl-1-picrylhydrazyl radical
$E^{0'}$ - Standard redox potential
EDTA - Ethylenediaminetetraacetic acid
Enz-I - Enzyme Inhibitor
EPR (ESR) - Electron Paramagnetic (Spin) Resonance
FID - Flame Ionization Detector
FRAP - Ferric-Reducing Antioxidant (Ability) Power
GC - Gas Chromatography
GSH - Glutathione reduced
HACCP - Hazard Analysis of Critical Control Points
HMBC - Heteronuclear Multiple Bond Correlation spectroscopy
HMQC - Heteronuclear Multiple Quantum Correlation spectroscopy
HPLC - High Pressure Liquid Chromatography
HS-GC - Head-Space Gas Chromatography
IP - Induction Period
IR - Infrared; **FT-IR** - Fourier Transformation Infrared
LC/MS - Liquid Chromatography-Mass Spectrometry; **LC/NMR** - Liquid Chromatography- Nuclear Magnetic Resonance

List of Abbreviations

LOD - Limit of Detection
LDL - Low Density Lipoprotein
M - Metal
M-D - Metal Deactivator
MDA - Minimum Detectable Amount; **MDC -** Minimum Detectable Concentration
MPLC - Medium Pressure Liquid Chromatography
MS - Mass Spectrometry; **FAB-MS** - Fast Atom Bombardment MS; **ESI-MS** - Electrospray Ionisation MS
ND - not done
NMR - Nuclear Magnetic Resonance
ORAC - Oxygen Radical Absorbance Capacity
PA - Polyamide 6S
PBS - Phosphate Buffered Saline
PEEK - Polyetheretherketones
PF - Protection Factor
PG - Propyl gallate
PTFE - Polytetrafluoroethylene
PUFA - Polyunsaturated Fatty Acids
PV - Peroxide Value
RA - Rosmarinic acid
ROESY - Rotational nuclear Overhauser Effect Spectroscopy
ROOH - Lipid Hydroperoxides
ROOH-D - Lipid Hydroperoxide Destroyer
RRH - Relative Rate of Hexanal formation
RSA - Radical Scavenging Activity
RSD - Relative Standard Deviation; **SD** - Standard Deviation
S/N - Signal-to-Noise ratio
SAR - Structure-Activity Relationship
Sens - Sensitizer
Seph - Sephadex LH-20
SiO_2- Silica gel
SOD - Superoxide Dismutase
TAA - Total Antioxidant Activity
TBA - 2-Thiobarbituric Acid; **TBARS** - 2-Thiobarbituric Acid Reactive Substances
TBHQ - *tert*-Butylhydroquinone
TEAC - Trolox Equivalent Antioxidant Capacity
TFA - Trifluoroacetic Acid
THF - Tetrahydrofuran
TLC - Thin Layer Chromatography
TOCSY - Total Correlation Spectroscopy
TOTOX - Total Oxidation
TRAP - Total Radical-Trapping Parameter
UV - Ultraviolet
Vis - Visible
VLC - Vacuum Liquid Chromatography

List of Publications

Publications related to the thesis

1. Koleva I.I., Niederländer H.A.G., van Beek T.A. (2000). "A rapid on-line HPLC method for detection of radical scavenging compounds in complex mixtures." Anal. Chem. **72:** 2323 – 2328 (**SCI:** 4.473).
2. Evstatitieva L.N., Koleva I.I. (2000). "Cultivation of *Sideritis scardica* Griseb." Arandelovac, Yugoslavia **May 29-June 03:** 189196.
3. Dapkevicius A., Koleva I.I., Pukalskas A., Niederländer, H.A.G., van Beek T.A. (2000). "Screening, isolation and identification of antioxidants from Lithuanian and Bulgarian herbs." in Flavour and Fragrance Chem. ch.19. (Eds. Lanzotti V., Taglialatela-Scafati O.), Kluwer Academic Publishers, Dordrecht, pp 187-199.
4. Koleva I.I., van Beek T.A., Niederländer H.A.G. (2001) "Application of ABTS radical cation for selective on-line detection of radical scavengers in HPLC eluates." Anal. Chem. **73:** 3373-3381 (**SCI:** 4.473).
5. Koleva I.I., van Beek T.A., Linssen J.P.H., de Groot Ae., Evstatieva L.N. (2002). "A comparison of three methods for screening of plant extracts for antioxidant activity." Phytochem. Anal. **13:** 8-17 (**SCI:** 1.198).
6. Koleva I.I., Linssen J.P.H., van Beek T.A., Evstatieva L., Kortenska V., Handjieva N. (2003). "Antioxidant activity screening of extracts from *Sideritis* species (Labiatae) grown in Bulgaria." J. Sci. Food Agric. **83:** 809-819 (**SCI:** 0.895).
7. Dobreva S., Koleva I. (2006). "Biologically active cosmetic formulation with antioxidant properties." Patent **Reg. Nr. 108967 U**/07.12.2004 (in press). Published in Official Bulletin v. 8/2006 "Polezni modeli" ("Useful models"), Razdel A.
8. Koleva I.I., de Waard P., van Beek T.A., de Groot Ae., Kovacheva E., Evstatieva L. "Antioxidative components of *Sideritis syriaca* and *Sideritis scardica* grown in Bulgaria." J. Nat. Prod., in preparation.
9. Koleva I.I., Barsitute A., van Beek T.A., Niederläender H.A.G. "Review of on-line HPLC antioxidant detection." TRAC, in preparation.

Other publications and presentations on symposia

10. Dobreva S., Demireva Z., Koleva I.I. "Cosmetic formulation with plant biologically active products", Patent **Reg. Nr. 98743/**29.04.1994.
11. Koleva I.I.*, Kovatcheva E., Pavlov A., Ilieva M., Michneva M. "Rosmarinic acid from *Lavandula vera* MM cell culture.", Proc. of the "Future Trends in Phytochemistry - A Young Scientists Symposium", May 12-15, 1996, Rolduc, The Netherlands.
12. Koleva I.*, Handjieva N. (1997). "A Study on the Iridoid Glycosides in *Sideritis* (Lamiaceae)." Sci. Works UFT-Plovdiv **XLII**: 75.

13. Koleva I. (1997). ”Separation Methods for Iridoid Glycosides” Herba Pol. **53**: 322-333.
14. Bahchevanska S.*, Koleva I.I., Jurukova V. (1997). “A study on some possibilities for isolation of bioflavonoids from *Sophora japonica* blossoms.” Proc. of the 11th Balkan Biochemical & Biophysical Days, May 15-17, Thessaloniki, Greece.
15. Bahchevanska S., Demireva Z., Koleva I.I.* "Natural antioxidants in cosmetics", Proc. of the "2000 Years of Natural Products Research - Past, Present and Future" - Joint Meeting of the ASP, AFERP, GA and PSE, July 26-30, 1999, Amsterdam, The Netherlands.
16. Koleva I.I.*, Kovatcheva E., Pavlov A., Ilieva M., Mincheva M. "Antioxidant activity of extracts from *Lavandula vera* MM cell cultures.", Proc. of the "2000 Years of Natural Products Research - Past, Present and Future" - Joint Meeting of the ASP, AFERP, GA and PSE, July 26-30, 1999, Amsterdam, The Netherlands.
17. Koleva I.I.*, Linssen J.P.H., van Beek T.A., Evstatieva L.N., Kortenska V. "Antioxidant activity of extracts from *Sideritis* species (Labiatae) grown in Bulgaria", Proc. of the "2000 Years of Natural Products Research - Past, Present and Future" - Joint Meeting of the ASP, AFERP, GA and PSE, July 26-30, 1999, Amsterdam, The Netherlands.
18. Dapkevicius A., Koleva I.I., van Beek T.A.*, Pukalskas A., Niederländer H.A.G. “Isolation, identification and evaluation of antioxidants from herbs.” Proc. of the Int. Symposium on Flavour and Fragrance Chemistry, January 13–16, 2000, Campobasso, Italy.
19. Niederländer H.A.G.*, Dapkevicius A., Koleva I.I., van Beek T.A. “Methodologies for on-line screening of antioxidant activity in HPLC.“ Proc. of the Int. Symposium on Trends in Medicinal Chemistry and Biocatalysis, January 26-29, 2000, Delhi, India.
20. Koleva I.I.*, Niederländer H.A.G., van Beek T.A. “A rapid on-line HPLC method for detection of radical scavenging compounds in complex mixtures”, Proc. of the Int. Symposium “Future Trends in Phytochemistry”, May 7–10, 2000, Rolduc, The Netherlands.
21. Koleva I.I.*, Linssen J.P.H., van Beek T.A., Evstatieva L.N., Kortenska V., Handjieva N. “*Sideritis* plants as a source for natural antioxidants.”, Proc. of the 2nd Int. Symposium “Novelties and strategy in the world essential oils and perfumery industry”, May 25–26, 2000, Plovdiv, Bulgaria.
22. Kovatcheva E., Koleva I., Pavlov A., Ilieva M., Michneva M. (2001). “Antioxidant activity of extracts from *Lavandula vera* MM cell culture.” Food Chem. **72:** 295-300.
23. Koleva I.I.*, Niederländer H.A.G., van Beek T.A. “On-line HPLC methods for detection of radical scavengers in complex matrices.“ Proc. of the Int. Symposium “Drie scheidende hoogleraren”, February 23, 2001, Wageningen, The Netherlands.
24. Pavlov A., Kovatcheva P., Georgiev V., Koleva I. (2002). “Biosynthesis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures.” Z. Naturforsch. **57c:** 1-5.
25. Mihalev K.*, Koleva A., Mollov P., Koleva I. (2003). “Effect of enzymatic mash treatment on the antioxidant potential of cloudy apple juices.” Sci. Works UFT-Plovdiv **L:** 19-24.

* - presenting person

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Curriculum Vitae

Irina Iordanova Koleva was born on June 23, 1965 in Choumen, Bulgaria. She attended primary school at the 6th Secondary School of Choumen from 1971-1978. From 1978-1983 she attended Grammar Secondary School with Extended Learning of Russian Language in Choumen which she left with a "Gold medal". Then she started studies at the University of Chemical & Metallurgical Technology in Sofia (former Higher Institute of Chemical Technology). When she was 3rd-year student she won the first place in the National Chemical Olympiad. She graduated in 1988 as a Chemical Engineer in Organic Fine Synthesis under the supervision of Prof. Dr. L. Yankov with the subject "Synthesis of Methyl Anthranilate". From 1988 till 1994 she worked as a technologist at the Research Institute of Perfumery, Cosmetics & Essential Oils in Plovdiv. From 1989-1991 she was on maternity leave. In 1992 she worked at Plovdiv University at the Central Research Laboratory under guidance of Prof. Dr. G. Andreev who supervised her in doing UV- and IR spectra of essential oils. In 1994 she passed the exams for assistant-professor at the University of Food Technology (former Higher Institute of Food & Flavour Technologies) in Plovdiv. In 1995 she spent 3 months in England in the framework of the TEMPUS programme. In 1996 she performed a 6-month research study in The Netherlands in the framework of the same programme.

On February 1998 she was admitted as a PhD-student to a so-called "sandwich" research project of the Bulgarian Academy of Sciences - Sofia, University of Food Technology - Plovdiv and two departments of Wageningen University, The Netherlands, namely the Department of Organic Chemistry and the Department of Food Chemistry. She obtained in 1999 and 2000 twice a grant of the Dutch Ministry of Agriculture and Fisheries (IAC grant). As a result of this work, she published in high-ranked journals which can be found in the List of publications.

In 2001 she had an accident with heavy cranial-cerebral trauma that made her temporarily stop her thesis work. From 2001-2005 she was constantly on sick-leave. After 4 years of revalidation, she decided to finish the thesis work she started in 1998. At present, she is Head Assistant Professor at the University of Food Technology – Plovdiv.

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