Screening, isolation and evaluation of antioxidative compounds from *Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides*

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Giedrius Miliauskas

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CONTENT

GENERAL INTRODUCTION

1.1 Problem definition

Electron transfer is one of the most fundamental processes in chemistry. The transfer of an electron from a donor (reducing species) to an acceptor (oxidizing species) results in a change in properties of both components participating in the reaction. In general oxidation can be defined as a conversion of a chemical substance into another having fewer electrons [1].

Food molecules (lipids, proteins, carbohydrates) are widely involved in oxidation reactions; these reactions also play an important role in the ageing processes of living organisms. Oxidation reactions can be induced by so called reactive oxygen species (ROS), which is a general term encompassing various reactive substances. Basically these are free radicals - organic substances with one or more unpaired electrons, such as hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxy ($RO^{\bullet}$), peroxonitrite ($ONOO^{\bullet}$), superoxide anion ($O_2^{\bullet -}$) and others. Other important ROS include singlet oxygen ($^1O_2$), ozone (O_3), nitrogen dioxide (NO_2) and hypochlorous acid (HOCl). Oxidation can also be induced and accelerated by metal ions, phagocyte derived ROS and some other reactive substances [2]. ROS are continuously generated in living organisms and are used in various beneficial physiological reactions, e.g. in signal transduction pathways in the body, immune defense, enzyme catalyzed oxidation [3], however in many cases ROS are harmful. Oxidative stress, caused by ROS in the living cell is associated with numerous diseases, like coronary heart disease, cancer, cataracts and age-related macular degeneration and ageing [4].

ROS are the major cause of lipid containing food products deterioration, commonly defined as rancidity. Significant changes can occur in product odour, taste, colour, texture, nutritive value. Progressing oxidation results in complete spoilage of foods.

Although protective mechanisms exist both in living cells (e.g. enzymes: superoxide dismutase, glutathione peroxidase) and in foods (e.g. tocopherols, vitamin E, C, flavonoids), in many cases there is a need to strengthen this mechanism. Use of antioxidants can postpone problems caused by ROS and thus they are frequently used to retard oxidation processes in the food industry. In recent years an increasing interest in natural formulations of food antioxidants and other additives has been observed in the food market. One of the reasons for this interest is an overall growth of living standards. For consumers who choose a product, the quality becomes the main priority determining this choice. Even though money-wise buying “natural” products may not be in their favour, natural

ingredients can have advantages. For instance some natural antioxidants, apart from the primary use in foods (i.e., to prolong the product shelf life), can possess additional beneficial biological activities. Flavonoids, a very widely spread group of compounds occurring in herbs, fruits and vegetables – are reported to have anticarcinogenic, antiinflammatory, antibacterial, antiviral, immune stimulating and antiallergic activities [5]. At the same time, the use of synthetic antioxidants in food products is strictly regulated due to uncertainty of their safety [6, 7].

A large number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties. Numerous studies were carried out on some of these plants, e.g. rosemary, sage, oregano, which resulted in the development of natural antioxidant formulations for food, cosmetic and other applications. However, scientific information on antioxidant properties of various plants, particularly those that are less widely used in culinary and medicine is still rather scarce. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants, functional foods and nutraceuticals.

1.2 Aims and scope of the work

Scarce information on bioactive compounds and their properties in many species, including those growing in Lithuania, as well as increasing demand for naturalness of food were important motivations to start this study. The general aim of this study was to find new natural antioxidants in medicinal and aromatic plants, cultivated in Lithuania, determination of their molecular structures, analysis of their properties and assessment of application possibilities of partially purified extracts containing these compounds.

The studies of aromatic and medicinal plants grown in Lithuania so far resulted in the evaluation of antioxidant properties of some less investigated plants [8-11] and identification of new antioxidants [12, 13]. These findings encouraged to initiate this work. The initial aim was to screen extracts isolated from some plants growing in Central and Eastern Europe (chapter 2 of this thesis) for radical scavenging activity. The following plants were selected: *Salvia sclarea*, *Salvia glutinosa*, *Salvia pratensis*, *Lavandula angustifolia* (all Lamiaceae), *Calendula officinalis*, *Matricaria recutita*, *Echinacea purpurea*, *Rhaponticum carthamoides* (all Asteraceae), *Juglans regia* (Juglandaceae), *Melilotus officinalis* (Fabaceae), *Geranium macrorrhizum* (Geraniaceae) and *Potentilla fruticosa* (Rosaceae). To my knowledge there are only few data on the radical scavenging and/or antioxidant

properties of these plants. Garden sage (*Salvia officinalis,* Lamiaceae) was used as a reference plant due to its well documented antioxidant properties.

Plant extract screening studies pointed out further investigations, as several promising plant materials with strong radical scavenging properties showed up. These were leaves of *Geranium macrorrhizum*, blossoms of *Potentilla fruticosa* and leaves of *Rhaponticum carthamoides*. The antioxidant activity of extracts of these plants was similar or higher to that of garden sage (*Salvia officinalis*), which is a thoroughly tested and widely used plant, containing powerful antioxidants. Moreover these plants have not been widely investigated before, especially not for their antioxidant properties. So the second general task (chapters 3, 4 and 5) was isolating and identifying the radical scavenging compounds in these plants, and determining their individual activity and comparing them to reference antioxidants.

In addition to studying the above mentioned parts from *Geranium macrorrhizum* and *Potentilla fruticosa* a quantitative comparison of the major radical scavenging compounds present in different botanical parts of *Geranium* and *Potentilla* was performed (chapter 3, section 3.3.5 and chapter 4, section 4.3.4).

The assessment of the antioxidant properties of the selected extracts from the studied plants was the following step of this study (chapter 6). A variety of different antioxidant activity evaluation tests were applied in this step in order to obtain as much as possible information on the properties of the selected extracts. The tests that were applied included several model radical tests and oil oxidation tests: measuring superoxide anion and hydrogen peroxide scavenging properties, β-carotene bleaching test, monitoring of hexanal, as a secondary oxidation product in the course of oxidation of edible oil, monitoring of peroxide formation, and the increase in UV absorbance in the oil samples with added extracts. Moreover oxidation tests with applied *G. macrorrhizum* and *P. fruticosa* extracts in real food system (fermented sausages) have been conducted.

Regardless of all the positive properties or effects that natural formulations derived from plant material can possess, they hardly would be approved as new food additives or supplements without an evaluation of their safety. For this reason a preliminary safety evaluation of the chosen plant extracts was another task of the study. Several tests assessing genotoxicity and mutagenicity are presented in section 6.3.5 of chapter 6. Safety aspects are discussed and preliminary results are presented.

To achieve the general aims of this thesis sophisticated analytical techniques, like LC-MS and LC-NMR were needed. Also some specific techniques were applied, e.g. on-line radical scavenging detection of individual plant constituents from complex plant mixtures. These methods have been

developed in the Laboratory of Organic Chemistry of Wageningen University [14, 15]. While using these methods in my own research, I was also aiming at improving these methods. Expansion of the on-line LC-DAD-DPPH/ABTS system by additionally coupling it to a mass detector was part of the methodological research and is described in chapter 7.

The hyphenated LC-DAD-SPE-NMR system is another powerful technique for compound isolation and structure elucidation from complex mixtures. The system works well on the analytical scale, however hyphenated 2D NMR experiments (like heteronuclear multiple bond correlation - HMBC), which are highly important for elucidation of structures of novel compounds, still require substantial amounts of analyte, which are difficult to obtain using analytical HPLC columns. The use of semi-preparative HPLC columns, as an alternative to analytical columns in LC-DAD-SPE-NMR is described in chapter 8.

An overall evaluation and concluding remarks are presented in chapter 9. Some important aspects and targets for future research are also proposed.

1.3 Literature survey

1.3.1 Classification of antioxidants

During recent years the most widely used definition of antioxidants was proposed by Halliwell and his coworkers: “antioxidants are substances that when present in low concentrations with those of an oxidisable substrate, significantly retard oxidation of that substrate” [16]. A very common way to classify antioxidants is to divide them into two mechanistically distinct groups: primary and secondary [17]. A similar classification of antioxidants is to divide them as (a) chain breaking (vitamin E, phenolics), (b) preventive (intracellular enzymes, such as catalase, superoxide dismutase (SOD) and others) and (c) complementary (vitamin C, β-carotene, flavonoids) [18].

Primary antioxidants delay or inhibit the initiation step and interrupt the propagation step of the radical chain reaction. Antioxidants act by transferring a hydrogen atom to the peroxy radical. The resulting radicals from the oxidized antioxidant are stabilized by resonance and are relatively un-reactive and therefore are not capable of initiating or propagating the oxidative reaction.

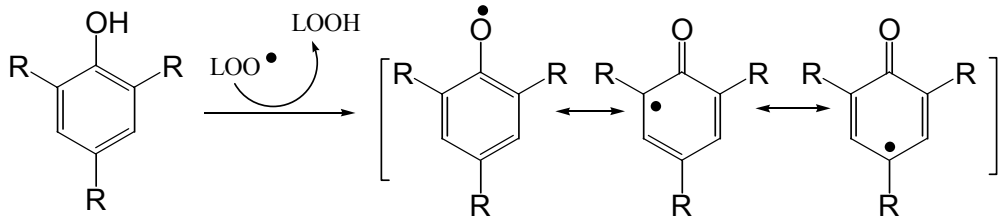


Figure 1.1 *Action of primary antioxidants: transfer of hydrogen to the peroxy radical and formation of a stable aryloxy radical [19].*

Most of the antioxidants used in food protection are primary antioxidants. Basically they are different phenolic compounds with various ring substitutions: phenolic acids, catechins, flavonoids, anthocyanidins, lignans, tannins, coumarins. Synthetic antioxidants, like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propyl gallate (PG) also have a phenolic structure.

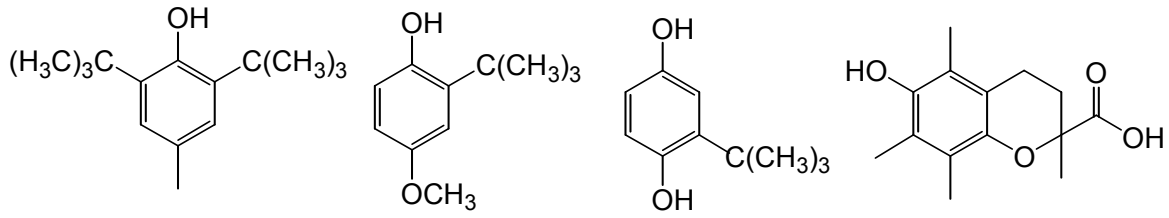
The effectiveness of the phenolic antioxidant depends on the resonance stabilization of the phenoxy radical. This is influenced by the substituents attached to the aromatic ring [20]. Substitution at *ortho* and *para* positions (relative to the hydroxyl group) increases the reactivity and formed radicals are more stable. Bulky substituents (e.g. the tertiary alkyl groups of BHA and BHT create steric hindrance and provide stability to the phenoxy radical, however they also lower the reaction rate with peroxy radicals.

Several antioxidants are used in combinations because of synergistic effects. For instance, because of the earlier described steric hindrance of BHA and BHT, they are often used in combination with other antioxidants, e.g. with PG and TBHQ [19]. Another class of antioxidants is the secondary or preventive antioxidants. They include metal chelating agents, singlet oxygen quenchers, peroxide destructors and some others [1].

Natural and synthetic antioxidants

Independently from other classifications all antioxidants can be classified into natural and synthetic ones. The best known synthetic antioxidants are BHA, BHT, gallates and TBHQ. The basic action mode of synthetic antioxidants has been described earlier.

The variety of natural antioxidants is much higher. For instance, if one considers the diversity of plant phenolics and the fact that most of them exhibit some radical scavenging properties, the number of possible natural antioxidants becomes huge.



BHT BHA TBHQ Trolox

Figure 1.2 *Chemical structures of some synthetic antioxidants.*

The biggest advantage of synthetic antioxidants is related to their low cost. Other advantages are their well studied chemical and technological properties, which in most cases meet the demands of producers. That makes synthetic antioxidants dominating in the world market. Among natural antioxidants, however, only a small percentage has been thoroughly analyzed and even fewer are actually being used. Up to now only tocopherols, carotenoids, ascorbic acid and its derivatives, as well as extracts from rosemary and sage have been industrially applied in foods [17].

Tocopherols. Tocopherols are very important natural antioxidants. They can be divided into two groups: tocols and tocotrienols. Cereals and legumes are rich sources of tocols [21]. Oils and green vegetables are also sources of tocopherols [22]. The antioxidant mechanism of tocopherols involves reactions with free radicals (especially the peroxyl radical), resulting in the formation of a relatively stable phenoxy radical. Another mechanism of tocopherols includes singlet oxygen scavenging and quenching.

Carotenoids. Carotenoids are yellow, orange or red pigments, which are found in high concentrations in certain edible fruits or roots (carrots, tomatoes, etc) [1]. Structurally they are long-chain polyisoprenes, with 40 carbon atoms.

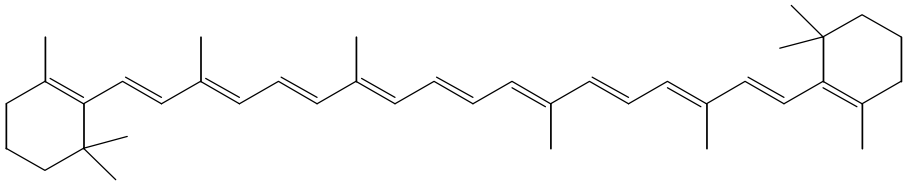


Figure 1.3 *Structure of β-carotene.*

Carotenoids are required dietary constituents for animals, as well as humans, since some of them are precursors of vitamin A. However these compounds also display other physiological properties, one of them being antioxidant activity. The radical trapping ability of carotenoids lies on the

delocalization mechanism of unpaired electrons over the conjugated polyene system [23], making it less likely for the formed radical to take part in chain processes.

Like tocopherols, carotenoids are effective $^1O_2$ quenchers. Quenching of singlet oxygen is due to an energy transfer from $^1O_2$ to the carotene [24]. Quenching also depends on the number of conjugated double bonds in carotene. This is linked with the fact that carotenoids with seven or fewer double bonds were unable to delocalize the unpaired electron gained from $^1O_2$ [25].

Ascorbic acid. This vitamin occurs in rather high concentrations naturally in many fruits and vegetables. Ascorbic acid acts as a multifunctional antioxidant and as a synergist for primary antioxidants. In the presence of higher concentrations of metal ions ascorbic acid can show pro-oxidant properties by reducing back oxidized metal ions after which they can initiate new free radical reactions [1, 26].

Phenolic compounds. Flavonoids. Flavonoids represent a large and diverse group of phenolic compounds derived from higher plants. These aromatic compounds are formed in plants from the aromatic amino acids, phenylalanine and tyrosine, and acetate units [27]. Flavonoids can display a wide range of substitution patterns and oxidation states, and are divided into flavonols, flavanonols, flavones, flavanones, catechins (flavan-3-ols) and anthocyanins.

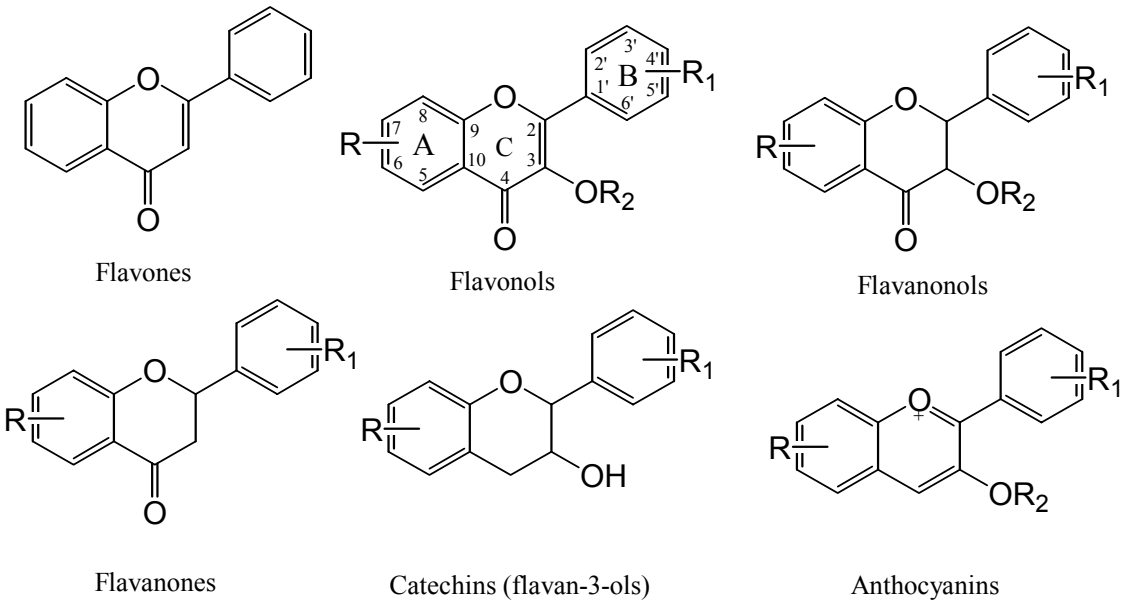


Figure 1.4 *Structures of flavonoids.*

Some flavonoids are responsible for the colours of plants. Many of them are glycosylated. They are known to display a multitude of pharmacological and biochemical actions. They have long been recognized as possessing anti-inflammatory, antiallergic, antimicrobial, hepatoprotective, antiviral,

antimutagenic/anticarcinogenic and many more properties [28]. Catechins have received much attention in recent years, being constituents of green tea, which has been reported to show potential anticancer properties [29, 30]. The antioxidant function and enzyme modifying actions of flavonoids could account for many of their pharmacological activities [31]. The compounds appear to possess variable mechanisms of action, which include radical scavenging and metal ion complexation. Numerous studies have been carried out to determine the necessary structural features for flavonoids to be effective radical scavengers [5, 32-34].

Many studies have shown that flavonoids having more hydroxyl groups, or hydroxyl groups *ortho* to one another, are more effective antioxidants. The B ring of flavonoids is more electron rich than the A and C rings. That is why the B ring is primarily attacked by radicals. These properties are consistent with oxidation mechanisms of phenols – hydroxyl groups act as electron donating substituents, and *ortho* hydroxylation helps to stabilize the phenoxyl radical [1]. Glycosylation of hydroxyl groups is reported to be linked with diminished antioxidant activity. The type of glycosyl moiety also seems to play a role in overall activity [35].

Phenolic acids. Phenolic acids are widely distributed in cereals and legumes. They act as free radical terminators. Phenolic antioxidants act to inhibit lipid oxidation by trapping the peroxyl radical. This radical abstracts a hydrogen atom (or electron after prior loss of a proton) from the antioxidant to yield a phenoxyl radical.

In plants, free phenolic acids occur as substituted benzoic and cinnamic acid derivatives. Tests comparing the effectiveness of cinnamic and benzoic acid compounds showed that cinnamic derivatives were superior probably because of the stability provided to intermediate free radical forms by the extended conjugation of the side chain [36].

Other natural antioxidants. Tannins (complex gallic acid esters) show antioxidant activity due to their ability to scavenge metal ions and free radicals [1]. Ellagic acid – an internal lactone, derived from gallic acid and protocatechuic acid is found in some fruits and vegetables and has been reported to possess antioxidant properties [37].

There are a number of other natural compounds that have been reported to show antioxidant properties: lignans, curcumin derivatives, hydroquinones, some essential oil components (e.g. thymol and carvacrol) and diterpenes (like carnosol, carnosic acid from rosemary).

1.3.2 Methods of antioxidant activity evaluation

Oxidation processes in foods can cause changes in organoleptic, physical and chemical properties. During these processes many different products are formed. Currently there is no single and uniform method to evaluate all of these changes. Chemical methods that are used to monitor lipid oxidation in food and biological systems may be divided into groups measuring primary changes and secondary changes of oxidation products [38].

1.3.2.1 Methods to evaluate primary changes

Methods to evaluate primary changes can be classified into those (1) quantifying loss of reactants (unsaturated fatty acids or fatty acid composition [39]); (2) measuring addition of oxygen (e.g. weight gain method); (3) measuring formation of primary oxidation products (hydroperoxides), conjugated dienes.

Peroxide value (PV). The hydroperoxide content is determined by an iodometric method [40]. The method is based on the titration of iodine released from potassium iodide by the peroxides in a biphasic system with a thiosulfate solution. One of the drawbacks of this method is the difficulty to adequately measure low PV's, because of difficulties in the end point determining of the titration. Derivatized peroxidation products can also be analyzed and quantified by HPLC or GC-MS techniques [41, 42].

Measurement of conjugated dienes. Lipids containing methylene-interrupted dienes or polyenes show a shift in their double bond position during oxidation due to isomerization and conjugate formation [43]. The resulted conjugated dienes exhibit an absorbance at 234 nm; similarly conjugated trienes absorb at 268 nm. A spectrophotometric method for measuring primary and secondary oxidation products (dienes and trienes) is based on these properties [9].

1.3.2.2 Methods to evaluate secondary changes

While primary oxidation products are quite unstable, colourless and flavourless, secondary products, which are derived from primary products are generally flavour active and more stable: aldehydes, ketones, hydrocarbons, alcohols. This improves the reliability of measurements of secondary changes.

The 2-thiobarbituric acid (TBA) value. Malondialdehyde (MDA) is relatively minor product of oxidation of polyunsaturated fatty acids. It gives a coloured complex (λ_{max} = 530 nm) with the TBA reagent. By measuring the intensity of the colour, the extent of oxidation can be assessed [44]. Other products of lipid oxidation, such as aldehydes, alkenals and 2,4-alkadienals may also react with TBA to produce coloured complexes [45]. Therefore the term "thiobarbituric acid reactive substances" (TBARS) is commonly used [46].

p-Anisidine value. This method is based on the formation of a coloured (yellowish) product by the reaction of unsaturated aldehydes with *p*-anisidine reagent under acidic conditions [47].

Amount of carbonyls. The analysis of individual compounds is another approach for measuring the extent of oxidation. Two aldehydes - hexanal and pentanal are usually the major volatiles of secondary oil oxidation processes. The amount of volatile carbonyls in the headspace of oxidized samples can be measured by gas chromatography [48]. Apart from aldehydes, saturated hydrocarbons (e.g. propane, pentane) as oil decomposition products can also be measured [49].

Fluorescence methods. These methods are based on the formation of fluorescent products from the reaction of malonaldehyde with amino compounds, such as proteins and nucleotides [50].

1.3.2.3 Other tests to evaluate radical scavenging or antioxidant activity

Over the last few decades there has been a significant increase in the development of advanced techniques for monitoring either primary or secondary oxidation products. Pulse radiolysis [51] or electron spin resonance (ESR) [52] facilitated the detection of very short-lived radicals, like $OH^{\bullet}$. These techniques are based on monitoring microwave absorbance which comes from promotion of an electron spin to a higher energy level in a variable magnetic field.

Relatively new methods for measuring radical scavenging detection ability have been created using stable free radical species like 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical or 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation. $DPPH^{\bullet}$ can accept an electron or hydrogen radical from an antioxidant and therefore lose its light absorbance at 515 nm [53]. Differently from $DPPH^{\bullet}$ (it is acquired directly without preparation), $ABTS^{\bullet+}$ must be generated enzymatically (using peroxidase) or chemically (manganese dioxide, potassium persulfate) [54]. Another important difference is that $DPPH^{\bullet}$ can only be dissolved in organic solvents (e.g. methanol), while $ABTS^{\bullet+}$ is soluble in both aqueous and in organic media. So the ABTS test can be performed in both aqueous and lipophilic systems [55]. Model radical tests, like

the DPPH$^{\bullet}$ scavenging test are simple, fast, reproducible and their mechanism of action is known. That is why these tests are convenient, e.g. for a preliminary screening of many samples. A drawback of the DPPH test, as well as for all methods using artificial radicals, is the lack of a direct correlation between the established antioxidant activity and its protective properties in real food systems [56].

Other antioxidant activity evaluation tests are based on a competition of an antioxidant and an oxidisable indicator for free radicals formed during oxidation of simple lipids [17]. Examples of such systems are the β-carotene bleaching test [57], the ferric thiocyanate method [58], the linoleic acid [59] or methyl linoleate oxidation tests [60], chemiluminescence test (based on luminol/isoluminol oxidation in presence of catalysts) and the Oxygen Radical Absorbance Capacity (ORAC) Assay.

1.3.3 Sources of natural antioxidants

Aromatic and medicinal plants are used since ancient times for different purposes, e.g. for flavouring of foods, for preservation and for disguising unpleasant odours in non-fresh meat or fish. Moreover, nearly all folk medicine remedies included the formulation of plants. The fragrant or spicy herbs are still treasured nowadays. However in many countries the number of used medicinal plants considerably shrank in comparison to the middle ages. Mostly this is linked with the discovery of synthetic drugs, which usually are more effective. Nevertheless a big part of the world population still widely relies on traditional herbal medicine. The demand for raw material of natural origin for the production of food supplements, neutraceuticals, and cosmetic products is growing.

A first systematic study on antioxidant properties of herbs goes back to the fifties, when Chipault and co-authors performed a screening of 72 different spices for their antioxidant activity in various substrates [61, 62]. Rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) were found to be effective sources of antioxidants. These herbs received a lot of attention in the past decades. Numerous further studies have proven the effectiveness of these antioxidative plants (see table 1.1 for references) and resulted in the commercial applications of these plants [63].

A lot of other spices common in our diet have also been extensively studied with respect to their antioxidant activity. Chipault and co-authors have found strong antioxidant activity in oregano, thyme, nutmeg, mace, turmeric and bell peppers [61, 62]. A few years later Sethi and Aggrawal reported the antioxidant effects of cinnamon, clove, turmeric, nutmeg and some other spices [64]. Allspice, aniseed, basil, cardamom, cinnamon, ginger and pepper are other examples of spicy plants

possessing antioxidative properties [65]. These properties in spices sometimes are attributed to their essential oils, although in general essential oils are not good sources of antioxidants. Additionally the application of essential oils is almost impossible because of their strong odours. Spices remain one of the most promising sources of natural antioxidants [66]. Active compounds present in spices and showing antioxidative properties are phenolic acids, flavonoids, natural pigments (the phenolic structure turmerin, curcumin, capsaicin) and terpenes (e.g. rosmanol, carnosol, carnosic acid, epirosmanol, isorosmanol) from rosemary and sage [67].

Fruits, berries, vegetables, cereals, legumes, oils (especially from olive), oilseeds and tea have also been extensively explored and their composition is well established as these products are common in the human diet. Active antioxidant components in these sources are often reported to be polyphenolic compounds: phenolic acids, flavonoids, anthocyanidins (in fruits, berries, cereals, legumes), tocols, phospholipids (especially in cereals, legumes), carotenoids (oils, oilseeds), ascorbic acid, chalcones and some amino acids [21, 68].

Tea extracts have received a lot of attention in recent years. Numerous studies have proven that the biologically active components of the extracts (epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate) possess strong radical scavenging effects *in vivo* [30, 69]. A similar story is true for red wines, whose moderate consumption is associated with a lower risk of coronary heart disease and other beneficial effects. Dolora *et al.* reported protective effects of wine polyphenols against colon carcinogenesis in rats [70].

Apart from the mentioned spicy plants, there is still a multitude of unexplored species some of which may be good sources of antioxidants. Herbs that are not used in the human diet have been much less investigated.

Many less known plants are investigated nowadays for their beneficial properties, including antioxidant activity. This is largely stimulated by the increasing shift of consumers towards “natural” labeled alimentary products. Moreover food supplements or cosmetic products with natural plant extracts or other natural formulations are very popular nowadays and this popularity seems to increase. Keyword hits containing plant family names in literature databases of recent years (2001 and earlier of National Library of Medicine [MEDLINE]) have revealed that research is being carried out with almost all entered plant family names. Moreover keyword hits combining family names and the word “antioxidant” in a majority of cases also resulted in positive publication findings. This clearly shows the interest in new sources of natural antioxidants. From the same preliminary literature search it was observed that the Fabaceae, Lamiaceae, Asteraceae and

Rosaceae are the most widely studied plant families. Keyword hits within the publications of the last three years resulted in hundreds of records for these families and in dozens of records including the word "antioxidant".

Table 1.1 *List of some widely investigated herb material, possessing antioxidant properties*

Plant name	Family	Identified compounds with antioxidant properties	References
Rosemary (*Rosmarinus officinalis*)	Lamiaceae	Carnosol, carnosic acid, rosmanol, epirosmanol, isorosmanol, rosmariquinone, rosmaridiphenol, rosmarinic acid;	[65, 71-78]
Sage (*Salvia officinalis*)	Lamiaceae	Carnosol, rosmanol isomers, rosmadial, methyl carnosate;	[67, 74, 75, 79]
Oregano (*Origanum vulgare*)	Lamiaceae	Essential oil, apigenin, eriodictyol, dihydrokaempferol, dihydroquercetin, protocatechuic acid, caffeic and rosmarinic acids;	[72, 80-82]
Summer savoury (*Satureja hortensis*)	Lamiaceae	Rosmarinic acid, caffeic acid;	[83-85]
Thyme (*Thymus vulgaris*)	Lamiaceae	Essential oil: carvacrol, thymol, p-cymene-2,3-diol; eriodictyol, rosmarinic acid, 3'-O-(8"-Z-caffeoyl)-rosmarinic acid, taxifolin, luteolin glucuronide;	[17, 86, 87]
Basil (*Ocimum basilicum*)	Lamiaceae	Flavonoid glycosides, phenolic acids	[88-90]
Nutmeg (*Myristica fragrans*)	Myristicaceae	Essential oil;	[91]
Clove (*Eugenia caryophyllata*)	Myrtaceae	Essential oil, gallic acid;	[92, 93]
Ginger (*Zingiber officinalis*)	Zingiberaceae	Curcuminoids, gingerol compounds, diarylheptanoids;	[58, 94-96]
Licorice (*Glycyrrhiza glabra*)	Leguminosae	Flavonoids;	[97]

Although the research of various plants is quite wide, food application examples of antioxidants from less known plants are very few. This is caused by the fact that complex and expensive research is needed in order to create a new, effective and safe product. The economical interest in such products however is in the majority of cases negligible. Many technological and related questions should be answered before new natural antioxidants can be utilized as food additives. What oxidation mechanism is going to take place? Will antioxidants be soluble in the used media? Will they be stable? Will they have any impact on the product flavour or colour? What is the proper concentration needed? These and a number of related questions are important for food producers. In many cases antioxidants of natural origin fail to answer positively to some of these questions

(solubility, odour and colour are the most common limitations). Another important question is: natural = safe? It is well known that this not always the case; therefore the assessment of safety aspects is a necessity, even though this type of research is expensive. Moreover, natural formulations of antioxidants are usually complex, this makes toxicological and other investigations even more complicated. Complex additives can give a wide range of interactions with the product, and this should be taken into account.

Apart from the mentioned questions while looking for new sources of natural antioxidants, plant cultivation or climatic conditions should be taken into account. These factors influence the plant compound composition and therefore its properties too.

Nevertheless the research should continue on finding promising sources of antioxidants or carrying out detailed investigations of promising ones, on the optimization of isolation techniques, on biochemical studies towards safety of newly discovered sources, and the bioavailability of active compounds.

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SCREENING OF RADICAL SCAVENGING ACTIVITY OF SOME MEDICINAL AND AROMATIC PLANT EXTRACTS*

2.1 Introduction

From the huge variety of aromatic, spicy or medicinal plants, only a few have found a niche for application as antioxidants in food products. Information about plants that are less widely used in food or as medicines is scarce and consequently they are not used. To untap this vast source of potentially useful plants, the assessment of their properties is a first task. Therefore this part of the work was aimed at a preliminary screening for radical scavenging activities of extracts isolated from some plants growing in Central and Eastern Europe.

2.2 Review of the screened in this study plants

A literature survey of aromatic and medicinal plants grown in Lithuania provided results on the antioxidant properties of marjoram (*Majorana hortensis*), catnip (*Nepeta cataria*), oregano (*Origanum vulgare*), hyssop (*Hyssopus officinalis*) [1], horehound (*Marrubium vulgare*) [2] sweet grass (*Hierochloe odorata*), costmary (*Chrysanthemum balsamita*) [3, 4], thyme (*Thymus vulgaris*) [5], peril (*Perilla frutescens*), Moldavian dragonhead (*Dracocephalum moldavica*), Roman chamomile (*Anthemis nobilis*) [6]. These successful studies prompted further research and several more plants grown in Lithuania were initially selected for investigation in this study: *Salvia sclarea*, *Salvia glutinosa*, *Salvia pratensis*, *Lavandula angustifolia*, *Calendula officinalis*, *Matricaria recutita*, *Echinacea purpurea*, *Rhaponticum carthamoides*, *Juglans regia*, *Melilotus officinalis*, *Geranium macrorrhizum* and *Potentilla fruticosa*.

Salvia species are widespread plants in many countries. Clary sage *(Salvia sclarea)* is commercially cultivated for production of essential oil [7]; meadow sage (*Salvia pratensis)* is used in cosmetics and possesses some medicinal properties [8]. *Salvia glutinosa* was tested in enzyme-dependent and enzyme-independent systems of lipid peroxidation and found to possess some antioxidant properties [9]. In general, data about antioxidant properties of these plants are scanty

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[10]. *Salvia officinalis* (Lamiaceae) is a well-known plant with widely documented antioxidant properties (see chapter 1, paragraph 1.3.3. for details and references).

Lavender (*Lavandula angustifolia*) is an important source of a thoroughly studied essential oil, while antioxidant properties of this plant are much less documented. Reports on the antioxidant properties of this plant are somewhat contradictory, most likely due to the differences in the assessment methodology. For instance, Dapkevicius *et al.* [1] did not detect antioxidant activity in lavender extracts in the model linoleic acid–β-carotene system, while Hohmann *et al.* [11] reported that aqueous methanolic extracts of lavender were effective in lipid peroxidation media.

The use of chamomile (*Matricaria recutita*) teas and medicinal preparations has a long tradition in various countries. Although chamomile contains a great number of phenolic compounds [12], it was reported that antioxidative properties of its extracts in rapeseed oil were not distinct [13].

The essential oil of pot marigold (*Calendula officinalis*) is used as medicine for soothing the central nervous system and it exhibits other useful healing properties. Pot marigold is also rich in carotenoids, used as a dye, as a lubricant and for other purposes [14].

Rhaponticum carthamoides (Asteraceae) is a widespread medicinal plant. Originally an endemic plant of southern Siberia, now it is widely grown in Central and Eastern Europe [15]. It is included in formulas of some beverages, medicinal-hygienic preparations, lubricants, creams and shampoos. It has been reported that fractions of the plant possess some pharmacological properties, like haemorheological activity or CNS stimulating activity [16, 17]. The principal bioactive constituents of the whole plant are ecdysteroids and flavonoids. It also possesses a number of phenolic acids [18].

Purple coneflower (*Echinacea purpurea*) is a well-known medicinal plant possessing bactericidal, antiviral and antifungal effects. *Echinacea purpurea* and *Melilotus officinalis* were tested for antioxidant activity using $ABTS^{\bullet+}$ (generated by metmyoglobin and H_2O_2 in the presence of peroxidase) scavenging method and were found to be rather ineffective [19].

Sweet clover (*Melilotus officinalis*) is applied in the production of some beverages and foods [20]. Honey of *M. officinalis* obtained during the plant flowering period was found to possess quite high antioxidant activity as it reduced polyphenol oxidase [21].

The walnut tree (*Juglans regia*) has been thoroughly studied; most attention was paid to its nuts, and the properties of other parts are less known. It was found that the leaves of *J. regia* have antimicrobial activity against some bacteria [22]; they also possess some radical scavenging properties, that have been assessed by electron spin resonance (ESR) techniques [23].

The genus *Potentilla* is a member of the Rosaceae, shrubby cinquefoil (*Potentilla fruticosa)* being one of the few shrubs in this family [24]. It is a perennial plant native to North America [25]. It has spread throughout the world, but is most popular in temperate regions. Apart from being an ornamental, it also has medicinal properties. Moreover, the roots of some *Potentilla* species are edible, and leaves of *P. fruticosa* have some applications as food additives and in cosmetics [26]. Studies on the antioxidant properties of cinquefoil are scarce. It has been reported that shoot extracts inhibit lipid peroxidation and have some other protective effects *in vivo* [27]. A high antioxidant activity of extracts was determined using a photochemiluminescence method [28]. Further, more comprehensive studies on the antioxidant properties of *P. fruticosa* are of interest for its potential application in foods.

Geranium macrorrhizum is a grassy perennial plant with long roots. Balkan countries are the main habitat of this plant, however it is widely found in other European countries too [29, 30]. *G. macrorrhizum* is used for the production of essential oil (named 'zdravets' oil in Bulgarian, what means "healthy"), containing mainly the sesquiterpene germacrone. The plant is known to be rich in tannins and its extracts were reported to possess a broad spectrum of antimicrobial activities. It also has strong hypotensive, astringent activity, as well as cardiotonic, capillary and sedative properties [30, 31].

2.3 Experimental

2.3.1 Reagents

The following reagents were used for the assessment of radical-scavenging activity: 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) (95%, Sigma-Aldrich, Steinheim, Germany), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Fluka, Buchs, Switzerland), KCl, NaCl, NaH_2PO_4, $K_2S_2O_8$ (Merck, Darmstadt, Germany), KH_2PO_4 (Jansen Chimica, Beerse, Belgium). For measuring the amount of phenolic compounds 2.0 M Folin-Ciocalteu phenol reagent and gallic acid (Sigma-Aldrich Chemie) were used. Acetone was from OBR PR (Plock, Poland), ethyl acetate (99.7 %) and methanol (99.5 %), were from Lachema (Brno, Czech Republic).

Screening of radical scavenging activity

2.3.2 Plant material

The following plants (table 2.1) were harvested from the collection of medicinal plants in the experimental fields of Kaunas Botanical Garden (Vytautas Magnus University, Lithuania) at different vegetation phases during May–August, 2000.

Table 2.1 *Investigated plants*

Plant name	Anatomical part	Harvesting stage
Salvia officinalis	leaves and stems	initial bloom stage
Salvia sclarea	leaves and stems	full bloom stage
Salvia glutinosa	leaves	full bloom stage
Salvia pratensis	leaves and stems	full bloom stage
Lavandula angustifolia	leaves, stems and blossoms	full bloom stage
Calendula officinalis	perianths	full bloom stage
Matricaria recutita	blossoms	full bloom stage
Echinacea purpurea	leaves, stems and blossoms	full bloom stage
Rhaponticum carthamoides	leaves and stems	growing stage
Juglans regia	leaves and stems	growing stage
Melilotus officinalis	leaves, stems and blossoms	initial blossom stage
Geranium macrorrhizum	leaves	growing stage
Potentilla fruticosa	blossoms	full bloom stage

The freshly cut plants were sorted, dried in the drying room with active ventilation at ambient temperature, packed in paper bags and stored at ambient temperature (< 30 ºC) for 3-5 months before use.

2.3.3 Extraction procedures

Dried plants were milled with a sample mill (300 Waufn S2, Germany) and separately extracted with three solvents: acetone (analytical grade), ethyl acetate (99.7 %) and methanol (99.5 %). A two-step extraction was applied by shaking flasks with 6-10 g (± 0.01 g) of plant material and 100 ml (2 × 50 ml) of solvent in a shaking machine (Sklo Union LT, Teplice, Czech Republic). Each extraction step was completed in 2 hours. The extracts were filtered and concentrated in a rotary evaporator (Büchi, Flawil, Switzerland) at approx. 40 °C.

2.3.4 Radical scavenging assays

2.3.4.1 DPPH radical scavenging assay

Radical scavenging activity of plant extracts against the stable DPPH radical was determined spectrophotometrically. The colourimetric changes (from deep–violet to light–yellow), when $DPPH^{\bullet}$ is reduced, were measured at 515 nm on a UV/visible light spectrophotometer (Spectronic Genesys 8, Rochester, USA).

DPPH radical scavenging activity of extracts was measured by a slightly modified method of Brand-Williams *et al.* [32], as described below. Extract solutions were prepared by dissolving 0.025 g of dry extract in 10 ml of MeOH. Acetone and ethyl acetate extracts were not fully soluble in methanol (even after treating solutions for 5 min in an ultrasonic bath), therefore they were filtered and only the soluble part was further analyzed. A fresh solution of $DPPH^{\bullet}$ in MeOH (6×10^{-5} M) was prepared daily, before VIS measurements. Three ml of this solution were mixed with 77 µl (38 or 19 µl in additional assays) extract solution in 1 cm path length disposable microcuvettes (final mass ratio of extracts to $DPPH^{\bullet}$ was approximately 3:1, 1.5:1, 0.75:1). The samples were kept in the dark for 15 min at room temperature and then the decrease in absorbance was measured. Absorbance of blank sample containing the same amount of MeOH and $DPPH^{\bullet}$ solution was prepared and measured daily. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Reduction of absorbance } = [(A_B - A_A) / A_B] \times 100, \quad (1)$$

where: A_B – absorbance of blank sample (t= 0 min); A_A – absorbance of tested extract solution (t= 15 min).

2.3.4.2 ABTS radical cation scavenging assay

ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, radical cation decolourisation test is also a spectrophotometric method widely used for the assessment of radical scavenging activity of various substances. The experiments for screening analysis were carried out using an improved ABTS decolourisation assay, described by Re *et al.* [33]. It is applicable for both lipophilic and hydrophilic compounds. $ABTS^{+\bullet}$ was generated by oxidizing ABTS with potassium

persulfate (BDH, Poole, Great Britain). Three ml of ABTS cation solution were mixed with 30 µl of methanolic extract solution in a 1 cm path length disposable microcuvette and the decrease of absorbance was measured at 734 nm during 6 min. All determinations were carried out in triplicate.

2.3.4.3 Measuring the amount of phenolic compounds

The content of total phenolic compounds in plant methanolic extracts was determined by the Folin-Ciocalteu method [34]. For the preparation of the calibration curve 1 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml gallic acid solutions in EtOH were mixed with 5 ml of Folin-Ciocalteu reagent (diluted ten-fold) and 4 ml of sodium carbonate solution (75 g/l). The absorbance was read after 30 min at 20 ºC at 765 nm and the calibration curve was constructed. One ml of a methanolic plant extract (10 g/l) was mixed with the same reagents as described above, and after 1 hour the absorbance was measured for the determination of total plant phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c \times V / m, \qquad (2)$$

where: C= total content of phenolic compounds in mg/g plant extract, in GAE; c= the concentration of gallic acid established from the calibration curve in mg/ml; V= the volume of extract in ml; m= the weight of plant methanolic extract in g.

2.3.4.4 Statistical analysis

Correlation coefficients (R) to determine the relationship between two variables (between different radical scavenging tests; radical scavenging tests and content of total phenolic compounds) were calculated using MS Excel software (CORREL statistical function).

2.4 Results and discussion

2.4.1 Evaluation of radical scavenging activity by model tests

The selected plants were extracted with three different solvents (methanol, ethyl acetate and acetone). Radical scavenging activities of these extracts were measured using model colourimetric tests: DPPH radical scavenging test and ABTS radical cation scavenging test. The results are summarized in table 2.2 and figure 2.1 respectively.

Table 2.2 *Decrease of DPPH absorbance (%) by plant extracts isolated with ethyl acetate, methanol and acetone. Mass ratio of extracts to DPPH$^{\bullet}$ was approximately 3:1*

Plant material	**DPPH absorbance reduction, %**		
	Ethyl acetate extract	Acetone extract	MeOH extract
Salvia officinalis	91.7 ± 0.5	92.6 ± 0.6	92.3 ± 0.5
Salvia sclarea	21.5 ± 1.8	17.8 ± 3.4	92.9 ± 0.4
Salvia glutinosa	16.2 ± 1.5	41.0 ± 0.9	91.5 ± 0.5
Salvia pratensis	17.2 ± 1.4	26.0 ± 0.3	93.0 ± 0.5
Lavandula angustifolia	2.5 ± 1.4	7.4 ± 1.3	35.4 ± 1.7
Calendula officinalis	1.6 ± 1.4	2.6 ± 1.0	12.9 ± 0.8
Matricaria recutita	6.4 ± 1.7	8.2 ± 1.1	44.7 ± 2.6
Echinacea purpurea	3.5 ± 0.1	14.2 ± 1.8	6.8 ± 1.5
Rhaponticum carthamoides	11.4 ± 1.8	30.0 ± 0.3	87.6 ± 1.1
Juglans regia	33.9 ± 2.3	25.3 ± 1.6	67.8 ± 0.2
Melilotus officinalis	8.1 ± 1.7	7.6 ± 5.2	75.9 ± 1.8
Geranium macrorrhizum	26.9 ± 1.4	44.6 ± 1.2	91.7 ± 0.6
Potentilla fruticosa	46.3 ± 0.5	93.0 ± 0.2	93.9 ± 0.7

Methanolic extracts were the most effective DPPH radical scavengers. Seven of the investigated methanolic extracts decreased the DPPH absorbance to almost zero (*Salvia sclarea* – 92.9 %, *Salvia glutinosa* – 91.5 %, *Salvia pratensis* – 93.0 %, *Rhaponticum carthamoides* – 87.6 %, *Geranium macrorrhizum* – 91.7 % and *Potentilla fruticosa* – 93.9 %). These percentages can be considered as total scavenging of DPPH radicals, because after completing the reaction the final solution always possesses some yellowish colour and therefore the decrease of absorbance cannot reach 100 %. The

residual absorbance is approximately 7 % of the initial value. The extracts of *Juglans regia* and *Melilotus officinalis* were also good radical scavengers with absorbance decrease of 67.8 % and 75.9 % respectively. The extracts of *Echinacea purpurea* and *Calendula officinalis* contained lower amounts of radical scavenging compounds.

Ethyl acetate and acetone extracts were considerably less effective radical scavengers than methanolic extracts. However, the radical scavenging activity of the ethyl acetate and the acetone extracts of *Salvia officinalis* and the acetone extract of *Potentilla fruticosa* was similar to the radical scavenging activity of methanol extracts of these plants. The acetone extract of *Echinacea purpurea* was more effective than its methanolic extract; however all the extracts from this plant were very weak radical scavengers. It should be pointed out that ethyl acetate and acetone extracts during the test were dissolved in methanol, and this procedure could have had some effect on the measurements of the radical scavenging activity as not all extracts were fully soluble in methanol. Acetone extracts were more active against the DPPH radical than ethyl acetate extracts except for *Juglans regia* and *Salvia sclarea*, therefore it can be concluded that the use of ethyl acetate for extraction of radical scavenging compounds from the selected plants was not effective.

For further DPPH scavenging assessment the most effective extracts were diluted two or four fold. The final mass ratio of extracts with $DPPH^{\bullet}$ was 1.5:1 and 0.75:1 (Table 2.3).

Table 2.3 *Decrease of DPPH absorbance (%) by plant methanol and acetone extracts*

Plant material	**Decrease of DPPH absorbance, %**			
	MeOH extr.	Acetone extr.	MeOH extr.	Acetone extr.
	$[M_{extract}]:[M_{DPPH}]=1.5:1$		$[M_{extract}]:[M_{DPPH}]=0.75:1$	
Salvia officinalis	91.1	87.8	57.5	45.9
Salvia sclarea	74.5	n.d.*	n.d.	n.d.
Salvia pratensis	80.3	n.d.	n.d.	n.d.
Salvia glutinosa	77.6	n.d.	n.d.	n.d.
Potentilla fruticosa	92.5	92.4	79.6	91.4
Geranium macrorrhizum	92.3	n.d.	89.0	n.d.
Rhaponticum carthamoides	66.9	n.d.	n.d.	n.d.

n.d. – values were not determined

The results demonstrate that the most active radical scavengers were the methanolic extract of *Geranium macrorrhizum* and the acetone extract of *Potentilla fruticosa*. The radical scavenging activity of these extracts after their four-fold dilution remained the same as that of the more

concentrated extracts, while the activity of *Salvia officinalis* under the same conditions was approximately twice reduced. The methanolic extract of *Potentilla fruticosa* was also a strong radical scavenger.

Another antioxidant activity screening method, applicable for both lipophilic and hydrophilic antioxidants - ABTS radical cation decolourisation assay, showed quite similar results compared to those obtained in the DPPH assay.

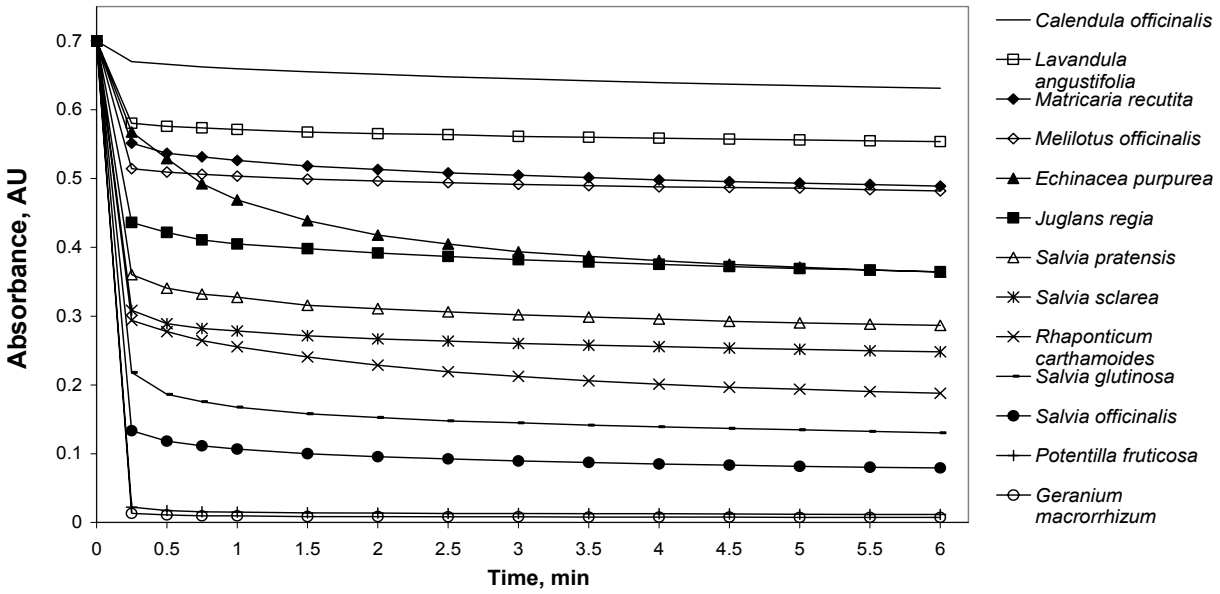

Figure 2.1 *Decrease of $ABTS^{+\bullet}$ absorbance by methanolic plant extracts.*

The extracts of *Geranium macrorrhizum* and *Potentilla fruticosa* were the most active: they nearly fully scavenged $ABTS^{+\bullet}$ (the absorbance after 6 min. was close to 0 AU). In the ABTS assay the activity of *Salvia officinalis* extracts was slightly lower: the absorbance after 6 min. decreased to 0.08 AU, i.e. by 89 % (figure 2.1). The extracts of *Salvia glutinosa*, *Rhaponticum carthamoides* and *Salvia sclarea* also possessed strong activity: the absorbance was decreased by 81 %, 72 % and 64 % respectively by these extracts. It should be noted that the reaction with $ABTS^{+\bullet}$ was quite fast and in almost all cases was completed within 0.25 - 0.5 minutes. During the remainder of the reaction time the changes in absorbance were negligible, except for *Echinacea purpurea*, where the absorbance continued to decrease during 3 min.

The results of these two radical scavenging assays show a good correlation (correlation coefficients between DPPH and ABTS assays were 0.83 and 0.76 for acetone and methanol extracts respectively). *Geranium macrorrhizum*, *Potentilla fruticosa* and *Salvia officinalis* possessed the

highest activity in both tests. Quite similar results were obtained also for other plants, except for *Echinacea purpurea.* The activity of its extracts in the ABTS assay was higher than in the DPPH test. On the other hand a slow reaction was observed in this case with *Echinacea*.

2.4.2 Determination of the amount of phenolic compounds

As plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, the total amount of phenolic compounds in the selected plant extracts was determined using the Folin-Ciocalteu method [34].

Table 2.4 *Total amount of plant phenolic compounds*

Plant extracts	**Total phenolic compounds, mg/g plant extract (in GAE)**
Salvia officinalis	22.6 ± 0.9
Salvia sclarea	24.0 ± 1.1
Salvia glutinosa	17.1 ± 0.6
Salvia pratensis	9.7 ± 0.4
Lavandula angustifolia	5.4 ± 0.2
Calendula officinalis	6.6 ± 0.3
Matricaria recutita	7.5 ± 0.1
Echinacea purpurea	4.1 ± 1.2
Rhaponticum carthamoides	13.3 ± 0.3
Juglans regia	11.5 ± 1.0
Melilotus officinalis	4.3 ± 0.6
Geranium macrorrhizum	25.9 ± 0.2
Potentilla fruticosa	37.9 ± 2.1

The content of phenolic compounds (mg/g) in methanolic extracts varied between 4.1 – 37.9 mg/g. The content of phenolic compounds was determined from the regression equation of a calibration curve ($y = 10.738x + 0.061$, $R^2 = 0.98$) and expressed as gallic acid equivalents (GAE).

The highest amounts were found in the extracts of *Potentilla fruticosa* and *Geranium macrorrhizum*. A high content of phenolic compounds was also found in all *Salvia* species and in *Rhaponticum carthamoides*. It can be observed that the content of phenolics in the extracts

correlates with their radical scavenging activity (e.g. correlation coefficient between data of ABTS assay and total phenolic compounds is 0.84), confirming that phenolic compounds contribute to the radical scavenging activity of these plant extracts.

After the screening analysis, which revealed a high radical scavenging activity of extracts from *Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides*, these plants were selected for detailed analysis: to identify the major radical scavenging compounds.

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ISOLATION AND IDENTIFICATION OF RADICAL SCAVENGERS FROM *GERANIUM MACRORRHIZUM*[*]

3.1 Introduction

In a screening of several plants grown in Eastern and Central Europe for their radical scavenging properties (chapter 2), it was found that extracts of *Geranium macrorrhizum* L. possessed strong radical scavenging capacity. The activity of some extracts was higher than that of extracts isolated from sage (*Salvia officinalis*), a thoroughly tested and widely used plant, containing strong antioxidants.

The primary aim of this chapter was to identify the major active compounds in extracts of *Geranium macrorrhizum* and to determine their radical-scavenging activities relative to the reference antioxidant, rosmarinic acid (RA). A second aim was to compare the presence of the major radical scavenging compounds in different botanical parts of this plant.

3.2 Materials and methods

3.2.1 Reagents

The following reagents and antioxidants were used for radical-scavenging activity evaluation: DPPH (95%, Sigma-Aldrich), ABTS (Fluka), KCl, NaCl, NaH_2PO_4 and $K_2S_2O_8$ (Merck), KH_2PO_4 (Jansen Chimica), RA (Extrasynthese, Genay, France), and Trolox 97% (Sigma-Aldrich).

The following solvents were used in the extraction and fractionation steps: *tert*-butyl methyl ether (tBuMeO, Fluka), ethanol (EtOH, technical grade, distilled prior to use), 1-butanol (BuOH, analytical grade, LabScan, Stillorgan, Ireland) and ultra pure water, prepared in a combined Seradest LFM 20 and Seralpur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). HPLC grade methanol (MeOH, Fluka), acetonitrile (MeCN, Sigma-Aldrich) and formic acid (98–100%, Merck) were used for analytical HPLC analysis. Technical grade MeOH was distilled prior to use for preparative chromatography. Deuterated solvents - CD_3OD, D_2O and DMSO-d_6 (Acros, Geel, Belgium) were used for NMR analysis.

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Radical scavengers from Geranium macrorrhizum

Extraction procedures for compositional analysis. Dry leaves of *Geranium macrorrhizum* (harvested from the collection of medicinal plants in the experimental fields of Kaunas Botanical Garden, Vytautas Magnus University, Lithuania in May 2001) (100 g) were ground in a Moulinex sample mill (Erevete, France) and extracted with 2× 500 ml of tBuMeO at room temperature for 2× 12 h under constant stirring with a magnetic stirrer Ikamag RTC (IKA Labortechnik, Staufen, Germany). The *tert*-butyl methyl ether extract was filtered through a middle porosity filter paper and the solvent was evaporated with a Büchi RE rotary evaporator connected to a Vacuubrand CVC2 vacuum pump and a Büchi 461 water bath (Vacuubrand, Wertheim, Germany). The residue of plant material remaining after extraction with tBuMeO was re-extracted under the same conditions with EtOH and finally with H_2O. The extracts obtained with EtOH and H_2O were further partitioned between water and BuOH resulting in ethanol-butanol (EB), ethanol-water (EW), water-butanol (WB) and water-water (WW) fractions. EW and WW fractions were freeze-dried with a Christ Alpha 1-2 Freeze drier (Christ Gefriertrocknungsanlagen, Osterode, Germany), equipped with a Vacuubrand rotary vane vacuum pump.

3.2.2 Radical scavenging assays

Evaluation of $DPPH^{\bullet}$ and $ABTS^{+\bullet}$scavenging activity of all *Geranium macrorrhizum* fractions and isolated compounds from these fractions was carried out using procedures described by Re *et al.* and von Gadow *et al.* [1, 2]. A Lambda 18 spectrophotometer (Perkin-Elmer, Ueberlingen, Germany) with 1 cm path-length, disposable, plastic half-micro cuvettes (Greiner Labortech, Alphen aan den Rijn, The Netherlands) were used for absorbance measurements. The decrease of absorbance in the $DPPH^{\bullet}$ scavenging assay was measured 30 minutes after mixing of the solutions. Two molar concentration ratios of $DPPH^{\bullet}$·versus the compound to be tested (TC) were chosen (2:1 and 10:1). In the ABTS assay Trolox equivalent antioxidative capacity (TEAC) values were determined for isolated compounds. The decrease of absorbance versus concentration of antioxidant compound was plotted and TEAC values were calculated by dividing the slope of the antioxidant compound by that of Trolox. The activities of compounds were compared with that of rosmarinic acid. The most active fractions were also monitored by on-line HPLC-DPPH/$ABTS^{\bullet+}$ assay methods.

On-line HPLC–DPPH/ABTS assays. The assays were performed as described by Dapkevicius *et al.* [3] and Koleva *et al.* [4]. The set-up for on-line assessment of radical scavenging activity consisted of a Waters 600 E HPLC eluent pump (Millipore, Waters Chromatography, Milford, USA), a Model 231 autosampler (Gilson, Middleton, USA), and a Waters 994 photodiode array detector. A linear binary gradient was used at a flow rate of 0.8 ml/min. Solvent A was a 2% MeCN solution in water, and solvent B was 100% MeCN. Gradient conditions for the WW fraction were as follows: 0 to 10 min B = 0%, from 10 to 16 min B reached 10% and was kept constant till 40 min, from 40 to 50 min B reached 100%. The compounds were separated on an Alltima C18 analytical column, 5 μm, 250×4.6 mm i.d. (Alltech Associates, Deerfield, USA). After the separation and detection, a 45 ml laboratory-made syringe pump (Free University, Amsterdam, The Netherlands) was used for delivery of free radical solution at a flow rate of ca. 0.3 ml/min to a 15 m (13.7 m for ABTS assay) length PEEK tubing reaction coil. The decrease in absorbance at 515 nm for $DPPH^{\bullet}$·(734 nm for $ABTS^{+\bullet}$) was measured with a 759A UV-Vis detector (Applied Biosystems, Foster City, USA), equipped with a tungsten lamp and connected to a recorder BD 40 (Kipp & Zonen, Delft, The Netherlands).

3.2.3 Procedures for isolation of active compounds

Compounds from the WW fraction after reversed phase medium pressure liquid chromatography (RP-MPLC) pre-separation were finally purified by semi-preparative RP-HPLC techniques on a C18 column, 5 μm, 250×10 mm i.d., 300 Å (Rainin, Emeryville, USA). RP-MPLC consisted of a Gilson 802C pump, a Jobin Yvon axial compression system (I.S.A. Jobin Yvon, Longjumeau, France), a Gilson 202 Fraction Collector, a Gilson 201–202 Fraction Controller and a Gilson 111 LC UV detector (254 nm) connected to a Kipp & Zonen BD 40 recorder. The separation was carried out on a 46×2 cm i.d. column containing 60 g Baker Bond Phase C-18 stationary phase (Mallinckrodt Baker, Deventer, The Netherlands) with two eluents - A (10% MeOH in H_2O) and B (MeOH) at the following elution conditions: 0–15 min 0% B, 15– 40 min B increasing to 100% and continued isocratically for 5 min. Compounds from the EB and WB fraction were also isolated by RP-MPLC techniques. Other compounds from the EB fraction were isolated on a 40–63 μm silica gel column (Fluka Chemie) with $CHCl_3$-MeOH-H_2O (60:22:4), and purified on a Sephadex LH-20 (Pharmacia, Stockholm, Sweden) column with MeOH-H_2O (50:50) (compounds **5**, **6** and **7**, see the Results and Discussion for details and structures) or on polyamide column with MeOH. The fractionation

conditions for silica gel column chromatography were worked out on Silica gel 60 F_{254} (Merck, Darmstadt, Germany) TLC plates (5×10 cm). The activities of different fractions after their separation on RP-MPLC or silica gel columns were tested by spotting a few microliters of each collected fraction on a Merck silica gel 60 F_{254} TLC plate and spraying with 0.1% DPPH·solution. Active fractions reduce $DPPH^{\bullet}$, causing a colour change from deep-purple to light yellow.

3.2.4 Structure elucidation of isolated compounds

The structures of the purified compounds were elucidated by various spectroscopic methods: UV, ESI-MS, ^{1}H NMR, ^{13}C NMR and 2D NMR, and by comparison with reference compounds (when available). UV spectra were measured with a Lambda 18 UV/Vis spectrophotometer. (PerkinElmer, Ueberlingen, Germany). ^{1}H NMR spectra were recorded on a Bruker DPX 400 or a Bruker AC-E 200 spectrometer (Bruker, Rheinstetten, Germany). ^{13}C and 2D spectra (COSY, TOCSY, COLOC and HMBC) were recorded on a Bruker DPX 400. Mass spectra were recorded on a Finnigan LCQ spectrometer (Thermo Finnigan, San Jose, CA) equipped with an ESI probe.

3.2.5 Quantitative comparison of major radical scavenging compounds in different botanical parts

Leaves and roots of *Geranium macrorrhizum* were compared using HPLC-UV-MS. Ethanol extracts from *Geranium* leaves and roots were prepared by a cold extraction procedure: 20 g of milled plant material was extracted with 2× 200 ml of EtOH for 2 hours with constant shaking. The extracts were filtered and concentrated with a rotary evaporator (Büchi, Flawil, Switzerland) at approx. 40 °C. For chromatographic analysis 1% methanolic solutions of these extracts were prepared.

The HPLC-UV-MS setup consisted of the following parts: Waters 1525 binary HPLC pump (Millipore, Waters Chromatography, Milford, USA); Merck L-7400 UV detector (LaChrom, Tokyo, Japan) and Waters Micromass ZQ mass detector. The linear binary gradient was used at a flow rate of 0.8 ml/min. Analytes were injected using a Rheodyne manual injector (loop volume 20 µl). Solvent A consisted of 10 % (v/v) MeOH and 1 % HOAc (v/v) solution in water, solvent B was 100% MeOH. Gradient conditions were as follows: 0 to 30 min B increased from 30 to 100 % and kept constant till 33 min; 33-36 min B decreased from 100 % to 30%. The compounds were

separated on a Phenomenex Synergi MAX-RP analytical column, 4 μm, 250×4.6 mm i.d. (Phenomenex, Torrance, USA). The UV detector was operating at 254 nm. The MS detector was operating using an electrospray interface (ESI), in positive and negative ionization modes. After HPLC separation, the eluent flow was split into equal parts using a T connector, directing 0.4 ml/min of total flow to the ESI probe and other 0.4 ml/min to the waste. UV-MS data were processed with MassLynx 4.0 (Waters Micromass Ltd) integration software.

3.3 Results and discussion

3.3.1 Extraction process and assessment of radical scavenging activity of obtained fractions

A scheme of the extraction process and yields of fractions is presented in figure 3.1. Using tBuMeO a considerably lower amount of substances was extracted from *Geranium macrorrhizum* leaves than using the polar solvents EtOH and H_2O. The latter two were subsequently used to extract the plant residue remaining after the tBuMeO extraction. This indicated that the plant contains high amounts of polar compounds.

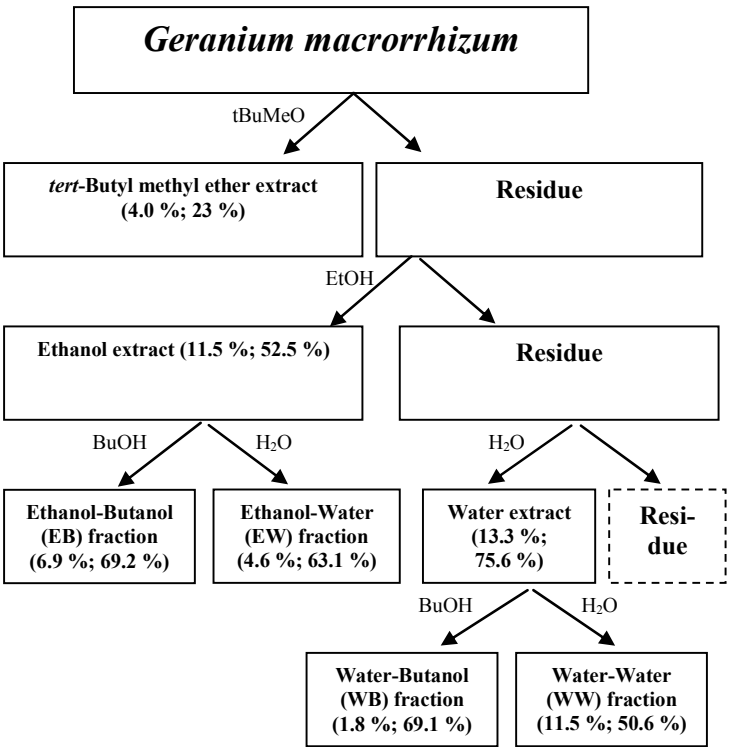


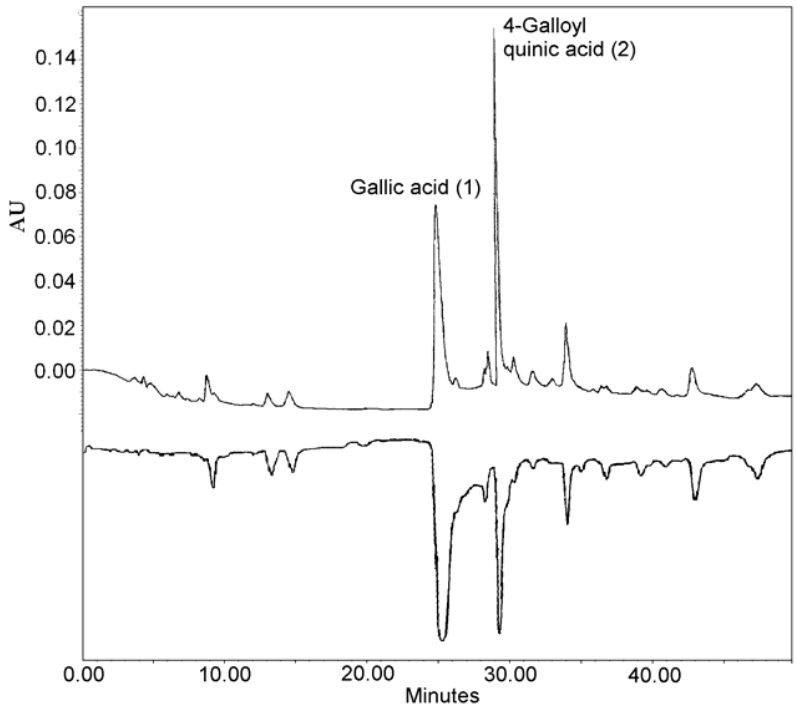
Figure 3.1 *Extraction - fractionation scheme of Geranium macrorrhizum leaves and the radical scavenging activity evaluation of the obtained fractions; in parentheses: yield, %; decrease in absorbance (%) 30 min after initial mixing of extract and DPPH$^{\bullet}$ solutions (final mass ratio between them was 3:1 for tert-butyl methyl ether fraction and 0.75:1 for others).*

Radical scavengers from Geranium macrorrhizum

The assessment of DPPH radical scavenging capacity of these extracts demonstrated that most of the active compounds occurred in the ethanol and water fractions. The radical scavenging capacity of the aqueous fraction was the highest (decrease in absorbance was 75.6 %) followed by the ethanol fraction (52.5 % decrease); while the *tert*-butyl methyl fraction, applied in 4 fold higher concentration showed a very weak radical scavenging capacity (23.0 % decrease). Ethanol and water extracts were further partitioned between BuOH and H_2O, and the activities of the obtained fractions were again determined with the $DPPH^{\bullet}$ assay. The activities distributed nearly equally among these fractions (decrease in absorbance 63.1 - 69.2 %) with a bit lower value for the WW fraction (50.6 %).

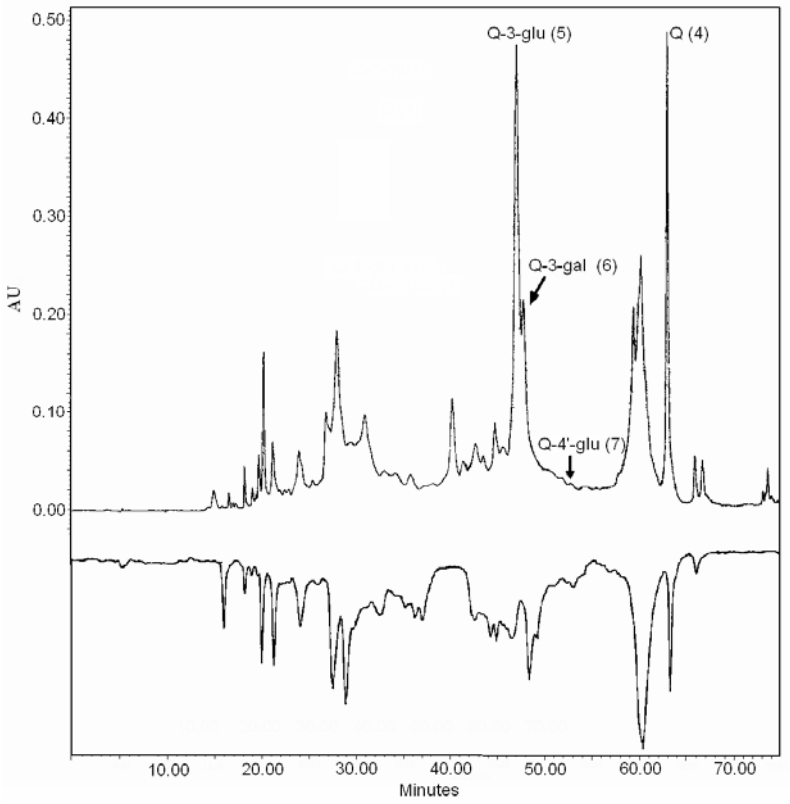
3.3.2 On-line HPLC–DPPH/ABTS assays for detection of active radical scavengers

On-line combination of HPLC separation and $DPPH^{\bullet}$ or $ABTS^{+\bullet}$ scavenging assays is very useful in assessment the activity of detectable plant components in a mixture and in this manner guides the further isolation process. All fractions obtained from *Geranium macrorrhizum* were monitored by these on-line assays. Examples of some chromatograms are shown in figure 3.2 a,b. Negative peaks in the $ABTS^{+\bullet}$ reduction profile indicate active compounds.



a

Figure 3.2 *On-line HPLC–UV–$ABTS^{+\bullet}$ scavenging assay profile of G. macrorrhizum WW fraction (a) and EB fraction (b); upper profile: UV signal at 254 nm, lower profile: $ABTS^{+\bullet}$ reduction signal (734 nm).*



b

On-line radical scavenging assays showed that the highest number of active compounds was present in the ethanol and water extracts, with major active compounds concentrated in EB and WW fractions. Two major peaks were present in the HPLC profile of the WW fraction (figure 3.2 a), and both of them, judging by the intensity of their negative counterparts, represented components possessing strong scavenging activity.

3.3.3 Isolation, purification and structure elucidation of active compounds

Seven compounds were identified in different fractions of *Geranium macrorrhizum* (figure 3.3). Two of them, gallic acid **1** and quercetin **4** have already been reported to be constituents of this plant [5, 6], while compounds **2**, **3**, **5**, **6** and **7** were isolated from *Geranium macrorrhizum* for the first time.

Gallic acid **1** and 4-galloylquinic acid **2** were identified in the WW fraction on the basis of their ^{1}H NMR, ESI-MS data, UV spectra and R_t value (for **1**). Both acids were the most polar compounds relative to other identified components. An HMBC NMR experiment allowed identifying the esterification site of the compound **2** fully elucidating the structure as 4-galloyl quinic acid. By

various separation techniques quercetin **4**, quercetin-3-*β*-glucopyranoside **5**, quercetin-3-*β*-galactopyranoside **6** and quercetin-4'-*β*-glucopyranoside **7** were isolated from the EB fraction.

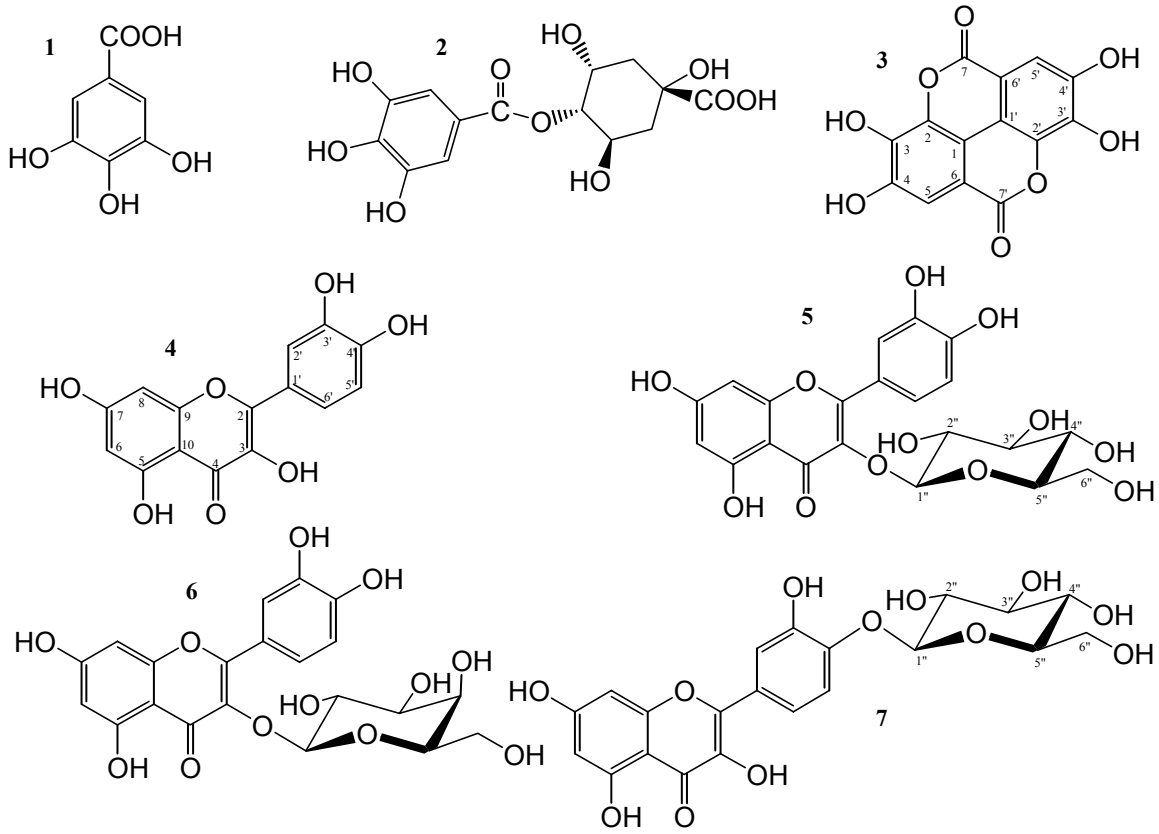


Figure 3.3 *Radical scavenging compounds identified in G. macrorrhizum leaves.*

Quercetin-4'-*β*-glucopyranoside was present in the EB extract in lower concentration relative to the other identified glycosides. Structures of these compounds were elucidated by ^{1}H NMR, ESI-MS, UV data and R_t value (for **4**). In addition, ^{13}C NMR was used for the identification of compounds **5** and **6**. The glycosylation site for these compounds was determined using long-range ^{1}H-^{13}C (COLOC) NMR spectroscopy. Separation of the WB fraction yielded one major radical scavenger that was poorly soluble in water or alcohol. ESI-MS showed a molecular weight of m/z 302 ([M-H]$^{-}$ at m/z 301) suggesting it to be ellagic acid **3**. The ^{1}H NMR, ESI-MS-MS, UV data and its R_t confirmed this hypothesis. For all the isolated compounds the NMR assignments were in accordance with literature data [7-13].

All of the isolated compounds (except 4-galloyl quinic acid) were already known as effective antioxidants. They are all of high to moderate polarity, and can be extracted effectively with cheap

and food-grade solvents, like H_2O or EtOH. The spectral data of all isolated compounds is presented below.

Compound 1 (gallic acid): UV (MeCN) λ_{max} 270 nm; ESI-MS (negative ion mode) *m/z* 169 $[M-H]^-$; ^1H NMR data (D_2O) δ (ppm) 6.88 (2H, s, H-2 and H-6);

Compound 2 (4-galloyl quinic acid): UV (MeCN) λ_{max} 273 nm; ESI-MS (negative ion mode) *m/z* 343 $[M-H]^-$; ^1H NMR data (D_2O) δ (ppm) 7.00 (2H, s, H-2 galloyl and H-6 galloyl), 4.76 (1H, dd, *J* = 3.1, 9.1 Hz, H-4), 4.08-4.20 (2H, m, H-3 and H-5), 1.80-2.08 (4H, m, H-2 and H-6); ^{13}C NMR data (D_2O) δ 178.5 (COOH), 168.0 (-COO-), 144.9 (2C, C-3 galloyl and C-5 galloyl), 138.8 (C-4 galloyl), 120.5 (C-1 galloyl), 110.5 (2C, C-2 galloyl and C-6 galloyl), 78.0 (C-4), 75.7 (C-1), 68.0 (C-5), 64.8 (C-3), 40.4 (C-6), 37.3 (C-2);

Compound 3 (ellagic acid): UV (MeOH) λ_{max} 253, 367 nm; ESI-MS (negative ion mode) *m/z* 301 $[M-H]^-$; ^1H NMR data (CD_3OD) δ (ppm) 7.53 (2H, s, H-5 and H′-5);

Compound 4 (quercetin): UV(MeOH) λ_{max} 254, 354 nm; ESI-MS (positive ion mode) *m/z* 303 $[M+H]^+$; ^1H NMR data (CD_3OD) δ (ppm) 7.73 (1H, d, *J* = 2.1 Hz, H-2′), 7.62 (1H, dd, *J* = 2.1, 8.5 Hz, H-6′), 6.87 (1H, d, *J* = 8.5 Hz, H-5′), 6.37 (1H, d, *J* = 2.1 Hz, H-8), 6.17 (1H, d, *J* = 2.1 Hz, H-6);

Compound 5 (**quercetin-3-O-*β*-glucopyranoside)**: UV(MeOH) λ_{max} 254, 356 nm; ESI-MS (negative ion mode) *m/z* 463 $[M-H]^-$; ^1H NMR data (CD_3OD) δ (ppm) 7.84 (1H, d, *J* = 2.1 Hz, H-2′), 7.58 (1H, dd, *J* = 2.2, 8.5 Hz, H-6′), 6.86 (1H, d, *J* = 8.4 Hz, H-5′), 6.38 (1H, d, *J* = 1.9 Hz, H-8), 6.19 (1H, d, *J* = 1.9 Hz, H-6), 5.24 (1H, d, *J* = 7.6 Hz, H-1″ glucosyl), 3.77-3.18 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″$_{A,B}$); ^{13}C NMR data (CD_3OD) δ 179.4 (C-4), 166.6 (C-7), 163.1 (C-5), 158.9 (C-2), 158.5 (C-9), 149.9 (C-4′), 145.9 (C-3′), 135.6 (C-3), 123.2 (C-6′), 123.1 (C-1′), 117.5 (C-5′), 116.0 (C-2′), 105.6 (C-10), 104.3 (C-1″), 100.1 (C-6), 94.8 (C-8), 78.4 (C-5″), 78.1 (C-3″), 75.7 (C-2″), 71.2 (C-4″), 62.6 (C-6″);

Compound 6 (quercetin-3-O-*β*-galactopyranoside): UV(MeOH) λ_{max} 254, 356 nm; ESI-MS (negative ion mode) *m/z* 463 $[M-H]^-$; ^1H NMR data (CD_3OD) δ (ppm) 7.70 (1H, d, *J* = 2.2 Hz, H-2′), 7.58 (1H, dd, *J* = 2.2, 8.5 Hz, H-6′), 6.86 (1H, d, *J* = 8.4 Hz, H-5′), 6.38 (1H, d, *J* = 1.9 Hz, H-8), 6.19 (1H, d, *J* = 1.9 Hz, H-6), 5.16 (1H, d, *J* = 7.6 Hz, H-1″ galactosyl), 3.87-3.18 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″$_{A,B}$); ^{13}C NMR data (CD_3OD) δ 179.3 (C-4), 167.6 (C-7), 162.9 (C-5), 158.8 (C-2), 158.6 (C-9), 149.9 (C-4′), 145.8 (C-3′), 135.7 (C-3), 123.0 (C-6′), 122.9 (C-1′), 117.7 (C-5′), 116.1 (C-2′), 105.6 (C-10), 105.2 (C-1″), 100.4 (C-6), 95.1 (C-8), 77.1 (C-5″), 75.1 (C-3″), 73.2 (C-2″), 70.1 (C-4″), 61.9 (C-6″);

Compound 7 (quercetin-4´-O-*β*-glucopyranoside): UV(MeCN) λ_{max} 252, 365 nm; ESI-MS (positive ion mode) *m/z* 465 $[M+H]^+$; ^{1}H NMR data (CD_3OD) δ (ppm) 7.79 (1H, d, *J* = 2.1 Hz, H-2´), 7.74 (1H, dd, *J* = 2.4, 8.8 Hz, H-6´), 7.32 (1H, d, *J* = 8.8 Hz, H-5´), 6.41 (1H, d, *J* = 2.0 Hz, H-8), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 4.97 (1H, d, *J* = 12.0 Hz, H-1″ glucosyl), 3.96-3.32 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″$_{A,B}$); ^{13}C NMR data (CD_3OD) δ 177.4 (C-4), 165.7 (C-7), 161.5 (C-5), 157.4 (C-9), 147.1 (C-2), 147.0 (C-3´), 146.8 (C-4´), 137.9 (C-3), 126.7 (C-1´), 120.2 (C-6´), 116.7 (C-5´), 115.5 (C-2´), 103.4 (C-10), 102.5 (C-1″), 98.6 (C-6), 93.6 (C-8), 77.4 (C-5″), 76.6 (C-3″), 73.8 (C-2″), 70.3 (C-4″), 61.4 (C-6″).

3.3.4 Assessment of radical scavenging activity of isolated compounds

The activity of purified compounds was measured by two off-line techniques ($DPPH^•$ and $ABTS^{•+}$ scavenging assays) and the results were compared with those of a well-known reference antioxidant, rosmarinic acid (table 3.1).

Table 3.1 *Radical scavenging activity of compounds isolated from Geranium macrorrhizum*

Compound	TEAC value at:		Decrease in absorbance (%) at:	
	1 min	6 min	$[M_{DPPH}]/[M_{TC}]=2$	$[M_{DPPH}]/[M_{TC}]=10$
Rosmarinic acid	1.48	1.52	95	95
Gallic acid (**1**)	1.51	1.63	94	93
4-Galloyl quinic acid (**2**)	0.99	1.01	68	8
Ellagic acid (**3**)	1.58	1.81	95	75
Quercetin (**4**)	1.61	1.74	96	36
Quercetin-3-glucoside (**5**)	1.64	1.84	93	85
Quercetin-3-galactoside (**6**)	1.64	1.84	93	85
Quercetin-4'-glucoside (**7**)	1.42	1.49	86	35

Trolox Equivalent Antioxidant Capacity (TEAC), estimated at *t=1 min* and *t=6 min*; $[M_{DPPH}]/[M_{TC}]$ – weight ratio of $DPPH^•$ reagent and tested compound.

Although some discrepancies were found, the results for the most active compounds correlated quite well. In the $ABTS^{•+}$ assay (TEAC values) the most active compounds were the quercetin-3-glycosides and in the $DPPH^•$ assay - gallic acid. In the $ABTS^{•+}$ assay most of the isolated compounds reacted within 1 minute and during the next five minutes the TEAC value increased only slightly.

Flavonoid glycosylation is usually linked with diminished scavenging activity of flavonoids due to disappearance of hydroxyl groups and by steric hindrance by sugar moieties on neighbouring hydroxyl groups. However, some factors like type of glycoside group or glycosylation site may distinctly influence this effect. The results show, that glycosylation of the flavonoid quercetin at the 4′ position decreased the antioxidant activity of quercetin, suggesting that the OH group in this position influenced the antioxidant activity of quercetin. However attachments of glucose and galactose to the 3-OH position of quercetin did not have any negative effect on the antioxidant capacity of this compound in this assay. In fact, these glycosides were even more active radical scavengers, than their aglycones. These results are in accordance with literature data [14, 15] suggesting that glycosylation of the 3-OH group is not important for antioxidant capacity.

In the DPPH$^{\bullet}$ assay nearly all compounds (at concentrations of 1:2 with DPPH$^{\bullet}$) fully reduced the DPPH$^{\bullet}$ solution. At a concentration ratio of 1:10 (compound to be tested : DPPH$^{\bullet}$) more informative results were obtained. Gallic acid **1** showed an activity comparable to rosmarinic acid. High activity was shown by quercetin-3-glycosides and ellagic acid **3**. 4-Galloyl quinic acid **2** was the least active scavenger among the isolated compounds with a TEAC value of approx. 1.

In summary: the radical-scavenging capacity (according to TEAC values) of the major plant constituents increases in the order: 4-galloyl quinic acid **2** < quercetin-4′-*β*-glucopyranoside **7** < gallic acid **1** < ellagic acid **3** < quercetin **4** < quercetin-3-*β*-glucopyranoside **5** ≈ quercetin-3-*β*-galactopyranoside **6**.

3.3.5 Quantitative comparison of major radical scavenging compounds in different botanical parts

After the identification of the main radical scavenging compounds in the various plant extracts, their concentration in different botanical parts (leaves, roots) was compared. This was done by comparing peak areas from total ion current (TIC) signals (in arbitrary units) in MS analysis.

Four major compounds were detected and quantified in ethanolic extracts of both leaves and root extracts of *Geranium macrorrhizum*: quercetin-3-O-*β*-glucopyranoside **5**, quercetin-3-O-*β*-galactopyranoside **6**, ellagic acid **3** and quercetin **4** (table 3.2). Quercetin glucoside and galactoside are very similar in their structure and their separation was insufficient to obtain reliable integration results. Therefore the sum of these two compounds was used for comparing purposes.

Radical scavengers from Geranium macrorrhizum

The amounts of these radical scavengers in *G. macrorrhizum* leaf extract were significantly higher than in root extract. The difference in the concentration of ellagic acid in roots and leaves was small compared to quercetin and its glycosides. It can be concluded that leaves are a better source of radical scavenging compounds than *G. macrorrhizum* roots.

Table 3.2 *Relative peak areas (and their ratio) of corresponding major radical scavenging compounds from ethanolic extracts of G. macrorrhizum leaves and roots*

Compound	TIC area (in arbitrary units)		
	Leaves extract	**Root extract**	**Ratio (leaves/roots)**
Quercetin-3-glucoside and galactoside (**5**,**6**)	2.2×10^5	4.8×10^3	**45**
Ellagic acid (**3**)	2.1×10^5	3.4×10^4	**6**
Quercetin (**4**)	1.5×10^6	3.7×10^5	**41**

3.4 References

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ISOLATION AND IDENTIFICATION OF RADICAL SCAVENGERS FROM *POTENTILLA FRUTICOSA*[*]

4.1 Introduction

Potentilla fruticosa was shown to possess strong radical scavenging properties in a preliminary screening of some aromatic and medicinal plants (chapter 2). Information on the composition of the antioxidant or radical scavenging compounds and on the antioxidant properties of this plant were quite sparse, therefore a more comprehensive study was initiated. The aim of this part of the work was to assess the radical scavenging properties of different extracts or fractions from *P. fruticosa*, to identify the major active components in these fractions and to determine their activity. Secondly, studies were aimed to conduct a quantitative comparison of major antioxidant compounds in different botanical parts of this plant.

4.2 Materials and methods

The reagents and most of the methods for the assessment of radical scavenging activity of *Potentilla fruticosa* extracts, fractions or individual compounds, structure elucidation of separated compounds were identical to those applied for the investigation of *Geranium macrorrhizum* (see chapter 3).

Plant material. Dry blossoms of *Potentilla fruticosa* were harvested from the collection of medicinal plants in the experimental fields of Kaunas Botanical Garden, Vytautas Magnus University, Lithuania in May 2002. Blossoms were dried in a room with active ventilation at ambient temperature (~ 30 ˚C) and stored in a paper bags for 3-4 months before use. Extraction-fractionation procedures of plant material were identical to those described in chapter 3.

Procedures for isolation of active compounds. Ethanol-butanol (EB) and water-butanol (WB) fractions (see figure 4.1 for details) of *P. fruticosa* were applied for separation on a silica gel (particle size: 40-63 μm) column with $CHCl_3$-MeOH-H_2O [60:22:4]. Collected fractions (35× 10 ml) were further separated on polyamide (with MeOH) or Sephadex LH-20 (H_2O:MeOH [50:50, v/v]) columns. Purification of compounds **13**, **14** and **15** (see results paragraph for details and

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structures), isolated from EB after silica gel column separation, was performed by semi-preparative RP-HPLC techniques (Xterra Prep. MS C18 column, 19×50 mm, 5 μm, Waters, Milford, USA) at a flow rate of 5 ml/min using an isocratic solvent system MeCN-H_2O-HCO_2H (27:72.9:0.1, v/v).

HPLC-SPE-NMR setup. An HPLC-SPE-NMR set-up with an Alltima C18 analytical column (5 μm, 250 × 4.6 mm i.d.) was used for the separation of some compounds from *Potentilla fruticosa* extracts. A linear binary gradient was used at a flow rate of 0.5 ml/min. Solvent A was 2% MeCN solution in water and solvent B was 100% MeCN. Gradient conditions for the EB fraction were as follows: 0 to 5 min B increased from 8% to 19% and kept constant till 15 min, 15 – 20 min B reached 30% and kept constant till 30 min, 30 – 40 min B reached 100%. Multiple peak trapping was done using an SPE unit Prospekt 2 (Spark Holland, Emmen, The Netherlands) with 10-12 μm Hysphere Resin GP cartridges. Loaded and dried (with nitrogen flow) cartridges were flushed with deuterated MeOH to transfer trapped compounds to the LC-NMR probe. For the detailed description of this technique and equipment used see chapter 8.

Procedures and equipment for the comparison of the content of major radical scavenging compounds in *P. fruticosa* leaves and blossoms were the same as those described in chapter 3, except that the EB fraction (not the raw ethanol extract) of *Potentilla* blossoms was compared to the ethanol leaf extract.

4.3 Results and discussion

4.3.1 Assessment of radical scavenging activity of various plant fractions

Extraction procedure and the yields of extracts from *Potentilla fruticosa* blossoms are shown in figure 4.1. Using tBuMeO a considerably lower amount of substances was extracted from *Potentilla fruticosa* blossoms than with the polar solvents EtOH and H_2O. This clearly indicates that the plant contains a higher amount of polar compounds.

The DPPH$^{\bullet}$ assay demonstrated that most of the active compounds were present in the ethanol and water fractions. Both ethanol fractions, EB and EW possessed the highest radical scavenging activity. A similar activity was measured for the WB fraction, while the activity of the water-water (WW) fraction was slightly lower. Taking into account that the concentration of the *tert*-butyl methyl ether extract in the assay was approximately 8 times higher than of the other fractions, the activity of this fraction was low.

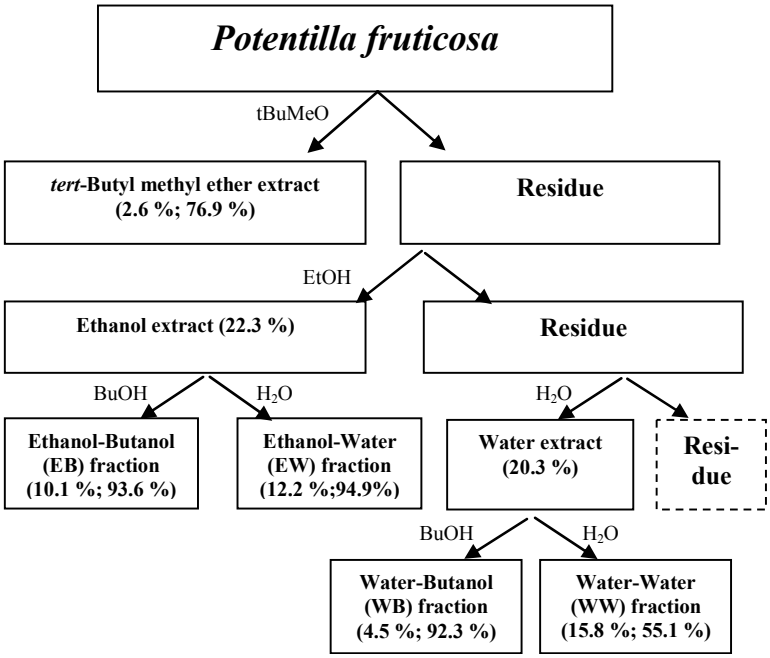


Figure 4.1 *Extraction - fractionation scheme for Potentilla fruticosa blossoms and radical scavenging activity evaluation of the obtained fractions; in parentheses: yield, %; decrease in absorbance (%) 30 min after initial mixing of extract and DPPH$^{\bullet}$ solutions (final mass ratio between them was 1.5:1 for tert-butyl methyl ether fraction and approx 0.19:1 for others.*

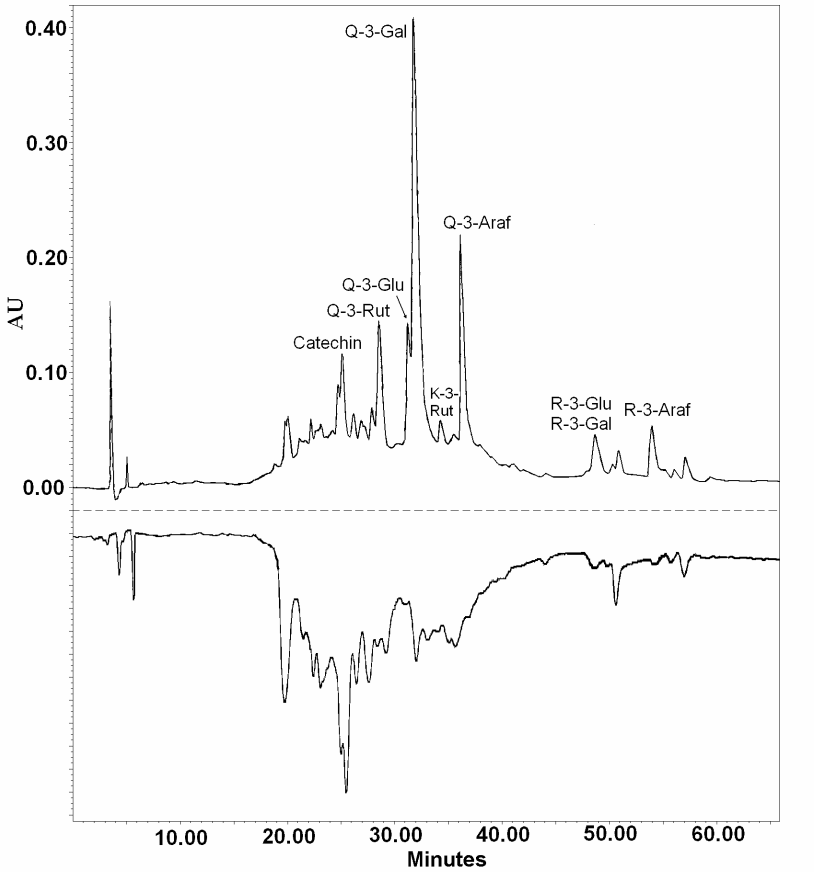


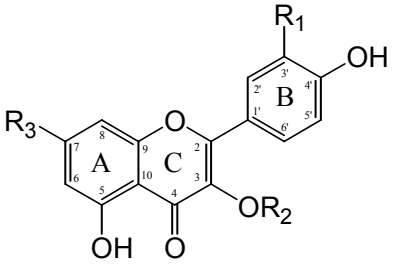
Figure 4.2 *On-line HPLC-UV-ABTS$^{+\bullet}$ scavenging assay profile of Potentilla fruticosa EB fraction (upper profile: UV signal at 254 nm, lower profile: ABTS$^{+\bullet}$ reduction signal (734 nm).*

Radical scavengers from Potentilla fruticosa

All active fractions were further monitored by on-line DPPH$^{\bullet}$ or ABTS$^{\bullet+}$ scavenging assays (see example in figure 4.2). Preliminary HPLC analysis suggested that the composition of the butanol and aqueous fractions for both ethanol and water extracts were quite similar with a different distribution of polar and less polar compounds. As the activity of the WW fraction was somewhat lower, for further analysis EB and WB fractions were used.

4.3.2 Isolation, purification and structure elucidation of active compounds

Twelve compounds were identified in different fractions of *Potentilla fruticosa* (figure 4.3).



	R_1	R_2	R_3
5	OH	β-glucopyranosyl	OH
6	OH	β-galactopyranosyl	OH
11	OH	β-glucopyranosyl	OCH_3
12	OH	β-galactopyranosyl	OCH_3
13	OH	α-arabinofuranosyl	OCH_3
14	H	6-α-rhamnosyl-β-glucopyranosyl	OH

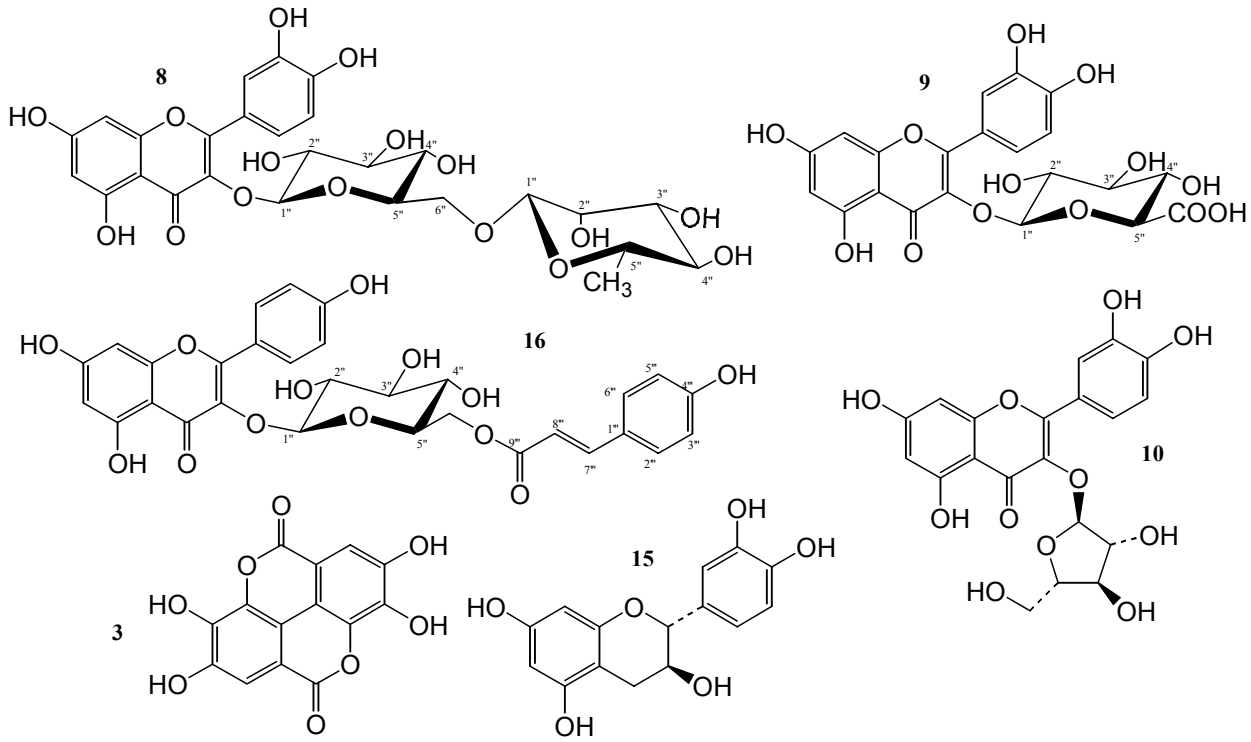


Figure 4.3 *Structures of radical scavenging compounds identified in Potentilla fruticosa.*

Like in *G. macrorrhizum*, the majority of radical scavengers in *P. fruticosa* were derivatives of the flavonoid quercetin. Quercetin-3-*α*-arabinofuranoside **10** was isolated from EB and WB fractions by silica gel column chromatography. Separation on polyamide yielded kaempferol-3-O-*β*-(6"-O-(E)-p-coumaroyl)glucopyranoside **16** from both fractions. Sephadex column chromatography resulted in the isolation of kaempferol-3-*β*-(6"-α-rhamnosyl)glucopyranoside **14**, quercetin-3-O-*β*-(6"-α-rhamnosyl)glucopyranoside (rutin) **8**, quercetin-3-*β*-glucopyranoside **5**, quercetin-3-*β*-galactopyranoside **6**, quercetin-3-*β*-glucuronopyranoside **9** and (+)-catechin **15** from EB fraction. Rhamnetin-3-*β*-glucopyranoside **11**, rhamnetin-3-*β*-galactopyranoside **12** and rhamnetin-3-*α*-arabinofuranoside **13** isolated from EB by silica gel column chromatography were purified by semi-preparative reversed phase HPLC techniques.

Three of the isolated compounds - ellagic acid **3,** (+)-catechin **15** and quercetin-3-*β*-galactopyranoside **6** have already been reported in this plant [1, 2], while the compounds **5**, **8**, **9**, **10**, **11**, **12**, **13**, **14** and **16** were identified from *Potentilla fruticosa* for the first time. All structures except for **3,** were elucidated on the basis of their ^{1}H NMR, MS and UV data. Glycosylation sites for all flavonoid glycosides, except for **9** and **14,** were determined using Heteronuclear Multiple Bond Correlation (HMBC) NMR experiments. The structure of kaempferol coumaroyl glucopyranoside **16** was also determined using an HMBC experiment. Structures of sugar moieties for all flavonoid glycosides were determined by ^{1}H NMR, 2D NMR correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and by ESI-MS data.

The isolation of sufficient amounts of ellagic acid **3** for NMR analysis was not successful. However, the structure was identified by ESI-MS, UV data and by comparison of its R_t with a reference compound. Moreover, the structure was proven by comparison of MS-MS spectra (collision energy 40%) of isolated and reference compounds. Additionally, compounds **5**, **6** and **15** were identified by LC-NMR analysis. Multiple peak trapping after several HPLC injections enabled the recording of their ^{1}H NMR spectra. The NMR assignments for compounds **5**, **6**, **8**, **9**, **10**, **14**, **15** and **16** were in agreement with literature data [3-9].

Rhamnetin-3-*β*-glucopyranoside **11** and rhamnetin-3-*β*-galactopyranoside **12** are rare flavonoids. Therefore a detailed description of their NMR assignments was not available in literature. For rhamnetin-3-α-arabinofuranoside only one reference was found: Mueller *et al.* detected it in *Euphorbia amygdaloides* in 1970 [10]. Detailed ^{1}H and ^{13}C NMR assignments of **11, 12** and **13** are presented in table 4.1.

Table 4.1 *^{1}H and ^{13}C NMR data of rhamnetin-3-β-glucopyranoside **11**, rhamnetin-3-β-galactopyranoside **12** and rhamnetin-3-α-arabinofuranoside **13** in DMSO-d_6 (δ = ppm)*

Position	^{1}H δ, multiplicity; coupling constant *J* (Hz)			^{13}C		
	11	**12**	**13**	**11**	**12**	**13**
2				156.8	156.8	157.2
3				133.3	133.1	133.6
4				177.2	177.2	177.5
5				160.7	160.7	160.6
6	6.37, d; 2.0	6.37, d; 2.0	6.38, d; 1.9	97.6	97.6	97.9
7				164.8	164.8	165.2
8	6.71, d; 2.4	6.71, d; 2.4	6.71, d; 2.4	91.9	91.9	92.2
9				156.0	156.0	156.3
10				104.7	104.7	105.1
1'				120.6	120.6	120.7
2'	7.62, d; 2.0	7.58, d; 2.0	7.54, d; 2.0	116.0	115.8	115.7
3'				144.7	144.7	145.3
4'				148.6	148.6	148.9
5'	6.84, d; 8.8	6.82, d; 8.8	6.86, d; 8.4	114.9	114.9	115.8
6'	7.61, dd; 2.0, 8.4	7.69, dd; 2.0, 8.4	7.56, dd; 2.0, 8.4	121.4	121.8	121.8
1"	5.48, d; 7.2	5.40, d; 7.6	5.61, s	100.8	101.4	107.9
2"	3.27, m	3.56, m	4.17, d; 3.3	73.8	70.9	82.0
3"	3.23, m	3.41, m	3.72, m	76.5	72.8	77.0
4"	3.09, m	3.65, m	3.59, m	69.6	67.8	85.9
5"	3.10, m	3.32, m	3.27, dd; 5.0, 12.0	77.4	75.6	60.7
			3.34, dd; 3.5, 12.0			
6"	3.30, dd; 7.6, 9.6	3.31, dd; 5.2, 9.6		60.7	59.9	
	3.56, dd; 7.6, 9.6	3.46, dd; 5.2, 9.6				
OCH_3	3.86, s (3H)	3.86, s (3H)	3.87, s (3H)	55.9	55.9	56.1

The spectral data of the compounds **8-16** are presented below:

Compound 8 (quercetin-3-O-β-rutinoside): UV (MeCN) λ_{max} 254, 355 nm; ESI-MS (positive ion mode) *m/z* 611 $[M+H]^+$; ^{1}H NMR data (CD_3OD) δ 7.67 (1H, d, *J* = 1.7 Hz, H-2′), 7.64 (1H, dd, *J* = 2.0, 8.4 Hz, H-6′), 6.85 (1H, d, *J* = 8.4 Hz, H-5′) 6.39 (1H, d, *J* = 1.8 Hz, H-8), 6.21 (1H, d, *J* = 1.7 Hz, H-6), 5.10 (1H, d, *J* = 7.6 Hz, H-1″ glucosyl), 4.52 (1H, s, H-1‴ rhamnosyl), 3.81-3.26 (10H, m, sugar protons), 1.12 (3H, d, *J* = 6.1 Hz, H-6‴); ^{13}C NMR data (DMSO d_6) δ 177.6 (C-4),

165.4 (C-7), 160.9 (C-5), 156.4 (C-2), 156.3 (C-9), 144.7 (C-3′), 140.5 (C-4′), 133.0 (C-3), 121.4 (C-6′), 120.7 (C-1′), 115.9 (C-2′), 115.1 (C-5′), 103.2 (C-10), 101.5 (C-1″), 100.8 (C-1‴), 98.9 (C-6), 93.6 (C-8), 77.5 (C-5″), 76.7 (C-3″), 74.4 (C-2″), 72.1 (C-4‴), 70.8 (C-3‴), 70.4 (C-2‴), 70.2 (C-4″), 68.2 (C-5‴), 67.1 (C-6″), 17.6 (C-6‴);

Compound 9 (quercetin-3-O-*β*-glucuronopyranoside): UV (MeCN) λ_{max} 254, 353 nm; ESI-MS (positive ion mode) *m/z* 479 $[M+H]^+$; ^{1}H NMR data (CD_3OD) δ 7.98 (1H, d, *J* = 2.0 Hz, H-2′), 7.49 (1H, dd, *J* = 2.2, 8.5 Hz, H-6′), 6.84 (1H, d, *J* = 8.5 Hz, H-5′) 6.36 (1H, d, *J* = 2.1 Hz, H-8), 6.17 (1H, d, *J* = 1.8 Hz, H-6), 5.40 (1H, d, *J* = 7.5 Hz, H-1″ glucuronosyl), 3.28-3.60 (4H, m, H-2″, H-3″, H-4″, H-5″); ^{13}C NMR data (DMSO d_6) δ 177.2 (C-4), 172.2 (C-6″), 166.2 (C-7), 160.7 (C-5), 157.0 (C-2), 156.4 (C-9), 148.3 (C-4′), 144.7 (C-3′), 133.8 (C-3), 120.2 (C-6′), 120.2 (C-1′), 117.8 (C-2′), 115.3 (C-5′), 102.3 (C-10), 102.3 (C-1″), 99.2 (C-6), 93.8 (C-8), 76.5 (C-4″), 73.9 (C-2″), 73.9 (C-5″), 71.6 (C-3″);

Compound 10 (quercetin-3-O-*α*-arabinofuranoside): UV (MeCN) λ_{max} 254, 352 nm; ESIMS (positive ion mode) *m/z* 435 $[M+H]^+$; ^{1}H NMR data (CD_3OD) δ 7.52-7.45 (2H, m, H-2′, H-6′), 6.89 (1H, d, *J* = 8.3 Hz, H-5′) 6.37 (1H, d, *J* = 1.9 Hz, H-8), 6.18 (1H, d, *J* = 1.8 Hz, H-6), 5.45 (1H, s, H-1″ arabinosyl), 4.33 (1H, m, H-2″), 3.93-3.80 (2H, m, H-3″, H-4″), 3.49 (1H, m, H-5″); ^{13}C NMR data (CD_3OD) δ 179.9 (C-4), 166.0 (C-7), 163.0 (C-5), 159.4 (C-2), 158.5 (C-9), 149.8 (C-4′), 146.3 (C-3′), 134.8 (C-3), 123.0 (C-6′), 123.0 (C-1′), 116.8 (C-2′), 116.4 (C-5′), 109.4 (C-1″), 105.6 (C-10), 99.9 (C-6), 94.8 (C-8), 87.8 (C-4″), 83.3 (C-2″), 78.6 (C-3″), 62.4 (C-5″);

Compound 11 (rhamnetin-3-O-*β*-glucopyranoside): UV (MeCN) λ_{max} 256, 353 nm; ESI-MS (negative ion mode) *m/z* 477 $[M-H]^-$; for ^{1}H NMR and ^{13}C NMR data see table 4.1;

Compound 12 (rhamnetin-3-O-*β*-galactopyranoside): UV (MeCN) λ_{max} 256, 353 nm; ESI-MS (negative ion mode) *m/z* 477 $[M-H]^-$; for ^{1}H NMR and ^{13}C NMR data see table 4.1;

Compound 13 (rhamnetin-3-O-*α*-arabinofuranoside): UV (MeCN) λ_{max} 256, 351 nm; ESI-MS (negative ion mode) *m/z* 447 $[M-H]^-$; for ^{1}H NMR and ^{13}C NMR data see table 4.1;

Compound 14 (kaempferol-3-O-*β*-rutinoside): UV (MeCN) λ_{max} 265, 361 nm; ESI-MS (positive ion mode) *m/z* 595 $[M+H]^+$; ^{1}H NMR data (CD_3OD) δ 8.06 (2H, d, *J* = 8.9 Hz, H-2′, H-6′), 6.80 (2H, d, *J* = 8.9 Hz, H-3′, H-5′), 6.37 (1H, d, *J* = 2.0 Hz, H-8) 6.18 (1H, d, *J* = 2.0 Hz, H-6), 5.10 (1H, d, *J* = 7.3 Hz, H-1″ glucosyl), 4.50 (1H, s, H-1‴ rhamnosyl), 3.81-3.21 (10H, m, sugar protons), 1.11 (3H, d, *J* = 6.2, H-6‴ rhamnosyl);

Compound 15 ((+)-catechin): UV (MeCN) λ_{max} 278 nm; ESI-MS (negative ion mode) *m/z* 289 $[M-H]^-$; ^{1}H NMR data (CD_3OD) δ 6.84 (1H, d, *J* = 2.0 Hz, H-2′), 6.75 (1H, d, *J* = 8.2 Hz, H-5′),

6.72 (1H, dd, *J* = 2.0, 8.2 Hz, H-6′) 5.93 (1H, d, *J* = 2.2 Hz, H-8), 5.86 (1H, d, *J* = 2.3 Hz, H-6), 4.56 (1H, d, *J* = 7.5 Hz, H-2), 3.98 (1H, m, H-3), 2.85 (1H, dd, *J* = 5.4, 16.1, H-4), 2.51 (1H, dd, *J* = 5.4, 16.1, H-4_A);

Compound 16 (kaempferol-3-O-*β*-(6''-O-(E)-*p*-coumaroyl)glucopyranoside): UV (MeCN) λ_{max} 265, 313 nm; ESI-MS (negative ion mode) *m/z* 593 $[M-H]^-$; ^{1}H NMR data (CD_3OD) δ 7.98 (2H, d, *J* = 8.9 Hz, H-2′, H-6′), 7.39 (1H, d, *J* = 16.0 Hz, H-7‴ coumaroyl), 7.30 (2H, d, *J* = 8.6 Hz, H-2‴, H-6‴), 6.81 (2H, d, *J* = 8.9 Hz, H-3′, H-5′), 6.79 (2H, d, *J* = 8.7 Hz, H-3‴, H-5‴), 6.30 (1H, d, *J* = 2.0 Hz, H-8), 6.12 (1H, d, *J* = 2.0 Hz, H-6), 6.07 (1H, d, *J* = 16.0 Hz, H-8‴), 5.22 (1H, d, *J* = 7.5 Hz, H-1″), 4.27 (1H, dd, *J* = 2.2, 11.8 Hz, H-6″$_A$), 4.18 (1H, dd, *J* = 6.6, 11.8 Hz, H-6″$_B$), 3.47-3.29 (4H, m, H-2″, H-3″, H-4″, H-5″); ^{13}C NMR data (DMSO d_6) δ 180.0 (C-4), 168.9 (C-9‴), 167.2 (C-7), 163.0 (C-5), 161.4 (C-4′, C-4‴), 159.3 (C-2), 158.6 (C-9), 146.7 (C-7‴), 135.2 (C-3), 132.2 (C-2′, C-6′), 131.3 (C-2‴, C-6‴), 127.2 (C-1‴), 122.8 (C-1′), 116.9 (C-3′, C-5′), 116.1 (C-3‴, C-5‴), 114.8 (C-8‴), 105.4 (C-10), 104.1 (C-″), 100.5 (C-6), 95.2 (C-8), 78.1 (C-3″), 75.8 (C-2″, C-5″), 71.8 (C-4″), 64.5 (C-6″).

Most of the isolated *P. fruticosa* compounds (**3**, **5**, **6**, **8** and **15**) were already reported to be effective antioxidants. They are all of high to moderately high polarity, and like *G. macrorrhizum* compounds can effectively be extracted with cheap and food-grade solvents, such as H_2O or EtOH.

4.3.3 Assessment of radical scavenging activity of isolated compounds

Procedures for the radical scavenging activity assessment of *Potentilla* compounds were similar to those for *Geranium*: two off-line methods with $DPPH^•$ and $ABTS^{•+}$ were used and the activity was compared with the well-known antioxidant rosmarinic acid (table 4.2); the $ABTS^{•+}$ scavenging efficiency was expressed in TEAC units, estimated at t = 1 and t = 6 min of reaction time between radical and compound. A TEAC value 1 means that the antioxidant capacity of the compound is the same as that of the reference antioxidant Trolox.

A difference in the outcome of these two radical scavenging assays was observed for kaempferol-3-O-*β*-(6"-O-(E)-*p*-coumaroyl)glucopyranoside **16**: its $ABTS^{•+}$ scavenging efficiency was much higher than its $DPPH^•$ scavenging. For other compounds the results from both assays were similar. The highest radical scavenging activities were measured for catechin **15** and ellagic acid **3**. Based on HPLC peak areas these two compounds together with quercetin-3-*β*-

glucopyranoside **5**, quercetin-3-*β*-galactopyranoside **6** and quercetin-3-*β*-rutinoside **8** are the major active compounds in this plant.

Table 4.2 *Radical scavenging activity of isolated compounds*

Compound	**TEAC**		**Decrease of absorbance (%) at:**	
	1 min	6 min	$[M_{DPPH}/M_{TC}]=2$	$[M_{DPPH}/M_{TC}]=10$
Trolox	1.00	1.00	96	38
Rosmarinic acid	1.37	1.44	97	38
Quercetin-3-*β*-glucopyranoside **(5)**	1.53	1.95	91	27
Quercetin-3-*β*-galactopyranoside (**6**)	1.53	1.95	91	27
Quercetin-3-*β*-rutinoside (**8**)	1.25	1.63	89	10
Quercetin-3-*β*-glucuronopyranoside **(9)**	0.26	0.38	36	0
Quercetin-3-*α*-arabinofuranoside (**10**)	0.91	1.18	80	17
Rhamnetin-3-*β*-glucopyranoside (**11**)	0.85	1.22	92	16
Rhamnetin-3-*β*-galactopyranoside (**12**)	0.85	1.22	92	16
Rhamnetin-3-*α*-arabinofuranoside (**13**)	0.49	0.65	81	5
Kaempferol-3-*β*-rutinoside (**14**)	0.84	1.13	26	6
Catechin (**15**)	1.54	1.57	96	80
Ellagic acid (**3**)	1.40	1.83	95	77
Kaempferol-3-O-*β*-(6''-O-(E)-*p*-coumaroyl)glucopyranoside (**16**)	1.12	1.43	1	0

Trolox Equivalent Antioxidant Capacity (TEAC), estimated at t=1 min and t=6 min; $[M_{DPPH}]/[M_{TC}]$ = concentration ratio of $DPPH^{\bullet}$ reagent and tested compound.

The antioxidant activity of flavonoids has been reported in numerous publications and structure activity relationships have been proposed to explain the results (see chapter 1 and 3 and references there for details). Bulky groups (e.g. glycosides) or methoxylation of hydroxyl groups of flavonoids (particularly in the B ring) significantly reduce the antioxidant activity of the molecule [11-12]. No literature data were found on the influence of different sugars on the same position of a flavonoid ring on the antioxidant activity. In the study of *Potentilla compounds* it can be observed that quercetin-3-glucoside **5**, -galactoside **6** and -rutinoside **8** possess almost similar high radical scavenging activity, while quercetin-3-α-arabinofuranoside **10** and glucuronopyranoside **9** showed much lower activity. Böhm *et al.* in his study on vegetable flavonoids also concluded that a 3-O glycosidic substituent is important for the radical scavenging activity of flavonoids [13]. Another

interesting observation, suggesting a relationship between the nature of the sugar moiety and the antioxidant activity, is that the relative activity of rhamnetin glycosides differs in the same manner as that of quercetin analogues: glucopyranoside and galactopyranoside showed higher activity than arabinofuranoside both in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays.

Slightly different from the *G. macrorrhizum* compounds, where the reaction between all tested compounds and $ABTS^{\bullet+}$ was almost finished during the first reaction minute, for some *P. fruticosa* compounds (quercetin-3-*β*-glucuronopyranoside **9,** rhamnetin-3-*β*-glucopyranoside **11** and rhamnetin-3-*β*-galactopyranoside **12**) distinct increases in TEAC values were observed during the entire measurement time – up to 6 min. This suggests that these compounds exhibit slower reaction kinetics.

Most of the compounds in the $DPPH^{\bullet}$ scavenging assay (at molar ratios of 1:2 with $DPPH^{\bullet}$) fully reduced the $DPPH^{\bullet}$ solution. More informative results were obtained when the concentration ratio of the compound and $DPPH^{\bullet}$ was changed to 1:10. Similar to the $ABTS^{\bullet+}$ assay, the highest activity was shown by ellagic acid and catechin, followed by quercetin-3-glucoside, -galactoside and -rutinoside.

Summarizing the obtained results, the radical scavenging activity of isolated compounds (according to TEAC values) from *Potentilla fruticosa* in increasing order was: quercetin-3-*β*-glucuronopyranoside **9** < kaempferol-3-O-*β*-(6"-O-(E)-*p*-coumaroyl)glucopyranoside **16** < rhamnetin-3-*α*-arabinofuranoside **13** < rhamnetin-3-*β*-glucopyranoside **11**, rhamnetin-3-*β*-galactopyranoside **12** < kaempferol-3-*β*-rutinoside **14** < quercetin-3-*α*-arabinofuranoside **10** < quercetin-3-*β*-rutinoside **8** < quercetin-3-*β*-glucopyranoside **5** ≈ quercetin-3-*β*-galactopyranoside **6** < catechin **15** < ellagic acid **3**.

Three compounds – ellagic acid, quercetin-3-glucoside and quercetin-3-galactoside have been isolated and identified in both plants - *Geranium macrorrhizum* and *Potentilla fruticosa.* Slight differences have been observed in the radical scavenging assay results for the same compounds, but isolated from either *Geranium* or *Potentilla*. As identical methods and equipment were used for running the radical scavenging tests, the differences are probably caused by differences in the purity of isolated compounds, reagents' freshness or other stochastic errors.

4.3.4 Quantitative comparison of major radical scavenging compounds in different botanical parts

The relative concentration of the active constituents in botanical parts of *Potentilla fruticosa* was compared. The comparison of ethanolic extracts from leaves and EB fractions of the blossoms was performed by determining peak areas from total ion current (TIC) signal (in arbitrary units) during MS analysis (table 4.3).

On average the relative amount of the major radical scavenging compounds in the *P. fruticosa* blossoms fraction was more than twice as big as in the leaves extract. Kaempferol-3-coumaroylglucoside was not detected in the ethanolic extract of leaves at all.

Table 4.3 *Peak areas and their ratio (in arbitrary units) of corresponding radical scavenging compounds in ethanol-butanol fraction of P. fruticosa blossoms and ethanol extract of leaves*

Compound	**TIC area (in arbitrary units)**		
	Blossom extract	**Leaves extract**	**Ratio (blossoms/leaves)**
Catechin (**15**)	1.86×10^{6}	9.77×10^{5}	**1.91**
Quercetin-3-rutinoside (**8**)	1.31×10^{5}	5.20×10^{4}	**2.53**
Quercetin-3-glucoside and galactoside (**5**,**6**)	7.22×10^{5}	2.76×10^{5}	**2.62**
Quercetin-3-glucuronoside (**9**)	4.38×10^{5}	2.29×10^{5}	**1.91**
Quercetin-3-arabinoside (**10**)	4.02×10^{5}	2.17×10^{5}	**1.85**
Kaempferol-3-rutinoside (**14**)	2.13×10^{4}	1.36×10^{4}	**1.57**
Kaempferol-3-coumaroylglucoside (**16**)	1.50×10^{5}	-	**-**
Rhamnetin-3-glucoside and galactoside (**11**, **12**)	1.78×10^{5}	2.37×10^{5}	**0.75**
Rhamnetin-3-arabinoside (**13**)	1.10×10^{5}	1.14×10^{5}	**0.97**

The amounts of some minor compounds, like rhamnetin glycosides were bigger in leaves than in blossoms. It should be noted that the comparison of different *P. fruticosa* botanical parts was not completely precise, as extracts from them were produced using slightly different procedures. Nevertheless the ethanol extract of crude leaves contained lower amounts of active compounds in comparison to the ethanol fractions of blossoms. In summary it can be concluded, that blossoms of *Potentilla fruticosa* are a richer source of radical scavenging compounds than leaves.

4.4 References

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ISOLATION AND IDENTIFICATION OF RADICAL SCAVENGERS FROM *RHAPONTICUM CARTHAMOIDES* [*]

5.1 Introduction

Studies of aromatic and medicinal plants grown in Lithuania have provided information on the antioxidant properties of some less investigated plants [1-4] and led to the identification of new antioxidants [1, 2]. These findings encouraged further investigations and several new plants grown in Eastern and Central Europe were screened for their antioxidant properties (chapter 2). The extracts of *Rhaponticum carthamoides*, similarly to extracts of *Geranium macrorrhizum* (chapter 3) and *Potentilla fruticosa* (chapter 4) were shown to possess strong radical scavenging capacity.

This part of the work was aimed at the rapid identification of the major radical scavenging compounds in *R. carthamoides* extracts by using a hyphenated liquid chromatography set-up coupled to a solid phase extraction unit and an NMR detector (LC-DAD-SPE-NMR). The advantages of this set-up in combination with a semi-preparative column are described in chapter 8.

5.2 Materials and methods

5.2.1 General Experimental Procedures

All solvents used in HPLC analysis were of analytical grade. The melting point was measured with a Buchi 510 apparatus (Buchi, Flawil, Switzerland) and is uncorrected. The optical rotation was measured with a Perkin-Elmer 241 polarimeter at 589 nm in a 10 cm 1 ml cell. UV and IR spectra were recorded on Perkin-Elmer Lambda 18 UV/Vis (Perkin-Elmer, Ueberlingen, Germany) and Vector 22 FT-IR (Bruker, Billerica, MA) spectrometers respectively. Exact mass measurements were performed on a Micromass Q-Tof Ultima API mass spectrometer (Micromass, Manchester,

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UK). Mass spectra were recorded on a Finnigan LCQ spectrometer (Thermo Finnigan, San Jose, CA) in ESI or APCI mode. All NMR spectra were recorded on a Bruker DPX 400 spectrometer (Bruker, Rheinstetten, Germany) except for the HMBC spectrum of compound **5** (see results chapter for details), which was recorded on a Bruker AMX 500 spectrometer. HMBC spectra of all compounds were recorded using SHIGEMI tubes (Campro Scientific, Veenendaal, The Netherlands).

5.2.2 Plant Material and Extraction Procedures

Aerial parts (leaves and stems) of *Rhaponticum carthamoides* were harvested in June 2003 in the experimental garden of medicinal plants of Kaunas Botanical Garden at the Vytautas Magnus University (Lithuania) and a voucher specimen (V01213) is deposited in the local herbarium. Leaves and stems were dried in a drying room with active ventilation at ambient temperature (< 30 °C) and stored in paper bags for 3 - 4 months before use. Extraction-fractionation procedures of plant material were identical to those described in chapter 4.

5.2.3 Radical Scavenging Assays

Off-line radical scavenging activities of different fractions (compounds) were determined by using an off-line $DPPH^{\bullet}$ assay method [5]. The reagents and the equipment were the same as described in chapter 4. The most active fractions were further monitored by on-line RP-HPLC-$DPPH^{\bullet}$ or $ABTS^{\bullet+}$ (Fluka Chemie, Buchs, Switzerland) assay methods, which were performed as described by Dapkevicius *et al.* and Koleva *et al.* [6, 7]. The equipment set-up for the on-line assessment of radical scavenging activity was also identical to that described by Koleva *et al*. On-line assays were also carried out in combination with the LC-DAD-SPE-NMR set-up (*vide infra*).

5.2.4 LC-DAD-SPE-NMR Instrumental Setup

The scheme of the experimental set-up is presented in Figure 5.1. The HPLC system was the same as described in chapter 4. An Xterra semi-preparative column (MS C18, 7.8×100 mm, 5 μm, Waters, Milford, USA) was used for the separation and a binary gradient with 0.5 ml/min flow rate

was used. Solvent A was a 1% MeCN solution in H_2O acidified with 0.1% TFA (trifluoroacetic acid); B was 100 % MeCN. The gradient conditions for the EB or WB fractions were as follows: 0 - 10 min B – 12%, 10 – 50 min B increased to 35%, 50 – 55 min B reached 100% and was kept constant till 60 min, 60 - 65 min B decreased till 12%. The gradient conditions for the WW fraction were almost the same as for EB and WB fractions, except that the first 10 minutes the % of B was 5% instead of 12%.

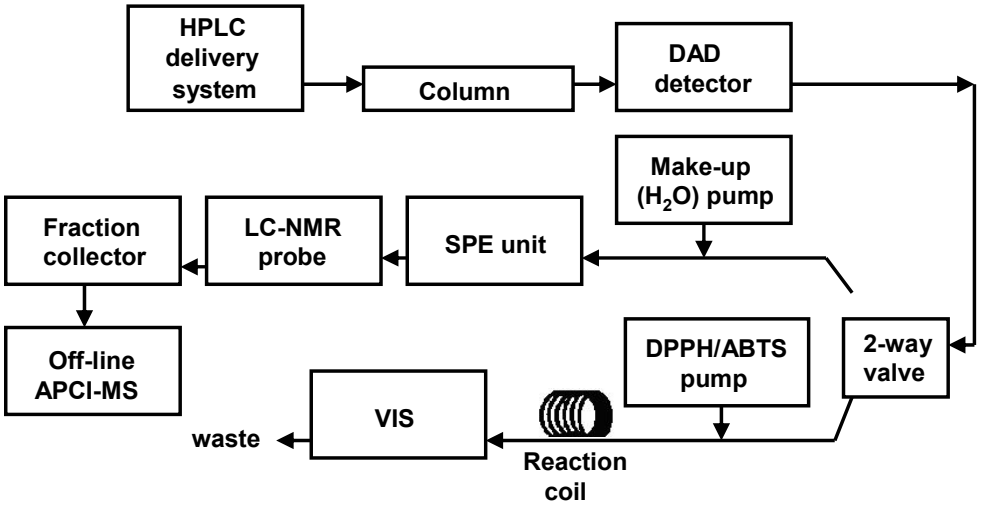


Figure 5.1 *Scheme of experimental LC-DAD-SPE-NMR set-up. Depending on the position of the 2-way valve either radical scavenging detection or LC-NMR occurs.*

After the HPLC separation, compounds were detected with a diode array detector. The total flow was then directed to either (i) the radical scavenging detector or (ii) the trapping (solid phase extraction - SPE) unit. Thus, two separate runs were carried out. In the first run radical scavenging activity of separate constituents in the extract mixture was determined by on-line methodology (see paragraph above and references noted there for details). In the second run peaks of interest were trapped with the SPE unit (Prospekt 2, Spark Holland, Emmen, The Netherlands) according to the UV signal and their radical scavenging activity determined in the first run. 10 × 2 mm Hysphere Resin SH cartridges (packed with 10-12 μm particles, Spark Holland) were used for trapping. Afterwards SPE cartridges were dried (with nitrogen flow) and active compounds transferred to a Bruker 4 mm inverse $^{1}H/^{13}C$ pulse-field gradient flow probe operating at 400 MHz (detection volume 120 μl) using 100 % deuterated solvents (CD_3OD or CD_3CN). After NMR peaks were collected for additional measurements, e.g. MS analysis.

5.3 Results and Discussion

The scheme of the extraction – fractionation procedure of the plant material is shown in figure 5.2. The plant was rich in polar substances as the water fraction was the most abundant fraction. Off-line evaluation of DPPH radical scavenging activity of all obtained fractions provided data about the distribution of the active components in the plant.

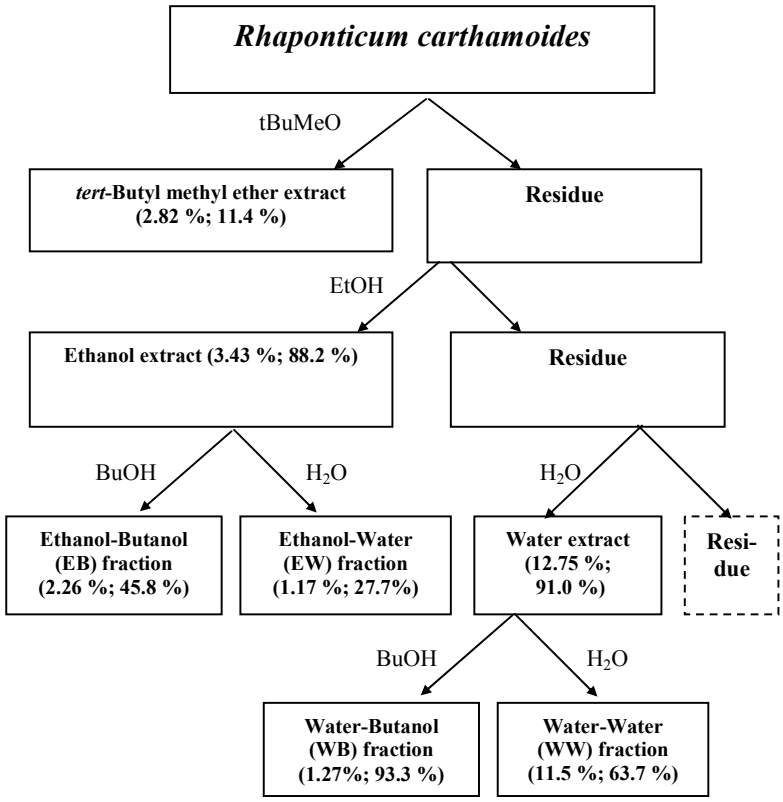
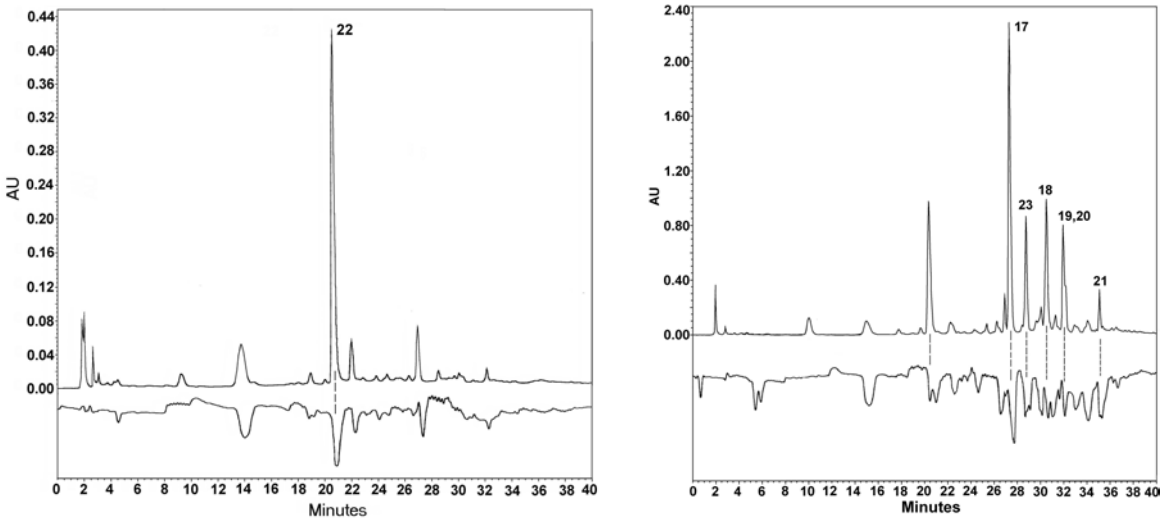


Figure 5.2 *Extraction - fractionation scheme of R. carthamoides and the radical scavenging activity evaluation of the obtained fractions. In parentheses: yield, %; decrease in absorbance (%) 30 min after initial mixing of extract and DPPH$^{\bullet}$ solutions. Final mass ratio between them was 3:1 for tert-butyl methyl ether, ethanol, water extracts and water-water fractions and 0.75:1 for others.*

The ether extract was the least active against DPPH$^{\bullet}$ radicals. The water-water (WW) fraction also showed somewhat lower activity in comparison to the ethanol-butanol (EB), ethanol-water (EW) and water-butanol (WB) fractions. For further analysis all EtOH and H_2O fractions have been investigated using on-line LC-DPPH$^{\bullet}$/ABTS$^{\bullet+}$ scavenging tests [6, 7].

On-line tests indicated that the WB and EB fractions of the plant possessed a similar profile of compounds, with slightly higher amounts of polar compounds in the WB fraction. Both fractions contained several major constituents (detected at 254 nm, see Figure 5.3 b), showing radical scavenging activity. From the WB chromatogram one can also observe that minor compounds in that fraction, judging from the corresponding negative signals, possessed quite high activity, similar

to that of major compounds. The HPLC profile of the WW fraction showed that it consisted of one major and a few minor active compounds (Figure 5.3 a).



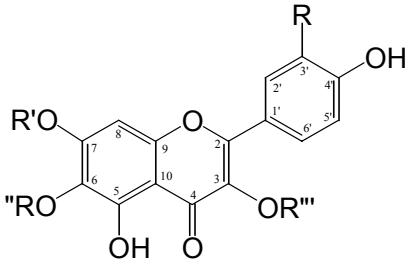
a b

Figure 5.3 *On-line HPLC-UV-ABTS$^{\bullet+}$ scavenging assay profiles of Rhaponticum carthamoides WW fraction (a) and WB fraction (b). Upper profiles - UV signal at 254 nm, lower profiles (negative peaks, λ = 734 nm) - ABTS$^{\bullet+}$ reduction signal. Numbers at the top of the peaks correspond to the compounds: quercetagetin-7-β-glucopyranoside (**17**), 6-hydroxykaempferol-7-β-glucopyranoside (**18**), quercetagetin-7-β-(6″-acetylglucopyranoside) (**19**), 6-methoxy-kaempferol-3-β-glucopyranoside (**20**), 6-hydroxykaempferol-7-β-(6″-acetylglucopyranoside) (**21**), chlorogenic acid (**22**), β-ecdysone (**23**).*

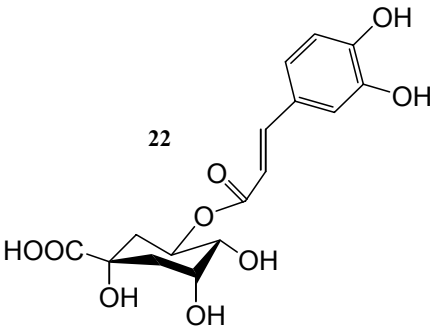
Next the HPLC set-up was connected to a solid phase extraction unit that was in turn coupled to an NMR detector. Studies using the described LC-DAD-SPE-NMR set-up have shown its potential: a significant speed-up of the isolation-identification process, as time and labour-intensive chromatographic steps can be avoided [8]. A limitation is still relatively poor sensitivity of the NMR detector, especially for recording 2D NMR spectra like HMBC. Therefore, in order to obtain substantial amounts of compounds a semi-preparative HPLC column was used. More sample could be loaded on such a column and consequently higher amounts of compounds were trapped. The larger injected volumes did not pose a problem because of the focusing effect of the SPE unit. Multiple peak trapping of the same analyte by repeated LC injections on a column were used in some cases.

Under optimized conditions of the LC-SPE-DAD-NMR set-up (0.5 ml/min flow rate of HPLC pump and 1.0 ml/min of water make-up pump; proper cartridges) five relatively rare flavonoid glycosides, namely quercetagetin-7-*β*-glucopyranoside (**17**), 6-hydroxykaempferol-7-*β*-glucopyranoside (**18**), quercetagetin-7-*β*-(6″-acetylglucopyranoside) (**19**), 6-methoxykaempferol-3-*β*-glucopyranoside (**20**) and 6-hydroxykaempferol-7-*β*-(6″-acetylglucopyranoside) (**21**) were

isolated and identified from the EB and WB fractions. Two of these glycosides (**17** and **18)** have been reported previously as constituents of *R. carthamoides* [9]. *β*-Ecdysone (**23**) was isolated and identified in the EB (WB) fraction. From the chromatographic profile (see Figure 5.3 b), it appeared that *β*-ecdysone possesses radical scavenging properties, however this could also be due to some minor phenolic compound co-eluting at the same time. DAD signal has also indicated the presence of some phenolic structure. Chlorogenic acid (**22**) was the major radical scavenger present in the water-water fraction.



	R	R′	R″	R‴
17	OH	*β*-glucopyranoside	H	H
18	H	*β*-glucopyranoside	H	H
19	OH	*β*-(6′′-acetyl) glucopyranoside	H	H
20	H	H	CH_3	*β*-gluco-pyranoside
21	H	*β*-(6′′-acetyl) glucopyranoside	H	H



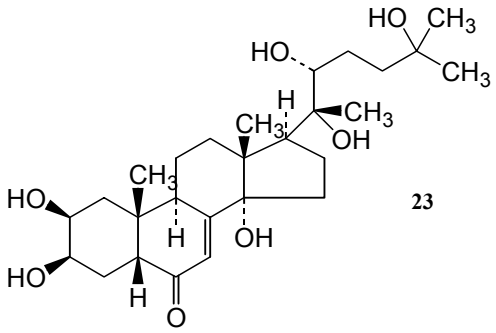


Figure 5.4 *Compounds identified in Rhaponticum carthamoides.*

Structures of the flavonoid glycosides **17**-**21** were elucidated by UV spectra, ^{1}H, ^{13}C and 2D NMR techniques and MS. Structures of chlorogenic acid and *β*-ecdysone were determined by ^{1}H NMR, UV and MS. ^{1}H NMR spectra of all compounds were recorded directly (on-line) after drying cartridges and transferring compounds to the LC-NMR probe. The amounts of compounds trapped on one cartridge were insufficient for heteronuclear NMR experiments, therefore analyte trapping was continued on separate cartridges in repeated runs (two or three). Analytes obtained from these runs were combined, re-dissolved and ^{13}C or 2D (HMBC) spectra (off-line) were recorded in SHIGEMI tubes using a standard (5 mm i.d.) NMR probe. The magnetic susceptibility matching solvent properties of these tubes enables NMR measurements using a smaller (more concentrated) sample volume, which leads to improved signal to noise ratio and reduced acquisition time.

The ^{1}H NMR spectra of **21** with 2H doublets at δ 8.0 and 6.9 suggested a kaempferol type of flavonoid. Multiple peaks between δ 3.2 - 4.0, a 1H singlet at δ 6.9 and 3H singlet at δ 2.00 in

combination with the absence of other peaks in the low field area suggested an acetylated 6-substituted kaempferol glycoside (see figure 5.5). APCI-MS gave as molecular weight of the compound 506 Da. An intense fragment at *m/z* 301 confirmed the presence of a hydroxy-substituted kaempferol structure (286 [kaempferol] + 16 [hydroxyl group] – H = 301), while the acetyl group should be attached to the sugar.

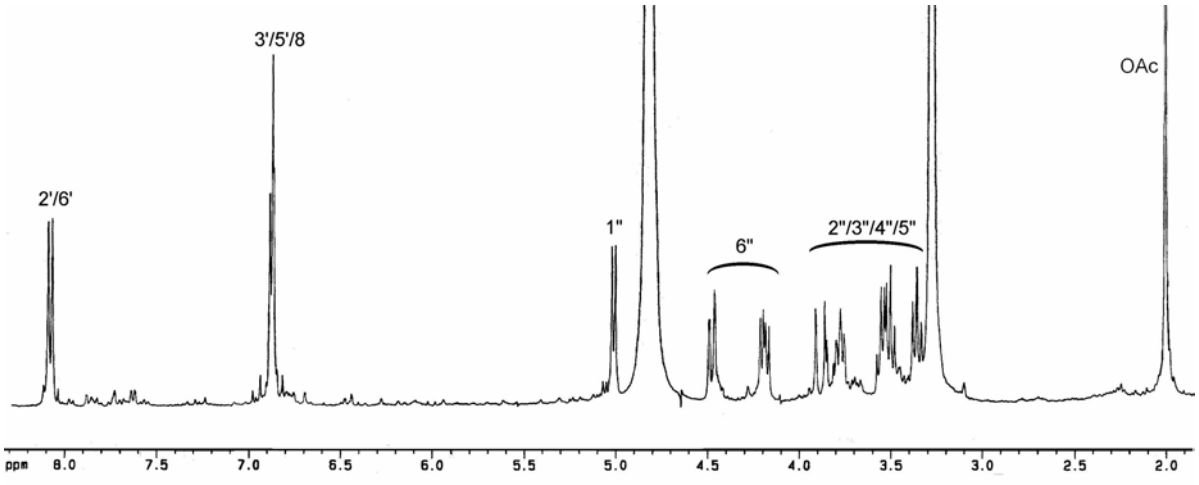


Figure 5.5 *^{1}H NMR spectrum (recorded with on-line LC-SPE-NMR in CD_3OD) of 6-hydroxykaempferol-7-(6''-acetyl-β-glucopyranoside) (**21**).*

Accurate mass measurements by ESI-TOF-MS technique provided the elemental composition $C_{23}H_{22}O_{13}$. The HMBC spectrum allowed the total elucidation of the structure. The 6″- position of the glucopyranosyl moiety was acetylated and the sugar was linked to position 7 of 6-hydroxykaempferol. This compound has not been reported before as a natural product. Harborne *et al*. mentioned an acetylated glycoside of 6-hydroxykaempferol from *Chrysantinia mexicana* [10], however the structure of the sugar moiety was not fully resolved.

Spectral data of identified compounds **17**-**23**:

Quercetagetin-7-*O*-*β*-glucopyranoside (17): yellow powder; UV(MeOH) λ_{max} 258, 274, 355 nm; ^{1}H NMR data (DMSO *d*-6) see table 5.1; ^{13}C NMR data are in agreement with those published [11]; APCI-MS (positive ion mode) *m/z* 481 $[M+H]^+$.

6-Hydroxykaempferol-7-*O*-*β*-glucopyranoside (18): yellow powder; UV(MeOH) λ_{max} 255, 269, 350 nm; ^{1}H NMR data (DMSO *d*-6) see table 5.1; ^{13}C NMR data are in agreement with those published [12]; APCI-MS (positive ion mode) *m/z* 465 $[M+H]^+$.

Quercetagetin-7-*O*-*β*-(6”-*O*-acetyl-*β*-glucopyranoside) (19): yellow powder; UV(MeCN) λ_{max} 262, 346 nm; ^{1}H NMR data (DMSO *d*-6) see table 5.1; ^{13}C NMR data are in agreement with those published [13]; APCI-MS (positive ion mode) *m/z* 523 $[M+H]^+$.

Table 5.1 *^{1}H NMR data for compounds **17-21** and ^{13}C data for compound **21** in DMSO-d6 (δ = ppm)*

Position	^{1}H δ, mult; J (Hz)					^{13}C, δ
	17	**18**	**19**	**20**	**21**	**21**
2						147.5
3						135.6
4						170.2
5						145.5
6						131.4
7						151.4
8	6.92, s	6.96, s	6.87, s	6.51, s	6.89, s	93.5
9						148.1
10						105.2
1'						121.7
2'	7.71, d; 1.8	8.06, d; 8.8	7.74, d; 1.9	8.03, d; 8.8	8.04, d; 8.9	129.6
3'		6.94, d; 8.8		6.93, d; 8.8	6.92, d; 8.9	115.4
4'						159.4
5'	6.88, d; 8.6	6.94, d; 8.8	6.91, d; 8.8	6.93, d; 8.8	6.92, d; 8.9	115.4
6'	7.53, dd; 1.8, 8.5	8.06, d; 8.8	7.53, dd; 2.0, 8.5	8.03, d; 8.8	8.04, d; 8.9	129.6
1"	5.01, d; 7.4	4.99, d; 7.3	5.05, d; 7.3	5.27, d; 7.1	5.03, d; 7.4	100.7
2"	3.37, m	3.37, m	3.40, m	3.40, m	3.39, m	73.1
3"	3.38, m	3.33, m	3.37, m	3.26, m	3.38, m	75.6
4"	3.21, m	3.20, m	3.18, m	3.09, m	3.20, m	69.9
5"	3.48, m	3.48, m	3.76, m	3.08, m	3.76, m	74.1
6"	3.51, dd; 6.1, 12.2 3.74, dd; 1.7, 12.1	3.57, dd; 5.6, 12.1 3.77, dd; 1.4, 12.3	4.06, dd; 7.4, 11.9 4.39, dd; 1.4, 11.8	3.30, dd; 1.9, 11.8 3.58, dd; 7.6, 11.9	4.06, dd; 7.6, 11.9 4.39, dd; 1.4, 11.9	63.5
OAc			2.00, s (3H)		2.00, s (3H)	20.6
6-OMe				3.88, s (3H)		
$\underline{C}O_2Me$						176.3

6-Methoxykaempferol-3-*O*-*β*-glucopyranoside (20): yellow powder; UV(MeCN) λ_{max} 263, 350 nm; ^{1}H NMR data (DMSO *d*-6) see table 5.1; ^{13}C NMR data are in agreement with those published [14]; APCI-MS (positive ion mode) *m/z* 479 $[M+H]^{+}$.

6-Hydroxykaempferol-7-*O*-*β*-(6"-*O*-acetyl-*β*-D-glucopyranoside) (21): yellow powder: mp 173-176 ºC; $[\alpha]^{22}_{D}$ –87º (*c* 0.07, MeOH); UV(MeOH) λ_{max} 257, 274, 348 nm; IR (DMSO *d*-6 ν_{max} 3432, 3295, 2928, 1737, 1660, 1595, 1484, 1370, 1285, 1243, 1196 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO *d*-6), see table 5.1; APCI-MS (positive ion mode) *m/z* 507 $[M+H]^+$, 303 $[M–acetylglucose+H]^+$; ESI-TOF MS (negative ion mode) *m/z* 505.0987 $[M–H]^-$ (calculated for $C_{23}H_{22}O_{13}$ - H was 505.0982).

Chlorogenic acid (22): ESI-MS (negative ion mode) *m/z* 353 $[M-H]^-$; UV (MeOH) λ_{max} 257 nm, ^{1}H NMR (CD_3OD) δ 7.56 (1H, d, *J* = 16 Hz, H-7), 7.05 (1H, d, *J* = 2 Hz, H-2), 6.95 (1H, dd, *J* = 2, 8 Hz, H-6), 6.77 (1H, d, *J* = 8 Hz, H-5), 6.28 (1H, d, *J* = 16 Hz, H-8), 5.32 (1H, m, H-3´), 4.17 (1H, m, H-5´), 3.72 (1H, dd, *J* = 3, 8 Hz, H-4´), 2.16-2.25 (2H, m, H-6´), 2.02-2.11 (2H, m, H-2´).

***β*-Ecdysone (23):** APCI-MS (positive ion mode) *m/z* 481 $[M+H]^+$; ^{1}H NMR (CD_3OD) data is in agreement with those published [15].

The activity of **21** was tested off-line against the $DPPH^{\bullet}$ radical and compared to that of two reference antioxidants - rosmarinic acid and Trolox. Two molar concentration ratios of DPPH versus the tested compound were chosen (2:1 and 5:1). At a ratio of 2:1, 6-hydroxykaempferol-7-acetylglycoside (**21**) gave a 75 % decrease of the absorbance of DPPH, while at the same concentration reference antioxidants rosmarinic acid or Trolox gave a 98 % decrease. At a ratio of 5:1, **21** still showed good radical scavenging activity, although it remained lower than that of reference compounds: 36 % against 89 % for rosmarinic acid and 79 % for Trolox.

At this point of the study it can be concluded that the majority of the active constituents (glycosides and other derivatives of the flavonoids quercetin and kaempferol; phenolic acids, like gallic acid, ellagic acid, chlorogenic acid and others) from the investigated plants (*Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides*) are abundant and well known compounds in many vegetables, fruits, and medicinal and aromatic herbs. These compounds are known to possess high antioxidant capacity (see chapter 1 for some references). The high concentrations of these compounds detected in these plants gave a positive impulse for the perspectives of these plants as a source of antioxidants. Moreover these compounds are of high to moderate polarity and can be effectively extracted with water or ethanol. The compounds can be expected to be readily soluble in polar media and less or non-soluble in hydrophobic media.

In the next phase of this study only *Geranium macrorrhizum* and *Potentilla fruticosa* – plants which possessed the highest radical scavenging activity – were chosen for practical evaluation tests.

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PRACTICAL EVALUATION OF THE ANTIOXIDATIVE PROPERTIES AND PRELIMINARY SAFETY STUDIES OF SELECTED PLANT EXTRACTS

6.1 Introduction

After the analysis of the major radical scavenging compounds in various plant extracts from *Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides*, the following step of this study was the assessment of the antioxidant properties of selected extracts. Several model radical scavenging tests have been applied previously (see chapters 3, 4 and 5) to determine the radical scavenging properties of plant extracts. Even though these assays are quick, reproducible and usually correlate well with antioxidant properties, nevertheless they do not reflect real oxidation conditions and cannot be used to predict activity of extracts in real foods. In order to evaluate antioxidant properties oxidation tests involving real (i.e. as encountered in food products) radical species should be used. Moreover to obtain reliable results the reaction medium should also be closer to that of real food systems. Finally real food-systems should be used.

Here several antioxidant activity evaluation tests involving different reaction media (oil, emulsion or real food system (fermented sausages)), reactive species, reaction phases (monitoring primary or secondary oxidation products) were used to obtain more detailed information on the properties of the extracts from *Geranium* and *Potentilla*. In the final step of the study, preliminary safety evaluation tests were conducted with selected extracts from *Geranium* and *Potentilla*. Such tests are important as plant-based formulations cannot be approved as new food additives or supplements without their prior safety evaluation, regardless of all the positive properties or effects that they possess.

6.2 Materials and methods

6.2.1 Determination of superoxide anion radical scavenging properties

Superoxide anion scavenging and hydrogen peroxide scavenging tests were performed as described by Wettasinghe *et al.* [1]. The superoxide radical was generated in an enzymatic reaction.

Practical evaluation

Hypoxanthine (99%), xanthine oxidase (from buttermilk), diethylenetriamine-pentaacetic acid and nitro blue tetrazolium (NBT) were from Sigma. The reaction mixture contained 0.5 ml of 3 mM hypoxanthine, 0.5 ml of xanthine oxidase (50 mIU), 0.5 ml of 12 mM diethylenetriamine-pentaacetic acid, 0.5 ml of 178 μM NBT and 0.5 ml of measured extract. Final concentration of the extracts in the reaction mixture was 200 ppm or 50 ppm. All reagents and extract solutions were prepared in 0.1 M phosphate buffer (pH 7.4). *tert*-Butyl methyl ether extracts were insoluble in phosphate buffer, thus their superoxide scavenging or hydrogen peroxide scavenging properties could not be determined with these methods. Absorbances of solutions were measured with a Spectronic Genesys 8 UV/Vis spectrophotometer (Rochester, USA) at 560 nm. Absorbance values were corrected by subtracting 0 min readings from subsequent readings. The experiment was carried out in duplicate.

6.2.2 Determination of hydrogen peroxide scavenging properties

The extract solutions (50 ppm or 33.3 ppm) were prepared by dissolving extracts in 0.1 M phosphate buffer (pH 7.4). For each sample 2.55 ml of this solution was mixed with 0.45 ml of 43 mM hydrogen peroxide (Lachema, Brno, Czech Republic) solution prepared in the same buffer. UV absorbance (230 nm) was measured at 0 min and then every 10 min up to 40 min. For each concentration a blank sample (devoid of hydrogen peroxide) was used for background subtraction. Each experiment was carried out in triplicate. As a reference compound rosmarinic acid (RA, 16.67 ppm) was used. By means of a calibration curve the hydrogen peroxide concentration in the reaction media could be calculated.

6.2.3 Linoleic acid *ß*-carotene oxidation assay

The linoleic acid *ß*-carotene oxidation assay [2] was performed according to the procedure described by Koleva *et al.* [3]. *trans*-*ß*-Carotene (95%) and linoleic acid (99%) were from Aldrich Chemical Co. (Milwaukee, USA) and the emulsifier Tween 40 was from Sigma (St. Louis, MO, USA). *ß*-Carotene (1.0 mg) was dissolved in 5 ml chloroform and linoleic acid (25 μl) and Tween 40 (200 mg) were added to 1 ml of this solution. The chloroform was evaporated under vacuum at 40°C and oxygenated ultra-pure H_2O (50 ml, obtained by bubbling air through the H_2O for 15 min) was added. The mixture was vigorously shaken. Plant extract solutions (0.1%, w/w), reference

compounds (0.01% solutions of BHT and RA) or commercially available rosemary extracts (spray-dried and fat soluble) were prepared in EtOH. Rosemary extracts (RE I and RE II; final concentration 0.1%) were prepared in two different ways: directly by dissolving spray-dried extract in EtOH (RE I) or by partitioning fat soluble extract between hexane and MeOH; then the methanol fraction was dried and finally dissolved in EtOH (RE II). Aliquots (250 µl) of the *ß*-carotene : linoleic acid emulsions were distributed in 96-well microtitre plates and ethanolic solutions of the test extract samples (30 µl) were added. EtOH was used for the blank sample. Four replicates were prepared for each sample. The microtitre plates were incubated at 55°C temperature, and absorbances were measured using a µQuant universal microplate spectrophotometer (BIO-TEK, Vinooski, USA) at 490 nm. Readings of all samples were performed immediately after mixing an aliquot of the emulsion with ethanolic extract samples and after 105, 210 and 315 min of incubation. The residual absorbance was used for antioxidant activity comparison of different samples.

6.2.4 Determination of peroxide value

Commercial refined rapeseed oil without added antioxidants (Obeliu aliejus, Vilnius, Lithuania) was used for peroxide value (PV) determination and UV absorbance measurements. Chloroform, glacial acetic acid (99.9%), potassium iodide (99.5%) and sodium thiosulphate were from Lachema (Brno, Czech Republic).

Peroxide values were monitored in oil samples with added plant extracts (0.1% w/w) and with reference antioxidant BHT (0.01%). A blank sample was run without any additives. Most extracts were hydrophilic and their homogenization in the oil was rather difficult. Therefore the extracts were dissolved in a small amount of EtOH (approx. 5% of the oil mass) and this solution was introduced in the oil by vigorous mixing. The samples (approximately 25 g) were placed in open beakers (250 ml volume) and kept in a ventilated thermostat (VEB, MLW Labortechnik, Ilmenau, Germany) at 55 °C. PV measurements were performed and values determined by AOCS official method [4]. Oil samples (approximately 1 g) were taken from the beakers, accurately weighed and dissolved in 25 ml of a chloroform/acetic acid mixture (3:2). Then 0.5 ml of saturated potassium iodide solution in H_2O was added, the samples were shaken for 1 min and 25 ml of distilled H_2O was added. The liberated iodine was titrated with 0.01 M sodium thiosulphate solution using a 1 % starch solution as indicator. Peroxide values (meq/kg oil) were calculated using the formula:

Practical evaluation

$$PV = 0.01 \times N \times 1000 / m, \quad (3)$$

where N is the volume of sodium thiosulphate used for the titration of a sample in ml and m is the mass of oil sample in g.

Antioxidant efficiency (AE) was calculated using the following formula:

$$AE = IP_A/IP_B, \quad (4)$$

were $IP_{A,B}$ are the induction periods (the time in days needed to reach a PV of 20 meq/kg oil) of test and blank samples. Results are presented as a mean of two replicates.

6.2.5 UV absorbance test

Approximately 0.02 g of oil sample prepared and handled in the same way as for PV measurements was weighed accurately into a 25 ml volumetric flask, diluted with hexane and properly mixed. UV absorbance at 232 and 268 nm was measured in 1 cm long quartz cells, with hexane as a reference. The absorbance (E) values were recalculated as for 1% solution:

$$E_{1\%} = A_\lambda \times c^{-1} \times d^{-1}, \quad (5)$$

where A_λ is the absorbance measured at given wavelength λ; c is the sample concentration in the solution (g/100 ml); d is the cell length in cm.

Results are presented as a mean of two replicates.

6.2.6 Measurement of hexanal using static headspace GC (HS-GC) method

Commercial cold-pressed safflower oil without added antioxidants (Natufood, Harderwijk, the Netherlands) was used for headspace-gas chromatographic (HS-GC) analysis. The oil was stored under nitrogen in the dark at –20 °C prior to analysis. Reference hexanal was from Acros Organics (96%, Geel, Belgium). Methanolic solutions of the extracts and BHT were added to approx. 0.2 g of safflower oil. The final extract and BHT concentrations in the samples were 0.1% w/w and 0.01% w/w respectively. A blank sample was prepared with an equal amount of MeOH. Samples were placed in headspace vials (10 ml), sealed with a TFE/butyl rubber liner and incubated at 55°C in an oven. Sampling was carried out approximately every second day. The amount of hexanal formed in

the course of oil oxidation was measured using a Carlo Erba (Milan, Italy) GC 8000 TOP gas chromatograph equipped with a COMBI-PAL (CTC Analytics, Zwingen, Switzerland) headspace sampler and a wide-bore thick-film capillary DB-WAX column (1 μm, 30 m; 0.54 mm i.d.; J&W Scientific, Folsom, USA). The temperature programme was the following: an isothermal hold at 50 °C for 5 min, then increase to 150 °C at 10 °C/min. The samples were incubated at 60 °C for 10 min in a 2000 rpm agitator operating with a 10 s run/10 s stop cycle prior to injection. The volatiles were cold-trapped using an MFA 815 cold trap (Carlo Erba, Milan, Italy) at −110°C and injected ballistically into the column at 240°C. The headspace sample volume was 2000 μl; the helium pressure was 30 kPa; the injection syringe temperature was 70°C; the injector temperature was 200°C; and the flame ionization detector (FID) temperature was 225°C. GC data was processed with Chrom Card (Carlo Erba, Milan, Italy) integration software. The experiment was carried out in duplicate.

The antioxidant efficiency of extracts was evaluated by dividing the induction period of a plant sample (IP_A) by that of a blank sample (IP_B). The induction periods were defined as the time (in days) after which the hexanal formation sharply increased.

6.2.7 Evaluation of antioxidative capacity of plant extracts during accelerated oxidation of fermented sausages

Production of cervelat sausage. The ingredients for the production of sausages (traditional Dutch style cervelat sausage) were obtained from the Department of Public Health and Food Safety (Utrecht University, the Netherlands). Standard ingredients are listed in table 6.1.

Ingredient	g/kg		g/kg
Beef	655	Ascorbate (Sodium salt)	0.5
Lard	300	Bell pepper (red) powder	1.0
Nitrite salt	25	White pepper	1.2
Glucose	4.0	Milled pepper	1.0
Glutamate	2.0	Clove	0.15
Starter sausage	10	Garlic powder	0.15

Table 6.1 *Standard recipe of Dutch cervelat sausage*

Practical evaluation

Sausages (with and without ascorbate) with plant extracts were prepared according to the same recipe as standard sausages without using spices. Commercially available rosemary extract was from Robertet (Grasse, France). The used amount of extract (spray dried rosemary extract, EB fraction of *Potentilla* extract or WW fraction of *Geranium* extract) was 1 g per kg of sausage; the amount of ascorbate (if used) was 0.5 g/kg of sausage. Manganese (0.033 g/kg of sausage) was used in order to enhance acid production by meat starter bacteria [5].

The beef meat was minced in the cutter, ingredients added and properly mixed. The temperature during the production was around –2 °C. The prepared meat for the sausages was put into artificial casings. Sausages were fermented for three days at 25°C and dried for 14 days (not smoked). Sausages were then cut in equal pieces and packed under a high oxygen/carbon dioxide atmosphere (60/25%) in order to accelerate the oxidation processes. During the storage of fermented sausages, primary (peroxides) and secondary (malondialdehyde and hexanal) oxidation products were monitored and the effectiveness of added extracts has been determined.

6.2.7.1 Peroxide value measurements

25 g pieces of sliced sausage were placed in Erlenmeyers, 200 ml of acetone was added, the flasks were closed and incubated in a shaker for 2 hours. Afterwards the mixture was filtered. PV values (meq/kg sausage) were estimated by the AOCS official method [4]. Measurements (in duplicate) were conducted within the period of 49 days (at 14^{th}, 26^{th}, 39^{th} and 49^{th} day).

6.2.7.2 Determination of TBARS

The following reagents were used: thiobarbituric acid (TBA, 0.02M), malondialdehyde-bis-(diethylacetal), 7.5 % solution of trichloroacetic acid (TCA), 0.1% propylgallate and 0.1% ethylendiaminetetraacetic acid (EDTA, all from Merck). Sulfanilamide solution was prepared in the following way: 0.5 g sulfanilamide (Fluka) was mixed with 40 ml of distilled H_2O, 54 ml of 37 % HCl (Riedel Haën, Seelze, Germany) was added and then the solution was diluted with H_2O up to 100 ml. Four measurements within the 39 day oxidation period were conducted for each sample and five measurement for samples with slower oxidation (within 49 days).

Preparation of calibration curve for determination of malondialdehyde. The following concentrations of malondialdehyde in TCA were prepared: 8 µM, 6.67 µM, 5µm, 3.33 µM and 2

μM. Three ml of MDA solution were mixed with 3 ml TBA solution. The blank sample contained 3 ml of TCA and 3 ml of TBA. The solutions were properly mixed and then incubated for 30 minutes in a boiling water bath. Afterwards they were cooled with cold H_2O to room temperature and the absorbance was measured at 532 and 600 nm with a Shimadzu UV Mini 1240 spectrophotometer (Shimadzu, Duisburg, Germany). The difference in absorbances ($A_{532nm} - A_{600nm}$) was calculated, where A_{600nm} was used to correct sample turbidity [6]. TBARS values, expressed as μmol malondialdehyde per kg meat were calculated using malonaldehyde-bis-(diethylacetal) as standard.

Measurements of TBARS in the process of the oxidation of fermented sausages. Approximately 7 g of sausage was weighed accurately; 25 ml TCA and 1 ml of sulfanilamide solution were added and properly mixed. Then the mixture was filtered and 3 ml of this solution was mixed with 3 ml of TBA. Solutions were incubated for 30 min in a boiling water bath, cooled, the absorbance measured and TBARS values calculated:

$$\text{TBARS (μg malondialdehyde/g of sausage)} = [((A_{532}-A_{600})/R) \times C] / G, \quad (6)$$

where A_{532}, A_{600} – absorbance at 532 or 600 nm; C – constant (slope) established from a calibration curve; C – constant (26/3) for the volume correction; G – mass of the sausage sample, g.

6.2.7.3 Determination of hexanal amount

Formation of hexanal was monitored using static headspace gas chromatographic (HS-GC) method. 1 g of sausage was placed in a headspace vial, sealed and incubated at 60 ºC in an oven. Sampling was carried out 4 times within 40 days. The amount of hexanal formed was measured using the same equipment and procedure as for the measurements of hexanal during oil oxidation (see section 6.2.6).

6.2.8 Preliminary safety evaluation studies

EB extract fraction of *Potentilla* and two fractions of *Geranium* (EB and WW) have been chosen for the following safety evaluation tests.

Practical evaluation

6.2.8.1 Investigation of the enzymatic oxidation of extracts

Polyphenols can be oxidized with hydrogen peroxide by peroxidases or with oxygen by tyrosinases as described by Folkes *et al.* and Jolley *et al.* [7, 8]. In order to assess the properties of new polyphenols in enzymatic reactions and to understand their oxidation mechanism, it is necessary to compare them with model compounds. For pure polyphenolic compounds so called catalytic constants can be determined. This parameter shows the number of polyphenol molecules that can be oxidized by one active enzyme centre (e.g. heme or diamagnetic Cu) within one second. For polyphenol rich extracts the oxidation of complex mixtures by an enzyme can be assessed only by the oxidation half-time ($t_{1/2}$). The solutions containing 200 μm H_2O_2 and 3 nM of peroxidase (from horseradish) or 0.6 μM tyrosinase (from mushrooms) were mixed with the 50-150 μg/ml of substrate (dry EB extract from *Potentilla fruticosa* and EB or WW extracts from *Geranium macrorrhizum*). Differences in absorbance changes during the oxidation were determined by measuring the spectrum of the solution (250-600 nm) and its changes 5 min after mixing. The wavelengths where the absorbance changes during the oxidation were the biggest (in comparison to the isosbestic point) were determined. Then the oxidation process was again monitored at the chosen wavelength and the oxidation half time was determined (an approximate middle point of the absorbance curve change over the oxidation time). The activity of extracts was compared with that of the reference compound 5,8-dihydroxycoumarin. Measurements were conducted at 25 $^{\circ}$C using a Hitachi-557 UV-Vis spectrophotometer (Hitachi Co, Japan) in 0.1 M potassium phosphate buffer with 1 mM EDTA.

6.2.8.2 Protective effects of extracts against singlet oxygen caused erythrocyte hemolysis

A suspension of human erythrocytes was obtained from the Blood Transfusion Center in Vilnius, Lithuania. In the course of photohemolysis the erythrocyte suspension (diluted 20 fold with buffer solution, containing 10 mM glucose, pH 7.4, and 10 μM photosensibilizator phthalocyanine tetrasulfonate (AlPcS4)) was irradiated (25 W/m^2, $\lambda > 590$ nm). Later this solution was 100-fold diluted in a cuvette and its absorbance at 740 nm measured. It was assumed that hemolysis was 100% complete when the absorbance of the investigated solution was the same as that of pure buffer [9]. Concentrations of extracts ($c_{1/2}$), which twice doubled the lysis time, were determined according to the erythrocytes lysis half-time dependence on extract concentration.

6.2.8.3 Mutagenicity evaluation of plant extracts (somatic mutations and recombinations test in *Drosophila melanogaster* wing cells)

The experiment was conducted according to U. Graf [10]. Two *Drosophila melanogaster* strains were used (obtained from *Umea Drosophila Stock Center*, Umea, Sweden): (1) *mwh* (multiple wing hairs; homozygotic by highlighted *mwh* gene line [homozygoticity determines appearance of cells with multiple setas]) male strains; (2) virgin females from the strain *ORR (1)/ORR (2); flr^3 /In (3LR) TM3, ri p^p sep l(3) 89 Aa bx^{34e} e^s Bd^S*, having a highlighted *flr^3* (flare) gene, which determines the appearance of misshapen (shorter, longer) setas. *flr^3* gene in homozygotic state is lethal, therefore for balancing *TM3, ri p^p sep 1(3) 89 Aa bx^{34e} e^s Bd^S* chromosome is introduced. The marker *Bd^S* determines pattern of fly wings. *flr^3/TM3,Bd^S* line virgin females were crossed with *mwh* line males. For growing *Drosophila* Instant Drosophila Medium (Sigma, USA) nourishment medium at 25 °C was used. Duration of exposition with ethanolic solutions of extracts was: (1) 120 hours, i.e. the whole evaluation period from egg to pupation of larvae; (2) 48 hours - influence of added extract solutions were observed on rolled 72±5 h age larvae. 1 ml of extract solutions were added to the surface of medium. Rolled drosophilas were sorted by phenotype, leaving for further analysis trans-heterozygoted ones (lacking *Bd^S* marker). The wing preparations were mounted on glass slides in *Faure* solution and analyzed with a microscope at 300 times magnification for the presence of single (*mwh* or *flr^3* phenotype) or twin (neighboring *mwh* or *flr^3* clone) spots. These spots appear due to genotoxic events (mutations or mitotic recombination). For each aliquot at least 40 wings have been observed. Data was processed statistically with GraphPad InsStat v2.02 program. Reliability of differences in results was checked with Student t-test ($P < 0.05$).

6.3 Results and Discussion

6.3.1 Antioxidant activity evaluation of selected extracts from *Geranium macrorrhizum*

Oxidation of lipids or other products is a complex process, resulting in a big variety of oxidation products. Many factors, particularly temperature, light, the presence of catalysts (metals, enzymes) and others influence the oxidation process and the development of reaction products. For this reason different methods are needed for monitoring the oxidation processes in order to get detailed information on the efficiency of added antioxidants.

6.3.1.1 Determination of superoxide anion and hydrogen peroxide scavenging properties

Superoxide radical ($O_2^{\bullet -}$) is a type of reactive oxygen species that is mainly formed in living cells. Although the reactivity of superoxide anion as well as H_2O_2 is limited, they can both damage some cellular targets [11]. In this study the superoxide anion radical generated in the xanthine/xanthine oxidase system was effectively scavenged by all *Geranium* extracts applied at 200 ppm concentration (figure 6.1).

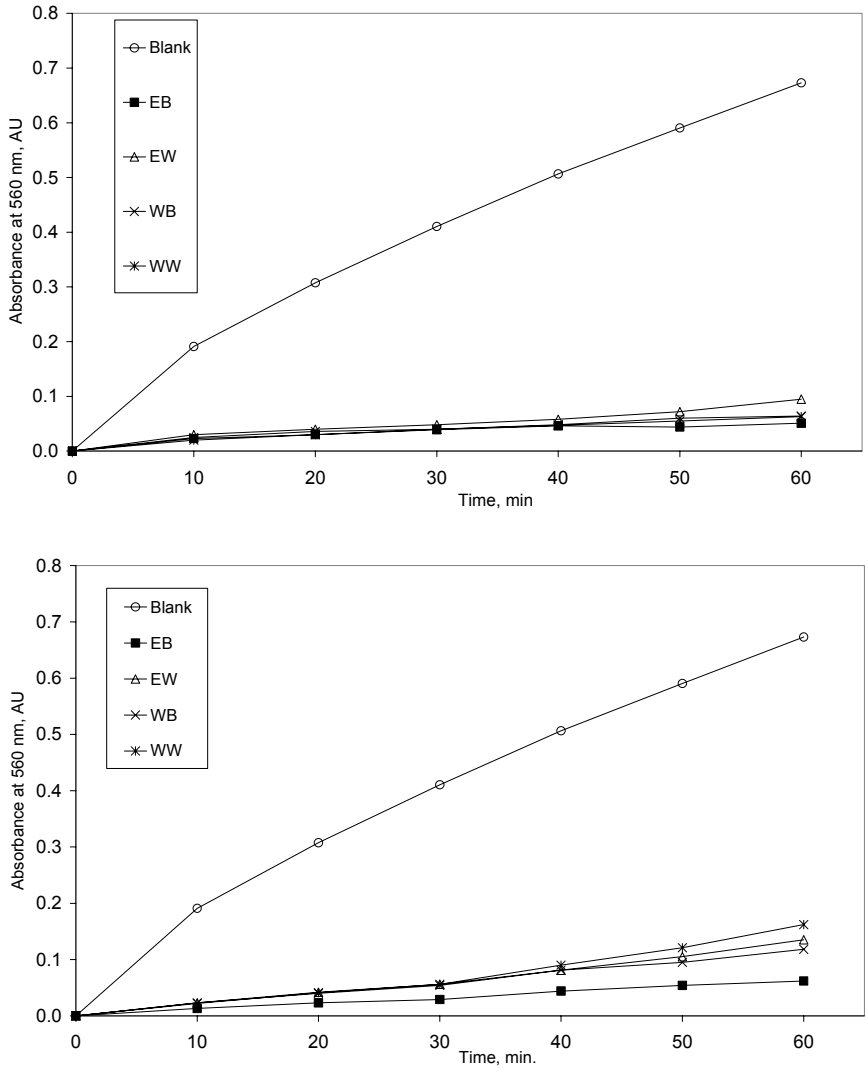


Figure 6.1 *The ability of Geranium extracts to scavenge superoxide radical anion, expressed as the intensity of reduced NBT indicator: extract concentrations were 200 ppm (a) and 50 ppm (b).*

This effect remained almost the same after a 4 fold dilution of extracts (50 ppm). The characteristic ink blue color of the reduced NBT was clearly observed for the blank sample and it was gradually increasing over the whole reaction period. The increase in absorbance for the samples

with extracts was minor compared to the blank sample. After dilution of the extracts (figure 6.1 b), it was observed that the EB fraction possessed the strongest superoxide scavenging properties.

The hydrogen peroxide scavenging curves are provided in figure 6.2. Comparing the curves in figures 6.1 and 6.2 it can be observed that hydrogen peroxide scavenging activities of the extracts varied in a wider range than the superoxide scavenging activities, which were quite similar for all the extracts.

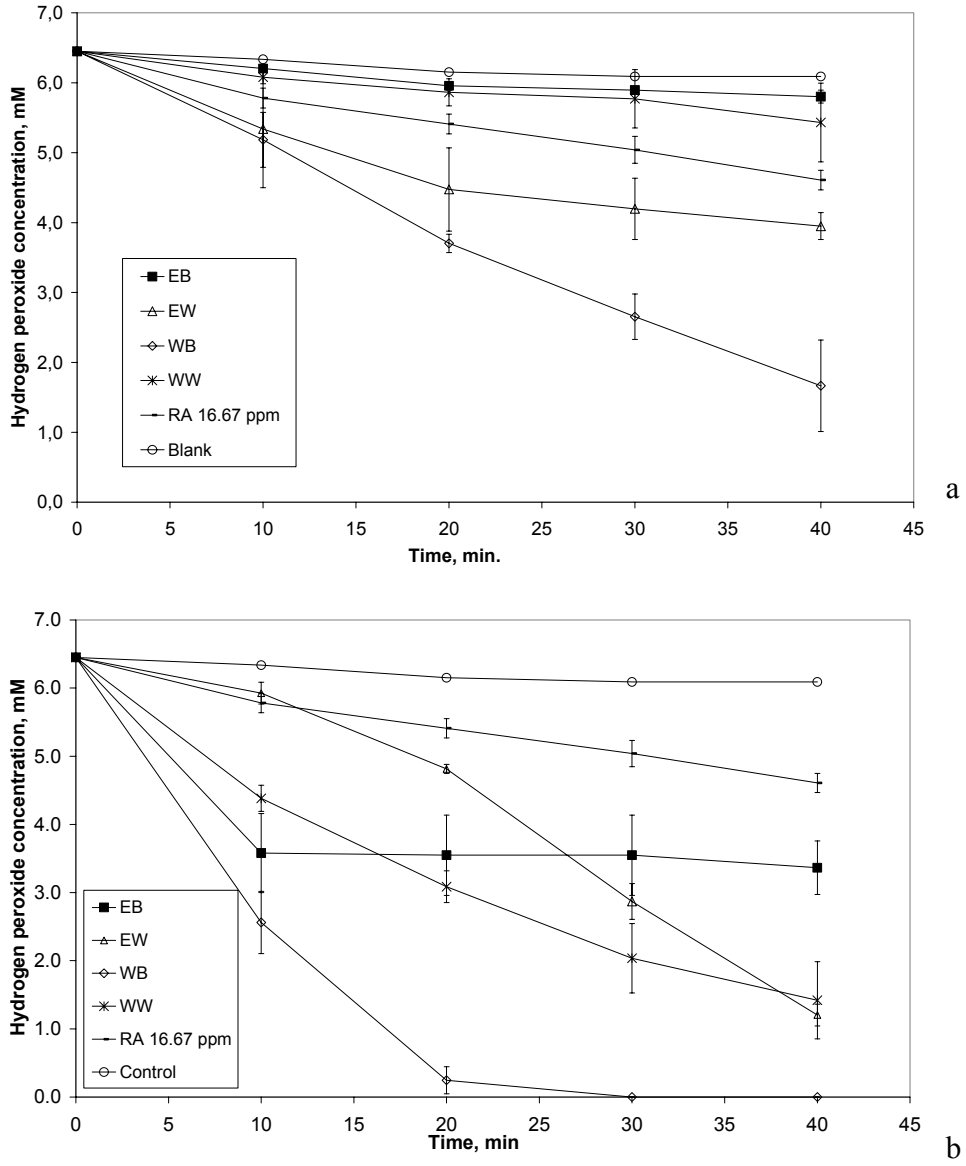


Figure 6.2 *Hydrogen peroxide scavenging activity of Geranium extracts: a – 33.3 ppm extract solutions; b – 50 ppm.*

The water-butanol fraction (WB) was the most effective hydrogen peroxide scavenger followed by ethanol-water fraction (EW). Ethanol-butanol (EB) fraction, being the strongest superoxide

scavenger, was considerably less effective in the hydrogen peroxide scavenging test. Water-water (WW) and ethanol-water fractions were better soluble in aqueous buffer, while a less hydrophilic extract, such as EB was less soluble in the same assay medium and consequently its activity was lower. On the other hand its activity in oxidation tests performed with oil was the highest. It was observed that EB extracts (50 ppm concentration) rapidly reduced the concentration of hydrogen peroxide within the first 10 min of reaction and after this time further decrease was not distinct. Similar behaviour was also observed by Wettashinghe *et al.* with borage extracts [1]. In the samples with other *Geranium* extracts the decrease of hydrogen peroxide was gradual over the whole reaction period. It suggests that possibly different compounds are present in the fractions obtained with different solvents and that they possess different reaction kinetics.

6.3.1.2 Linoleic acid-β-carotene coupled oxidation assay

In the linoleic acid-β-carotene coupled oxidation assay various radicals formed during the oxidation of linoleic acid oxidize ("bleach") β-carotene and in this way the antioxidant activity of various added substances can be monitored.

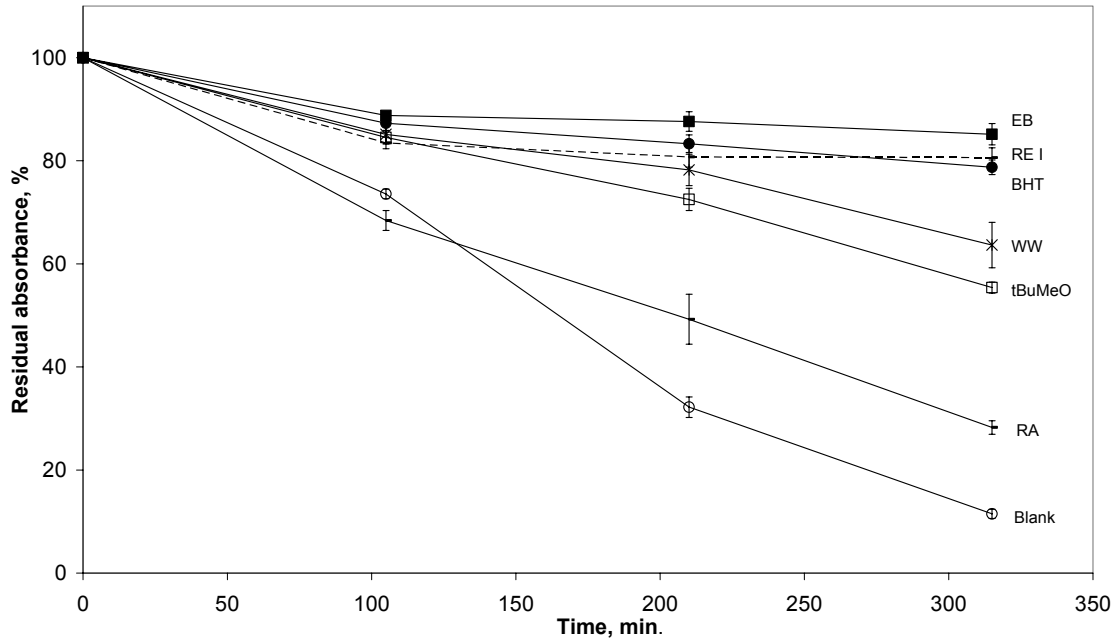


Figure 6.3 *Antioxidant activities of various Geranium extracts assessed in the linoleic acid-β-carotene coupled oxidation assay. The absorbance changes of EW, WB extracts from Geranium and RE II extract from rosemary were close to those of EB or RE I and are not shown.*

According to some authors the oxidation process in this assay reaches a *plateau* phase after 90-120 min of reaction time, after which the decrease in absorbance is no longer significant [1, 12].

However in my experiment the absorbance did not stabilize after this period, therefore the measurements were continued for more than 5 hours (figure 6.3). In order to compare the results for all different extracts and fractions the residual absorbance of all samples was calculated. The higher the residual absorbance, the more active the extract is. From figure 6.3 one can observe a low activity for rosmarinic acid (RA), which was added at a 10 times lower concentration than *Geranium* extracts. Apparently this amount of RA was insufficient to protect the emulsion from oxidation. The effect of BHT at the same concentration (0.01 %) was substantial. All *Geranium* extracts retarded *ß*-carotene bleaching, however the effect of tBuMeO and WW was weaker compared to the other fractions, which considerably delayed oxidation of *ß*–carotene: after 320 min from the beginning of the reaction the residual absorbance was still approximately 80 %. The effect was similar to that of commercial rosemary extracts (RE I and II).

6.3.1.3. Determination of peroxide value (PV)

The oxidation of commercial rapeseed oil expressed as PV is presented in figure 6.4. On the basis of the presented curves the induction periods (IP) and antioxidant efficiency (AE) of *Geranium* extracts has been calculated (table 6.2).

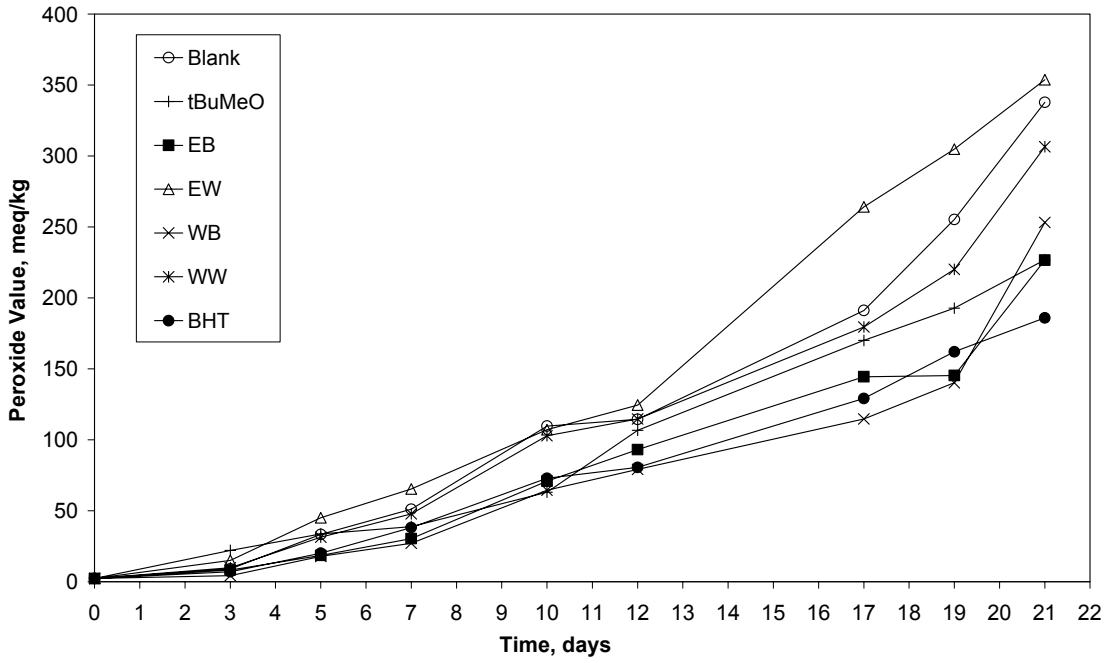


Figure 6.4 *Effect of Geranium extracts on lipid oxidation of rapeseed oil at 55 °C.*

Practical evaluation

It should be emphasized that plant extracts were prepared with polar solvents (EtOH or H_2O). These polar extracts are not convenient for such a lipophilic medium as rapeseed oil. Most likely due to this reason, the most polar fractions WW and EW did not show any antioxidant activity in the initial oxidation stage. However both butanol fractions (EB and WB) although also being poorly soluble in oil gave more promising results (AE was 1.33). The AE of BHT that was added at a 10 times lower concentration was 1.25. The *tert*-butyl methyl ether extract of the plant was non-polar and soluble in rapeseed oil, but did not show any antioxidant activity, showing that it doesn't contain any valuable antioxidants.

Extract sample	IP (hr)	AE
tBuMeO	79	0.83
EB	127	1.33
EW	84	0.88
WB	127	1.33
WW	96	1.00
BHT	120	1.25

Table 6.2 *Induction periods (IP) and antioxidant efficiency values (AE) of Geranium extracts in rapeseed oil (IP_{blank} = 96 h, AE = 1)*

6.3.1.4 UV absorbance test

Conjugated dienes are primary and trienes secondary oil oxidation products. By measuring the UV absorbance of oxidisable substrate at certain wavelengths information about the decomposition of an oxidized product and the efficiency of antioxidants added to it can be obtained. The effect of *Geranium* extracts on the formation of dienes and trienes in rapeseed oil at 55 °C expressed as UV absorbance at 232 and 268 nm is presented in figure 6.5. No significant differences could be observed among the tested samples for conjugated dienes during the oxidation process (figure 6.5 a). Usually changes in peroxide value correlate with differences in conjugated diene in oil formation [13]. However in this study the effect of the extracts on the formation of primary oxidation products in oil was too small to detect.

Conjugated trienes are characteristic secondary degradation products, therefore their increase during the oxidation process started only after approx. 12 days (figure 6.5 b). As it was expected, the slowest formation of secondary products was observed in the sample with BHT. All *G. macrorrhizum* extracts (except EW) showed antioxidant activity in this assay. It is interesting to note that EW demonstrated slightly pro-oxidant properties in this test as well as in PV assay.

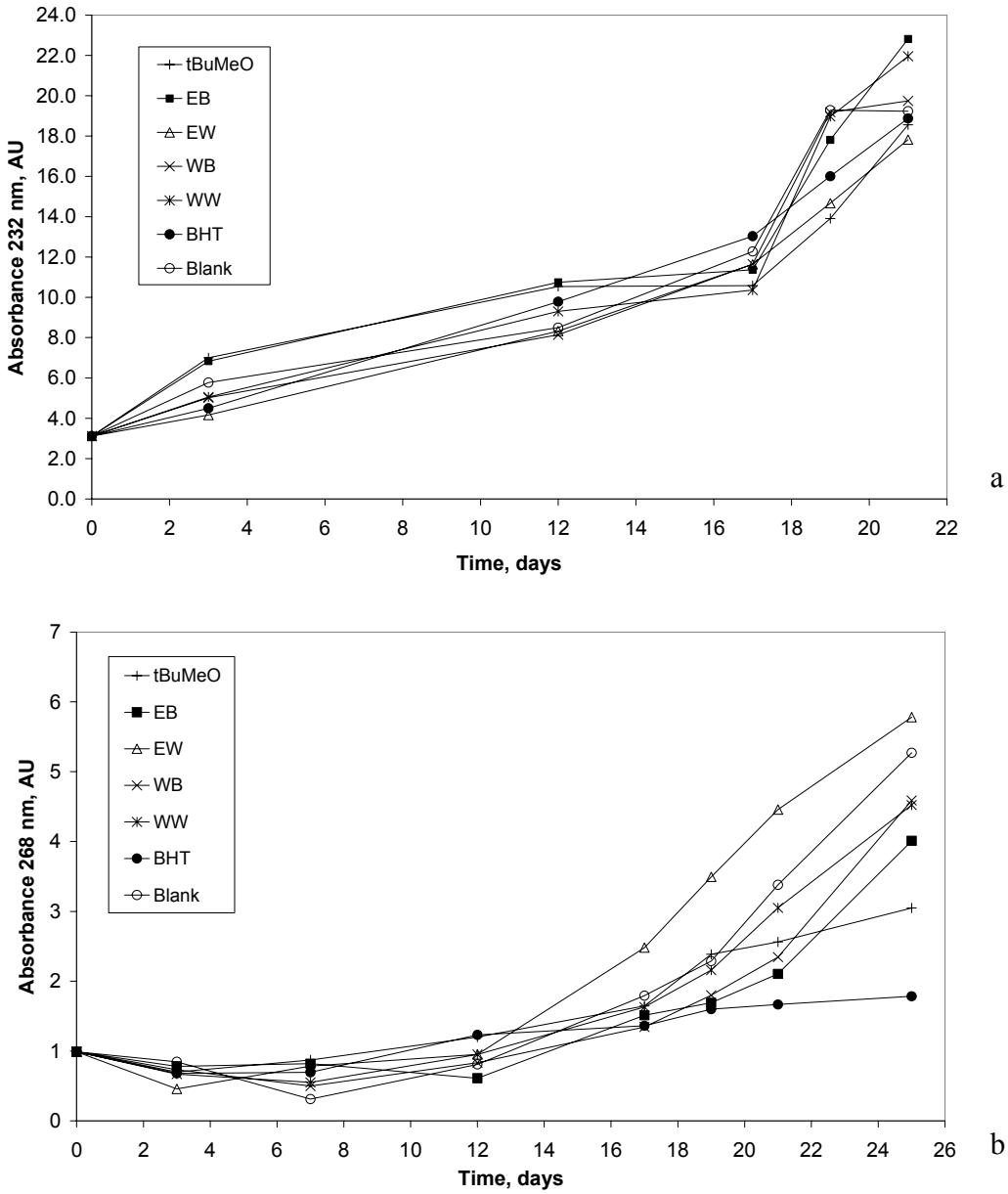


Figure 6.5 *Effect of Geranium extracts on the formation of conjugated dienes (a) and trienes (b) in rapeseed oil at 55℃. Plotted absorbance units are recalculated as for 1 % extract samples.*

6.3.1.5 Effect of extracts on the formation of hexanal

Two aldehydes - hexanal and pentanal usually are the major volatiles of secondary oil oxidation processes. The amount of produced hexanal correlates well with the decomposition of polyunsaturated fatty acids [14]. As the amount of pentanal formed during the oxidation is usually significantly lower than that of hexanal, the latter compound was selected for monitoring in this

study. Hexanal is a secondary oxidation product, therefore its rapid increase during the oxidation process was observed after a certain lag time (induction period).

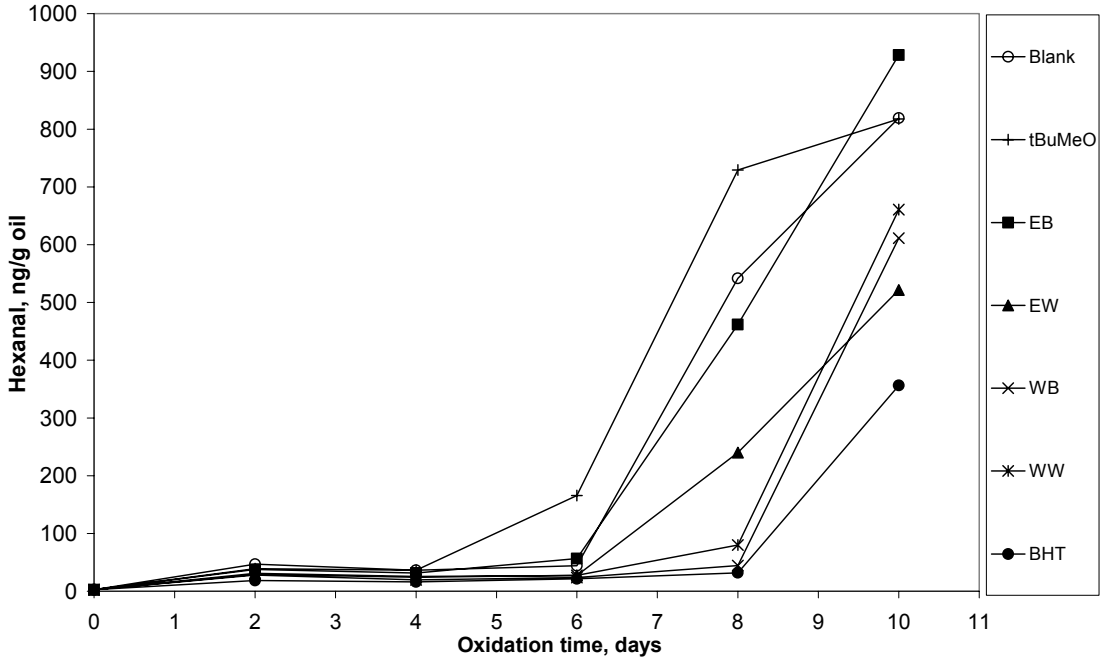


Figure 6.6 *Effect of various G. macrorrhizum extracts on the formation of hexanal during the oxidation of safflower oil at 55 °C.*

The antioxidant efficiency (AE) of the plant extracts was calculated by dividing the IP of the sample with additive by that of the blank. The AE for all tested *Geranium* extracts is presented in table 6.3. The results show that both *Geranium* ethanol fractions were more effective than the others. The AE of ethanol fractions was equal to that of synthetic antioxidant BHT. Fractions of the water extract did not show any activity in safflower oil (AE = 1) and the tBuMeO extract even exhibited pro-oxidant activity (AE < 1).

Tested *Geranium* extract	Antioxidant efficiency (AE)
tBuMeO	0.83
EB	1.33
EW	1.33
WB	1.00
WW	1.00
BHT	1.33
Blank	1.00

Table 6.3 *Antioxidant activity of Geranium extracts, measured by the HS-GC method*

Some disagreement can be observed when comparing the hexanal and conjugated triene results for the EW fraction (see figure 6.5). This fraction reduced the amount of hexanal and increased the UV absorbance, which can be attributed to the formation of conjugated trienes. Actually, these results again prove the complexity of the oxidation process, especially at its later phases.

6.3.2 Antioxidant activity evaluation of selected extracts from *Potentilla fruticosa*

6.3.2.1 Determination of superoxide anion and hydrogen peroxide scavenging properties

Similarly to *Geranium* extracts, *Potentilla* extracts also acted very effectively against superoxide anion radicals, generated in a xanthine/xanthine oxidase system.

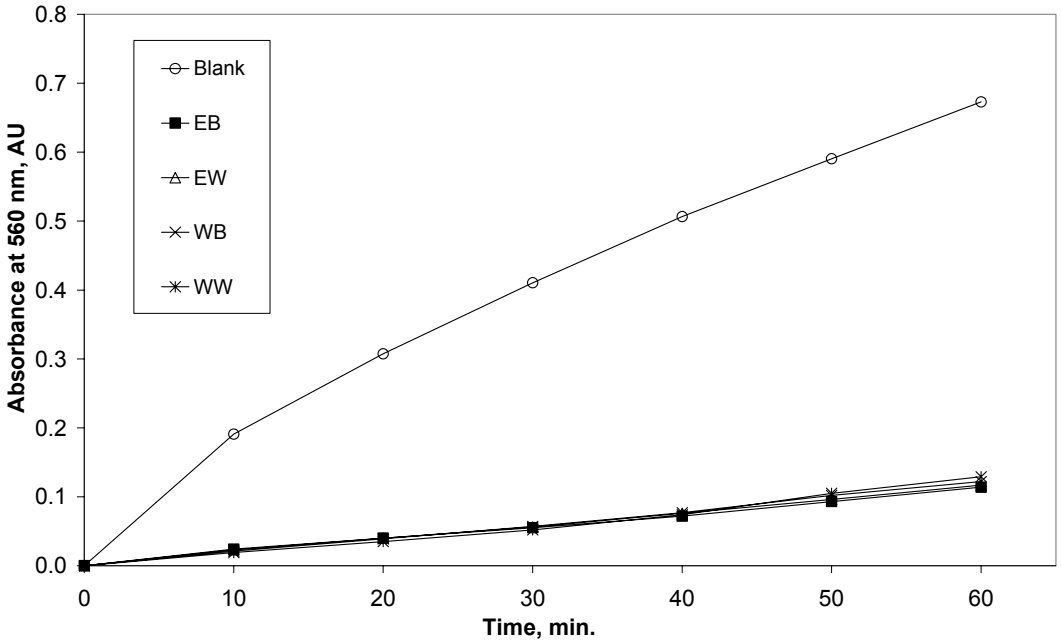


Figure 6.7 *Ability of P. fruticosa extracts (50 ppm) to scavenge superoxide anion radical, expressed as the intensity of reduced NBT indicator.*

Superoxide radicals were effectively scavenged by all *P. fruticosa e*xtracts applied at 200 ppm. This effect remained almost the same after diluting extracts 4-fold (50 ppm; figure 6.7). It should be noted, that compounds present in *G. macrorrhizum* and *P. fruticosa* extracts could also act as direct inhibitors of the enzyme necessary for superoxide generation.

The hydrogen peroxide scavenging curves are depicted in figure 6.8. All *P. fruticosa* extracts showed effective hydrogen peroxide scavenging properties. The scavenging process continued

throughout the whole reaction period and after 40 min almost all of the hydrogen peroxide was scavenged. RA was tested in a 3-fold lower concentration in comparison to the extracts as it was a pure compound. However at this concentration its activity was much lower than that of extracts.

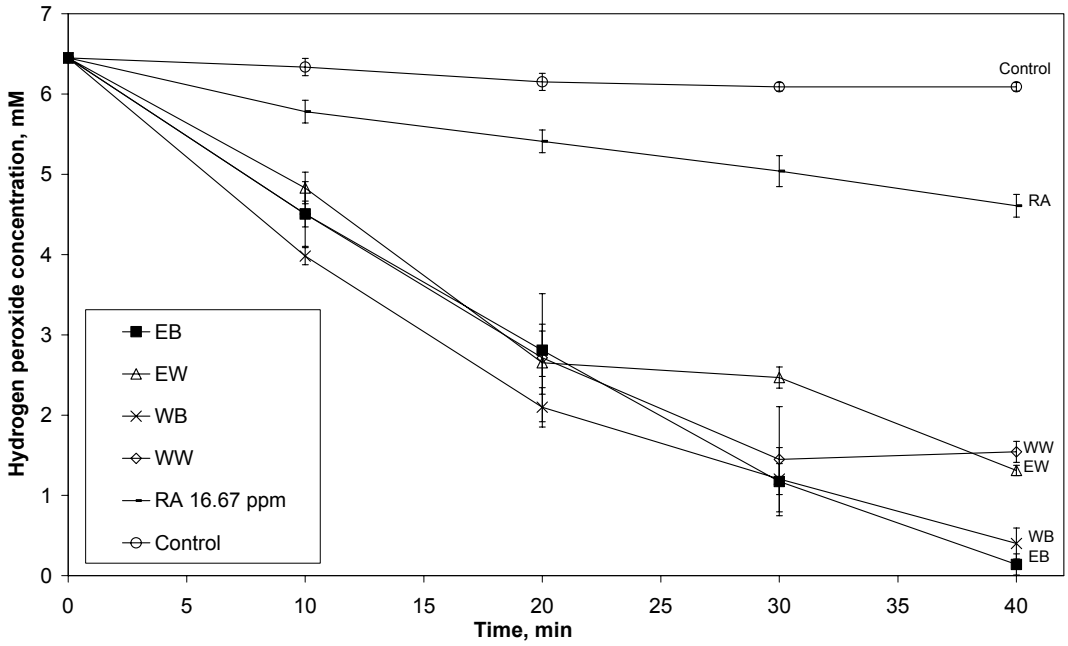


Figure 6.8 *Hydrogen peroxide scavenging activity of Potentilla extracts.*

6.3.2.2 Linoleic acid-β-carotene coupled oxidation assay

The effect of various *Potentilla fruticosa* extracts is shown in figure 6.9. All extracts effectively retarded the oxidation of β-carotene in the emulsion. Even after 5 hours the absorbance was still around 80 % of initial value for the most active *Potentilla fruticosa* fractions. The activities of all other extracts were very similar (residual absorbance values were 78 - 81 % after 315 min.). The effect was similar to that of commercial extracts of rosemary. The activity of BHT tested in a 10-fold lower concentration (0.01%) was also high; rosemarinic acid (RA) at this concentration showed only a slight effect.

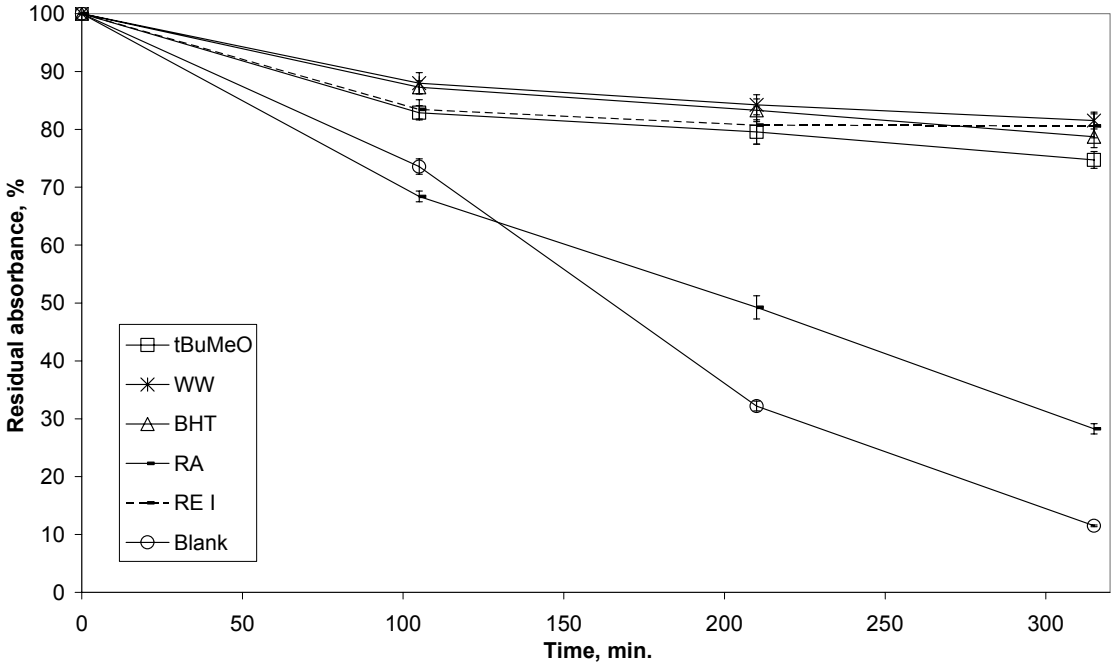


Figure 6.9 *Antioxidant activities of various Potentilla fractions as assessed with the linoleic acid-β-carotene oxidation assay. RA – rosemarinic acid; the absorbance changes of EB, EW, WB extracts from Potentilla and RE II extract from rosemary were close to those of WW or RE I and are not shown.*

6.3.2.3 Determination of peroxide value

The oxidation of commercial rapeseed oil expressed in PV is summarized in figure 6.10. The calculated induction periods (IP) and antioxidant efficiencies (AE) of tested extracts are presented in table 6.4. Most *Potentilla* extracts were prepared using polar solvents (ethanol or water) which complicated their application in such a lipophilic media as rapeseed oil. Most likely due to that reason polar fractions (EW, WW and WB) did not show any significant antioxidant activity. The activity of the less polar fraction EB was almost similar to that of polar fractions. The non-polar extract tBuMeO dissolved well in rapeseed oil and showed a bit higher antioxidant activity (AE = 1.13). BHT applied in a 10 times lower concentration gave the best results (AE = 1.25).

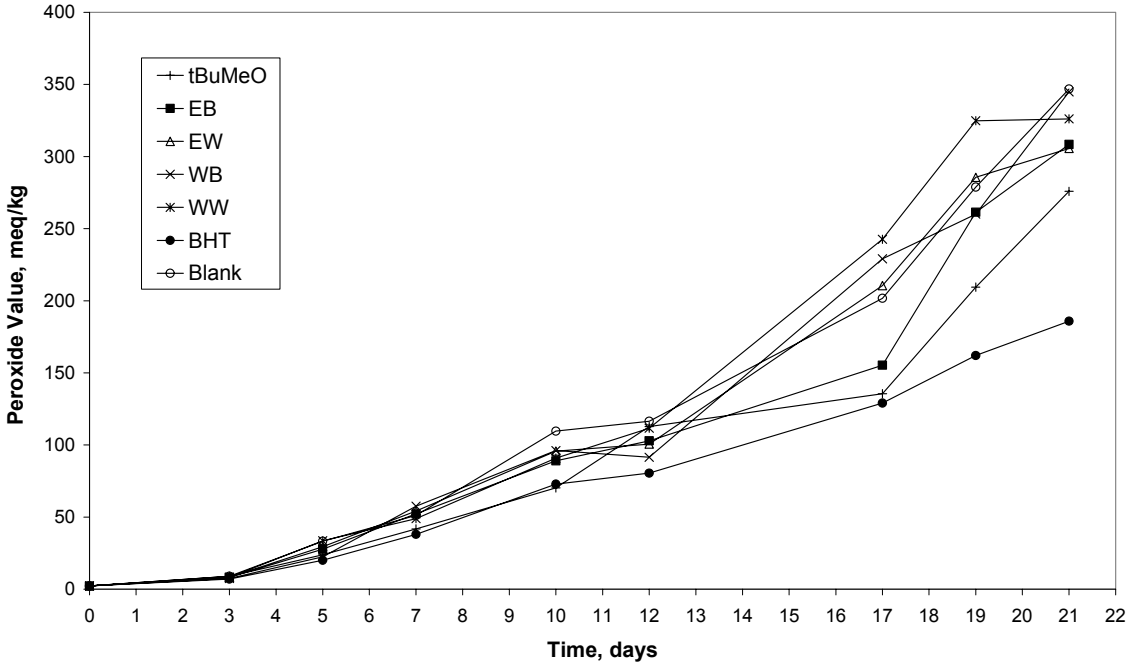


Figure 6.10 *Effect of Potentilla extracts on the oxidation of rapeseed oil at 55°C.*

Sample	IP (h)	AE
tBuMeO	108	1.13
EB	101	1.05
EW	98	1.03
WB	113	1.18
WW	96	1.00
BHT	120	1.25

Table 6.4 *Induction periods (IP) and antioxidant efficiency values (AE) of Potentilla extracts in rapeseed oil (IP_{blank} = 96 h, AE = 1)*

6.3.2.4 UV absorbance test

No distinct differences could be observed among the *Potentilla fruticosa* samples in the conjugated diene formation assay. Rapid formation of conjugated trienes, which can be assessed by the UV absorbance at 268 nm, started after approximately 12 days of oil storage (figure 6.11). The slowest formation of trienes (secondary products) was observed in the sample with BHT. tBuMeO, which is the only extract soluble in the oil, possessed some antioxidant activity, although no active radical scavenging compounds have been identified in this extract. The changes in absorbance of oil samples with polar *Potentilla* extracts were similar to the blank, which is probably due to their insolubility in the oil.

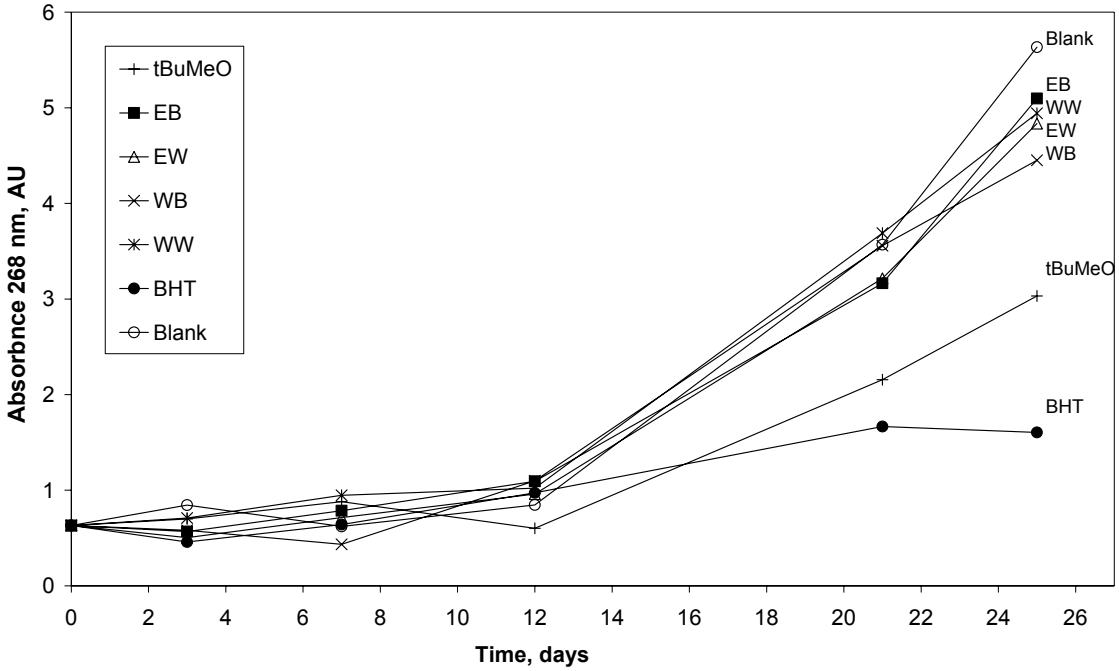


Figure 6.11 *Effect of Potentilla extracts on the formation of conjugated trienes in rapeseed oil at 55℃. Plotted absorbance units are recalculated as for 1 % extract samples.*

6.3.2.5 Measurement of hexanal using static head space GC (HS-GC) method

Hexanal formation curves are presented in figure 6.12.

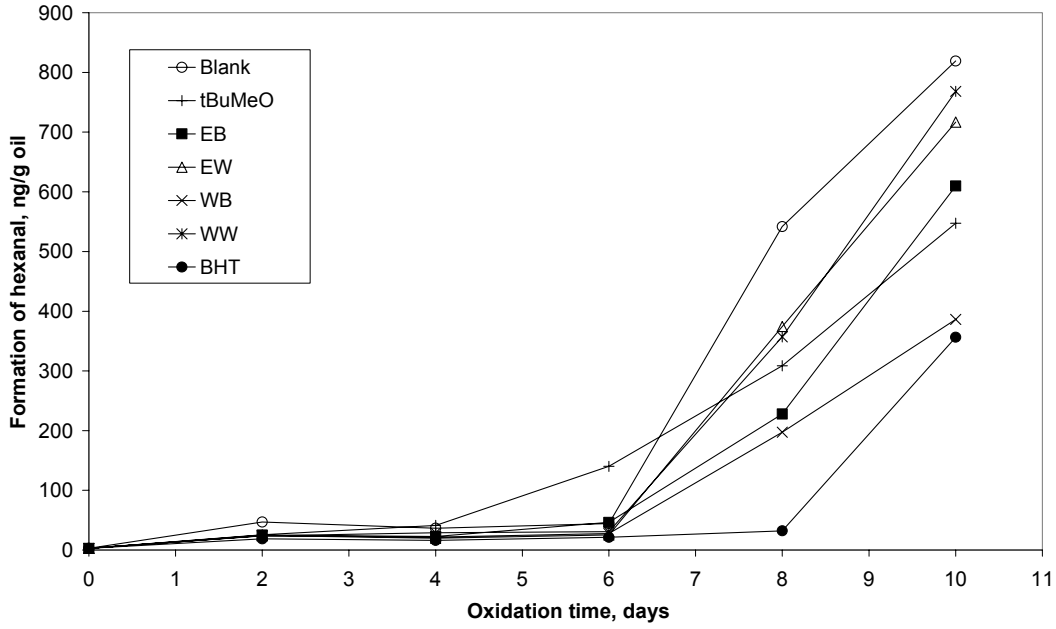


Figure 6.12 *Effect of various P. fruticosa extracts on the formation of hexanal during the oxidation of safflower oil at 55 °C.*

Practical evaluation

The induction period for most tested extracts was 6 days. The induction period of the tBuMeO extract was shorter than that of the control sample (4 days). Calculated antioxidant efficiency parameters were the same for all tested samples with added *Potentilla* extracts, as well for the control sample. Although the rate of hexanal formation for extract samples after the induction period was lower relative to the control sample, the differences were not significant and it cannot be concluded that the investigated extracts possessed some antioxidant activity in this test. The longest induction period (8 days) was shown by the sample with BHT.

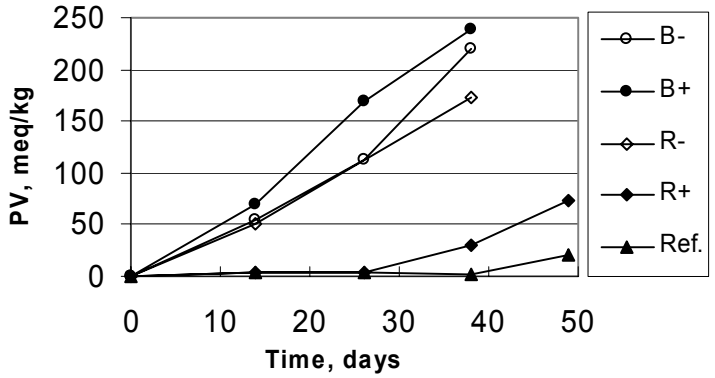
6.3.3 Evaluation of the activity of selected extracts from *G. macrorrhizum* and *P. fruticosa* during the oxidation of fermented sausages

During the storage of sausages the formation of primary (peroxides) and secondary (MDA and hexanal) oxidation products have been monitored and the influence of added extracts was determined. The pH changes for all sausage samples during the fermentation and maturation process were negligible (pH varied from 4.35 to 4.55).

6.3.3.1 Determination of peroxide value

The peroxide value changes in the fermented sausage samples with selected plant extracts from *G. macrorrhizum* and *P. fruticosa* are shown in figure 6.13 (a, b and c). These samples can be compared with the reference sausage sample, prepared with a standard mix of spices. The reference sausage sample showed the slowest formation of peroxides. Rosemary extracts (in combination with ascorbate) showed the highest antioxidant activity in this test. *Potentilla* extract (with ascorbate) also showed some activity. The *Geranium* extract was weakest and its activity was close to the blank sample. Although the amount of polyphenols in the extracts of *Potentilla* and *Geranium* is high (see chapter 2), the activity of these extracts in this test was relatively low. This suggests that more lipophilic compounds, like the terpenes from rosemary are better soluble in sausage fatty phase and act as better antioxidants in this test than the hydrophilic polyphenols from *Potentilla* and *Geranium*. It also should be noted that the amount of spices (bell pepper, pepper, clove, garlic) in the reference sausage sample prepared according to the standard recipe was more than 3 g per kg, while the amount of extracts in the samples was only 1g/kg. This could also explain the faster oxidation of these samples.

a



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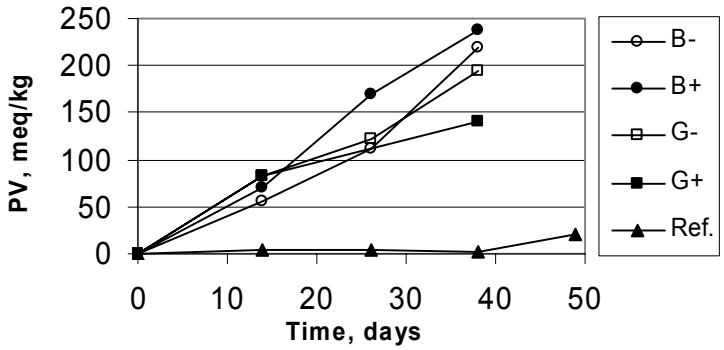

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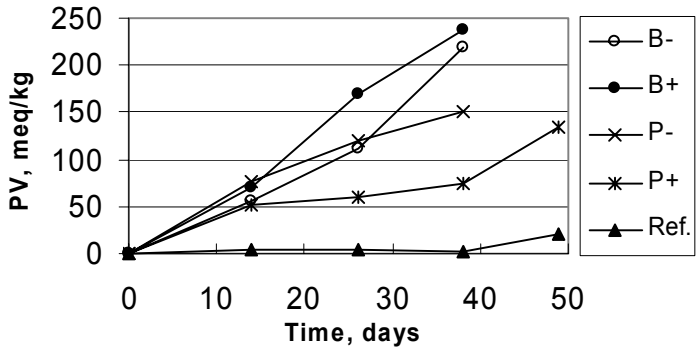


Figure 6.13 *Peroxide value changes (PV, meq /g fat) during the storage of sausages (B - blank, Ref - reference, R – rosemary, G – Geranium, P – Potentilla, "+" - samples with ascorbate, "-" samples without ascorbate).*

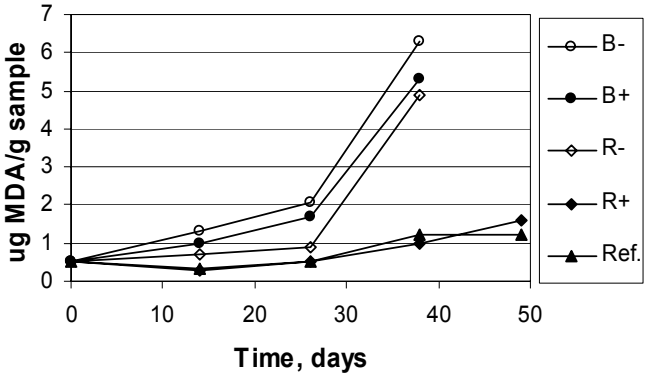
In the figures the activities of extracts without ascorbate can be seen. They were compared with blank and the reference sausage samples. In all the samples quick formation of peroxides was observed. The combination of ascorbate and extracts resulted in a higher antioxidative effect. The lowest amount of peroxides if used with ascorbate was found in the sample with rosemary extract, followed by the samples with *Potentilla* and *Geranium* extracts. However in comparison to the reference sausage sample, the activity of *Geranium* and *Potentilla* extracts was low. Curves of blank samples with and without ascorbate show that ascorbate alone has little effect on the rate of oxidation. After 40 days the peroxide values in blank samples (with and without ascorbate) were

very close, however the combination of the plant extracts and ascorbate in most cases showed a synergistic effect. It was especially distinct in the sample with rosemary.

6.3.3.2 Determination of TBARS

In this method the effect of extracts on the formation of secondary oxidation products (particularly malondialdehyde) during the storage of sausages was monitored (figure 6.14 a,b and c).

a



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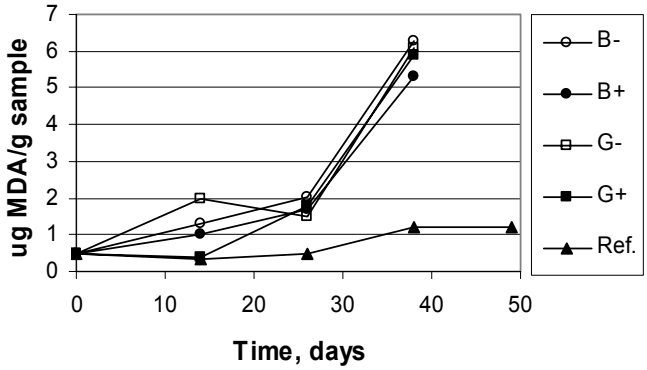

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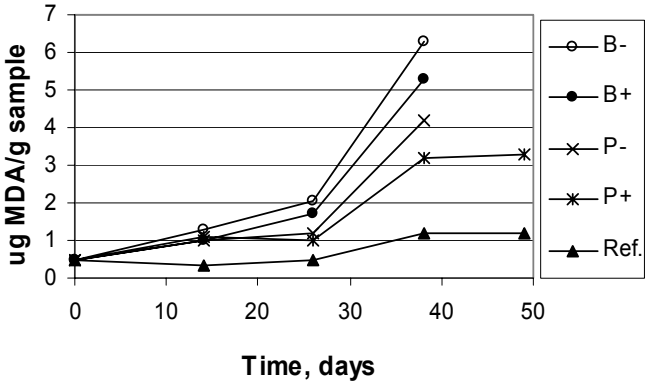


Figure 6.14 *Formation of malondialdehyde (μg per g sausage) during the oxidation process (B – blank sample, Ref - reference, R – rosemary, G – Geranium, P – Potentilla, "+" - samples with ascorbate, "-" samples without ascorbate).*

The slowest formation of MDA was observed in the reference sausage sample, followed by the rosemary and *Potentilla* samples. The activity of rosemary in a combination with ascorbate was identical to the activity of the standard spice mix in the reference sausages. *Geranium* extracts showed no activity, as the formation of MDA was close to that of the blank sample.

In this assay again clear synergistic effects between ascorbate and rosemary extract were observed. Synergistic effect with *Potentilla* was weaker. At the same time ascorbate alone did not show strong activity. *Geranium* extracts showed no activity in this assay neither with nor without ascorbate.

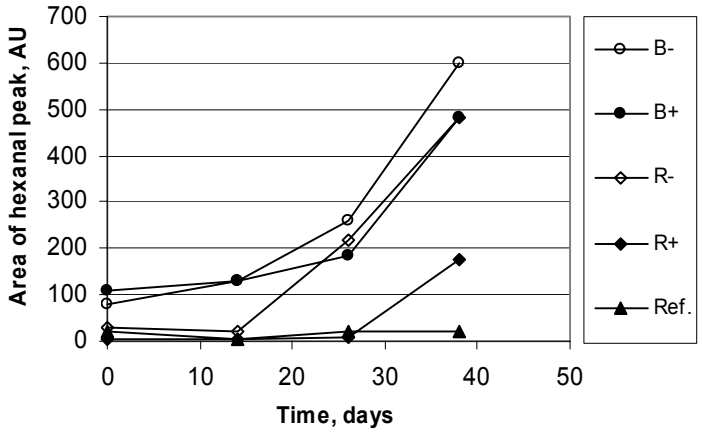
6.3.3.3 Measurement of hexanal formation

The amount of hexanal formed during the storage of fermented sausages is shown in figure 6.15 (a, b and c). The slowest formation of hexanal was in the reference sausage sample, followed by the samples with rosemary and *Potentilla* extracts. *Geranium* extracts did not retard hexanal formation. The results correlated well with the peroxide and TBARS formation results (figures 6.13 and 6.14). For rosemary and *Potentilla*, the addition of ascorbate markedly improved the activities of extracts although ascorbate itself did not show strong antioxidant properties. This again proved the synergistic effect of extracts with ascorbate. Especially distinct differences for the samples with and without ascorbate were observed for the rosemary samples, and this is in agreement with the TBARS and peroxide formation tests. No such effect was observed for the *Geranium* extract.

The synergistic effects observed between rosemary extracts and ascorbate (or weaker synergistic effect between *Potentilla* extracts and ascorbate) is most likely due the ability of ascorbate to regenerate oxidized primary antioxidants from extracts (like polyphenols or polyphenolic diterpenes from rosemary) back to the reduced state.

Generally it can be concluded that the antioxidant activity of tested extracts from *Potentilla* and *Geranium* if applied in lipophilic media is somewhat insufficient. Extracts proved to be rich in polyphenols (see chapter 2, 3 and 4), they show remarkable radical scavenging (antioxidant) activity in the tests with hydrophilic media, however their activity in test with lipophilic media (like bulk oil or fermented sausages) is low or they do not show any activity at all. Therefore it can be concluded that such extracts can only be successfully applied in more hydrophilic products or in emulsions and not in lipophilic products.

a



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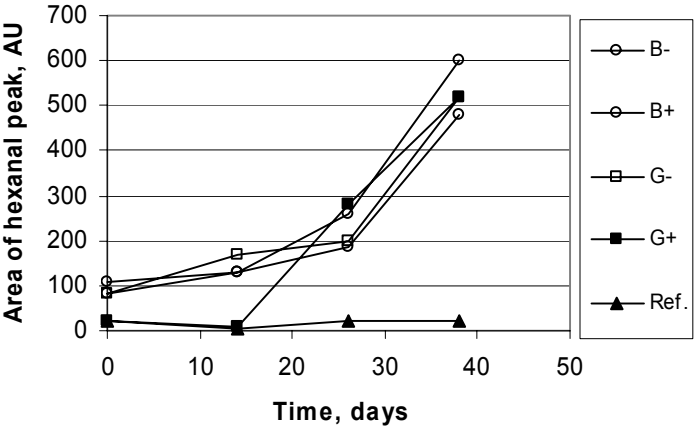

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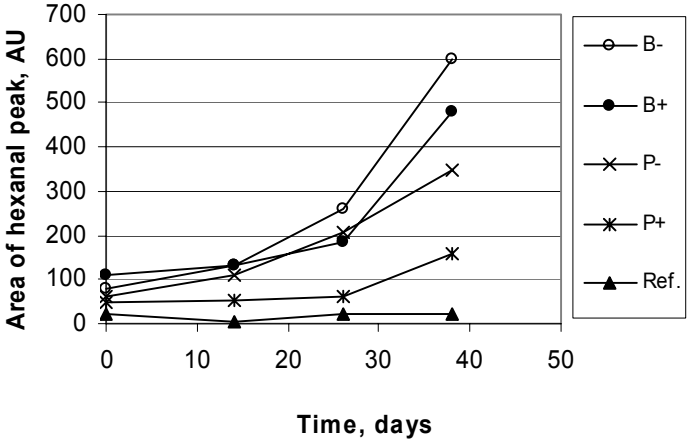


Figure 6.15 *The relative amounts of formed hexanal (expressed as the area of hexanal peak from gas chromatographic profile) during the oxidation of sausage samples (B – blank sample, Ref - reference, R – rosemary, G – Geranium, P – Potentilla, "+" - samples with ascorbate, "-" samples without ascorbate).*

A very high antioxidant capacity of the standard spices mix was found in this study. It was significantly higher than that of commercial rosemary extract with its well known antioxidant properties. Although the spices mix was used in a thrice higher concentration than the other extracts,

this finding raises some questions towards the identity of the active principles of this mix. Preliminary literature search on antioxidants from these spices revealed a broad array of constituents with antioxidant properties to be present. Gallic acid and essential oils are reported to be responsible for antioxidant activity in clove [15]. Capsaicinoids and flavonoids are reported in pepper and bell pepper, active organosulphur compounds in garlic [16, 17]. The joint effect of these antioxidants is likely to be responsible for the high antioxidant capacity of this spice mix. Further studies on this spice mix seem worthwhile.

6.3.4 Comparison of antioxidant activities between *Geranium* and *Potentilla* extracts

Although a comparison of *Geranium* and *Potentilla* extracts was not the primary objective of this study, nevertheless such a comparison is of interest from a practical point of view. After reviewing the results of all radical scavenging (antioxidant) activity evaluation assays it can be concluded that the activities of corresponding extracts and fractions from both plants were similar and it is quite difficult to discriminate between the two plants. In the superoxide anion scavenging test all fractions showed effective superoxide anion scavenging properties. In the hydrogen peroxide scavenging test the WB fraction from *Geranium* possessed higher activity relative to the other fractions. In the linoleic acid - β-carotene oxidation assay a bit lower activity of the tBuMeO and WW fractions from *Geranium* was observed. In the UV absorbance test (formation of conjugated trienes) the highest activity was demonstrated by tBuMeO extracts from both plants. In the fermented sausages oxidation assays only extracts prepared in different ways from *Geranium* and *Potentilla* can be compared. It is clear that the polar WW fraction from *Geranium* is not effective in these assays, while the less polar *Potentilla* EB fraction showed some antioxidant activity.

6.3.5 Preliminary safety evaluation of chosen plant extracts

The work of this thesis was mainly focused on the chemical aspects of finding and using plant extracts as natural antioxidants in foods. Nevertheless the possibility of a practical application of plant extracts was also considered. This follow-up research requires research not only by chemists, but also by agrotechnologists, nutritionists, biochemists and even economists.

With the help of partners and colleagues from the Institute of Biochemistry, Sector of Xenobiotics Biochemistry (Vilnius, Lithuania) and the Department of Botany and Genetics, faculty

of Natural Sciences from Vilnius University (Lithuania), preliminary genotoxicity, cytotoxicity and mutagenicity tests of the selected extracts from *Geranium macrorrhizum* and *Potentilla fruticosa* were conducted.

6.3.5.1 Enzymatic oxidation of polyphenols from plant extracts with peroxidase and tyrosinase[1]

Oxidation with peroxidase. High doses of polyphenolic antioxidants can be geno- or cytotoxic, as one of the main mechanisms of cytotoxicity is the ability of products derived from polyphenol oxidation to cause so called oxidative stress. E.g. polyphenols can act as substrates for peroxidases and other metalloenzymes, yielding quinone- or quinomethide- prooxidant and/or alkylating products [18, 19]. In mammalian cells, polyphenols can be oxidized with oxygen by tyrosinases or with H_2O_2 by peroxidases [8, 20]. These enzymes are also abundant in plant tissues, where they can cause some undesirable effects, e.g. enzymatic browning. Therefore in order to determine potential side effects of polyphenol rich plant extracts (e.g. as a possible source of food additives) it is important to examine their oxidation catalyzed by peroxidase and tyrosinase.

In order to assess the behaviour of new polyphenols in enzymatic reactions and to understand their oxidation mechanism, it is necessary to compare them with model compounds. Although the oxidation of polyphenols by peroxidases is being extensively investigated [20], structure activity relationships have not been completely established.

The major polyphenols in my extracts have been identified (see chapters 3 and 4), however the full composition of extracts is not known. The common procedure in such a case is to assess the oxidation of these complex mixtures by peroxidase by measuring the oxidation half-time ($t_{1/2}$). The oxidation $t_{1/2}$ of *Potentilla* EB, *Geranium* EB and WW fractions (50-150 μg/ml) in the presence of 3.0 nM peroxidase and 200 μM H_2O_2 in all cases was around 80 ± 15 s. By comparing the determined half-time of the mentioned extracts with reference compounds, the oxidation half time can be assessed as long (e.g. $t_{1/2}$ of the flavonoid quercetin is around 2 s [21], similar to the reference 5,8-dihydroxycoumarin used in this study), so it can be concluded that the relative reactivity of extracts against peroxidase is low.

[1] Enzymatic oxidation experiments and singlet oxygen caused erythrocyte hemolysis tests were conducted by Dr. Audronė Marozienė and Dr. Narimantas Čėnas from the Institute of Biochemistry and results are presented with their permission.

Oxidation with tyrosinase. When oxidizing extracts with tyrosinase the changes were analogous to the ones caused by peroxidase. Observed half-times of *Potentilla* EB (at 290 nm), *Geranium* EB (at 385 nm) and WW fractions (at 325 nm) in the presence of 0.6 μM tyrosinase were 100 ± 20, 108 ± 22 and ≥150 s respectively. The maximum ratio of absorbance increase was 1 : 0.6 : 0.15, i.e. it was the same as in the case with peroxidase oxidation. Therefore it can be stated that compounds from these extracts do not inactivate tyrosinase or peroxidase in the reaction process. In comparison, the oxidation half-time of the reference 5,8-dihydroxycoumarin is longer (≥ 600 s) and therefore extracts could be easier oxidized by tyrosinase than 5,8-dihydroxycoumarin. However some other well known antioxidants, like catechol, quercetin, caffeic acid, chlorogenic acid [21] are much more active (have higher catalytic constants) towards tyrosinase than 5,8-dihydroxycoumarin [22]. The activity of investigated extracts towards tyrosinase falls in the range of the above mentioned reference antioxidants and therefore it can be concluded that these extracts are non-genotoxic according to this assay.

6.3.5.2 Protective effects of plant extracts against singlet oxygen caused erythrocyte hemolysis

One of the properties of phenolic antioxidants is singlet oxygen scavenging. The ability of extracts to neutralize the cytotoxic effect of singlet oxygen and in this way to protect erythrocytes from singlet oxygen caused hemolysis has been examined.

During the irradiation of an erythrocyte suspension in the presence of aluminum phthalocyanine tetrasulfonate (AlPcS4) the formed singlet oxygen ($^1O_2$) causes lysis of erythrocytes. Polyphenols slow down this process and extend the erythrocytes lysis half-time ($t_{1/2}$) [9]. During the test the lysis half-time of erythrocytes without any additives was 55-65 min. The effect of the plant extracts was as follows: $c_{1/2}$ (concentration of extract which doubled the erythrocyte lysis half-time) of *Potentilla* EB extract fraction was 38 ± 5 μg/ml, > 300 μg/ml for *Geranium* EB fraction and >380 μg/ml for *Geranium* WW fraction. The $c_{1/2}$ of the most active flavonoids is 36 ± 4 μg/ml (morin), 45 ± 6 μg/ml (kaempferol), 60 ± 8 μg/ml (quercetin) [9]. Therefore it can be stated that the EB fraction of *Potentilla* effectively protected erythrocytes from singlet oxygen impact. *Geranium* fractions also exhibited singlet oxygen scavenging effects, but it was several times lower in comparison to *Potentilla* EB fraction or reference compounds.

6.3.5.3 Mutagenicity evaluation of plant extracts (somatic mutations and recombinations test in *Drosophila melanogaster* wing cells) [2]

Some plants can contain toxic, carcinogenic or mutagenic metabolites. Moreover a beneficial effect of useful compounds is also associated with proper dose. Not optimal doses can eliminate positive effects and be very harmful. Apart from the acute toxic effects there also are chronic effects that appear only when a product has been consumed for a certain time. This effect is typical for compounds with carcinogenic and genotoxic properties. In the literature there are a lot of examples about mutagenic properties of some plant extracts or plant derived compounds on certain cells or organisms [23-26].

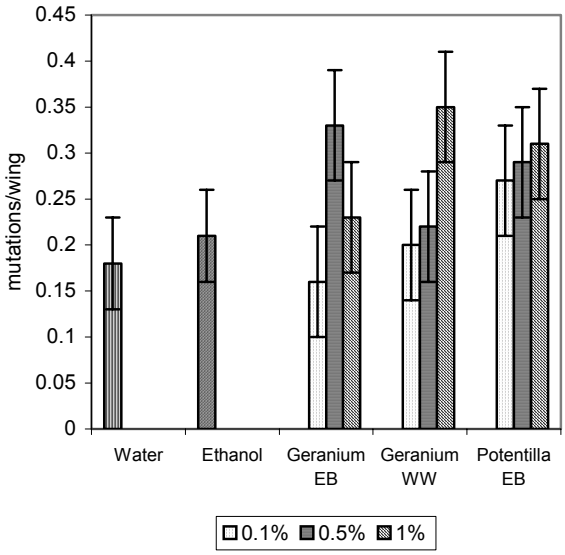
Most genotoxic investigations are performed using various bacterias or with *in vitro* assays. Results obtained during these investigations are of course valuable, but it is not always easy to extrapolate them to the human situation. Human enzymatic systems can metabolize chemical substances from the inactive to the genotoxically active (mutagenic) state or vice versa.

A *Drosophila melanogaster* somatic mutations and recombinations test is useful from the point that it allows determining mutagenic and recombinogenic activity of compounds after their metabolic activation. With this assay the genotoxicity of extracts from *Geranium macrorrhizum* (EB and WW) and *Potentilla fruticosa* (EB fraction) was determined (figure 6.16). After a 120 hour Drosophilas exposure (evolutionary period from fly egg till case-worm) to the nourishment media containing plant extracts a significant increase in the number of somatic mutations was observed only for the *Geranium* WW sample at 1 % concentration (0.35 vs. 0.18 mutations/wing, $P < 0.05$). For the other extracts no statistically significant increase was observed. In almost all cases discrete small spots (1-2 mutated cells) formed the biggest part of mutations. Only in the case of the 0.5 % *Geranium* EB fraction more twinned spots were observed. After 48 hours of exposure (72 ± 5 h age larvae were exposed) no sample induced a statistically significant increase in the amount of somatic mutations. As a positive control in somatic mutation test of *Drosophila melongaster* benzopyrene (2 mM) can be used. Under identical experimental conditions it gives in average 1 spot (mutation) per wing [24]. Therefore it can be concluded that the tested extracts from *Geranium macrorrhizum* and *Potentilla fruticosa* do not show toxic properties in the *Drosophila melanogaster in vivo* mutagenicity assay.

[2] Tests were conducted by Dr. Gražina Slapšytė, Dr. Liucija Dimanskienė and Prof. Juozas Lazutka from Vilnius University (Lithuania) and are presented here with their permission.

These were very preliminary safety test on *Geranium* and *Potentilla* extracts. Further, more comprehensive studies of course would be necessary before any practical application of these extracts. At this point it can be concluded that extracts from *Geranium macrorrhizum* and *Potentilla fruticosa* are not toxic in the above three tests and that their application in respect to their safety would be promising.

a



b

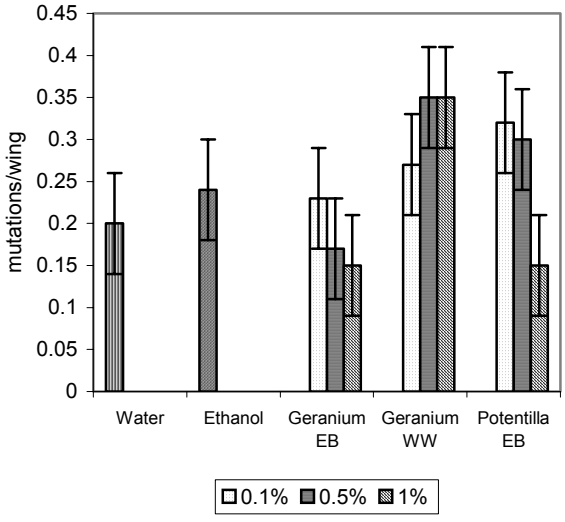


Figure 6.16 *Inductions of G. macrorrhizum and P. fruticosa extract fractions on the frequency of somatic mutations in Drosophila melanogaster wing cells; a – exposition duration 120 h; b – 48 h.*

6.4 References

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HYPHENATED ON-LINE METHOD (HPLC-DAD-MS-DPPH/ABTS) FOR DETECTION OF RADICAL SCAVENGING COMPOUNDS IN COMPLEX EXTRACTS

7.1 Introduction

During the last few decades there has been a steady progress in the development of analytical techniques for the separation of complex mixtures and the structure elucidation of the separated compounds. Analytical equipment for isolation/purification/analysis (LC, GC, CE) coupled to various detectors (UV, diode array (DAD), IR, electrochemical (ECD), light scattering, MS, NMR, chemiluminescence (CL)) [1-10] or even combinations of them have gradually replaced classical, more time- and labour-intensive procedures and have become conventional tools in many labs. Moreover miniaturization of developed techniques up to the chip scale is also an on-going process providing new possibilities and benefits [11-13].

Isolated and identified compounds are usually further investigated (e.g. determination of antioxidant properties). On-line coupling of various assays with the separation-structure elucidation set-ups is also becoming a common approach. The research on radical scavenging (antioxidants) compounds of natural origin during recent years resulted in the development of several on-line methods, like LC-UV-DPPH, LC-UV-ABTS, LC-UV-CL, LC-UV-NMR [10, 14-17] and a few others [18, 19]. Some of these methods have been developed and widely used in the Laboratory of Organic Chemistry of Wageningen University. These techniques, particularly LC-UV-DPPH and LC-UV-ABTS were used throughout my research period. A continuous aim was to improve these methods. The primary goal of this part of the study was to expand the scope of these analytical set-ups by coupling them to a mass spectrometer. This could significantly speed up the compound identification process and avoid replication of isolating already known compounds. Developing and optimization of this set-up is described below. The method was evaluated by investigating *Ginkgo biloba* and rosemary (*Rosmarinus officinalis*) extracts.

7.2 Materials and methods

Ginkgo biloba extracts (powder) were obtained from (i) Chemco Industries (Los Angeles, CA; enriched in flavonol glycosides (24 %) and ginkgolides and bilobalide (6 %)) and (ii) from Zeller

(full extract rich in biflavones; Romanshorn, Switzerland). Rosemary (*Rosmarinus officinalis*) extract (spray dried powder) was from Robertet (Grasse, France). 1% solutions were prepared by dissolving extracts in a MeOH-H_2O mixture (1:1) and filtering them.

7.2.1 On-line HPLC-DAD-MS-DPPH/ABTS analytical setups

The scheme of the HPLC-DAD-MS set-up with on-line radical scavenging detection is given in figure 7.1. The HPLC system consisted of a SpectraSYSTEM P4000 quaternary pump, SCM1000 solvent degasser**,** AS 3500 autosampling injector (all from Thermo Finnigan, San Jose, CA), Alltima C18 analytical column (5 μm, 250 mm length, 4.6 mm i.d., Alltech Associates, Deerfield, IL.). A binary gradient (vide infra) was used at a flow rate of 1.0 ml/min. Analytes were first detected with a Dionex UVD 340U Photodiode Array Detector (Dionex, Sunnyvale, CA), then the flow was split into two streams using a passive T-splitter. 0.2 ml/min was directed to a Finnigan LCQ mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with ESI or APCI probes (in positive or negative ionization modes). The remainder of the flow (0.8 ml/min) was used for on-line radical scavenging detection. A 45 ml laboratory-made syringe pump (Free University, Amsterdam, The Netherlands) was used for adding free radical solution (flow rate of the syringe pump was approximately 0.3 ml/min when pumping DPPH radical solution and approximately 0.5 ml/min when pumping ABTS radical cation solution). The reaction between separated compounds and DPPH radical took place in a 15 m length (13.7 m for ABTS assay), 0.25 mm i.d. PEEK reaction coil. The decrease in absorbance at 515 nm for DPPH and at 734 nm for ABTS, was measured with a 759A UV-Vis detector (Applied Biosystems, Foster City, CA), equipped with a tungsten lamp and connected to a BD 40 recorder (Kipp & Zonen, Delft, The Netherlands).

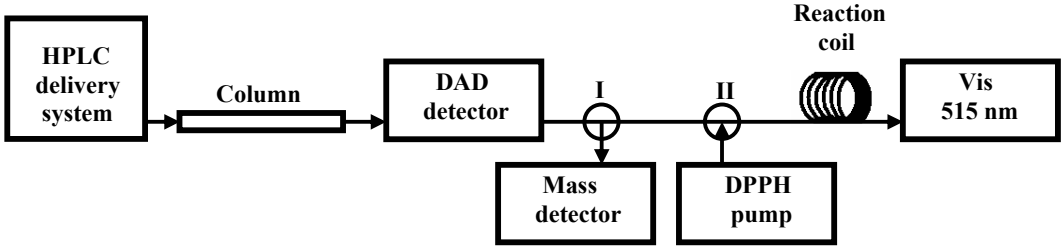


Figure 7.1 *Scheme of the experimental setup.*

Gradient conditions for *Ginkgo biloba* extracts (Chemco) were as follows (A = H_2O-MeCN-HCOOH (97.9:2:0.1), B = 100 % MeCN): 0 to 7 min B increased from 0 to 16 % and kept constant till 20 min, 20-22 min B increased to 21 % and kept constant till 30 min, 30-35 min B increased to 35% and kept constant till 39 min, 39-42 min B increased 100%, 42-45 min B decreased to 0%.

Gradient conditions for rosemary extract (A = H_2O-MeCN-HCOOH (97.9:2:0.1), B = 100 % MeCN) were as follows: 0 to 15 min B increased from 0 to 50 %, 15-50 min B increased to 100 %, 50-55 min B decreased to 0%.

7.3 Results and Discussion

7.3.1 Development of the experimental setup

On-line radical scavenging detection methods (HPLC-UV-DPPH/ABTS) have proven to be effective tools for the detecting of active compounds in complex samples. The techniques have been widely used for a quick determination of the number of active components in the investigated extract or fraction and for preliminary assessment of their relative activity. However, for the identification of active components in the majority of cases further isolation and structure elucidation steps were necessary.

The potential of MS detection for structure elucidation is significantly higher than that of UV absorbance or photodiode array detectors. MS detectors are widely used nowadays and they can be coupled to a liquid chromatographic set-up. They can be very sensitive and can provide information about the molecular weight and induced fragmentation and in many cases enable identification of compounds even if they are not well separated during the chromatographic run (MS/MS techniques). Because of the mentioned advantages, it was attempted to couple an MS detector to the on-line radical scavenging detection set-up. Detailed mass information, together with DAD data might lead to rapid identification of the separated compounds.

For coupling of the on-line LC-DAD-DPPH/ABTS set-up to the MS detector, a stable splitting of the flow is a prerequisite. One part should be directed to the mass detector and the other part to the on-line radical scavenging assay. Flow splitting can be accomplished either by using passive splitting, i.e. using a T-splitter or using an active splitting device. The main drawback of passive splitting is that the flow cannot be easily changed. Another drawback of a fixed splitter is that when the HPLC analysis is performed using gradient conditions, the solvent composition and consequently the viscosity changes during the chromatographic run and this could influence the split ratio to some extent. An active splitting device can provide a broad range of reproducible split ratios; it is unaffected by changes of the mobile phase and viscosity, tubing length and temperature changes and is not susceptible to clogging. However such a solution demands additional equipment (active splitter) and is therefore more expensive. In this study a fixed (passive) splitter was installed.

Extended on-line method

The flow rate during the chromatographic separation in the original on-line radical scavenging methods [14, 15] was approximately 0.7-0.8 ml/min. To keep this flow rate in the same range in the expanded system, the HPLC flow rate was increased to 1.0 ml/min and after splitting 0.8 ml/min was used for radical scavenging detection, while the remainder (0.2 ml/min) was directed to the MS detector. The splitting was achieved by installing PEEK tubing of the necessary length and i.d. The coil for the on-line radical scavenging activity was kept the same as before (15 m, 0.25 mm i.d. of PEEK tubing for DPPH radical scavenging assay and 13.7 m for ABTS assay). The desired split ratio (1:4) was achieved by installing 4.5 m of 0.005 inch (0.125 mm) i.d. PEEK tubing between the splitter and MS detector. The stability of the flow rates using this fixed splitter was evaluated under-both isocratic and gradient conditions using different solvents (MeOH or MeCN). The flow stability was good: the influence of eluent changes on the flow rate was less than 1%.

When analyzing phenolic or carboxylic acids, a low pH of the HPLC eluent is favourable as it improves retention by retarding dissociation of compounds. In addition a small amount of acid in the solvent improves the "ionization" process in the MS interface (in ESI probes, positive ionization mode). On the other hand when running radical scavenging assays (e.g. DPPH), the optimum pH in the reaction coil is 5.0 - 6.5 [20]. This means that either the eluent should be only slightly acidified or buffers should be added to the DPPH reagent. However the coil in these assays is relatively long, therefore inorganic buffers should be used with caution (especially with MeCN), because of possible precipitation of the buffer salts, which may clog the tubing and damage the DAD detector cell.

7.3.2 Evaluation of the hyphenated LC-DAD-MS-DPPH/ABTS methods with *Ginkgo biloba* and *Rosmarinus officinalis* extracts

Identification of radical scavengers in Ginkgo biloba extract. Two different extracts of *Ginkgo biloba* were analyzed with the above system (see Materials and Methods section for details). The chromatographic profile of a *Ginkgo* extract (by Chemco Industries) is shown in figure 7.2.

Based on the signals from the DAD and MS detector (pseudomolecular ion peaks and fragmentation) and literature [21] twelve compounds were identified (see table 7.1, figures 7.2, 7.5 and 7.6). The *Ginkgo biloba* extracts were simultaneously investigated with the on-line LC-DAD-MS-DPPH assay for identification of active radical scavengers.

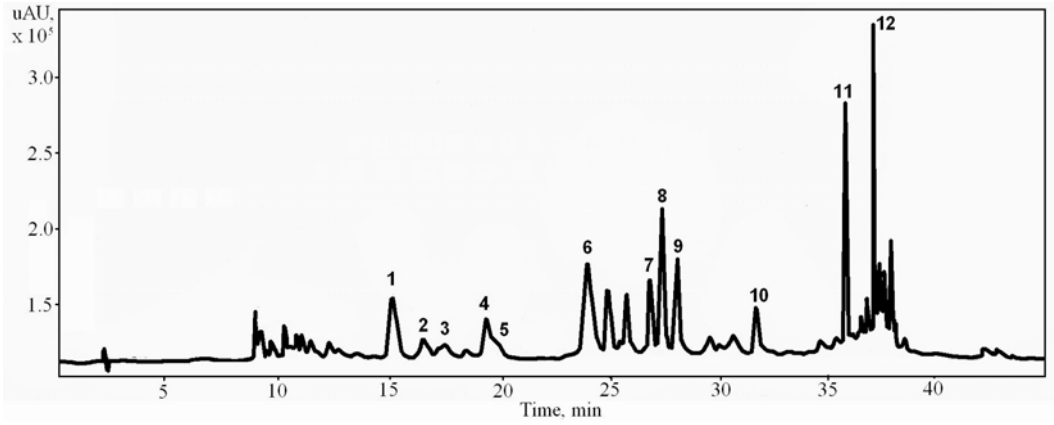


Figure 7.2 *Chromatographic profile of Ginkgo biloba extract (Chemco, λ = 350 nm). For gradient conditions see the Materials and Methods paragraph. Peaks labeled with numbers are listed in table 7.1.*

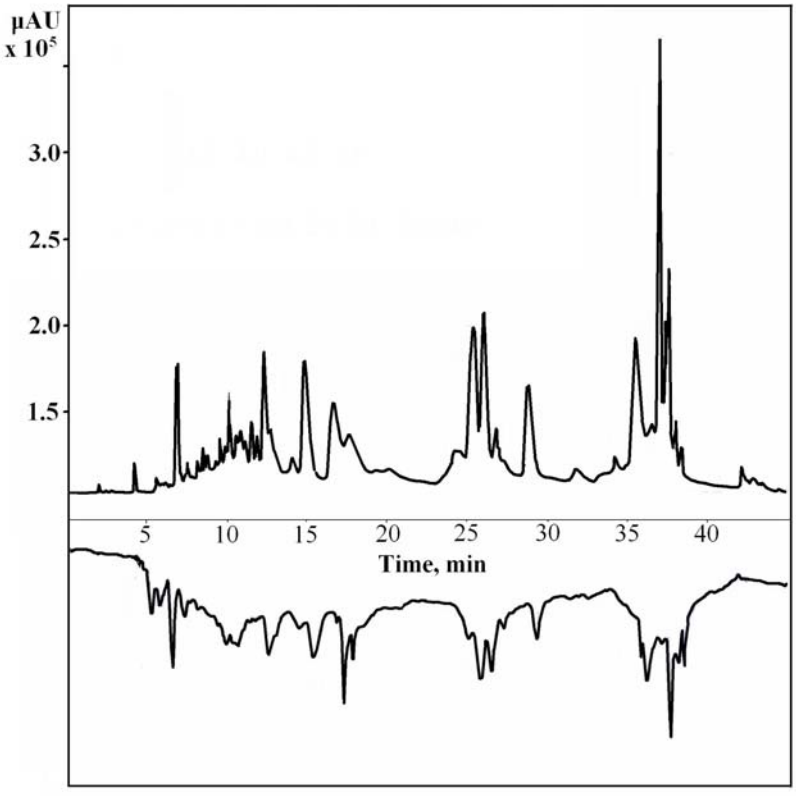


Figure 7.3 *On-line HPLC-DAD-MS-DPPH scavenging assay profile of Ginkgo biloba extract (Chemco). Upper profile - UV signal at 350 nm, lower profile DPPH• reduction signal (515 nm). Gradient conditions (only without using acid) were the same as for the run depicted in figure 7.2).*

As the eluent acidity negatively affected on-line DPPH assay and use of strong buffers may cause clogging of tubing, no acid in the eluents was used while performing the on-line assay. Other separation conditions were identical to those described in the materials and methods paragraph. The profile of the chromatographic run with radical scavenging detection is shown in figure 7.3.

Extended on-line method

1 **4**

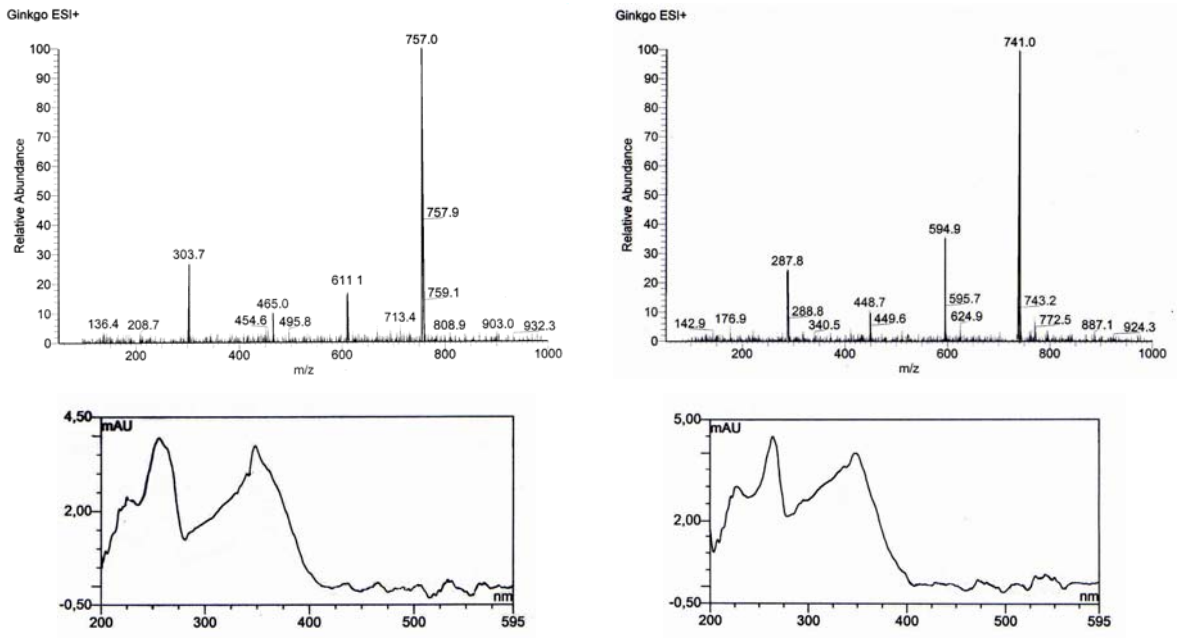
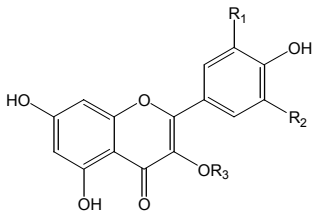


Figure 7.4 *ESI-MS (in positive mode) and UV spectra of 3-O-[2-O,6-O-bis(α-L-rhamnosyl)-β-D-glucosyl] quercetin (**1**) and 3-O-[2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl] kaempferol (**4**).*



1-12

	Compound name	R_1	R_2	R_3
1	3-O-[2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl]quercetin	OH	H	2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl
2	3-O-[6-O-(α-L-rhamnosyl)-β-D-glucosyl]myricetin	OH	OH	6-O-(α-L-rhamnosyl)-β-D-glucosyl
3	3-O-[2-O-(6-O-{*p*-[β-D-glucosyl]coumaroyl)-β-D-glucosyl)-α-L-rhamnosyl]quercetin	OH	H	2-O-(6-O-{*p*-[β-D-glucosyl]coumaroyl)-β-D-glucosyl)-α-L-rhamnosyl
4	3-O-[2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl]kaempferol	H	H	2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl
5	3-O-[2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl]isorhamnetin	OCH_3	H	2-O, 6-O-bis(α-L-rhamnosyl)-β-D-glucosyl
6	3-O-[6-O-(α-L-rhamnosyl)-β-D-glucosyl]quercetin	OH	H	6-O-(α-L-rhamnosyl)-β-D-glucosyl
7	3-O-[2-O-(β-D-glucosyl)-α-L-rhamnosyl]quercetin	OH	H	2-O-(β-D-glucosyl)-α-L-rhamnosyl
8	3-O-[6-O-(α-L-rhamnosyl)-β-D-glucosyl]kaempferol	H	H	6-O-(α-L-rhamnosyl)-β-D-glucosyl
9	3-O-[6-O-(α-L-rhamnosyl)-β-D-glucosyl]isorhamnetin	OCH_3	H	6-O-(α-L-rhamnosyl)-β-D-glucosyl
10	3-O-[2-O-(β-D-glucosyl)-α-L-rhamnosyl]kaempferol	H	H	2-O-(β-D-glucosyl)-α-L-rhamnosyl
11	3-O-[2-O-(6-O-{*p*-coumaroyl-β-D-glucosyl)-α-L-rhamnosyl]quercetin	OH	H	2-O-(6-O-{*p*-coumaroyl-β-D-glucosyl)-α-L-rhamnosyl
12	3-O-[2-O-(6-O-{*p*-coumaroyl}-β-D-glucosyl)-α-L-rhamnosyl]kaempferol	H	H	2-O-(6-O-{*p*-coumaroyl}-β-D-glucosyl)-α-L-rhamnosyl

Figure 7.5 *Flavonol glycosides **1-12** identified in the extracts of Ginkgo biloba.*

The separation profile (peak widths and elution times) without using acid is different. However based on the data from the MS detector it was relatively easy to identify the same major compounds. Moreover according to the lower chromatographic profile (radical scavenging assay) it was observed that many of the *Ginkgo biloba* compounds possess radical scavenging activity. Glycosides of kaempferol and quercetin, including those with coumaroyl moieties, were strongly contributing to this effect [22, 23]. The ESI-MS and UV spectra of bi-rhamnosyl-glycosides of quercetin and kaempferol (compounds **1** and **4)** can be seen in figure 7.4.

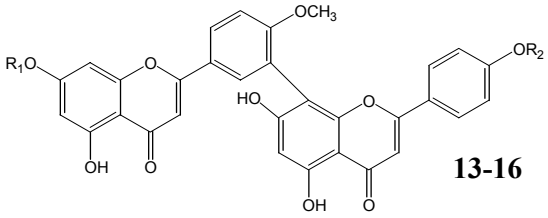
Table 7.1. *The flavonoids and biflavones identified in extracts of Ginkgo biloba, their molecular ions, major fragments and UV maxima*

Peak	R_t, min	Compound	$[M+H]^+$ *m/z*	Fragments *m/z*	UV λ_{max}, nm
1	15.3	3-O-[2-O, 6-O-bis(*α*-L-rhamnosyl)-*β*-D-glucosyl]quercetin	757.0	303.7; 465.0; 611.1	255; 349
2	16.7	3-O-[6-O-(*α*-L-rhamnosyl)-*β*-D-glucosyl]myricetin	626.9	319.7; 480.7	256; 349
3	17.7	3-O-[2-O-(6-O-{*p*-[*β*-D-glucosyl]coumaroyl)-*β*-D-glucosyl)-*α*-L-rhamnosyl]quercetin	918.9	303.8	270; 320
4	19.5	3-O-[2-O, 6-O-bis(*α*-L-rhamnosyl)-*β*-D-glucosyl]kaempferol	741.0	287.8; 448.7; 549.9	265; 347
5	20.1	3-O-[2-O, 6-O-bis(*α*-L-rhamnosyl)-*β*-D-glucosyl]isorhamnetin	770.9	317.2; 625.1	265; 347
6	24.3	3-O-[6-O-(*α*-L-rhamnosyl)-*β*-D-glucosyl]quercetin	611.1	303.9; 465.1	256; 349
7	27.0	3-O-[2-O-(*β*-D-glucosyl)-*α*-L-rhamnosyl]quercetin	610.8	303.9	255; 347
8	27.4	3-O-[6-O-(*α*-L-rhamnosyl)-*β*-D-glucosyl]kaempferol	594.8	287.7	265; 347
9	28.2	3-O-[6-O-(*α*-L-rhamnosyl)-*β*-D-glucosyl]isorhamnetin	624.9	317.0	254; 348
10	31.8	3-O-[2-O-(*β*-D-glucosyl)-*α*-L-rhamnosyl]kaempferol	594.9	287.3; 466.3	264; 346
11	35.8	3-O-[2-O-(6-O-{*p*-coumaroyl-*β*-D-glucosyl)-*α*-L-rhamnosyl]quercetin	756.9	303.9; 449.2	266; 313
12	37.1	3-O-[2-O-(6-O-{*p*-coumaroyl}-*β*-D-glucosyl)-*α*-L-rhamnosyl]kaempferol	740.9	287.3	266; 313
13*	45.6	Bilobetin	553.1		267; 322; 346
14*	49.8	Ginkgetin	567.1		268; 330; 347
15*	50.2	Isoginkgetin	567.1		269; 329; 347
16*	53.4	Sciadopitysin	581.1		270; 323; 346

* -were only identified in the *Zeller* extract of *Ginkgo biloba*.

Collision induced dissociation of the pseudomolecular ion shows fragments at *m/z* 611.1 and 465.0 for compound **1** and at *m/z* 594.9 and 448.7 for compound **2** (see figure 7.4). This is indicative

of fragments lacking one or both of rhamnosyl moieties. After loss of the glycosyl moieties, ions corresponding to the protonated aglycones of quercetin (*m/z* 303.7) and kaempferol (*m/z* 287.8) can be observed.



13-16

Peak	Compound name	R_1	R_2
13	Bilobetin	H	H
14	Ginkgetin	Me	H
15	Isoginkgetin	H	Me
16	Sciadopitysin	Me	Me

Figure 7.6 *Biflavones* ***13-16*** *identified in the extracts of Ginkgo biloba.*

The other *Ginkgo biloba* extract (rich in biflavones extract from Zeller) was investigated too. The chromatographic profile of this extract was similar to the one described above (chromatogram is not shown). Additionally four biflavones - bilobetin, ginkgetin, isoginkgetin and sciadopitysin (see figure 7.6 for structures) were identified in this extract. They were more retained than the flavonoid glycosides and they also possessed radical scavenging properties.

Identification of radical scavengers in Rosemary extract

By using the same set-up and based on the data from MS and DAD detectors and literature [17] four compounds (epiisorosmanol, carnosol, carnosic acid, methoxycarnosic acid) were identified in the extract of rosemary (see figure 7.7 for the chromatographic profile and figure 7.8 for structures). All four compounds according to corresponding negative peaks in the radical cation scavenging chromatograms possessed radical scavenging properties, however the activities while determined in the $ABTS^{+\bullet}$ scavenging assay were quite different from those determined in the $DPPH^{\bullet}$ assay. From

the $ABTS^{\bullet+}$ scavenging assay (first lower chromatographic profile in figure 7.7) one could say that carnosol **2** is the compound contributing most to the radical scavenging activity of the extract.

12-Methoxycarnosic acid **4**, co-eluting with some other compound also shows strong radical scavenging activity. However in the on-line $DPPH^{\bullet}$ scavenging assay (second lower chromatographic profile) carnosic acid **3** was by far the most active compound, while activity of 12-methoxycarnosic acid was negligible. Similar results (in the on-line $DPPH^{\bullet}$ scavenging assay) were also demonstrated in the work of Pukalskas *et al.* [17]. Thus the kinetics of rosemary constituents towards these two radical species is different.

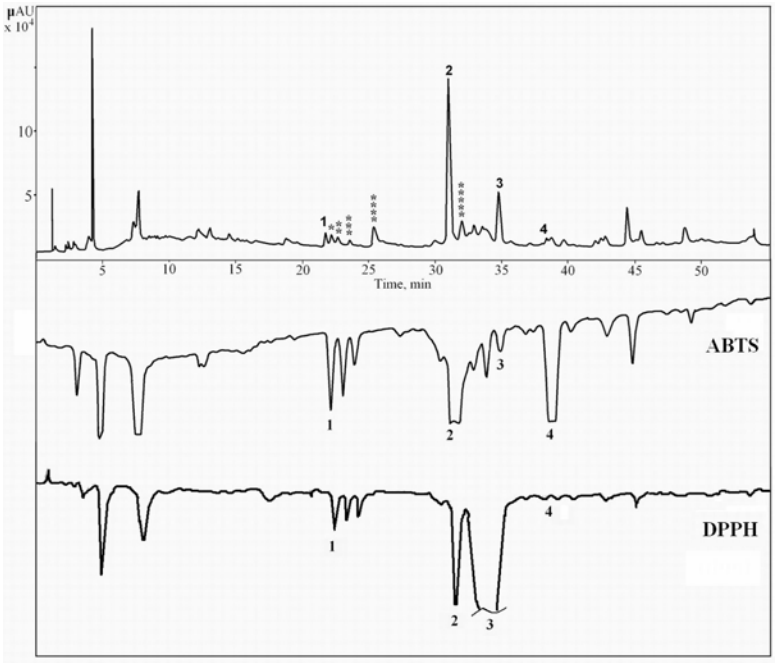


***Figure** 7.7 On-line HPLC-DAD-MS-ABTS$^{+\bullet}$/DPPH$^{\bullet}$ profile of rosemary extract. Upper profile UV signal at 254 nm, first lower profile ABTS$^{+\bullet}$ reduction signal, second lower profile DPPH$^{\bullet}$ reduction signal. Compounds labeled with "*" were non-identified.*

***Table 7.2.** The molecular ions and major fragments of detected rosemary compounds.*

Peak	R_t, min	Compound	$[M-H]^-$	$[fragments-H]^-$
1	21.6	Epiisorosmanol	345.0	301.4; 283.3
*	22.1	? isorosmanol	345.0	301.4; 283.8
**	22.6	? epirosmanol	345.0	283.3
***	23.5	? rosmanol	345.0	283.3
****	25.8	?	345.1	301.6; 283.1
2	31.0	Carnosol	329.0	285.1
*****	31.9	?	331.1	
3	34.7	Carnosic acid	331.1	289.3
4	38.7	12-Methoxycarnosic acid	345.1	301.1

The MS data indicated that four active compounds (at approx. 21-24 min) belonged to epiisorosmanol or related structures (rosmanol, epirosmanol, isorosmanol), as all of them showed similar UV spectra and had identical masses ($[M-H]^-$ = 345.0). According to the literature [17, 24] the first eluting compound was identified as epiisorosmanol. It was difficult to discriminate which of the following ones could be rosmanol, epirosmanol or isorosmanol. A non-identified compound eluting at 25.8 min had the same mass as the rosmanol derivatives ($[M-H]^-$ = *m/z* 345.0), however this compound did not show radical scavenging activity. The pseudomolecular ion peaks and the most distinct fragment ion peaks of identified as well of non-identified compounds are listed in table 7.2. Fragments at *m/z* 301.4 for **1**, *m/z* 285 for **2**, and *m/z* 301.1 for **4**, were caused by loss of carbon dioxide.

Extended on-line method

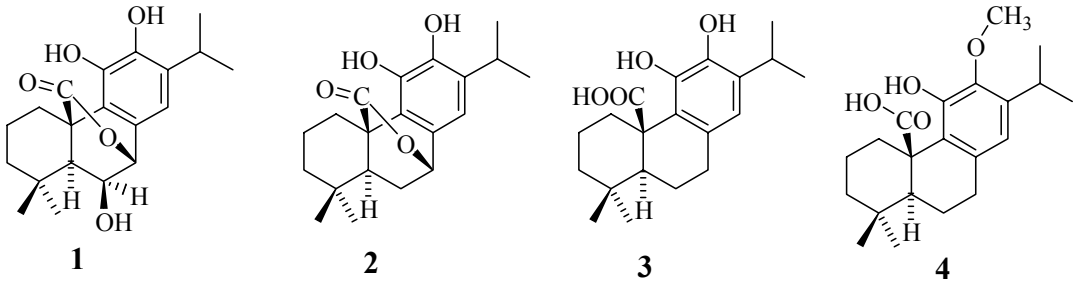


Figure 7.8 *Phenolic diterpenes identified in rosemary extracts:* ***1*** *– epiisorosmanol,* ***2*** *– carnosol,* ***3*** *- carnosic acid,* ***4*** *- 12-methoxycarnosic acid.*

Using a fixed splitter, coupling of an MS detector to an on-line LC-DAD-DPPH/ABTS radical scavenging set-up is possible. The hyphenated system enabled a rapid analysis (isolation-identification and detection of radical scavenging activity) of complex Ginkgo and Rosemary extracts. Mass and diode array detectors proved to be effective tools for identification of several major compounds present in these extracts. With only a UV detector this would not have been possible. The hyphenated method accelerates the compound identification process and helps to avoid the replication of isolation and identification of already known structures.

7.4 References

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A COMPARISON BETWEEN ANALYTICAL AND SEMI-PREPARATIVE COLUMNS FOR LC-SPE-NMR*

8.1 Introduction

Often at some stage in natural products research a structure elucidation step is necessary. Usually this involves UV, IR, NMR and MS. Although in comparison to UV or MS, NMR is a relatively insensitive technique, on the other hand its advantages for precise structure elucidation in many cases are impossible to replace. NMR is universal and non-destructive, and yields detailed structural information including the stereochemistry around individual carbon atoms. In recent years there has been a steady progress in the development of NMR spectrometers and machines with more than 20 T field strength are now available on the market. As the sensitivity increases with the field strength to the power 7/4, this has significantly increased the sensitivity. Other approaches for improving NMR sensitivity include the use of cryogenic probes [1-3] which increased the sensitivity with a factor 4.

Similar to LC-UV or LC-MS hyphenation, NMR spectrometers can also be coupled on-line with liquid chromatography. During the last decade LC-NMR has gradually evolved to a mature technique and nowadays it is important for analysing natural products, drug metabolites and in combinatorial chemistry [3-6]. Avoiding laborious and time-consuming isolation and purification steps is the main advantage of LC-NMR and leads to a rapid high-throughput screening. Additional advantages include less chance of decomposition of sensitive products and introduction of impurities, and an increase in sensitivity. When sample size is a limiting factor capillary LC-NMR is a good choice [7, 8]. However, in its traditional form (i.e. on-flow, stop-flow or loop-storage LC-NMR) this technique also has distinct disadvantages. The necessity to use deuterated solvents in the separation step makes every run expensive. Additionally, on-flow LC-NMR is very insensitive and stop-flow LC-NMR negatively affects the separation.

Development and application of the NMR in direct combination with on-line solid-phase extraction (HPLC-SPE-NMR) is a new approach aiming at eliminating some of the above mentioned problems associated with LC-NMR [3, 9]. The SPE interface between HPLC and NMR

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permits the use of regular protonated solvents during the reversed-phase separation. After that water is added to the HPLC eluent to lower its elution strength. The analytes are temporarily "parked" on small hydrophobic SPE cartridges, the non-deuterated solvents are evaporated and then subsequently a 100 % deuterated solvent is used to transfer the pure analyte to the NMR probe. Therefore, the need for solvent suppression techniques is reduced and a larger range of the NMR spectrum is available for interpretation. The advantages of the new SPE form of LC-NMR has already been demonstrated in several publications [10-14] and holds considerable promise for future research applications [15, 16]

Regardless of the advantages of HPLC-SPE-NMR, hyphenated two dimensional (2D) experiments (like heteronuclear multiple bond correlation—HMBC), which are highly important for elucidation of structures of novel compounds, still require substantial amounts of analytes, e.g. ≈ 1 mg for 400MHz NMR and 120 μL measuring volume. If the compound is a major constituent of a mixture, the total injected amount of this mixture on the HPLC column should be in the range of a few milligrams. Normally an analytical HPLC column cannot handle such large amounts. The multiple peak trapping option of HPLC-SPE-NMR system provides the possibility to overcome this problem to some extent [3]. The compound of interest can be concentrated in repeated runs before the detection and then, if necessary, 2D NMR experiments can be performed. However, it is technically more complicated and takes more time. Also the volume of the HPLC-NMR probe should match the SPE elution volume and the larger amount of analyte should still be soluble. Nevertheless, if multiple peak trapping works, the decrease in NMR measurement time far outweighs the additional separation time.

As an alternative for multiple peak trapping on a 4.6-mm diameter analytical column, it should - in theory - be possible to perform one run on a semi-preparative HPLC column, i.e. with a diameter between 7–10 mm. The total peak volume for one analyte should be approximately the same for four runs on a 4.6-mm column and one run on a 9.2-mm column with a four times higher injection volume. In fact, as long as there is no volume breakthrough and all analytes are focussed on the SPE cartridge the peak volume plays no role at all. Volume breakthrough is expected for more polar compounds that elute already at low percentages of organic modifier from the HPLC column. The effect of diluting the HPLC eluent with two parts of water to lower its elution strength is much more effective at 60% acetonitrile (20% acetonitrile after dilution) than at 15% acetonitrile (5% after dilution) as the capacity factor increases about a factor 3 for every 10% of organic modifier being less present. For very polar compounds, adding water will even work counter-productive once the increase in volume due to dilution outweighs the small effect of lowering the elution strength. The

successful approach of using semi-preparative columns for HPLC-SPE-NMR is demonstrated by Xu and Alexander [17]. They implemented an on-line device (high capacity retention and mixing system) to isolate, dilute and deliver the analytes of interest to an SPE column. However, again this approach is technically more complicated and demands substantial investments.

In this chapter, the separation and loadability of various model compounds on analytical and semi-preparative columns are compared and the maximum focussing on various SPE cartridges is determined. Based on these results some general recommendations for when to use and not use semi-preparative columns (or multiple peak trapping on analytical columns) as a function of the polarity of analytes are made.

8.2 Materials and Methods

8.2.1 Reagents

All solvents used were of HPLC grade (LabScan Analytical Sciences Ltd., Dublin, Ireland). Formic acid (98-100 %) was from Merck, Darmstadt, Germany. Ultra pure H_2O was prepared in a combined Seradest LFM 20 and Seralpur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). CD_3OD (99.5 %, Acros Organics, Geel, Belgium) or CD_3CN (Sigma-Aldrich, St. Louis, USA) were used for NMR analysis.

Reference compounds. Gallic acid monohydrate, caffeine, d(–)-amygdalin and sinigrin monohydrate were from Janssen Chimica (Beerse, Belgium). Santonin was from ICN Biomedicals (Aurora, USA), methyl salicylate from Roth (Karlsruhe, Germany), rutin and quercetin from Sigma (Deisenhofen, Germany) and geranic acid from Fluka (Buchs, Switzerland).

Two mixtures with compounds of different polarity were prepared. The first mixture (I) included compounds of high to middle polarity: sinigrin, gallic acid, amygdalin and caffeine, while the second (II) included middle polar to non-polar compounds: rutin, santonin, quercetin, methyl salicylate and geranic acid. Octanol/water partition coefficients (K_{OW}) for compounds were estimated using KOWWIN software [18], and some values were available from the literature [19]. The log K_{OW} (= log P) values are as follows: amygdalin (–4.34), sinigrin (–4.18), rutin (–2.02), caffeine (–0.07), gallic acid (0.70), quercetin (1.48), santonin (1.78), methyl salicylate (2.55) and geranic acid (3.70).

A 1% (w/v) stock solution of each compound was prepared. Compounds for mixture I were dissolved in MeOH - H_2O (2:8) and compounds for mixture II in MeOH - H_2O (5:5). All solutions

were filtered through Spartan 13/20 H.H μm filters (pore size 0.2 μm, Schleicher & Schuell, Dassel, Germany) and equal volumes of the stock solutions were mixed to give I and II.

A commercially available extract of rosemary (powder, Robertet, Grasse, France) was used for real extract analysis. A 1% (w/v) extract solution was prepared by dissolving extract in MeOH–H_2O (5:5) and filtering it. The Tagetes oil was steam distilled locally in Nepal.

8.2.2 LC-DAD-SPE-NMR instrumental setup

The scheme of the experimental set-up is presented in figure 8.1. The HPLC consisted of the following parts: Bruker HPLC pump (LC 22), LC 225 gradient former, Bruker diode array detection (DAD) system (all from Bruker, Rheinstetten, Germany). Analytes were injected with a Rheodyne manual injector. An XTerra column (semi-preparative, MS C18, 100 mm × 7.8 mm and analytical 100 × 4.6 mm, both 5 μm, Waters, Milford, MA, USA) were used for the separation. Binary gradients at 0.5 ml/min were used for both columns. Solvent A was 10% MeOH in water acidified with 0.1% formic acid; B was MeOH with 0.1% of formic acid. The gradient conditions for analysis of compounds on the columns were as follows: 0 - 30 min (for the 4.6 mm I.D. column) and 0 - 90 min (for the 7.8 mm I.D. column) B increased from 0 to 100 %. The gradient conditions for analysis of extract on the 4.6 mm I.D. column were: 0 - 10 min B increased from 0 to 50 %, 15 - 50 min up to 100 %; on 7.8 mm I.D. column: 0 - 30 min B increased from 0 to 50 %, 30 - 150 min to 100%. Tagetes oil was separated isocratically with MeCN - H_2O (7:3).

After the separation, an additional 'make-up' pump (Knauer D-14162, Berlin, Germany) was used to add extra water (acidified with 0.1% formic acid, 1 mL/min) to the eluent in order to reduce its eluting power and to increase retention of analytes. The in-line pulse dampener (toroid mixer) connected to the make-up water pump was from Scientific Systems (State College, PA). Peaks of interest were trapped with an SPE unit (Prospekt 2, Spark Holland, Emmen, The Netherlands) according to the UV signal. For the trapping the following 10 × 2 mm cartridges were used: Hysphere Resin SH (particle size 15-25 μm), Resin GP (5-15 μm), C18 (8 μm), C8 (8 μm), C2 (8 μm, all from Spark Holland, Emmen, The Netherlands) and Oasis MCX, dual mode ion-exchange/reversed phased cartridges (Waters, Milford, MA). The efficiency of the cartridges to trap the injected compounds was additionally monitored by installing a second UV detector after the SPE unit ('breakthrough' monitoring). SPE cartridges were dried with a nitrogen flow (25 mL/min) and afterwards the compounds were transferred to a Bruker 4 mm inverse $^{1}H/^{13}C$ pulse-field gradient

flow probe operating at 400 MHz (total volume 240 µL, actual detection volume 120 µl) using 500 µL of CD_3OD (or CD_3CN). The signal to noise ratio (S/N) after 8 scans in NMR measurements was determined using XWIN-NMR 2.6 software (Bruker). After the NMR measurements compounds from plant extracts were collected for ESI-MS infusion.

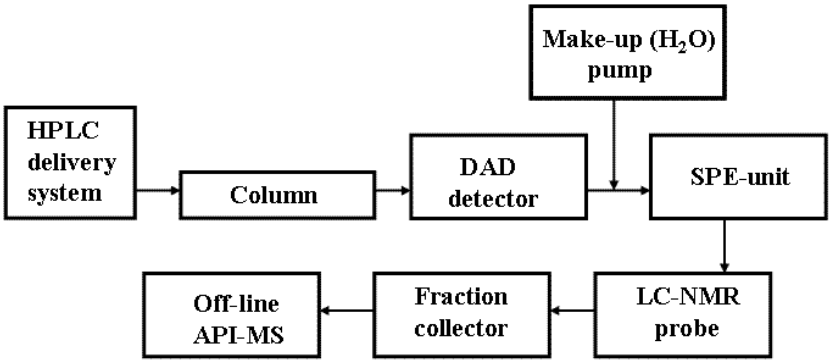


Figure 8.1 *Scheme of LC-DAD-SPE-NMR set-up.*

8.2.3 Efficiency testing

For testing the separation efficiency of the columns a mixture of four reference compounds (acetone, phenol, *p*-cresol and anisole, all from Sigma-Aldrich) was used. Column efficiencies at different flow rates have been compared by determining the theoretical plate height (HETP) dependency on the eluent flow rate (F) [HETP = *f* (F)].

HETP (in mm) = L/N,
where L – column length (in mm); N – plate number.

$$N = 5.54 \left(\frac{t_R}{w_{1/2}} \right)^2$$

where t_R – peak retention time in min; $w_{1/2}$ – peak width at half height in min (was calculated using chromatographic software HyStar 2.3 from Bruker, Rheinstetten, Germany).

8.3 Results and discussion

The general aim of this work was to find out whether – under certain conditions – a semi-preparative column could have an advantage over an analytical column in combination with an HPLC-SPE-NMR set-up. A secondary aim was to be able to predict *a priori* for which analytes a semi-preparative column would be advantageous. Initially the optimal conditions for both columns

within certain constraints had to be determined. One clear constraint was that the maximum flow rate through an SPE trapping cartridge is approximately 1.5 mL/min. Higher flows lead to back-pressure problems. According to the guidelines of the equipment supplier best results for trapping are achieved when the water make-up flow rate is 4 fold higher than the HPLC flow, e.g. 0.3 mL/min HPLC flow and 1.2 mL/min water make-up flow. For reversed phase liquid chromatography, water is the weakest eluent, therefore the higher the percentage of water in the eluent, the more an analyte is retained on the hydrophobic stationary phase. However in my previous work (see chapter 5) with analytical columns good results were obtained with a water make-up flow of 1 mL/min and an HPLC flow of 0.5 mL/min. This eluent flow rate is fixed for both the analytical (4.6 mm) and the semi-preparative column (7-10 mm) and for semi-preparative column leads to longer separation times. Normal flow rates for these columns are 1.0 and 4.0 mL/min respectively. However the long separation times on the semi-preparative column are easily compensated by the possibility of performing only one HPLC run and much shorter measuring times on the NMR spectrometer.

Column efficiency parameter HETP is dependent on the flow rate and such dependency curves were experimentally determined for both columns. The selected RP-C18 columns were 100 x 4.6 mm and 100 x 7.8 mm filled with an identical 5 μm stationary phase. A 7.8 mm I.D. is a compromise between separation time and loadability. The HETP dependency on the flow rate plot for the 7.8 mm column is depicted in figure 8.2. The optimal flow rate is around 0.8 mL/min but the HETP at the chosen flow rate of 0.5 mL/min is certainly acceptable and considerably better than the HETP at the standard flow rate of 4 mL/min for this column. For the analytical column an optimal flow of 0.1 mL/min was determined with a corresponding optimal HETP of 0.03 mm, i.e. similar to that of the 7.8 mm column. Thus the maximum performance of both columns is similar and the low flow rate of 0.5 mL/min does not lead to adverse effects for either of the columns from a chromatographic point of view.

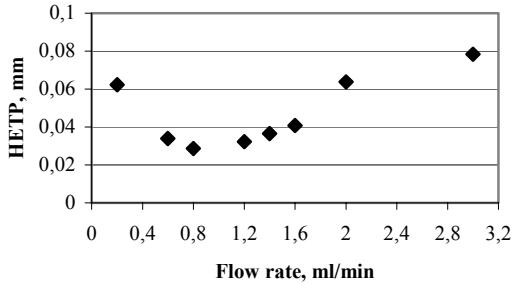


Figure 8.2 *Theoretical plate height (HETP) dependency on the eluent flow rate using a semi-preparative column.*

Before comparing both columns under actual HPLC-NMR conditions with real samples, a number of parameters related to the trapping processes were determined or optimised:

1. choice of stationary phase in SPE cartridges;
2. maximum load in mg of compound per SPE cartridge;
3. influence of the amount organic modifier in eluent on breakthrough;
4. SPE cartridge drying time.

For this purpose two sets of model compounds were analysed. Mixture (I) contained more polar compounds (sinigrin, gallic acid, amygdalin and caffeine) and test mixture (II) more non-polar compounds (rutin, santonin, quercetin, methyl salicylate and geranic acid). The variety of cartridges that can be found on the market is broad, and new types, including various mixed-mode cartridges, are released continuously by different manufacturers. This variety of the cartridges and their properties is determined by the different application fields and compounds of interest. The chosen approach to find the most suitable cartridges for my set-up was to determine the breakthrough volume, i.e. the volume that causes a compound to elute from the cartridge. The HPLC flow rate was fixed at 0.5 mL/min, the eluent was H_2O - MeOH (7:3), acidified with 0.1 % formic acid, the flow rate of the make-up water pump (also containing 0.1% formic acid) was 1 mL/min and no HPLC column was used in this experiment. An extra UV detector was installed after the SPE unit to monitor any breakthrough. The higher the breakthrough volume, the better the trapping ability of the cartridge. SPE cartridges that have been evaluated were: SH, GP, C18, C8, C2 and Oasis MCX mixed-mode cartridges. The results are presented in table 8.1. The most effective cartridge was the SH cartridge (SH = strongly hydrophobic). These modified polystyrene-divinylbenzene based resin cartridges most strongly absorbed non-polar as well polar compounds with the exception of sinigrin, and were selected for further applications. Sinigrin is hardly retained by any cartridge. Amygdalin and sinigrin are by far the most polar compounds of those tested and are difficult to retain by van der Waals interactions. A bit unexpected was the low retention of the intermediately polar gallic acid. Probably 0.1% formic acid is insufficient to suppress the ionisation of this compound causing poor retention. For carboxylic acids 0.1% trifluoroacetic acid as modifier is probably a better choice. Surprisingly rutin was more retained than caffeine even though the latter compound is the less polar one according to their log P values. A possible explanation is the strong interaction of the aromatic rings of rutin with the aromatic rings present in the SH stationary phase. This shows that although the log P value is a useful parameter to predict to some extent the retention of analytes, compound

specific interactions with the stationary phase do play a role and such effects can change the order of retention expected on the basis of log P values.

Table 8.1 *Breakthrough volumes of the different cartridges for the 9 model compounds in combination with H_2O – MeOH with 0.1% FA (9:1).*

Compound	Breakthrough volumes in ml					
	SH	**GP**	**C18**	**C8**	**C2**	**Oasis MCX**
Sinigrin	0.45	0.57	0.67	0.62	0.55	0.67
Amygdalin	1.12	0.87	0.72	0.67	0.75	0.62
Gallic acid	0.87	0.75	0.62	0.67	0.70	0.72
Caffeine	1.25	1.12	0.75	0.65	0.70	0.72
Rutin	> 4.5	> 4.5	4.00	> 4.5	1.92	0.92
Quercetin	> 4.5	> 4.5	> 4.5	> 4.5	2.25	> 4.5
Santonin	> 4.5	> 4.5	1.87	2.55	1.52	0.65
Methyl salicylate	> 4.5	> 4.5	1.87	2.00	0.87	0.67
Geranic acid	> 4.5	> 4.5	> 4.5	> 4.5	2.47	0.75

Apart from the volume breakthrough, also the maximum loadable amount on one cartridge is important for HPLC-NMR experiments. The mass breakthrough of SH cartridges was determined by conducting a multiple peak trapping experiment using rutin and quercetin. 0.75 mg (a 75 µL injection of a 1% rutin solution, dissolved in MeOH - H_2O (5:5)) was trapped on an SH cartridge using as solvent water – methanol (7:3). Water was acidified with 0.1% formic acid, HPLC flow rate was 0.5 mL/min, make-up pump flow rate 1 mL/min. Peak trapping took approximately 30 s. With the first injection full trapping of the compound occurred, i.e. no breakthrough was observed. However the next attempt to trap the same amount of compound on the same cartridge resulted in a slight breakthrough of the compound. The third attempt resulted in intense breakthrough. The same effect was also observed in the experiment with quercetin. This showed that the loadability of the 2 mm cartridges is in the order of 1-1.5 mg of compound which is enough for all NMR experiments.

The trapping ability of SH cartridges is strongly dependent on the amount of organic modifier present. The influence of the percentage of methanol on the retention was tested for all 9 model compounds. No HPLC column was used in this experiment. Breakthrough volumes for all compounds as determined in this experiment are presented in table 8.2. These breakthrough volumes are not due to mass overload but to actual chromatographic migration over the small SPE cartridge (volume overload). To be able to predict which compounds can be trapped at a certain methanol

percentage the peak volume must be known. I have assumed approximate peak volumes of 0.7 and 2 mL for the 4.6 and 7.8 mm column respectively. If three-fold trapping would be performed on a 4.6 mm column the total peak volume would also add up to 2 mL. For calculating the total volume passing through the SPE cartridge these values have to be multiplied by 3 due to the addition of make-up water. When this minimum value of 6 mL is combined with the data in table 8.2, it can be seen that rutin, quercetin, santonin, methyl salicylate and geranic acid can all be focussed at 20% methanol. Thus if these compounds can be eluted from the HPLC column at 60% methanol or less, trapping is complete even with a peak volume exceeding 6 mL. After having already seen the data in table 8.1, the poor trapping of caffeine and gallic acid and the excellent trapping of rutin does not come as a surprise. For a discussion *vide supra*. If compounds can be eluted from the HPLC column with less than 30% methanol, the final methanol percentage will be reduced to 10%. Under these conditions some but still insufficient trapping of caffeine is observed. Not surprisingly, the SH cartridges could not trap any of the tested compounds at methanol percentages greater than 50%. However this does not pose a problem as the actual percentage of methanol in a real HPLC-NMR system can never exceed 33%, because even when the HPLC solvent consists of pure methanol, it is diluted twice by the 1 mL/min of the water make-up flow.

Table 8.2 *Breakthrough volumes for 9 model compounds on SH cartridges as a function of the percentage of organic modifier (MeOH) in the final eluent. 0.5 mg of each compound dissolved in 50 μL of eluent was trapped. Measurements were performed in triplicate*

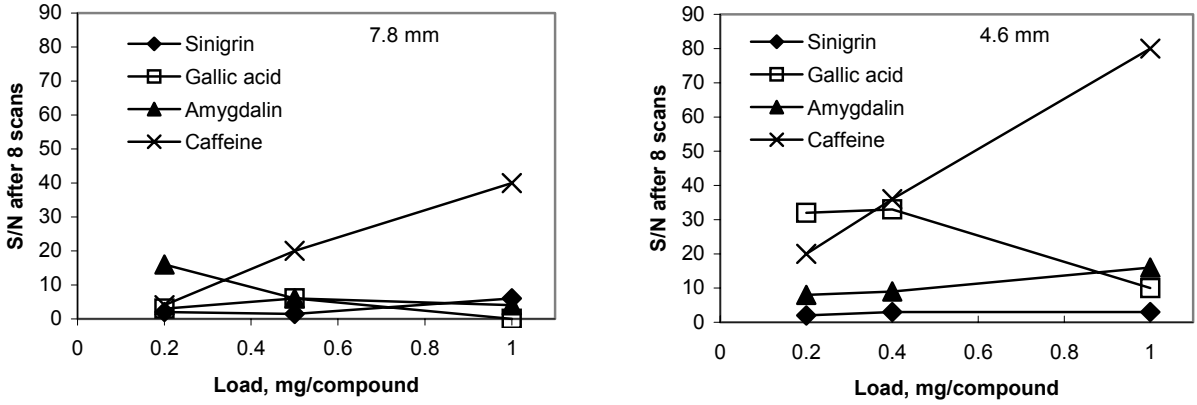
	Breakthrough volumes in ml at a given percentage of MeOH in the final eluent								
	0%	**10%**	**20%**	**30%**	**40%**	**50%**	**60%**	**70%**	**80-100%**
Sinigrin	0	0	0	0	0	0	0	0	0
Amygdalin	0.25	0.25	0.25	0.25	0.12	0	0	0	0
Gallic acid	0.18	0.18	0	0	0	0	0	0	0
Caffein	3.04	0.88	0.83	0.27	0.21	0.13	0	0	0
Rutin	> 10	> 10	> 10	> 10	1.38	0.1	0	0	0
Quercetin	> 10	> 10	> 10	> 10	> 10	3	1.50	0.25	0
Santonin	> 10	> 10	> 10	6.5	0.92	0	0	0	0
Methyl salicylate	> 10	> 10	> 10	7.13	1.92	0.83	0.22	0	0
Geranic acid	> 10	> 10	> 10	0.25	0	0	0	0	0

It is also clear that some highly polar compounds like sinigrin cannot be trapped under any conditions and thus cannot be investigated with the SPE unit in combination with hydrophobic cartridges. Such compounds should be investigated by loop-storage HPLC-NMR or possibly by

polar SPE cartridges through a normal phase trapping mode followed by elution with a more polar strong solvent, e.g. D_2O.

Another parameter that was optimised was the cartridge drying time. As protonated solvents are used in the separation and trapping steps, these solvents must be eliminated before the NMR measurement step as otherwise solvent suppression is necessary. Solvent suppression causes partial loss of the spectrum and decreases detection sensitivity. The water in the cartridges was removed by a flow of dry nitrogen gas. It was observed that the water signal in the NMR spectrum decreases by increasing the drying time up to 30 min. Additional drying of the cartridge up to 45 or 60 min did not further improve the spectra, suggesting that the sample was already dry. Hereafter a drying time of 30 min was used.

In the next step, the two HPLC columns were connected to the SPE-NMR system and different amounts of the two test mixtures were injected. Isocratic separation conditions were used: H_2O - MeOH (72:28) with 0.1% formic acid for mixture (I) and H_2O - MeOH (45:55) with 0.1% formic acid for mixture (II). Compounds were trapped on SH cartridges, subsequently dried and transferred to the HPLC-NMR probe. Spectra were recorded and signal to noise (S/N) ratios after 8 scans were determined (figures 8.3 and 8.4).



a b

Figure 8.3 *Measured NMR S/N values of the model compounds in the polar mixture (I) as a function of the amount of injected compound using (a) a 7.8 mm i.d. semi-preparative column, and (b) a 4.6 mm i.d. analytical column.*

As could be predicted from table 8.2, the trapping process using the above conditions is very inefficient for most compounds in mixture (I). Only caffeine is somewhat retained, resulting in higher S/N ratios when larger amounts are injected. When larger amounts of these compounds are injected, the trapped amount (as assessed by the S/N ratio) increases only slightly or even decreases. This is caused by the weak affinity of these compounds for the stationary phase, resulting in marked

volume breakthrough. Comparing the S/N ratios of the two columns, the higher values are observed with the analytical column. The reason for this phenomenon is the three times bigger peak volume for the semi-preparative column causing more volume breakthrough. Thus for polar compounds the use of a semi-preparative column has actually an adverse effect.

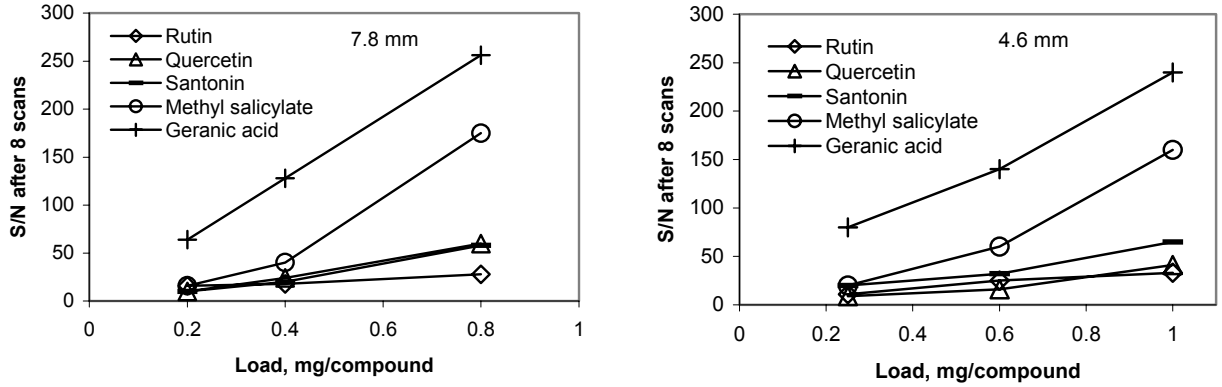


Figure 8.4 *Measured NMR S/N values of the model compounds in the non-polar mixture (II) as a function of the amount of injected compound using (a) a 7.8 mm i.d. semi-preparative column, and (b) a 4.6 mm i.d. analytical column.*

When the more non-polar model compounds were injected, trapped and measured, not unexpectedly similar results were obtained on the two columns. Now with the exception of the polar rutin all compounds were focussed on the SH cartridge and consequently higher S/N values were obtained with increasing sample load. The relatively low trapping of rutin is a bit unexpected and might be due to its precipitation over time. Of all compounds in mixture II, it is least soluble in methanol - water. Due to good focussing, the higher peak volumes of the semi-preparative column were not important. Santonin, quercetin and rutin are more polar than methyl salicylate and geranic acid, therefore some breakthrough was already observed for these compounds. As this was more obvious at higher amounts (0.6 mg and more) it is most likely due to a combination of mass and volume overload.

Although on the basis of these S/N ratios the use of a semi-preparative column instead of an analytical column seems to have no merit, this is not true. What these figures do not take into account is the chromatographic resolution and NMR spectral quality. In figure 8.5 the chromatograms of the separation of mixture II are depicted. It can be seen that under these conditions the analytical column was severely overloaded, resulting in significant overlap of the peaks of rutin, santonin and quercetin. Thus their selective trapping was impossible and the S/N determination experiments for these compounds on the analytical column were performed by

injecting and trapping compounds one by one. For the semi-preparative column this was not necessary. The run time of the semi-preparative column is approximately three times longer than on the analytical column due to its three times lower linear flow rate.

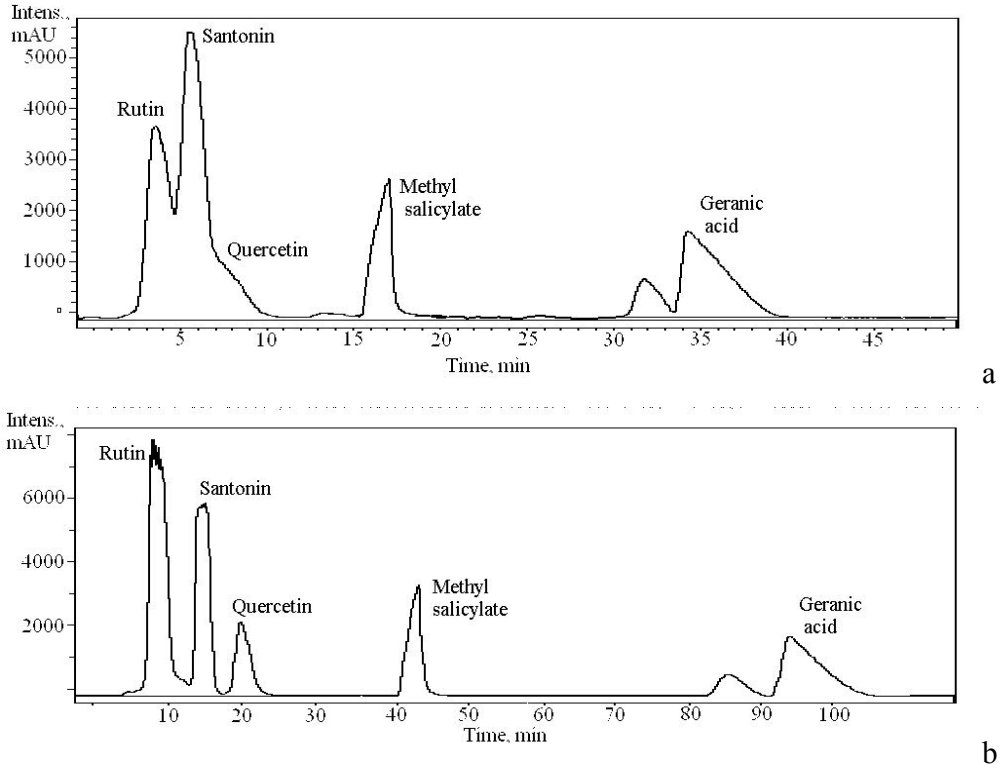


Figure 8.5 *Chromatographic profile (254 nm) of mixture II, separated under isocratic conditions using (a) analytical column, (b) semi-preparative column. The peak eluting just before geranic acid is an impurity of neric acid.*

The model compounds in test mixture (II) were also run with a linear gradient from 10% to 100% methanol. To obtain comparable elution conditions on both columns, the gradient took 30 min on the analytical column and 90 min on the semi-preparative column. Results are presented in table 8.3. Based on the retention times (a correction for the gradient delay time and the dilution with water was applied), the actual percentage of methanol at the SPE cartridge was calculated for each eluted compound. Subsequently this percentage was used in combination with the data in table 8.2 to predict the breakthrough volume for each compound. The estimated breakthrough volumes show that in a real HPLC-NMR experiment, rutin, santonin, quercetin and methyl salicylate can all be trapped on a 2 mm SH cartridge while using a semi-preparative column. The results would predict that geranic acid cannot be sufficiently trapped but this is most likely due to an insufficient concentration of formic acid in the make-up water. Otherwise adequate trapping can be achieved by slightly changing the ratio of eluent to make-up water, e.g. 0.4 to 1.2 mL/min.

The same phenomenon of a still adequate separation on the 7.8 mm column and a distorted chromatogram on the 4.6 mm column (results not shown) was observed when 14 mg of a commercial rosemary extract sample containing the potent antioxidants carnosol and carnosic acid were injected. These are quite non-polar constituents with estimated log P values of 4.9 and 3.7 and in the past it has been shown that such compounds can be well trapped on SH cartridges and measured by HPLC-SPE-NMR [20]. Thus it is an example of a sample where a semi-preparative column in combination with on-line SPE has a distinct advantage.

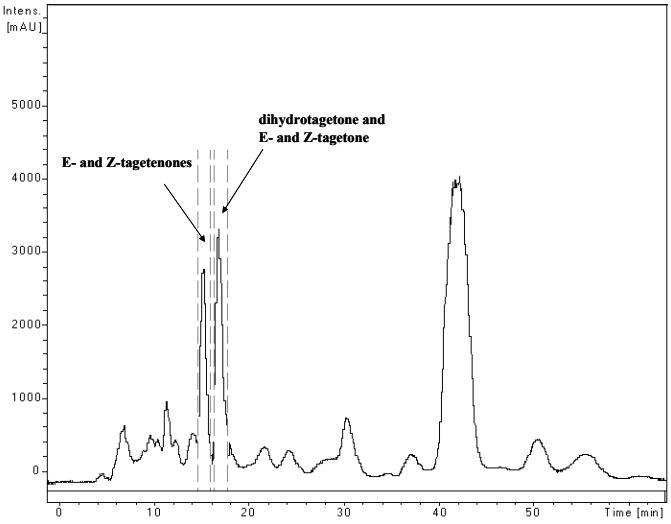
Table 8.3 *Comparison of the separation parameters (retention time; percentage of MeOH in the eluent at the respective retention time) and calculated breakthrough volumes of reference compounds using 30 min gradient for the analytical column and 90 min for the semi-preparative one.*

	Retention time, min		**Corresponding % of MeOH in SPE at given retention time**		**Predicted breakthrough volume in ml at given %**	
	Analytical	**Semi-prep.**	**Analytical**	**Semi-prep.**	**Analytical**	**Semi-prep.**
Sinigrin	3	8.7	3.3	3.3	0	0
Gallic acid	6.5	16.2	3.8	4.6	~ 0.2	~ 0.2
Amygdalin	12.7	30.6	10.0	10.5	0.25	0.25
Caffeine	13.7	32.6	11.0	11.2	~ 0.8	~ 0.8
Rutin	19.8	50	17.1	17.0	> 10	> 10
Santonin	21.6	54.8	18.9	18.6	> 10	> 10
Quercetin	23.5	60.7	20.8	20.6	> 10	> 10
Methyl salicylate	26.6	69.4	23.9	23.5	~ 8.0	~ 8.0
Geranic acid	29.4	77.1	26.7	26.0	~ 2.5	~ 2.5

In a final experiment a sample of Tagetes essential oil was separated by RP-HPLC on both the 7.8 and 4.6 mm columns. Important constituents of Tagetes oil are limonene, cis-β-ocimene, dihydrotagetone, cis-tagetone, cis-tagetenone and trans-tagetenone. It was tried to separate the two tagetenones from the remainder of the essential oil, to trap them on-line by SPE and to measure their NMR spectra. On the 7.8 mm column 50 µL of a 10% solution in methanol was injected (figure 8.6a) while on the 4.6 mm 17 µL and 50 µL of the same solution was injected (figures 8.6b and 8.6c). The solvent was MeCN - H_2O (7:3) isocratically. On the 7.8 mm column the two tagetenones (t_r ~ 15 min, ratio cis : trans = 3:5) were almost baseline separated from dihydrotagetone and the two tagetones (t_r ~ 17 min). When the injection volume was adapted for the smaller diameter (i.e. 17 µL), a similar separation was obtained on the 4.6 mm column (figure 8.6b). The retention times are thrice shorter as the absolute flow remained the same (0.5 mL/min for all separations). When an

identical volume was injected as on the 7.8 mm column (i.e. 50 µL) the separation deteriorated and the peaks were no longer baseline separated (figure 8.6c). In all cases both peaks were trapped and NMR spectra were recorded. In case of the 7.8 mm column a good ^{1}H-NMR spectrum of the tagetenones could be obtained in 96 scans (figure 8.7, upper spectrum) without visible impurities. An identical spectrum could be obtained of peak 1 in figure 8.6b but now 864 scans were necessary (result not shown). This fits exactly the 3 times smaller amount applied as the noise increases only with the square root of the number of scans (3^2 x 96 = 864). This also shows that the trapping in the case of the 7.8 mm was quantitative. The estimated log P value of the tagetenones is 2.7, i.e. they are moderately non-polar compounds that can be expected to be retained by SH cartridges. The NMR spectrum of peak 1 in figure 8.6c (50 µL injection and 4.6 mm I.D.) showed a clear 30% impurity of cis-tagetone at 7.65 ppm and 5.66 ppm (figure 8.7, lower spectrum). Also in the aliphatic part of the molecule several impurities including cis-tagetone were visible making the spectral interpretation more difficult. These relatively simple spectra show that for certain compounds the use of a semi-preparative column has an advantage. The advantages will be more substantial than in this example when the amounts are smaller (not in all cases it is possible to record 10 times more scans), when chromatographic resolution is poorer and when the NMR spectra are more complex.

It can be concluded that the use of a semi-preparative HPLC column in combination with an SPE-NMR set-up has some advantages over an analytical column when analysing mixtures consisting of middle polar to non-polar compounds, like quercetin, santonin, methyl salicylate, geranic acid (log P = 1.48 - 3.70). The higher peak volumes associated with semi-preparative HPLC columns do not play a role as long as all compounds are efficiently focussed on the SPE cartridge. In principle the same result can be obtained by multiple trapping on an analytical column but the latter is technically more demanding and takes at least as much time. Another point of consideration is the solubility of the analyte in the deuterated solvent that will be used to elute the SPE cartridge. If not all of the trapped compound can dissolve in the small volume of NMR solvent, multiple trapping or semi-preparative columns are not beneficial. Medium polar compounds like flavonoid glycosides (e.g. rutin) and caffeine ($-2 < \log P < 2$) are more critical and the efficient trapping of such compounds cannot be assumed *a priori*. The overall polar/non-polar character of a compound as characterised by its log P value does not allow making a fool-proof prediction of its trapping. Some polar aromatic compounds appear to be more efficiently trapped on SH cartridges than less polar non-aromatic compounds. Semi-preparative columns do not appear to have an advantage over analytical columns for the analysis of such compounds.



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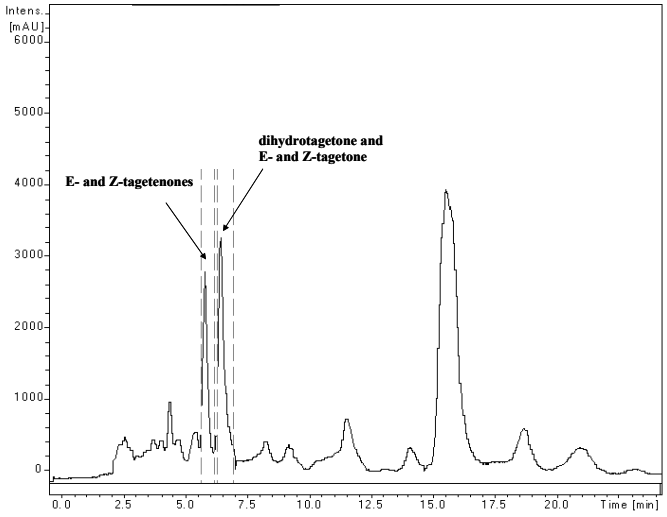

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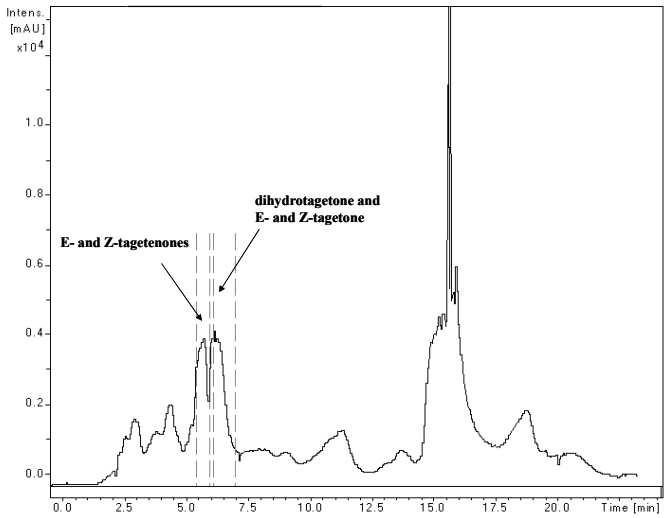

c

Figure 8.6 *The chromatographic profiles (210 nm) of a 10% solution of Tagetes essential oil dissolved in MeOH separated on 100 mm C18 columns with MeCN - H_2O (7:3) as eluent at 0.5 mL/min. (a) 50 µL injection on a 7.8 mm i.d. semi-preparative column; (b) 17 µL injection on a 4.6 mm i.d. analytical column; (c) 50 µL injection on a 4.6 mm i.d. analytical column. Dashed lines correspond with trapping on SPE unit.*

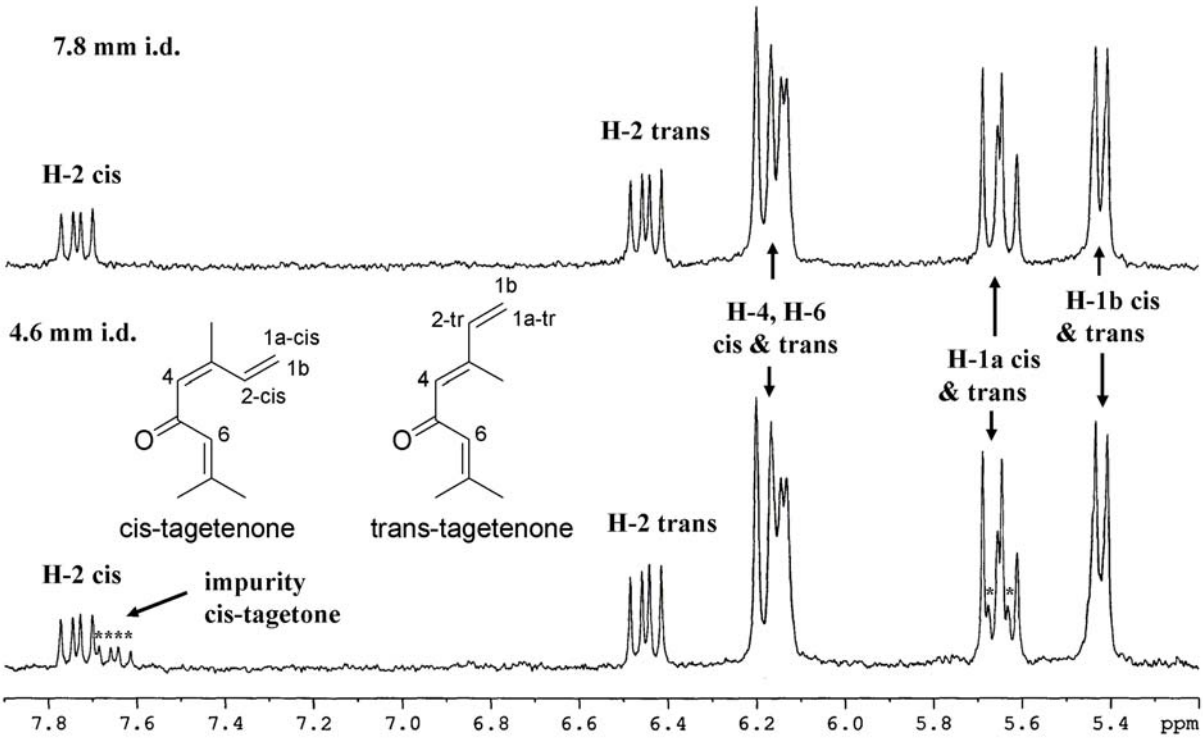

Figure 8.7 *Upper spectrum: olefinic and aromatic region of the ^{1}H-NMR spectrum of firstly trapped peak in Fig. 6a; lower spectrum: olefinic and aromatic region of the ^{1}H-NMR spectrum of firstly trapped peak in Fig. 6c. Asterisks (*) denote signals belonging to a 30% impurity of cis-tagetone. Both spectra were recorded in MeCN-d3 with a 120 µL LC-NMR probe at 400 MHz in 96 scans.*

If relatively large HPLC-NMR probes are used (60 - 120 µL active volume), the application of larger diameter SPE cartridges should be considered, i.e. 3 instead of 2 mm I.D. Such cartridges have a twice higher capacity and breakthrough volume. As long as all trapped analytes can be eluted with a volume smaller than the HPLC-NMR measuring volume, there should be a net gain. Still another way to improve the trapping of medium polar compounds is to increase the percentage of water during trapping. This can be accomplished either by lowering the ratio of column flow / water make-up flow. Unless larger particles (i.e. 40 µm instead of 15 µm) in the SPE cartridges are used allowing for a higher total flow, this can only be done at the expense of longer separation times. For smaller HPLC-NMR cells (20 - 30 µL active volume) 2 mm SPE cartridges are optimal. HPLC-SPE-NMR in combination with reversed phase cartridges is not suitable for the analysis of very polar compounds with log P values of –2 or lower. LC-NMR analysis of such compounds is better done by loop-storage or by LC-SPE-NMR with normal phase cartridges [21, 22]. The application of semi-preparative columns for such compounds will have an adverse effect due to the higher peak volumes.

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CONCLUSIONS AND GENERAL DISCUSSION

A great number of aromatic, spicy and medicinal plants contain compounds exhibiting antioxidant properties. A lot of research has been carried out on such plants, with most attention going to rosemary and sage (see paragraph 1.3.3 for details and references). However, scientific information on antioxidants from plants that are less widely used for culinary and medicinal purposes, including species growing in Lithuania is still rather scarce. This fact partially inspired this study. An increasing demand for naturalness of food was another important motivation. A third reason was to contribute to the development of faster analytical methods for investigation of antioxidants.

The selection of plants in this work was influenced by the institution that provided them (Botanical Garden of Vytautas Magnus University in Lithuania) and was primarily determined by the extent how much certain plants grown in Lithuania had been investigated for radical scavenging/antioxidant activity. Only after a successful preliminary screening, which revealed several promising plant species, growing in Central and Eastern Europe, further targets for the present study were formulated. These promising plant materials were leaves of *Geranium macrorrhizum* and blossoms of *Potentilla fruticosa*. Leaf extracts from *Rhaponticum carthamoides* and various Salvia species were also shown to possess strong radical scavenging properties. As the antioxidant properties of *Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides* had been little investigated before, they were selected for detailed investigations.

Most of the studies on natural antioxidants are initially attributed towards a particular herb, spice or mix of them. In many cases such studies carry a well defined practical task, like e.g. the shelf life of a certain food product needs to be extended. This study was carried out differently, as no practical - pointing towards application - task was initially formulated. The investigation of the chemical composition of plants in order to determine the active constituents and to understand the mode of action of these constituents was pursued after the initial screening studies.

The composition of radical scavenging compounds in these plants was analysed. The majority of identified compounds had not been reported before to be constituents of these plants and it was found that these compounds can be effectively extracted with cheap and food-grade solvents, like ethanol and water. All of the identified compounds except one were phenolics.

Next the antioxidant properties of selected extracts from *Geranium macrorrhizum* and *Potentilla fruticosa* were determined. The results showed that the plants are a rich source of hydrophilic phenols that possess strong radical scavenging properties in hydrophilic media in assays involving

model radical species. A poor solubility of extract constituents in lipophilic media was the likely reason behind the extract inactivity in such media. *A priori* it was expected that the polar extracts would perform better in these assays. The reason is the phenomenon called "polar paradox" [1], according to which hydrophilic antioxidants can show efficient antioxidant properties in bulk oil systems and lipophilic antioxidants in oil in water emulsions. However in this study this phenomenon was not observed possibly because of the total insolubility of the polar constituents in the bulk oil.

The activity of extracts in some assays was compared with that of well known rosemary extracts. Extracts of rosemary are used in some food products and widely used in cosmetic products. In the accelerated fermented sausage oxidation assay the activity of *Geranium* and *Potentilla* extracts was lower than that of rosemary extracts, however in the linoleic acid-*β*-carotene assay the antioxidant activity was close to that of rosemary.

Although initially not planned, preliminary safety evaluation studies of the selected plant extracts were performed. Such data on the investigated plants is scarce. Even though plant extracts are natural, this does not automatically imply that they are safe [2, 3]. More issues derive from the fact that climatic and soil conditions can also influence the quantitative and qualitative composition of plant constituents. Because of the above mentioned issues the regulations on food additives and supplements in many countries are quite strict and will get even stricter [4]. Preliminary safety tests have shown the investigated plants extracts to be safe with regard to genotoxicity and mutagenicity.

The studies have revealed the plant extracts to be rich in quercetin derivatives. Glycosides of this flavonoid are very common in many well-known vegetables, edible fruits, berries, spices and herbs. From this point it is likely that investigated extracts are potentially safe (GRAS status). Prior to using these extracts as food additives or supplements of course broader safety test remain necessary.

Generally after antioxidant activity evaluation assays it is still rather difficult to unambiguously identify investigated plant materials as a new source of antioxidants. The studies indicated that plant extracts from *Geranium* or *Potentilla* possess antioxidant properties in hydrophilic media. Their application in such media could be possible (e.g. salad dressings). However the application of the mentioned extracts in lipophilic food products, like e.g. pure oil most likely would not be successful. Moreover, similar antioxidants are available from other more abundant species, and this situation makes it less likely that *Geranium* and *Potentilla* extracts will be applied.

Continuation of this work could include finding an improved isolation procedure for the active extracts. However simple procedures usually yield crude extracts which because of their characteristic odour, colour, taste and unwanted constituents have limited application possibilities.

This is also true for extracts from sage and rosemary. Devising economic procedures which yield purified extracts necessitates more research.

Any practical application of plant extracts needs a wider variety of studies, and this involves not only chemists, but also nutritionists, agrotechnologists, and economists. Testing and comparing the activity of extracts from plants in targeted food systems with commercially used antioxidants, investigation of the stability of extracts during food processing, safety studies, agrotechnological investigations on cultivation conditions, economical feasibility studies and more issues derived from a practical application point of view need to be solved prior to any application. The experience with other natural antioxidative extracts i.e. rosemary and sage extracts shows that all the above mentioned steps towards commercial products are time-consuming and need detailed follow-up studies.

During the analysis of the radical scavengers in the selected extracts from *Geranium macrorrhizum* and *Potentilla fruticosa* on-line HPLC radical scavenging assays, like HPLC-UV-DPPH or HPLC-UV-ABTS proved useful [5, 6]. These methods are well suitable for the screening of complex mixtures, to determine the number of active components and their activity. However, for the identification of the active compounds in the majority of cases further isolation-structure elucidation steps remain necessary. For this reason there was a need to expand the scope of the above methods by coupling them to a mass spectrometer. This was achieved by using a simple passive splitter. The potential of the expanded system increased significantly because of the advantages provided by the MS detector. The developed system enabled a rapid analysis (detection and identification of radical scavengers) of complex Ginkgo and rosemary extracts. The expanded method accelerates the compound structure identification process and helps to avoid the replication of off-line isolation and identification of already known structures.

Another powerful technique, used for compound isolation and structure elucidation from complex mixtures is LC-DAD-SPE-NMR. This hyphenated technique proved itself in numerous applications [7, 8]. The isolation and structure elucidation procedures of some radical scavengers from *Potentilla fruticosa* were performed with this hyphenated set-up. The identification of the major constituents from *Rhaponticum carthamoides* was performed entirely by LC-DAD-SPE-NMR. Normally analytical HPLC columns are employed in LC-NMR. If higher amounts of sample are necessary for e.g. 2D NMR experiments, multiple compound trapping on the same SPE cartridge can be performed. To increase the amounts of trapped compounds in this study a semi-preparative HPLC column was evaluated. A semi-preparative HPLC column in combination with an SPE-NMR set-up was shown to have some advantages over an analytical column when analyzing mixtures

consisting of moderately polar to non-polar compounds. The higher loadability of a semi-preparative column leads to a better peak resolution when equal amounts are injected and this in turn leads to more pure NMR spectra. Because of the fixed maximum flow rate in an LC-SPE-NMR set-up, runs with semi-preparative columns take more time but this is compensated for by much shorter NMR measuring times. LC-SPE-NMR in combination with reversed phase cartridges is not suitable for the analysis of very polar compounds (e.g. sinigrin) with log P values of –2 or lower. Overall it is clear that good results in HPLC-SPE-NMR can only be achieved by a judicious choice of several parameters.

The conducted research has generated some further questions and tasks for future investigations. The influence of the particular glycosidic moiety on the radical scavenging activity of flavonoid glycosides is one the questions. The finding that the standard spices mix of pepper, clove, garlic and bell peppers is more active than any tested extract in the fermented sausage accelerated oxidation assay is a lead in itself and warrants further research with regard to the active principles and the mode of action of this spices mix.

Creation of new on-line assays using real life ROS is also of interest in antioxidant studies. In future while conducting similar studies it is perhaps not necessary to perform an array of various antioxidant assays. Practical evaluation assays (like the sausages accelerated oxidation assay in this work) are most important and decisive with regard to future applications of investigated extracts.

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SUMMARY

Food molecules (lipids, proteins, carbohydrates) can be widely involved in oxidation reactions. These reactions, caused by so called reactive oxygen species (ROS) are a major cause of food deterioration. In the case of lipid containing foods this process is defined as rancidity. Significant changes can occur in product colour, texture and nutritive value. Eventually the oxidation can result in complete spoilage. Oxidation reactions caused by ROS also play an important role in the ageing processes of living organisms and are associated with numerous diseases, like coronary heart disease, cancer, cataracts, ageing. Although protective mechanisms exist both in living cells and in foods, in many cases there is a need to strengthen this mechanism. A daily intake of antioxidants can prevent or delay problems caused by ROS.

The research described in this study is aimed at the evaluation of several plant extracts as a possible source of food antioxidants. A brief classification of antioxidants, their sources, a comparison of synthetic antioxidants with natural ones and methods for the evaluation of antioxidants are described in chapter 1.

An initial screening for radical scavenging activity of extracts from medicinal and aromatic plants namely *Salvia sclarea*, *Salvia glutinosa*, *Salvia pratensis*, *Salvia officinalis, Lavandula angustifolia*, *Calendula officinalis*, *Matricaria recutita*, *Echinacea purpurea*, *Rhaponticum carthamoides*, *Juglans regia*, *Melilotus officinalis*, *Geranium macrorrhizum* and *Potentilla fruticosa* has been performed using DPPH and ABTS radical scavenging assays (chapter 2). Of these extracts *Geranium macrorrhizum* and *Potentilla fruticosa* possessed the highest radical scavenging activity in both assays, higher than that of the *Salvia officinalis* extract. High radical scavenging activity was also shown by other *Salvia* species and *Rhaponticum carthamoides*. As *Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides* had been little studied, they were selected for further investigations. A correlation between the radical scavenging capacity of extracts and their total phenolic compound content was observed.

In further steps of this study the structures of the radical scavenging compounds present in leaf extracts of *Geranium macrorrhizum*, in blossoms extracts of *Potentilla fruticosa* and leaf extracts of *Rhaponticum carthamoides* were elucidated (chapters 3, 4 and 5). Seven compounds, namely gallic acid, ellagic acid, 4-galloyl quinic acid, the flavonoid quercetin and three of its glycosides, quercetin-3-*β*-glucopyranoside, quercetin-3-*β*-galactopyranoside and quercetin-4'-*β*-glucopyranoside were isolated and identified in various *Geranium macrorrhizum* fractions. All compounds

except gallic acid and quercetin were identified for the first time in this plant. Twelve compounds were isolated and identified from *Potentilla fruticosa* blossoms, namely ellagic acid, (+)-catechin, quercetin-3-*β*-glucopyranoside, quercetin-3-*β*-galactopyranoside, quercetin-3-*β*-rutinoside, quercetin-3-*β*-glucuronopyranoside, quercetin-3-*α*-arabinofuranoside, kaempferol-3-*β*-rutinoside, kaempferol-3-O-*β*-(6"-O-(E)-*p*-coumaroyl)glucopyranoside, rhamnetin-3-*β*-glucopyranoside, rhamnetin-3-*β*-galactopyranoside and rhamnetin-3-*α*-arabinofuranoside. All compounds except ellagic acid**,** catechin and quercetin-3-*β*-galactopyranoside had not been identified in *Potentilla fruticosa* before.

The radical scavenging activity of the isolated compounds from *Geranium macrorrhizum* and *Potentilla fruticosa* was measured using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays and compared with the activity of rosmarinic acid and Trolox. Quercetin-3-glucopyranoside, quercetin-3-galactopyranoside and ellagic acid showed the highest antioxidant capacity among *Geranium* compounds; quercetin-3-glycosides and catechin were found to be the most active radical scavengers in extracts of *Potentilla fruticosa.*

A quantitative comparison of the major antioxidant compounds in different botanical parts of *Geranium macrorrhizum* and *Potentilla fruticosa* was also made. Ethanolic extracts of leaves and roots from *Geranium* and ethanol-butanol fractions of blossoms and an ethanol extract from *Potentilla fruticosa* leaves were compared. The leaf extract of *Geranium* and the blossoms extract of *Potentilla* were markedly richer sources of radical scavenging compounds than extracts from other botanical parts.

A hyphenated LC-DAD-SPE-NMR set-up in combination with on-line radical scavenging detection was used for the identification of radical scavenging compounds from *Rhaponticum carthamoides* extracts. The technique enabled selective detection and identification of individual radical scavenging compounds without any prior off-line chromatographic steps. Seven compounds, namely, quercetagetin-7-*β*-glucopyranoside, quercetagetin-7-(6"-acetyl-*β*-glucopyranoside), 6-hydroxykaempferol-7-*β*-glucopyranoside, 6-methoxykaempferol-3-*β*-glucopyranoside, 6-hydroxy-kaempferol-7-(6"-acetyl-*β*-glucopyranoside), chlorogenic acid, and *β*-ecdysone, were identified in ethanolic or aqueous extracts. 6-Hydroxykaempferol-7-(6"-acetyl-*β*-glucopyranoside) was identified as a new natural compound. Its radical scavenging activity was tested against DPPH radical and was found to be weaker than that of the reference antioxidants rosmarinic acid and Trolox.

Antioxidant activity of all plant fractions from *Geranium macrorrhizum* and *Potentilla fruticosa* was assessed by model system assays and in real food systems consisting of edible oil or fermented sausages (chapter 6). The ethanol-butanol fraction of *Geranium macrorrhizum* possessed the highest

activity in most of the model tests and in the oil oxidation test, especially in retarding the formation of secondary oxidation products (hexanal and conjugated trienes). Most of the active radical scavengers were also identified in this fraction. The water-butanol fraction showed activity in retarding primary oxidation products (peroxide value test) and in model radical assays. The remainder of the extracts was less effective in oil oxidation tests.

Potentilla extracts were effective as antioxidants in assays with polar media (superoxide anion, hydrogen peroxide scavenging and *β*-carotene bleaching tests). *Potentilla* extracts showed limited activity in oil oxidation tests.

Geranium water-water fraction and *Potentilla* ethanol-butanol fractions were investigated for their antioxidative properties during oxidation of fermented sausages and compared to an extract of rosemary and the standard spice mix that is used for the production of Dutch style cervelat sausage. The oxidation was the slowest in the reference sausage sample with standard spices. That means that the spices present in the reference sausage in combination with ascorbate possessed stronger antioxidative capacity than rosemary and *Potentilla* extracts. The investigated *Geranium* fraction possessed the weakest activity. The most likely reason for this was a poor solubility of the fraction in the fat phase. The activity of *Potentilla* extracts in fermented sausage oxidation tests was higher than that of *Geranium* extracts, but lower than that of rosemary extracts. The overall activity of *Potentilla* extract and rosemary extract was higher if used in combination with ascorbate.

A preliminary safety evaluation of some plant extracts has been conducted (enzymatic oxidation assay; effects against singlet oxygen caused erythrocyte hemolysis; mutagenicity evaluation) and indicated the plant extracts not to be genotoxic or mutagenic.

Parallel to the investigation presented and discussed in this thesis, more research related to the application of extracts from *Geranium macrorrhizum* and *Potentilla fruticosa* has been carried out. E.g. results from microbiological tests showed that 1% ethanolic extract solutions of *Geranium macrorrhizum* leaves were active against some microorganisms (*Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus*). Similar extracts of *Potentilla fruticosa* were also active against *Salmonella* and *Bacillus* species, and showed strong bactericidal properties against *Hafnia alvei* and *Listeria monocytogenes* [*].

The same extracts were also tested in oil oxidation tests (determination of peroxide value) with refined rapeseed oil. The oil was refined to remove native antioxidative compounds, like tocopherols. In this way possible interactions between naturally present antioxidants and added ones were eliminated. It was observed that in the refined oil the activity of added plant extracts

[*] Personal communication of A. Šipailiene 2004, Kaunas University of Technology, Kaunas.

(expressed as induction periods), was the same as in tests where non-refined oil was used. This means that the plant extracts do not have a synergistic effect with naturally present tocopherols. Also the WB fraction of *Potentilla fruticosa* showed no synergistic effect. The EB fraction of *Geranium macrorrhizum* and the WB fraction of *Potentilla fruticosa* were also evaluated in the peroxide formation test by using a few different extract concentrations. 0.05 % *Geranium* extract showed antioxidant properties close to those of 0.1 % concentration. At 0.2 % extract concentration antioxidant properties were higher. In oxidation tests with *Potentilla* WB fraction the results were quite similar with concentrations of 0.05, 0.1 or 0.2 %.

Research on natural products, like plant antioxidants, demands the application of various analytical techniques. Apart from state-of-the-art analytical instruments, a lot of specific techniques needed to be applied at certain stages, e.g. on-line radical scavenging detection of individual plant constituents in complex plant mixtures. These DPPH/ABTS assays coupled on-line to liquid chromatography-diode array detection set-ups have been developed earlier at the Laboratory of Organic Chemistry of Wageningen University. Expansion of these assays by coupling a mass spectrometer to the system was one part of the methodological research (chapter 7). The coupling of the MS detector to the on-line set-up was possible by using a simple passive splitter. The developed system enabled a quick analysis (simultaneous separation, isolation-identification and detection of radical scavenging activity) of complex Ginkgo and rosemary extracts. A combination of mass and DAD detection proved to be effective for identification of the majority of the compounds present in extracts. The expanded method accelerates the compound structure identification process and avoids the replication of isolation and identification of already known structures.

The hyphenated LC-DAD-SPE-NMR system is another powerful technique for compound isolation and structure elucidation in complex mixtures. The investigation of the application of LC-DAD-SPE-NMR with a semi-preparative HPLC column and comparison of the obtained results with those of an analytical column was described in chapter 8. It was determined that the use of a semi-preparative HPLC column in combination with an SPE-NMR set-up (with reversed phase cartridges) has some advantages over an analytical column when analysing mixtures consisting of intermediately polar to non-polar compounds, like quercetin, santonin, methyl salicylate and geranic acid (log P = 1.48 – 3.70). The higher loadability of a semi-preparative column leads to a better peak resolution when equal amounts are injected and this in turn leads to better NMR spectra. The application of semi-preparative columns with polar compounds does not give any advantage, because of the weak affinity of the stationary phase for such compounds.

An overall discussion and concluding remarks of the current study and some ideas for future investigations are presented in chapter 9. It is concluded that extracts from *Geranium* and *Potentilla* are unlikely to be a new source of antioxidants for lipophilic food products. Extracts proved to be effective in assays with polar media, however their application in such products is also unlikely. Reasons are problems, like odour and colour of the extracts, and the complex extraction procedure.

Summary

SAMENVATTING

Bestanddelen van voedsel, zoals vetten, eiwitten en koolhydraten, reageren in contact met lucht (zuurstof) langzaam via complexe chemische processen. Bij deze processen ontstaan reactieve zuurstof intermediairen, die verder reageren tot componenten, die o.a. verantwoordelijk zijn voor het rans worden van vetten. Als gevolg van dit oxidatieproces kan de kleur, geur, smaak, consistentie en voedingswaarde van het voedsel geleidelijk veranderen. Uiteindelijk is het voedsel niet meer geschikt voor consumptie. De reactieve zuurstof intermediairen (meestal radicalen) spelen ook een belangrijke rol in ouderdomsprocessen van levende organismen. Bepaalde ziektes, zoals hart- en vaatziekten en kanker, worden hiermee in verband gebracht. Antioxidanten kunnen de radicalen omzetten in niet- of minder actieve componenten. Hoewel beschermingsmechanismen zowel in levende organismen als voedsel voorkomen, is het in vele gevallen nodig deze mechanismen te versterken door toevoeging van antioxidanten. Een dagelijkse inname van antioxidanten kan gezondheidsproblemen uitstellen of voorkomen en toepassing van antioxidatieve additieven in levensmiddelen kan de houdbaarheid daarvan verlengen.

In dit proefschrift wordt onderzoek beschreven naar het mogelijke gebruik van plantenextracten als antioxidanten in voedsel. In hoofdstuk 1 wordt een kort overzicht gegeven van de verschillende typen van natuurlijke en synthetische antioxidanten en methodes om de activiteit van antioxidanten te bepalen.

In eerste instantie zijn een aantal aromatische en medicinale kruiden oriënterend onderzocht op hun vermogen om radicalen te neutraliseren: *Salvia sclarea*, *Salvia glutinosa*, *Salvia pratensis*, *Salvia officinalis, Lavandula angustifolia*, *Calendula officinalis*, *Matricaria recutita*, *Echinacea purpurea*, *Rhaponticum carthamoides*, *Juglans regia*, *Melilotus officinalis*, *Geranium macrorrhizum* en *Potentilla fruticosa.* Dit wordt beschreven in hoofdstuk 2. Extracten van *Geranium macrorrhizum* en *Potentilla fruticosa* vertoonden de meeste antioxidatieve activiteit en waren zelfs actiever dan het *Salvia officinalis* referentie extract. Ook actief waren de andere Salvia extracten en het *Rhaponticum carthamoides* extract. Omdat *Geranium macrorrhizum*, *Potentilla fruticosa* en *Rhaponticum carthamoides* weinig onderzocht waren, werden deze extracten geselecteerd voor verdere studies. Er werd een verband gevonden tussen het vermogen om radicalen te neutraliseren en het totale gehalte aan fenolische verbindingen in deze extracten. Van fenolische verbindingen is bekend dat zij radicalen kunnen neutraliseren.

Samenvatting

In de volgende stap werden de moleculaire structuren van de actieve radicaalvangers in de bladextracten van *Geranium macrorrhizum* en *Rhaponticum carthamoides* en in het bloemextract van *Potentilla fruticosa* opgehelderd. Dit wordt beschreven in hoofdstukken 3, 4 en 5. Zeven verbindingen, nl. galluszuur, ellaginezuur, 4-galloyl-koffiezuur, het flavonoid quercetine en drie glycosiden daarvan (quercetine-3-*β*-glucopyranoside, quercetine-3-*β*-galactopyranoside en quercetine-4'-*β*-glucopyranoside) werden geïsoleerd en geïdentificeerd in verschillende *Geranium macrorrhizum* fracties. Al deze verbindingen met uitzondering van galluszuur en quercetine zijn nooit eerder in deze plant aangetoond. Twaalf verbindingen werden geïsoleerd en geïdentificeerd in de bloemen van *Potentilla fruticosa*, nl. ellaginezuur, (+)-catechine, quercetine-3-*β*-glucopyranoside, quercetine-3-*β*-galactopyranoside, quercetine-3-*β*-rutinoside, quercetine-3-*β*-glucuronopyranoside, quercetine-3-*α*-arabinofuranoside, kaempferol-3-*β*-rutinoside, kaempferol-3-O-*β*-(6"-O-(E)-*p*-coumaroyl)glucopyranoside, rhamnetine-3-*β*-glucopyranoside en rhamnetine-3-*β*-galactopyranoside. Alleen ellaginezuur, catechine en quercetine-3-*β*-galactopyranoside zijn eerder in deze plant aangetoond. De radicaal vangende activiteit van de verbindingen uit *Geranium macrorrhizum* en *Potentilla fruticosa* is bepaald met DPPH en ABTS radicaaltesten en vergeleken met de activiteit van de standaard antioxidanten rozemarijnzuur en Trolox (wateroplosbaar vitamine E). Quercetine-3-glucopyranoside, quercetine-3-galactopyranoside and ellaginezuur bezaten van alle *Geranium* verbindingen de hoogste antioxidatieve capaciteit. De quercetine-3-glycosides en catechine waren de meest actieve verbindingen aanwezig in *Potentilla fruticosa.*

De hoeveelheden van de belangrijkste antioxidanten in alcoholische extracten van de bladeren en wortels van *Geranium*, in alcohol-butanol fracties van de bloemen van *Potentilla fruticosa* en in een alcoholisch bladextract van dezelfde plant zijn bepaald. Het bladextract van *Geranium* en het bloemextract van *Potentilla* bevatten aanzienlijk meer radicaal vangende verbindingen dan extracten van andere plantendelen.

Hogedruk vloeistof chromatografie (HPLC) direct gecombineerd met UV-detectie, vastefase extractie (SPE) gekoppeld aan kernspinresonantie (NMR) detectie en een radicaal vangende activiteitsbepaling werd gebruikt voor een snelle identificatie van radicaal vangende verbindingen in *Rhaponticum carthamoides* extracten. Middels deze geavanceerde nieuwe techniek was het mogelijk pieken in het chromatogram direct te koppelen aan actieve verbindingen en vervolgens te identificeren zonder voorafgaande preparatieve kolomchromatografie. Zeven verbindingen, nl. quercetagetine-7-*β*-glucopyranoside, quercetagetine-7-(6"-acetyl-*β*-glucopyranoside), 6-hydroxy-kaempferol-7-*β*-glucopyranoside, 6-methoxykaempferol-3-*β*-glucopyranoside, 6-hydroxy-kaemp-

ferol-7-(6"-acetyl-β-glucopyranoside), chlorogeenzuur en β-ecdyson, werden geïdentificeerd in alcoholische en waterige extracten. 6-Hydroxykaempferol-7-(6"-acetyl-β-glucopyranoside) werd nooit eerder beschreven in de literatuur en is een nieuwe natuurstof. De DPPH-radicaal neutraliserende activiteit werd bepaald en deze was zwakker dan die van de referentiestoffen rozemarijnzuur en Trolox.

De antioxidatieve activiteit van alle *Geranium macrorrhizum* en *Potentilla fruticosa* fracties werd nader bepaald met behulp van testen die gebruik maken van modelradicalen, in plantaardige oliën en in echte voedselsystemen die gebaseerd zijn op de houdbaarheid van cervelaatworst (hoofdstuk 6). De ethanol-butanol fractie van *Geranium macrorrhizum* liet de hoogste activiteit zien in de radicaaltesten en in de plantaardige olie testen. Met name werd in laatst genoemde test een sterke vermindering in de vorming van secundaire oxidatie producten zoals hexanal en geconjugeerde triënen waargenomen. De meeste radicaal vangers in deze fractie konden worden geïdentificeerd. De water-butanol fractie was het meest actief in het verminderen van primaire oxidatie producten in de peroxide-vormings test en in de testen met modelradicalen. De overige extracten waren minder actief in de testen met plantaardige oliën.

Potentilla extracten waren effectief in antioxidatieve testen (neutralisatie van superoxide anion radicaal en waterstof peroxide en β-caroteen ontkleuring) in polaire media. *Potentilla* extracten waren niet erg effectief in testen met plantaardige oliën.

De *Geranium* water-water fractie en de *Potentilla* ethanol-butanol fracties werden onderzocht met betrekking tot hun vertragende effect op het oxidatieve bederf van gefermenteerde worsten. Dit effect is vervolgens vergeleken met dat van een rozemarijnextract en een mengsel van kruiden die gewoonlijk in Nederland aan cervelaatworst wordt toegevoegd. De houdbaarheid van de worsten met het standaard kruidenmengsel was het langste. Dit betekent dat deze kruiden, vooral in combinatie met ascorbinezuur, een betere antioxidatieve werking vertoonden dan het rozemarijnextract en de *Potentilla* extracten. De onderzochte *Geranium* fractie was het minst werkzaam. De meest waarschijnlijke reden voor deze matige activiteit is een slechte oplosbaarheid in de vetfase. *Potentilla* extracten waren weliswaar actiever dan de *Geranium* extracten, maar minder actief dan het referentie rozemarijn extract. De toevoeging van ascorbinezuur verhoogde de activiteit van *Potentilla* extract en rozemarijn extract.

Er werd een oriënterend onderzoek verricht naar de genotoxische en mutagene eigenschappen van sommige van de bovengenoemde extracten. De uitgevoerde testen omvatten een bepaling van de enzymatische oxidatieve activiteit, effecten tegen door singlet zuurstof veroorzaakte hemolyse

van erythrocyten en bepaling van de mutageniteit. Geen van de onderzochte planten extracten was actief in deze testen.

Parallel aan het antioxidatieve onderzoek van *Geranium macrorrhizum* en *Potentilla fruticosa* werd ook de antimicrobiële activiteit van 1% alcoholische extracten van *Geranium macrorrhizum* bladeren onderzocht. Er werd activiteit gevonden tegen *Salmonella typhimurium*, *Listeria monocytogenes* en *Bacillus cereus*. Vergelijkbare extracten van *Potentilla fruticosa* waren actief tegen *Salmonella* en *Bacillus* species, en lieten een sterke bactericide werking zien tegen *Hafnia alvei* en *Listeria monocytogenes*[*].

Dezelfde extracten werden ook getest in langdurige oxidatietesten met plantaardige oliën (bepaling van peroxides). Uit de gebruikte koolzaadolie werden eerst de natuurlijke antioxidanten (vitamine E) verwijderd. Dit voorkwam eventuele neveneffecten tussen toegevoegde antioxidanten en antioxidanten die van nature aanwezig zijn. Dit lijkt overigens niet het geval te zijn omdat geen verschil in oxidatiesnelheid werd gevonden tussen gezuiverde en niet gezuiverde olie. Ook de water-butanol fractie van *Potentilla fruticosa* liet geen neveneffect zien. De ethanol-butanol fractie van *Geranium macrorrhizum* en de water-butanol fractie van *Potentilla fruticosa* werden onderzocht op de vorming van peroxides. Verschillende concentraties van extract toevoeging werden getest. De *Geranium* fractie in een concentratie van 0.05% was bijna even actief als in een concentratie van 0.1%. In een concentratie van 0.2% was deze fractie significant actiever. De *Potentilla* water-butanol fractie liet een ongeveer gelijke activiteit zien onafhankelijk van de geteste concentratie van 0.05, 0.1 of 0.2%.

Het onderzoek naar natuurstoffen, zoals bijv. antioxidanten uit planten, vereist de toepassing van verschillende analytische technieken. Niet alleen is zeer geavanceerde apparatuur nodig, ook moeten veel specifieke technieken toegepast worden in verschillende stadia van het onderzoek. Een voorbeeld is hogedruk vloeistofchromatografie direct gekoppeld met de bepaling van radicaal vangende activiteit bij de aantoning van antioxidatieve verbindingen in ingewikkelde plantenextracten. Deze bepalingen die gebruik maken van DPPH en ABTS modelradicalen zijn eerder ontwikkeld binnen het Laboratorium voor Organische Chemie van Wageningen Universiteit. De uitbreiding van een dergelijk system met een massaspectrometer was een onderdeel van het onderzoek en wordt beschreven in hoofdstuk 7. De massaspectrometer was in te passen in bovengenoemde opstelling via een simpele passieve splitter. Met behulp van dit uitgebreide systeem was een snelle analyse van complexe Ginkgo en rozemarijn extracten mogelijk. Gelijktijdige scheiding, identificatie en bepaling van de radicaal vangende activiteit van afzonderlijke

[*] Persoonlijke mededeling van A. Sipailiene 2004, Technische Universiteit van Kaunas, Kaunas, Litouwen.

componenten werd mogelijk. In het bijzonder de combinatie van diode array detectie en electrospray massaspectrometrie was effectief voor de identificatie van het merendeel van de verbindingen. Deze uitgebreidere methode versnelt de structuuropheldering van componenten in complexe plantenextracten en vermijdt een moeizame isolatie en identificatie van al bekende verbindingen.

Het gekoppelde HPLC-DAD-SPE-NMR systeem is een andere krachtige techniek voor de scheiding en identificatie van verbindingen in mengsels. De mogelijke combinatie van LC-DAD-SPE-NMR met een semi-preparatieve HPLC kolom en vergelijking van de verkregen resultaten met die verkregen middels een meer gebruikelijke analytische kolom wordt beschreven in hoofdstuk 8. Gevonden werd dat het gebruik van een semi-preparatieve HPLC kolom in combinatie met SPE-NMR (met apolaire SPE concentreringskolommen) voordelen heeft boven het gebruik van een analytische kolom wanneer mengsels die bestaan uit middelpolaire tot apolaire verbindingen worden geanalyseerd. Voorbeelden van dergelijke verbindingen zijn quercetine, santonine, methyl salicylaat en geranylzuur (log P = 1.48 - 3.70). De hogere belaadbaarheid van een semi-preparatieve kolom geeft een betere HPLC scheiding indien gelijke hoeveelheden worden geïnjecteerd en dit leidt weer tot betere NMR spectra. De toepassing van semi-preparatieve kolommen heeft geen voordelen wanneer polaire verbindingen worden geanalyseerd omdat dergelijke verbindingen een lage affiniteit hebben voor de apolaire SPE concentreringskolommen en derhalve niet geconcentreerd kunnen worden.

Hoofdstuk 9 geeft een algemene discussie van alle verkregen resultaten en tevens enkele conclusies en aanbevelingen voor verder onderzoek. Het is onwaarschijnlijk dat *Geranium* en *Potentilla* extracten toegepast kunnen worden als antioxidatief additief in vetrijke levensmiddelen. De extracten waren wel effectief in testen met meer polaire media, echter ook daar lijkt een toepassing niet waarschijnlijk. Redenen zijn de geur en kleur van de extracten en de bewerkelijke isolatieprocedure.

SĄNTRAUKA

Maisto molekulės (lipidai, baltymai, angliavandeniai) gali plačiai dalyvauti oksidacijos reakcijose. Šios reakcijos, sukeliamos taip vadinamų reaktyvių deguonies darinių (RDD), yra svarbi maisto produktų gedimo priežastis. Jeigu maiste yra daug lipidų, gedimas apibūdinamas apkartimu. Reakcijų metu keičiasi produkto spalva, tekstūra ir mitybinė vertė. Galiausiai dėl oksidacijos produktas gali visiškai sugesti. RDD sukeltos oksidacinės reakcijos taip pat turi įtakos gyvų organizmų senėjimo procesams ir gali būti daugelio ligų, tokių kaip širdies vainikinių kraujagyslių susirgimų, vėžio, kataraktos priežastimi. Nors gyvose ląstelėse, taip pat ir maisto produktuose veikia apsauginiai antioksidaciniai mechanizmai, dažnai reikia juos stiprinti. Kasdienis antioksidantų vartojimas gali apsaugoti nuo RDD arba sulėtinti RDD sukeltų problemų atsiradimą.

Šio darbo tikslas yra įvertinti kelis tikslingai atrinktus augalus kaip galimus maisto antioksidantų šaltinius. 1-ame skyriuje pateikiama antioksidantų klasifikacija, aprašomi antioksidantų šaltiniai, palyginami sintetiniai ir natūralūs antioksidantai, taip pat aprašomi antioksidantų įvertinimo metodai.

DPPH ir ABTS radikalų sujungimo metodais (2-as skyrius) buvo atliktas pirminis šių Lietuvoje auginamų medicininių ir aromatinių augalų ekstraktų įvertinimas: *Salvia sclarea*, *Salvia glutinosa*, *Salvia pratensis*, *Salvia officinalis, Lavandula angustifolia*, *Calendula officinalis*, *Matricaria recutita*, *Echinacea purpurea*, *Rhaponticum carthamoides*, *Juglans regia*, *Melilotus officinalis*, *Geranium macrorrhizum* ir *Potentilla fruticosa.* Nustatyta, kad stambiašaknio snapučio (*Geranium macrorrhizum*) ir krūminės sidabražolės (*Potentilla fruticosa*) ekstraktai efektyviai sujungė radikalus: jų radikalų sujungimo geba buvo stipresnė negu vaistinio šalavijo (*Salvia officinalis*) ekstraktų. Kitų *Salvia* rūšių ir paprastojo rapontiko (šlamagraižės leuzėjos) (*Rhaponticum carthamoides*) ekstraktai taip pat pasižymėjo stipriomis antiradikalinėmis savybėmis. Kadangi stambiašaknis snaputis, krūminė sidabražolė ir paprastasis rapontikas iki šiol buvo mažai tyrinėti augalai, jie buvo atrinkti tolimesniems tyrimams. Augalų ekstraktų radikalų sujungimo geba koreliavo su bendru fenolinių junginių kiekiu ekstraktuose.

Kitas darbo etapas buvo skirtas stambiašaknio snapučio lapų, krūminės sidabražolės žiedų ir paprastojo rapontiko lapų ekstraktų antiradikalinių junginių struktūrų nustatymui (skyriai 3, 4 ir 5). Septyni junginiai identifikuoti įvairiose snapučio frakcijose: galo rūgštis, elago rūgštis, 4-galoilchinono rūgštis, flavonoidas kvercetinas ir trys jo glikozidai – kvercetin-3-*β*-gliukopiranozidas, kvercetin-3-*β*-galaktopiranozidas ir kvercetin-4'-*β*-gliukopiranozidas. Visi

junginiai, išskyrus galo rūgštį ir kvercetiną, identifikuoti šiame augale pirmą kartą. Dvylika junginių identifikuota sidabražolės žiedų ekstraktuose – elago rūgštis, (+)-katechinas, kvercetin-3-*β*-gliukopiranozidas, kvercetin-3-*β*-galaktopiranozidas, kvercetin-3-*β*-rutinozidas, kvercetin-3-*β*-gliukuronopiranozidas, kvercetin-3-*α*-arabinofuranozidas, kampferol-3-*β*-rutinozidas, kampferol-3-O-*β*-(6"-O-(E)-*p*-kumaroil) gliukopiranozidas, ramnetin-3-*β*-gliukopiranozidas ir ramnetin-3-*β*-galaktopiranozidas. Visi junginiai, išskyrus elago rūgštį, katechiną ir kvercetin-3-*β*-galaktopiranozidą nebuvo anksčiau identifikuoti sidabražolės žieduose.

Junginių, išskirtų iš stambiašaknio snapučio ir krūminės sidabražolės, radikalų sujungimo geba išmatuota taikant $DPPH^{\bullet}$ ir $ABTS^{\bullet+}$ sujungimo metodus; gauti rezultatai palyginti su rozmarinų rūgšties ir Trolokso geba sujungti radikalus. Kvercetin-3-gliukopiranozidas, kvercetin-3-galaktopiranozidas ir elago rūgštis pasižymėjo stipriausiomis antiradikalinėmis savybėmis tarp identifikuotų snapučio junginių; kvercetin-3-gliukozidas ir katechinas buvo stipriausi radikalų sujungėjai krūminės sidabražolės ekstraktuose.

Identifikuotų antioksidantų kiekiai palyginti skirtingose *Geranium macrorrhizum* ir *Potentilla fruticosa* anatominėse dalyse. Palyginus snapučio etanolinius lapų ir šaknų ekstraktus ir sidabražolės etanolinę-butanolinę žiedų ekstraktų frakciją bei lapų ekstraktą, nustatyta, kad antiradikalinių junginių kiekis snapučio lapų ir sidabražolės žiedų ekstraktuose buvo žymiai didesnis nei kitose augalų anatominėse dalyse.

Junginių iš paprastojo rapontiko ekstraktų identifikavimui panaudotas kompleksinis SC-DRD-KFE-BMR (LC-DAD-SPE-NMR) įrenginys, kuriuo galima įvertinti junginių antiradikalinį aktyvumą ekstrakto srauto analizės metu (*on-line*). Tokiu būdu pavyko selektyviai aptikti ir identifikuoti ekstrakto radikalų sujungėjus nenaudojant jokių išankstinių chromatografinių skirstymų. Etanoliniuose ir vandeniniuose augalo ekstraktuose identifikuoti septyni junginiai: kvercetagetin-7-*β*-gliukopiranozidas, kvercetagetin-7-(6"-acetil-*β*-gliukopiranozidas), 6-hidroksi-kampferol-7-*β*-gliukopiranozidas, 6-metoksikampferol-3-*β*-gliukopiranozidas, chlorogeno rūgštis, *β*-ekdizonas ir 6-hidroksikampferol-7-(6"-acetil-*β*-gliukopiranozidas). Pastarasis – pirmą kartą aptiktas natūralus junginys. Šio junginio antiradikalinis aktyvumas išmatuotas taikant DPPH radikalų sujungimo metodą ir nustatyta, kad jo antioksidacinės savybės silpnesnės negu palyginimui naudotų antioksidantų – rozmarinų rūgšties ir Trolokso.

Antioksidacinės stambiašaknio snapučio ir krūminės sidabražolės ekstraktų savybės tirtos modelinėse ir realiose maisto sistemose – aliejuje bei fermentinėse dešrelėse (6-as skyrius). Snapučio etanolinė-butanolinė frakcija pasižymėjo stipriausiu antioksidaciniu aktyvumu daugumoje

modelinių sistemų, o taip pat aliejuje, ypač stabdant antrinių oksidacijos produktų – heksanalio ir konjuguotų trienų susidarymą. Dauguma radikalų sujungėjų taip pat buvo aptikti šioje frakcijoje. Vandens-butanolinė frakcija ženkliai sulėtino pirminių oksidacijos produktų – peroksidų susidarymą, ji taip pat buvo efektyvi modelinėse reakcijose. Kiti ekstraktai buvo mažiau efektyvios medžiagos oksidacijos procesų atžvilgiu.

Sidabražolės ekstraktai pasižymėjo efektyviomis antioksidacinėmis savybėmis taikant metodus su poline reakcijos terpe (superoksido anijonų, vandenilio peroksido sujungimo testuose, *β*-karotino blukinimo teste). Sidabražolės ekstraktai pasižymėjo nestipriu antioksidaciniu aktyvumu aliejuje.

Snapučio vandeninės-vandeninės ir sidabražolės etanolinės-butanolinės frakcijų antioksidacinis aktyvumas išbandytas fermentuotose dešrelėse ir palygintas su rozmarinų ekstraktų ir tradicinių olandiškų servelato dešrelių gamyboje naudojamo prieskonių mišinio aktyvumu. Oksidacija lėčiausiai vyko bandinyje su prieskonių mišiniu: prieskoniai, naudojant juos kartu su askorbatu, pasižymėjo stipresniu antioksidaciniu aktyvumu negu rozmarinų ar sidabražolės ekstraktai. Tirtos snapučio frakcijos pasižymėjo silpniausiu aktyvumu, tikriausiai dėl nepakankamo ekstrakto frakcijos tirpumo riebalinėje dešrelės fazėje. Sidabražolės ekstrakto aktyvumas buvo didesnis nei snapučio, tačiau mažesnis nei rozmarinų ekstrakto. Bendras sidabražolės ir rozmarinų ekstraktų aktyvumas buvo didesnis naudojant juos kartu su askorbatu.

Darbe taip pat atlikti preliminarūs kai kurių ekstraktų toksikologiniai tyrimai taikant fermentinės oksidacijos testus bei įvertinant ekstraktų mutageniškumą ir aktyvumą prieš singletinio deguonies sukeltą eritrocitų hemolizę. Nustatyta, kad tirti ekstraktai nėra genotoksiški ar mutageniški.

Be tyrimų, kurie išsamiai aprašyti šiame darbe, taip pat atlikti papildomi tyrimai siekiant įvertinti stambiašaknio snapučio ir krūminės sidabražolės ekstraktų panaudojimo įvairiais tikslais galimybes. Mikrobiologiniai tyrimai parodė, kad 1% etanoliniai snapučio ekstraktų tirpalai slopino kai kurių patogeniškų mikroorganizmų (*Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus)* vystimąsi. Panašūs sidabražolės ekstraktai taip pat buvo veiksmingi prieš *Salmonella* ir *Bacillus* rūšis, o *Hafnia alvei* ir *Listeria monocytogenes* atžvilgiu pasižymėjo stipriu baktericidiniu aktyvumu[†].

Ekstraktai taip pat tirti rafinuotame aliejuje įvertinant jų poveikį aliejaus peroksidų skaičiui. Iš aliejaus buvo pašalinti jame natūraliai esantys antioksidantai tokoferoliai. Tokiu būdu išvengta galimų sąveikų tarp natūraliai esančių ir pridėtų antioksidantų. Nustatyta, kad išvalytame aliejuje augalų ekstraktų aktyvumas (išreikštas indukciniu periodu) buvo toks pats kaip ir nevalytame

[†] Iš asmeninio pasikalbėjimo su A. Šipailiene 2004, Kauno Technologijos Universitetas, Kaunas.

aliejuje. Tai reiškia, kad augalų ekstraktai nepasižymi sinergetiniu poveikiu su natūraliai aliejuje esančiais tokoferoliais. Sidabražolės vandeninė-butanolinė frakcija taip pat nepasižymėjo sinergetiniu poveikiu. Įvertintas augalų ekstraktų skirtingų koncentracijų poveikis peroksidų susidarymui (tirta etanolinė-butanolinė snapučio ir vandeninė-butanolinė sidabražolės frakcijos). Nustatyta, jog 0,05 ir 0,1 % koncentracijų snapučio ekstraktų antioksidacinis aktyvumas buvo labai panašus, tačiau esant 0,2 % koncentracijai antioksidacinis poveikis ženkliai padidėjo. Tuo tarpu 0,05, 0,1 ar 0,2 % sidabražolės ekstraktų priedų poveikis buvo beveik vienodas.

Natūralių junginių, tarp jų ir augalų antioksidantų, tyrimui naudojami įvairūs analitiniai instrumentai. Be serijiniu būdu gaminamų instrumentų tam tikrose tyrimų stadijose naudojama specialios paskirties aparatūra, pvz. skirta tiesioginiam (iš srauto) junginių antiradikaliniam aktyvumui įvertinti. Metodai greitam DPPH/ABTS radikalų sujungimui įvertinti (taikant efektyviosios skysčių chromatografijos su ultravioletinės absorbcijos diodų detektoriumi aparatūrą), buvo sukurti ir plačiai naudojami Vageningeno universiteto Organinės Chemijos laboratorijoje. Todėl viena šio darbo dalis buvo skirta minėtų metodų išplėtimui papildomai prijungiant prie naudojamo aparatūros komplekso masių spektrometrą (7-as skyrius). Masių detektorius buvo prijungtas naudojant paprastą inertišką srautų skirtuvą. Išplėsta sistema atvėrė galimybes greitai ištirti sudėtingus ginkmedžio ir rozmarinų ekstraktus, vienos analizės metu išskirstant daugumą ekstraktų junginių, juos identifikuojant ir įvertinant jų antiradikalinį aktyvumą. Masių ir UV diodų detektorių panaudojimas viename aparatūros komplekte yra efektyvus būdas daugumos ekstraktų junginių identifikavimui. Išplėstas metodas pagreitina identifikavimo procesus ir suteikia galimybę išvengti pakartotino jau žinomų junginių išskyrimo ir identifikavimo.

Kompleksinė SC-DRD-KFE-BMR (LC-DAD-SPE-NMR) sistema yra kita veiksminga priemonė ekstraktų junginių išskirstymui ir junginių struktūrų nustatymui. 8-ame skyriuje aprašytas SC-DRD-KFE-BMR sistemos įvertinimas naudojant pusiau-paruošiamąją ESC kolonėlę; gauti rezultatai palyginti su rezultatais gautais naudojant analitinę kolonėlę. Nustatyta, kad pusiau paruošiamoji kolonėlė KFE-BMR sistemoje kartu su atvirkštinių fazių sorbento šerdelėmis kietos fazės ekstraktoriuje yra pranašesnė nei analitinė kolonėlė tiriant vidutiniškai polinius arba nepolinius junginius, tokius kaip kvercetinas, santoninas, metilsalicilatas, gerano rūgštis (log P = 1.48 – 3.70). Didesnis galimas pusiau paruošiamosios kolonėlės apkrovimas, naudojant vienodus tiriamo mišinio kiekius turi įtakos efektyvesniam smailių išskirstymui; gaunamas geresnės rezoliucijos BMR spektras. Tiriant polinius junginius, pusiau paruošiamoji kolonėlė nėra pranašesnė, kadangi polinių junginių trauka kolonėlės nejudančiai fazei yra labai silpna.

9-ame skyriuje pateikiama apibendrinanti diskusija, baigiamosios darbo pastabos ir kelios idėjos ateities tyrimams. Prieita išvados, kad snapučio ir sidabražolės ekstraktai negalėtų būti nauju antioksidantų šaltiniu daug riebalų turinčiuose maisto produktuose. Nors ekstraktai yra efektyvūs polinėse terpėse, tačiau jų pritaikymas polinės terpės produktuose taip pat vargu ar įmanomas. Pagrindinė priežastis - intensyvi ekstraktų spalva, specifinis kvapas ir sudėtinga ekstrakcijos procedūra. Tačiau tokie ekstraktai galėtų būti toliau tiriami siekiant juos pritaikyti funkcinių maisto gaminių, maisto papildų, farmacijos bei kosmetikos preparatų gamyboje.

ABBREVIATIONS

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

APCI – atmospheric pressure chemical ionization

AE – antioxidant efficiency

BHA – butylated hydroxyanisole

BHT – butylated hydroxytoluene

BuOH – butanol

COLOC – correlation via long-range coupling

COSY – correlated spectroscopy

DAD – diode array detector

DPPH – 2,2- diphenyl-1-picrylhydrazyl hydrate (free radical)

EB – ethanol butanol

EDTA – ethylenediaminetetraacetic acid

ESI – electrospray ionization

ESR – electron spin resonance

EtOH – ethanol

EW – ethanol water

FID – flame ionization detector

GAE– gallic acid equivalents

GC – gas chromatography

HMBC – heteronuclear multiple bond coherence

HPLC – high performance liquid chromatography

HS-GC – head space gas chromatography

IP – induction period

LC – liquid chromatography;

MDA - malondialdehyde

MS – mass spectroscopy

NBT – nitro blue tetrazolium

NMR – nuclear magnetic resonance

MeOH – methanol

PG – propyl gallate

PV – peroxide value

RA – rosmarinic acid

RE I, II – rosemary extract

RE – rutin equivalent

ROS – reactive oxygen species

RP-HPLC – reversed phase high performance liquid chromatography

RP-MPLC – reversed phase medium pressure liquid chromatography

RSA – radical scavenging activity

R.T. – retention time

SOD – superoxide dismutase

SPE – solid phase extraction

TBA – thiobarbituric acid

TCA – trichloroacetic acid

TFA – trifluoroacetic acid

TBARS – thiobarbituric acid reactive substances

tBuMeO – *tert*-butyl methyl ether

TBHQ – tertiary butylhydroquinone

TEAC – Trolox equivalent antioxidant capacity

TIC – total ion current

TOCSY – total correlation spectroscopy

WB – water butanol

WW – water water

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Giedrius

CURRICULUM VITAE

Giedrius Miliauskas was born on February 23, 1976 in Kaunas, Lithuania in the family of Algirdas Miliauskas and Genovaitė Miliauskienė. During the years 1983 – 1994 he attended 38th Secondary School in Kaunas, Lithuania.

In 1994 he started studies at Kaunas University of Technology (KTU), Faculty of Chemical Technology. In 1998 he obtained the bachelor degree in Food Technology (specialization – fermentation technology). During 1998 - 2000 he continued university studies and obtained his M.Sc. degree in Food Chemistry and Technology of KTU.

In September 2000, he started Ph.D. studies in KTU, Departament of Food Technology. In September 2001 he was admitted as a Ph.D. candidate for a so-called "sandwich" research project between the Department of Food Technology of KTU and two laboratories (Laboratory of Organic Chemistry and Laboratory of Food Chemistry) in the Department of Agrotechnology and Food Sciences of Wageningen University, the Netherlands. The research area covered the investigation of biologically active substances (antioxidants) from aromatic and medicinal plants (investigation of composition, evaluation of antioxidant activity and application possibilities).

From June 2005 onwards he is working as a chemist-technologist in the Lithuanian company "Filtpakas".

LIST OF PUBLICATIONS

PUBLICATIONS RELATED TO THE THESIS WORK

1. Miliauskas, G., Venskutonis, P.R. and van Beek, T.A., *Screening of radical scavenging activity of some medicinal and aromatic plant extracts*. Food Chem., 2004. **85**: p. 231-237.
2. Miliauskas, G., van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H. and de Waard, P., *Antioxidant activity of Geranium macrorrhizum L*. Eur. Food Res. Technol., 2004. **218**: p. 253-261.
3. Miliauskas, G., van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H. de Waard, P. and E.J.R. Sudhölter., *Antioxidant activity of Potentilla fruticosa L*. J. Sci. Food Agric., 2004. **84**: p. 1997-2009.
4. Miliauskas, G., van Beek, T.A., de Waard, P., Venskutonis, P.R. and Sudhölter, E.J.R., *Identification of radical scavenging compounds in Rhaponticum carthamoides by means of LC-DAD-SPE-NMR.* J. Nat. Prod., 2005. **68**: p. 168-172.
5. Miliauskas, G., van Beek, T.A., de Waard, P., Venskutonis, R.P. and Sudhölter, E.J.R., *Comparison of analytical and semi-preparative columns for high-performance liquid chromatography–solid-phase extraction–nuclear magnetic resonance.* J. Chromatogr. A., 2006. **1112**: p. 276-284.

OTHER PUBLICATIONS

6. Miliauskas, G., Venskutonis, P.R. and Sivik, B., *Extraction of Aromatic and Medicinal Plants with Supercritical Carbon Dioxide*, in *Food Technology and Quality Evaluation*, R. Dris and A. Sharma, Editors. 2003, Science Publishers. p 209-213.
7. Miliauskas, G. and Venskutonis, P.R., *Investigation of Lemon Balm* (*Mellisa officinalis*) *extracts by LC-MS.* Maisto Chemija ir Technologija. 2004. p. 121-125.
8. Miliauskas, G. and Venskutonis, P.R., *Įvairiais metodais išskirtų melisos ir katžolės ekstraktų sudėtis [Composition of Lemon Balm and Catnip extracts obtained by various methods]*. Maisto Chemija ir Technologija. 2001. p. 47-51.
9. Miliauskas, G., Venskutonis, R. and Sivik, B., *Aromatinių ir vaistinių augalų ekstraktų išeigų, išskirtų skirtingais būdais, palyginimas [Isolation of extracts from different aromatic herbs by various methods]*. Maisto Chemija ir Technologija. 2000. p. 107-111.

10. Vinauskienė, R., Venskutonis, R. and Miliauskas, G., *Lakiųjų vištienos junginių tyrimo metodų įvertinimas [Evaluation of analysis methods of volatile poultry compounds]*. Maisto Chemija ir Technologija. 1999. p. 73-74.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

- International young scientists symposium "Future Trends in Phytochemistry". Poster presentation „Identification of radical scavenging compounds in *Rhaponticum carthamoides* by means of LC-DAD-SPE-NMR", Gargnano, Italy, 2004;
- „1st International Conference on Polyphenols and Health". Poster presentation „Radical scavenging compounds in *Potentilla fruticosa*", Vichy, France, 2003;
- "Food Chemistry and Technology", oral presentation, Kaunas University of Technology, Lithuania, 2003;
- "Annual NWO meeting Analytical Chemistry", Lunteren, The Netherlands, 2003;
- VLAG International Advanced Course "Chemistry and biochemistry of antioxidants, their effects on health and disease, and risk evaluation of their use as food ingredient", Wageningen, The Netherlands, 2002;
- VLAG International advanced course "Advanced Food Analysis", Wageningen, The Netherlands, 2002;
- International young scientists symposium "Future Trends in Phytochemistry". Poster presentation „Radical scavenging compounds in *Geranium macrorrhizum*", Gargnano, Italy, 2002;
- "Food Chemistry and Technology", oral presentation, Kaunas University of Technology, Lithuania, 2001;
- PhD meetings at Wageningen University and Kaunas University of Technology.

N.B. The two symposia in Italy have the character of a spring school. Attendance was supported by VLAG.

General courses

- SOCRATES short course "Identification of volatile and aroma active compounds in food", Kaunas University of Technology, Lithuania, 2002 and 2004;
- Short course "Introduction to Sensory Analysis", Kaunas University of Technology, the Food Institute, Lithuania, 2003;
- Socrates intensive programme "Agriculture: source of raw material for industry", Ghent University, Belgium, 2001.

Optional courses and activities

- PhD study tour to USA, California. Visiting University of California (Davis, Santa Cruz, Berkeley), Stanford University, companies, 2005;
- PhD study tour to Switzerland. Visiting Technology Institutes in Zürich, Lausanne, companies, 2003;
- Preparation of PhD research proposal.

Propositions

The finding that the standard spices mix of pepper, clove, garlic and bell peppers is more active – although at a thrice higher concentration – than the positive control or any tested extract in the fermented sausage accelerated oxidation assay warrants further research with regard to the active principles and the mode of action of this mix.

This thesis.

The overall polar/non-polar character of a compound as characterised by its octanol/water partition coefficient does not allow making a fool-proof prediction whether an analyte can be trapped on hydrophobic cartridges in an LC-SPE-NMR set-up.

This thesis.

The best way to get sufficient antioxidants is through the food you eat, rather than by taking supplements.

www.health.gov

The accuracy of the DPPH radical scavenging assay could be improved if reduced 1,1-diphenyl-2-picrylhydrazyl reagent would be available as a blank instead of methanol.

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

The equation proposed by Chevolleau *et al*. for the coupled β-carotene - linoleic acid antioxidant assay is inaccurate as the intrinsic colour of the plant extracts can distort the absorbance measurements.

Chevolleau, S., Mallet, J.F., Ucciani, E., Gamisans, J. and Gruber, M. Antioxidant activity in leaves of some Mediterranean plants. JAOCS, 1992. ***69****: p. 1269-1271.*

If one's Ph.D. period does not give sensational scientific results, it will almost surely give a lot of new friends.

Looking for peace of mind in life is also some kind of research.

Propositions belonging to the thesis entitled:
*"*Screening, isolation and evaluation of antioxidative compounds from *Geranium macrorrhizum*, *Potentilla fruticosa* and *Rhaponticum carthamoides"*
Giedrius Miliauskas, Wageningen, November 20, 2006