Isolation, identification and activity of natural antioxidants from sweet grass (*Hierochloe odorata*), costmary (*Chrysanthemum balsamita*) and horehound (*Marrubium vulgare*), cultivated in Lithuania

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Contents

1. Introduction

1.1. Oxidation and Autoxidation.

Electron transfer is one of the most fundamental processes in chemistry. The passage of an electron or a pair of electrons from a donor (reducing species) to an acceptor (oxidizing species) results in a change in properties for both partners in the reaction. Oxidation was once defined as the incorporation of oxygen into a substance, but now can be more precisely defined as the conversion of a chemical substance into another having fewer electrons. The propensity of chemical compounds to undergo reduction or oxidation has been studied for nearly 300 years, probably beginning with the Becher-Stahl theory of combustion, popularly known as the "phlogiston theory," [1] formulated in the early 18th century. Its followers believed that every combustible substance contained a "principle of fire," phlogiston, that was given up during burning. Oils, for example, burned almost completely and were therefore, in these terms, practically pure phlogiston. The theory was decisively overturned by the end of that century due to Lavoisier's quantitative demonstrations that products of combustion actually weighed more than the starting material, and Priestley's discovery of oxygen [1].

Virtually all substances made up entirely or in part of organic carbon decompose over time. Wood, plastics, petroleum, leather, paper, paints, waxes, etc., all undergo oxidative decomposition reactions at various rates. Oxidation induces many chemical and physical changes in a product, which may include changes in viscosity, brittleness, discolouration, surface cracking, and loss of impact or tensile strength. Oxidative decomposition may be initiated by many events: thermal processes, absorption of gamma rays, high-energy ultraviolet photons, ozone- or metal ion-induction. Additionally substances are differentially susceptible to oxidative damage. For food substances, one of the main problems of deterioration is the oxidation of lipids, and for this reason the topic of lipid oxidation has raised great interest in recent years.

1.2. Autoxidation of Lipids.

Oxidation of polyunsaturated fatty acids is one of the most fundamental reactions in lipid chemistry (Fig. 1.1). In the presence of initiators, unsaturated lipids (LH) form alkyl radicals ($L^{\bullet}$) and peroxyl radicals ($LOO^{\bullet}$), which propagate in the presence of oxygen by a free radical chain mechanism to form hydroperoxides (LOOH) as primary products of oxidation. In this propagation step another alkyl radical is produced, so the reaction is autocatalytic [2]. In the presence of light,

unsaturated fats can also form hydroperoxides by reacting with singlet oxygen produced by sensitized photooxidation, which is a non-radical process [3].

Lipid hydroperoxides readily decompose into a wide range of carbonyl compounds, hydrocarbons, and other compounds which are often responsible for the flavour deterioration of foods. Also these compounds may cause cellular damage in the body [4]. To decrease the rate of deterioration of food products due to oxidation of fats, antioxidants are used. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) or gallates (Fig. 1.2) have been widely used as food antioxidants for many years. An extensive review on the toxicity of BHA and BHT has been published [5] and it was concluded that BHA and BHT are not presenting any hazard to humans at current food additive levels. However, these synthetic antioxidants, when used at high concentrations had some tumour promoting activity in animals [5] and therefore in recent years the research on natural antioxidants has increased.

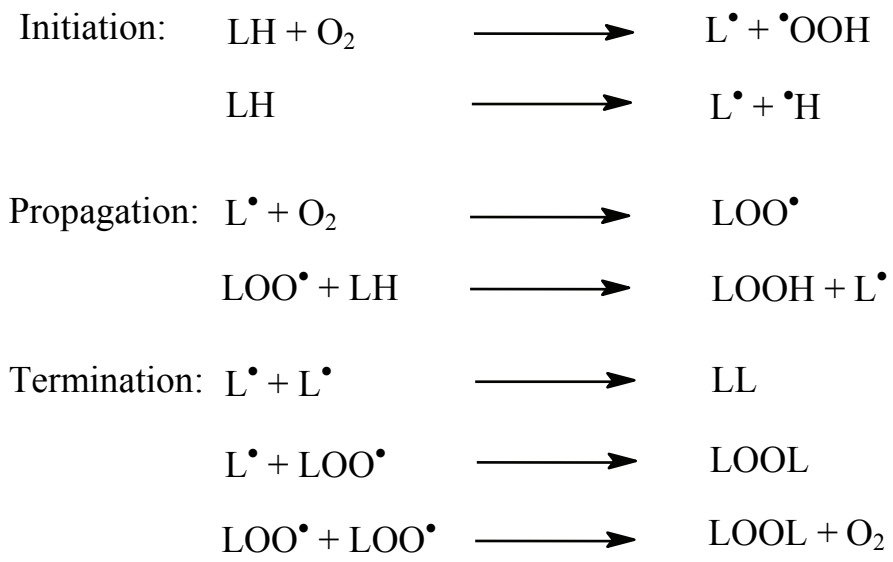


Fig. 1.1. Schematic representation of lipid oxidation

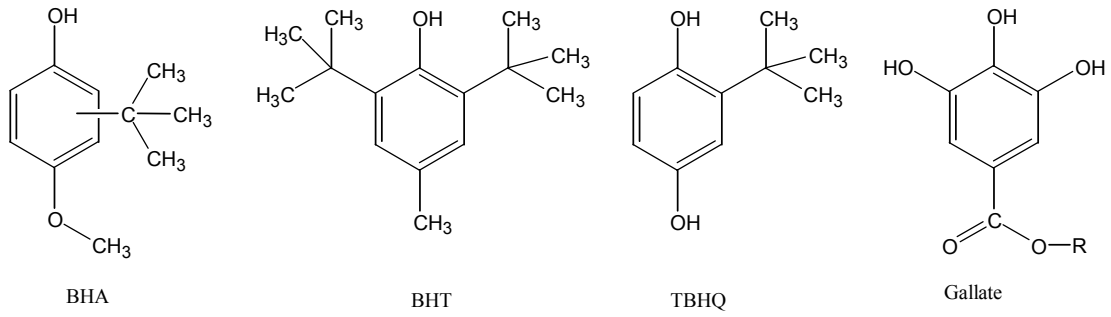


Fig. 1.2. Chemical structures of some synthetic antioxidants

1.3. Natural antioxidants.

All antioxidants can be broadly classified by their mechanism of action as primary antioxidants or as secondary antioxidants. Some antioxidants have more than one mode of action and are often referred to as multiple-function antioxidants.

Primary, type 1, or chain-breaking antioxidants are free radical scavengers that delay or inhibit the initiation step or interrupt the propagation step of autoxidation. In addition to this radical scavenging, primary antioxidants (AH) can reduce peroxyradicals to hydroperoxides compounds:

$$LOO^{\bullet} + AH \longrightarrow LOOH + {}^{\bullet}A$$

Secondary, or preventive, antioxidants slow the rate of oxidation by several different actions, but they do not convert free radicals to more stable products. Secondary antioxidants can chelate prooxidant metals and deactivate them, regenerate primary antioxidants, decompose peroxide radicals to non-radical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers. These antioxidants are often referred to as synergists because they enhance the antioxidant activity of primary antioxidants. Citric acid, ascorbic acid, ascorbyl palmitate, lecithin, and tartaric acid are good examples of such synergists. Beside these well known and commercially available antioxidants, there are still a lot of potential antioxidants under investigation. Most of them are phenolic compounds. They act by the mechanism shown in figure 1.3.

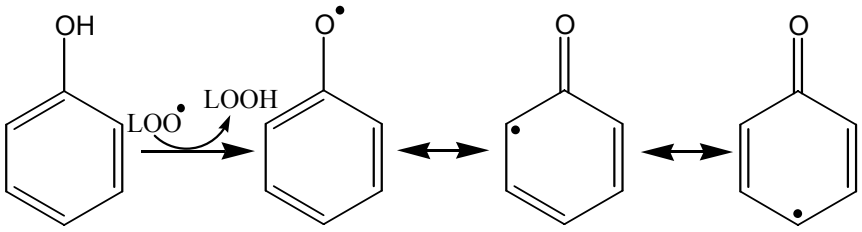


Fig. 1.3. Antioxidant mechanism of phenolic antioxidants

The most suitable oxidation inhibitors are common food ingredients, or plant extracts, as their use is not limited by legislation. Many foods contain compounds that possess antioxidant activity, but some of these additives are of limited use as they impart a specific flavour, aroma or colour to the finished product. Furthermore, compounds that have low antioxidant activity or low solubility in lipids are of limited use in the stabilization of edible oils and fats, although they may be used in other fat containing foods [6].

1.4. Natural sources of antioxidant compounds.

Many antioxidants such as vitamin C, vitamin E and carotenoids, occur as dietary constituents. There are a lot of strong antioxidant compounds found in fruits and vegetables [7-9] and in different beverages [10-16]. For example, fair antioxidants have been found in berries [17-21], apples [22-25], citrus [26] and in fruit juices [27-29]. High activity antioxidants were found in olives [30-32] and olive oil [33-39]. Activity changes during the processing of olive oil have been evaluated [40, 41]. Many studies were carried out on antioxidant research in fruits, and changes of antioxidants during fruit processing [27, 42, 43]. The effects of processing have been evaluated also on the changes of antioxidant activity in some roasted cereal products [44].

Red wines contain a variety of polyphenolic compounds, the most abundant being anthocyanins, and they have been shown to have high antioxidant activity [45-49]. However, not all polyphenols are extracted from grapes during the wine production process. Among the best known and most biologically active are resveratrol, quercetin and the catechins. It has been reported, that grape seeds [50] and grape pomace peels [51] still contain antioxidants, so wine production draff can be considered as a source of antioxidants. Antioxidant activity was also reported in whiskys [52, 53]. Green and black teas have been extensively studied for antioxidant properties [12, 14, 54-56]. The main compounds responsible for antioxidant activity were found to be catechins [16]. (–)-Epigallocatechin 3-gallate, (–)-epigallocatechin, (–)-epicatechin 3-gallate, (–)-epicatechin, (+)-gallocatechin and (+)-catechin were identified and their antioxidant activities have been studied [57-59].

Also herbs and spices are good sources of antioxidants [60-64]. Extensive research has been performed in this area, but only some extracts from rosemary and sage are available as commercial antioxidants [65, 66]. The main problem in the application of such extracts is that usually they have a specific odour, taste or colour, which in most cases is undesirable in the final product. Good examples of this are commercial products of garlic and ginger [67]. Therefore there have been attempts to deodorise extracts, to obtain odourless extracts having antioxidative properties [68, 69]. A great number of different spices and aromatic herbs have been tested for their antioxidant activity, with rosemary and sage being the most investigated [70-75]. However many more herbs and spices have never been examined in this respect.

Although microorganisms are among the most abundant species on earth, until the early 80's there was no interest in the search for antioxidant compounds in microorganisms. Since that time a number of investigations were carried out in this area and a number of antioxidants were found in Aspergillus species and subsequently evaluated [76-78]. Antioxidants were also detected in products fermented by Aspergillus [79]. Penicillium species also contain antioxidants [80].

Nowadays, consumers ask more for natural products, therefore research in the area of natural compounds is growing. It should be noted however, that natural is not identical with safe. Therefore also natural compounds must be tested for safety aspects before applying them in foods for human consumption.

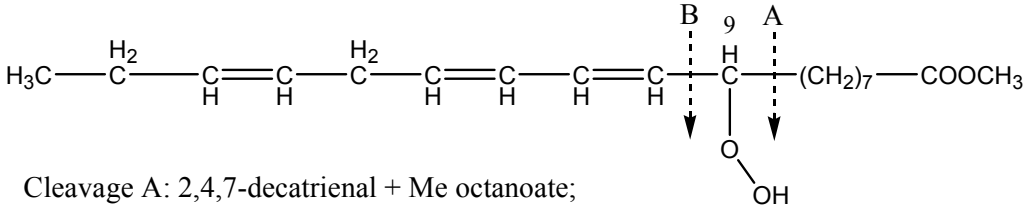
1.5. Methods for evaluation of antioxidants.

Antioxidants act by several mechanisms, therefore different methods are used for their detection and activity evaluation. This diversity of test methods makes a comparison of results obtained by different researchers difficult. Two main types of antioxidant activity testing can be distinguished: assays to evaluate oxidation of fats, oils and other fat containing foods; and assays to evaluate radical scavenging activity in model systems.

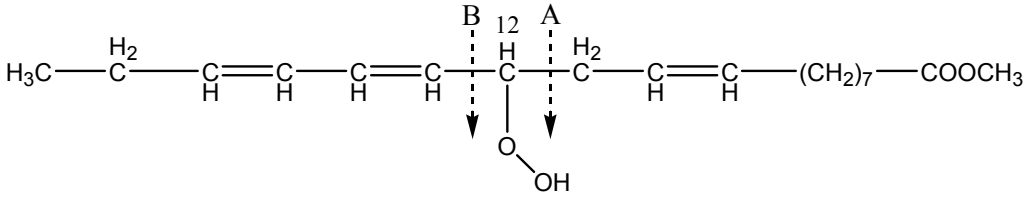
There are various methods available to measure lipid oxidation in foods. Changes in chemical, physical, or organoleptic properties of fats and oils during oxidation can be monitored; however, there is no single method for assessing all oxidative changes in different food systems. To determine primary oxidation of fats, changes of fatty acid composition [81], weight gain at different time intervals [82, 83], amount of hydroperoxides [84], or conjugated dienes, which correlate well with peroxide values [85], can be monitored. Addition of antioxidants decreases oxidation rates of samples and the decrease can be expressed as the antioxidant activity. These methods require a lot of time and therefore are not convenient for screening purposes. Nowadays accelerated methods, such as Rancimat, active oxygen method (AOM), or OXIPRES method, are used for assessing the oxidative stability of fats and oils [86].

When oxidation proceeds, quantification of secondary oxidation products is more appropriate to evaluate product deterioration. Secondary oxidation products include aldehydes, ketones, hydrocarbons and alcohols. Determining the thiobarbituric acid value is one of the methods to evaluate the formation of malonaldehyde, 2-alkenals and 2,4-dienals, as secondary oxidation products [87]. Epoxides are also formed during autoxidation of fats and oils, and these can be determined by titration with hydrobromic acid. This method is called the Oxirane value and was standardized by the American Oil Chemists' Society. However, the assay is not sensitive and lacks specificity. To measure the content of unsaturated aldehydes in fats, the *p*-anisidine method can be used [88]. Carbonyls can also be determined by other spectroscopic, or gas chromatographic methods. Usually the amount of hexanal is determined [89]. However, recent studies have shown that during oxidation of marine oils that are rich in polyunsaturated fatty acids of the ω-3 type, large amounts of propanal are formed [90]. The peroxidation of (ω-3) fatty acids (linolenate, eicosapentaenoic and docosahexaenoic acids) produces various compounds depending on the location of the hydroperoxy

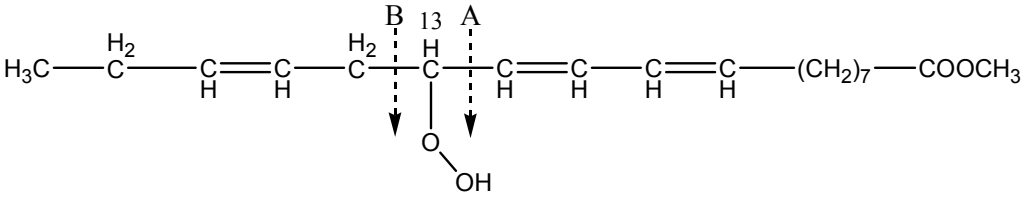
group in the primary oxidation products. The decomposition of these hydroperoxides can take place via homolytic or heterolytic fission. Thus, 9-OOH linolenate gives 2,4,7-decatrienal and 3,6-nonadienal, 12-OOH linolenate gives 2,4-heptadienal and 3-hexenal, 13-OOH linolenate gives 3-hexenal and 2-pentenal and, finally, 16-OOH linolenate gives propanal. Breakdown schemes of linolenate are shown in fig. 1.4.



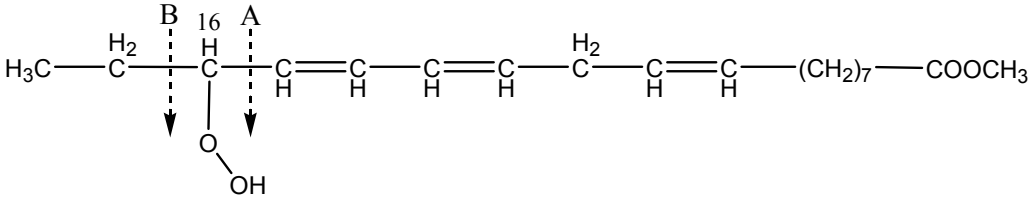
Cleavage A: 2,4,7-decatrienal + Me octanoate;
Cleavage B: 3,6-nonadienal + Me 9-oxononanoate;



Cleavage A: 2,4-heptadienal + Me 9-undecenoate;
Cleavage B: 3-hexenal + Me 12-oxo-9-dodecenoate;



Cleavage A: 3-hexenal + Me 12-oxo-9-dodecenoate;
Cleavage B: 2-pentene + 2-penten-1-ol + 2-pentenal + Me 13-oxo-9,11-tridecadienoate;



Cleavage A: propanal + Me 15-oxo-9,12-pentadecadienoate;
Cleavage B: ethane + Me 16-oxo-9,12,14-hexadecatrienoate;

Fig. 1.4. Cleavage products from 9-, 12-, 13- and 16-hydroperoxides formed by autoxidation of methyl linolenate [91].

All fat stability measuring methods are sample dependent, and the final result usually depends on sample type, amount and surface area. They require long sample storage times, so they are not applicable when results are needed fast (e.g. activity guided fractionation). For these purposes simpler model systems are used, as they provide fast, reproducible and equally informative results. Model systems can be divided into two groups: 1. partially simulating real systems; or 2. systems using synthetic reagents that normally do not occur in nature. The first ones mainly use linoleic acid as a substrate [92-94], a variety of oxidation acceleration factors and different detection techniques. The second ones mainly employ synthetic stable radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) [95], or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [96, 97]. Although these radicals do not exist in nature, it is assumed that compounds able to scavenge these radicals, possess also antioxidant activity. All strong natural antioxidants (vitamin C, vitamin E, carnosic acid) reduce DPPH and ABTS radicals. Additionally, antioxidants can be evaluated for scavenging superoxide radical [98], hydrogen peroxide [99, 100], hydroxyl radical [99, 101], hypochlorous acid [102], peroxynitrite [103], and artificially generated peroxyl radicals [98]. Some of these radical scavenging assays have been transformed to methods for on-line HPLC detection of radical scavenging compounds in complex plant extracts [104-106].

Because of the great diversity of methods for evaluating radical scavenging activity, there is significant need for standardisation in measuring antioxidant activity. This should be done considering such factors as the system composition, the substrate to be oxidised and the method for inducing oxidation. To select the proper method for measuring antioxidant activity, the oxidation target (lipids, proteins or DNA) should be chosen first.

1.6. The aims of this study

To screen some Lithuanian herbs for antioxidant activity, select the most promising ones, determine their antioxidant activities in different real food and model systems, isolate and identify the compounds in these herbs responsible for retarding lipid oxidation in foods, to determine their properties and application possibilities, and to develop a method for simultaneous detection and identification of compounds with radical scavenging properties.

The approach that has been followed to fulfil these aims is:

1) The production of acetone extracts from several herbs grown in Lithuania;

2) The evaluation of the obtained extracts to retard oxidation of rapeseed oil;

3) The production of acetone and methanol-water extracts from the most promising herbs and the preparation of fractions of different polarity;

4) The determination of the activities of the extracts and their fractions using different antioxidant activity assays;

5) The isolation and identification of natural antioxidants from the fractions possessing the highest antioxidant activities;

6) The determination of antioxidant activity of the isolated compounds using radical scavenging asays;

7) The investigation of the capabilities of different solvents to extract antioxidants from the chosen herbs.

8) The integration of on-line HPLC-DPPH radical scavenging method and HPLC-SPE-NMR.

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2. Preliminary Screening of the Antioxidant Activity of some Plant Extracts in Rapeseed Oil*

2.1. Introduction

Governmental medical authorities and consumers are concerned about the safety of their food and about potential effects of additives on their health. During the last few decades intensive safety testing of synthetic food additives has been carried out and some of them have been found to possess some toxicity [1]. For example, soy phytochemicals genistein and daidzein show estrogen-like biological activity [2] Consequently, the search for natural alternatives, which in most cases are considered as GRAS (generally recognised as safe), increased considerably. The number of reports about isolation and testing of natural antioxidants, mainly of plant origin, increased significantly during the last two decades [3]. The number of SciFinder Scholar database hits when searching for "natural antioxidants" is presented in figure 2.1. Research on natural antioxidants has led to the development of effective natural antioxidants from rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*), which are now commercially available [4-6]. Also a lot of research was carried out on antioxidants from tea [7-11] and olives [12-14]. Applications of tea antioxidants in frying oils, potato flakes, meat emulsions, mayonnaise, margarine, frozen fish, precooked cereals, chicken fat, pork, and cheese have been patented [15-17].

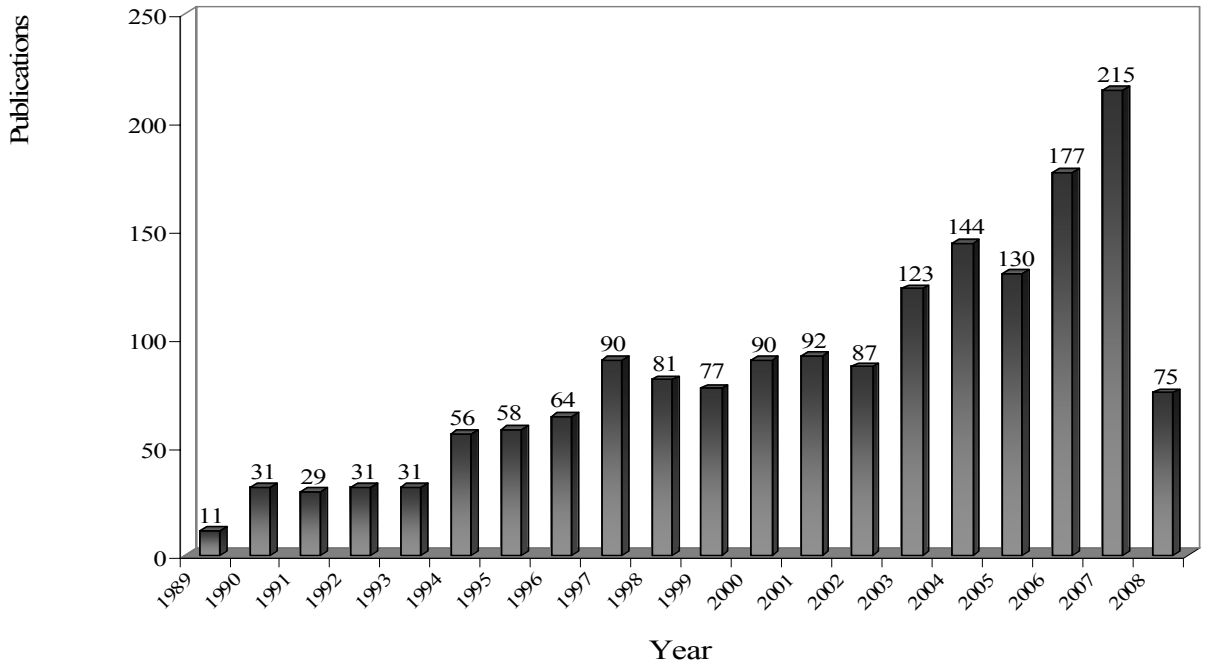

Fig. 2.1. Number of hits in SciFinder Scholar database for "natural antioxidants"

* This chapter is based on the paper: D. Bandonienė, A. Pukalskas, P. R. Venskutonis and D. Gruzdienė. Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil. International Food Research Journal, 33 (9), 785-791, 2000.

A great number of different spices and aromatic herbs have been tested for their antioxidant activity, however, there are still many plants, which have not been examined or the knowledge about their antioxidative properties is very scanty. Sweet grass (*Hierochloë odorata*), sea buckthorn leaves (*Hippophaë rhamnoïdes*), costmary (*Balsamita major*), Roman camomile (*Anthemis nobilis*), and tansy (*Tanacetum vulgare*) are among them. These plants have been investigated for other purposes, mostly for their medicinal properties, essential oil and flavonoid composition.

A large number of solvents and procedures have been used for the isolation of natural antioxidative substances, including polar ones such as ethanol [18, 19] and methanol [20] and non-polar ones, mainly hexane [20, 21]. Cuvelier [19] investigated 32 pilot-plant and commercial extracts from rosemary and sage isolated with hexane, supercritical carbon dioxide and ethanol. Significant differences between the antioxidant activities of the extracts were found, even when the same solvent was used. The authors concluded that these differences could depend on synergism and antagonism between extracted phenolic acids, diterpenoids and flavonoids, present in the extracts. The use of solvents with different polarities can provide useful information on the nature of the active constituents. Economou [20] concluded that acetone was the most efficient solvent for the extraction of compounds from sage and rosemary and other herbs with antioxidative activity [6, 22, 23].

In the present study, a preliminary screening of the antioxidant properties of some plants, grown in Lithuania, namely sweet grass, costmary, Roman camomile, sea buckthorn and tansy was carried out. To my knowledge there are no reports on the antioxidant properties of these plants. Acetone extracts obtained from these plants were added to rapeseed oil and oxidative deterioration (formation of peroxides) was measured at different time intervals during storage in an oven at 40 °C. BHT and sage extracts as well as pure natural antioxidants were used as references.

2.2. Materials and Methods

2.2.1. Materials

The following reagents were used: synthetic antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT) (Aldrich-Chemie, D-7924 Steinheim), acetone (pure, Poch, Poland), ethanol (rectified spirit 95%, Polmos, Poland), chloroform (pharm., Lachema, Czech Republic), acetic acid (98%, Lachema, Czech Republic), potassium iodide (Lachema, Czech Republic), Standard Folin-Ciocalteu Phenol reagent 2.0 M sodium carbonate, sodium thiosulfate and gallic acid (3,4,5-trihydroxybenzoic acid) (all from Sigma – Aldrich Chemie, Deisenhofen, Germany).

Roman camomile, tansy, sweet grass, costmary and sea-buckthorn were obtained from Kaunas Botanical Garden. Sage was obtained from the collection of aromatic plants of the Lithuanian

Institute of Horticulture in 1997. All herbs were harvested during full flowering. Stems and woody parts were separated and only the flowering parts and/or leaves were used for further analysis after drying in the shade in the open air.

The Company "Obeliu Aliejus" (Obeliai, Lithuania) donated fresh, fully refined, deodorised rapeseed oil, without synthetic antioxidants. The fresh rapeseed oil was of a good initial quality. The specifications are presented in table 2.1.

Table 2.1. Specifications of rapeseed oil used in antioxidant analysis

Specification	Value
Iodine value	115 g /100 g
Peroxide value	0.75 meq/kg
p-anisidine value	3.0
Erucic acid	0.5%
Linoleic acid	9.8%
Natural tocopherols	767 mg/kg
of which: α-tocopherol	228 mg/kg
β- and γ-tocopherol	539 mg/kg

2.2.2. Methods

2.2.2.1. Preparation of Plant Extracts.

The plants (leaves of sage, sea buckthorn and costmary, flowering parts of Roman camomile and tansy and aerial parts of sweet grass) were dried at 30 ± 2°C in a ventilated oven "Vasara" (Utena, Lithuania) for 24-36 h (depending on the plant material). Dried parts of the plants were ground (max. particle size 0.32 mm) and 15 g of comminuted material was extracted with 900 ml of acetone in a Soxhlet apparatus during 6 h. The solvent was evaporated in an R114 rotary evaporator by using a B480 water bath (60°C) and a B169 vacuum pump (Büchi, Switzerland). The extracts were finally dried in a SPT 200 vacuum dryer (Horyzont, Poland) at 25 ± 2°C and 0.08 MPa. Dry extracts were stored in a freezer below – 18°C until use. The yields of the plant extracts were as follows: sage (SE) – 14.8%, sea buckthorn (SBE) – 15.0%, costmary (CE) – 21.1%, Roman camomile (RCE) – 13.0%, sweet grass (SGE) – 9.4%, tansy (TE) – 14.1%.

2.2.2.2. Determination of the Total Phenolic Content.

Phenolic compounds were extracted from 0.5 g of ground raw material with 3 portions (30 ml each) of 80% ethanol in a round bottom flask with reflux on a heating stove (LTHS-1000, Druteva Brnenska, Czech Republic) at 50 °C for 1 hour. After each extraction the extract was filtered and collected into a 100 ml volumetric flask and finally diluted with 80% ethanol up to the mark. The

total amount of phenolic compounds in the extract was measured with standard Folin-Ciocalteu reagent [19]. A stock solution of the reagent was diluted with distilled water (1:10) and 4 ml were added to 1 ml of ethanolic plant extract solution. After adding 5 ml of a 7.5 % sodium carbonate solution in distilled water the absorbance of the colour development was measured after 30 min at 765 nm on a UV-VIS spectrophotometer (Specord M40, Carl Zeiss Jena, Germany). Gallic acid was used as the standard for the calibration curve. The total amounts of phenolic compounds in extracts were calculated by the following formula and expressed in mg/g on a dry weight of the herbs in gallic acid equivalents (GAE):

$$C = \frac{c \cdot V}{m};$$

where: C – concentration of total phenolics in extract, mg/g, in GAE;

c – concentration of gallic acid in sample (obtained from the calibration curve), mg/ml;

V – volume of plant extract, 100 ml;

m – weight of plant material, g.

All samples were analysed in triplicate.

2.2.2.3. Addition of the Extracts into the Oil.

Calculated amounts of extracts (varying from 0.0 to 0.2% of the oil weight) were mixed with 4 ml of absolute ethanol and added to 25 g of rapeseed oil. According to previous experiments it was the smallest amount of alcohol needed for an homogenous distribution of the extracts in the oil. The additive was mixed into the oil with a magnetic stirrer during 10 min at 50°C. The synthetic antioxidant, BHT, and the natural antioxidant, sage acetone extract (SE), were used as positive controls. The sage extracts were prepared in the same way as all other plant extracts used in this study. Ethanol was removed from the rapeseed oil in a vacuum oven during 12 hr at 35 °C and 0.05 bar.

2.2.2.4. Assessment of Oil Oxidation

The oil samples (25 g each) were placed in open 150 mL beakers. The oxidative deterioration of samples was determined by the Schaal oven test [20]. The experiments were carried out in duplicate. When the differences between the replicates were considerable the measurements were repeated, however this happened only rarely. The relative standard deviation was in all cases in the range of 3 to 10%. A blank sample was prepared under the same conditions, without adding any additives. The rate of autoxidation of rapeseed oil was estimated according to the increase of its

peroxide value (PV), which was determined by using the method Cd 8-53 of the American Oil Chemist's Society [24].

The changes in the induction period (IP) after the addition of each plant extract, was determined as a function of its concentration in the oil. The IP was determined as the number of hours needed before the PV of the sample reached a value of 20 meq/kg [25]. Protection factor (PF) values of rapeseed oil and antioxidant activities (AA) of the extracts were calculated with the following formulas:

$$PF = \frac{IP_X}{IP_K};$$

$$AA = \frac{IP_X - IP_K}{IP_{BHT} - IP_K};$$

where: IP_X – induction period of the sample with additive, h;

IP_K - induction period of sample without additive, h;

IP_{BHT} – induction period of sample with added synthetic antioxidant BHT, h.

The following scale was used for the interpretation of the protection factor (PF) values: 1.0 – 1.5 (very low), 1.5 – 2.0 (low), 2.0 – 2.5 (medium), 2.5 – 3.0 (high), >3.0 (very high) [26].

2.3. Results and Discussion

The amounts of total phenolic compounds in the herbs are presented in Table 2.2. The extract of sage possessed approximately twice-higher amounts of phenolics (47.7 GAE) than the other plant extracts except sea buckthorn leaves (32.1 GAE).

Table 2.2. The amount of total phenolic compounds, in mg/g of herbs on a dry weight basis, expressed as gallic acid equivalents (GAE)

Plant	Total phenolic compounds
Sage	47.7 ± 0.7
Sea buckthorn	32.1 ± 0.3
Roman camomile	24.8 ± 0.5
Sweet grass	22.0 ± 0.2
Tansy	18.6 ± 0.6
Costmary	22.0 ± 0.5

The results for rapeseed oil autoxidation, measured as a change in PV at 40 °C, after addition of extracts of sage, sweet grass, sea buckthorn, costmary, Roman camomile, and tansy, are presented in Table 2.3. The concentrations of the extracts in oil, calculated on a dry weight basis, varied from 0.00 to 0.20% (w/w). It is evident that all extracts in general showed some oil stabilising effect, which increased with increasing concentration of the extract in the oil.

The extracts obtained from sage and sweet grass were found to be the most effective natural antioxidants. The effect of sage (0.02%) and sweet grass (0.02%) extracts on the stability of rapeseed oil during accelerated oxidation was comparable with the effect of butylated hydroxytoluene (BHT) at the same concentration. The most important finding of this study was the strong antioxidant activity of the sweet grass extract, which was according to my knowledge, revealed for the first time. For instance, the PV of rapeseed oil with 0.10 and 0.20 % of sweet grass extract after 70 days of storage was approximately 20 meq/kg, whereas in the blank samples it increased to approximately 800 meq/kg, and in the samples with extracts from the other herbs to 350-926 meq/kg. Having in mind that BHT is a pure compound while the extracts are most likely complex mixtures containing ineffective substances in terms of antioxidative capacity or even some amount of prooxidant compounds it is clear that sweet grass contains constituents that strongly retard lipid peroxidation. Investigation of the structures of the active constituents in sweet grass will be a target for further studies.

Both Roman camomile at 0.05% and 0.1%, and tansy at 0.05%, showed an effect, which was almost similar to the effect of a smaller amount of BHT (0.0075%). When the concentration of sage extract was increased to 0.1% and that of sweet grass to 0.05, 0.1 and 0.2% the antioxidant effect in rapeseed oil at 40°C was very high. It is interesting to note that the activity of sweet grass at 0.1% concentration was approximately 1.3 times higher than that of the well-established sage extract at the same concentration.

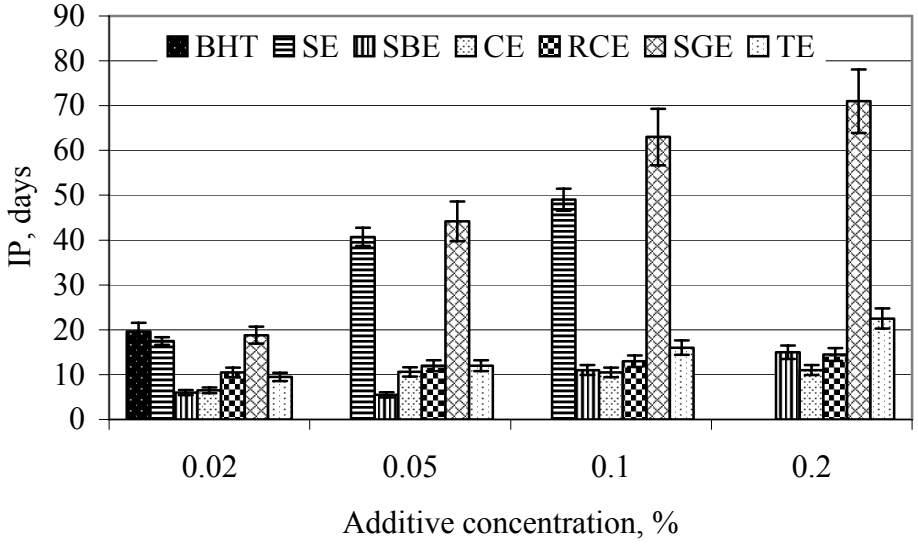

Fig. 2.2. Induction periods (IP) of rapeseed oil after addition of sage (SE), sea buckthorn (SBE), costmary (CE), roman chamomile (RCE), sweet grass (SGE), tansy (TE) extracts and BHT at concentrations varying from 0.02 to 0.2%

Table 2.3. Effect of various extracts on the formation of peroxides in rapeseed oil at 40°C

Additive	Concen-tration, %	Peroxide values (meq/kg) after different storage time (days)									
		0	3	7	14	21	28	35	49	63	70
Blank		0.7	7.7	22.0	42.3	72.9	97.00	120.8	232.1	818.9	793.7
BHT	0.0075	0.7	4.8	12.1	23.2	34.4	62.1	79.1	133.7	221.9	438.5
BHT	0.02	0.7	3.9	9.5	11.9	20.9	29.0	36.8	65.9	98.5	125.0
SE	0.02	0.7	9.3	9.4	14.9	24.0	39.6	63.8	125.5	210.0	434.4
SE	0.05	0.7	4.4	10.2	11.2	10.3	12.3	15.0	27.6	63.8	96.5
SE	0.10	0.7	5.7	8.0	9.0	10.0	11.1	12.9	20.1	29.4	45.0
SBE	0.02	0.7	11.7	22.8	52.0	79.4	100.6	131.1	196.1	692.7	926.3
SBE	0.05	0.7	14.0	23.1	48.8	74.0	105.8	124.3	227.4	705.9	882.1
SBE	0.10	0.7	8.9	10.7	27.4	48.3	78.0	92.6	145.9	429.0	855.3
SBE	0.20	0.7	11.5	10.8	24.9	43.0	76.3	100.9	87.3	313.2	705.4
CE	0.02	0.7	8.5	21.5	47.0	73.3	100.7	118.3	229.1	636.8	742.7
CE	0.05	0.7	4.5	12.5	29.1	47.8	83.6	117.0	171.7	427.3	729.5
CE	0.10	0.7	6.5	14.2	28.8	44.0	80.4	106.3	166.4	384.6	700.4
CE	0.20	0.7	8.9	13.4	25.6	46.0	75.6	101.4	151.3	342.8	658.3
RCE	0.02	0.7	3.2	8.4	25.7	44.3	76.7	93.4	160.3	585.6	918.1
RCE	0.05	0.7	4.5	5.2	22.0	38.5	67.2	90.0	137.0	471.1	854.2
RCE	0.10	0.7	5.2	5.8	21.1	33.2	63.0	85.1	140.6	381.9	807.5
RCE	0.20	0.7	6.1	5.8	18.6	35.6	61.4	82.7	136.4	270.9	674.0
SGE	0.02	0.7	7.3	6.6	14.8	22.2	43.8	63.6	117.3	200.0	222.1
SGE	0.05	0.7	4.9	6.9	10.4	12.8	10.9	12.7	23.4	34.9	57.7
SGE	0.10	0.7	4.7	5.6	10.3	9.5	8.0	7.9	16.5	19.9	22.1
SGE	0.20	0.7	5.1	7.9	14.0	7.4	7.3	8.2	16.8	16.1	19.3
TE	0.02	0.7	5.2	12.9	34.2	56.7	87.4	102.6	184.8	499.7	798.8
TE	0.05	0.7	4.9	6.2	24.7	44.8	73.7	93.2	158.5	558.7	819.8
TE	0.10	0.7	5.6	5.4	16.3	28.0	52.8	79.1	132.9	309.0	607.6
TE	0.20	0.7	6.1	9.4	12.9	16.8	39.1	63.6	115.4	177.2	349.2

BHT- butylated hydroxytoluene; SE - sage extract; SBE – sea buckthorn extract; CE – costmary extract; RCE – Roman camomile extract; SGE – sweet grass extract; TE – tansy extract

The relative antioxidant efficiencies of sage, sweet grass, sea buckthorn, costmary, Roman camomile and tansy are compared in Figure 2.2. The experimental data show that the rate of autoxidation in most samples increases much faster after the PV reaches 20 meq/kg. The data provided in Figure 2.2 also show that sage and sweet grass extracts are much more effective in stabilizing rapeseed oil than the other extracts used in this experiment. The effectiveness of the other plant extracts decreases in the following order: tansy > Roman camomile > sea buckthorn > costmary at a concentration of 0.1%. It is evident from Figure 2.2 that sweet grass extract is more efficient than sage at 0.05 and 0.1%, but at 0.02% slightly less effective than 0.02% BHT. The protection factors (PF) and antioxidant activities (AA) of the extracts are presented in Table 2.4. The effectiveness of the antioxidants was compared according to their stability values and protection factors.

Table 2.4. Antioxidant activity of investigated plant extracts and their effect on the stability of rapeseed oil

Additive	Concentration %	Protection factor (PF)**	Antioxidant activity (AA)*
Without additive	0.00	1.00	-
BHT	0.0075	1.82	-
BHT	0.02	2.97	1.00
Sage	0.02	2.65	0.84
Sage	0.05	6.17	2.62
Sage	0.10	7.42	3.26
Sea buckthorn	0.02	0.91	-0.05
Sea buckthorn	0.05	0.83	-0.09
Sea buckthorn	0.10	1.66	0.34
Sea buckthorn	0.20	2.27	0.65
Costmary	0.02	0.98	0.00
Costmary	0.05	1.59	0.30
Costmary	0.10	1.61	0.31
Costmary	0.20	1.66	0.34
Roman camomile	0.02	1.59	0.30
Roman camomile	0.05	1.82	0.42
Roman camomile	0.10	1.97	0.49
Roman camomile	0.20	2.20	0.61
Sweet grass	0.02	2.85	0.94
Sweet grass	0.05	6.69	2.89
Sweet grass	0.10	9.54	4.34
Sweet grass	0.20	10.76	4.95
Tansy	0.02	1.44	0.22
Tansy	0.05	1.82	0.42
Tansy	0.10	2.42	0.72
Tansy	0.20	3.41	1.22

* AA was calculated in comparison with BHT at the concentration 0.02%
** PF is the ratio of IP of the sample with additive with IP of the sample without additive

Sage extracts at 0.05 and 0.1%, sweet grass extracts at 0.05, 0.1 and 0.2%, and tansy at 0.2% concentrations exhibited a “very high” antioxidant activity (PF > 3). Sweet grass extracts at 0.02%, sage at 0.02% and BHT at 0.02% - showed “high” activity (PF of 2.5-3). Tansy at 0.1%, Roman camomile at 0.1% and sea buckthorn at 0.2% are “medium” active (PF of 2.0-2.5). Tansy at 0.05%, Roman camomile at 0.02 and 0.05%, costmary at 0.05, 0.1 and 0.2% and sea buckthorn at 0.1% exhibited “low” antioxidant activity (PF of 1.5-2) and tansy at 0.02% a “very low” activity (PF of 1-1.5). Costmary at 0.02% and sea buckthorn at 0.02 and 0.05% showed prooxidative effects in comparison with the control. A clear correlation between total phenolics and AA was not found. However, the content of phenolics in sweet grass, which gave a very strong antioxidative extract, was almost equal to the content of these compounds in other tested herbs. It is known that the AA of various phenolic compounds can differ significantly, therefore the content of total phenolics in herbs is not a very informative indicator of their AA. The structures of the individual constituents need to be elucidated and assessed in order to obtain more precise results.

2.4. Conclusions

The results of this study suggest that sweet grass acetone extract at 0.05, 0.1 and 0.2%, sage extract at 0.05 and 0.1% and tansy extract at 0.2% possess a higher AA than BHT at 0.02% when tested in rapeseed oil at 40°C. The lowest concentrations of the extracts showing a significant effect in retarding rapeseed oil oxidation are as follows: sweet grass and sage – 0.05%, tansy – 0.2%. Concentrations of costmary, sea buckthorn and Roman camomile extracts should be higher than those used in this study to exert any effect. The strong antioxidant activity of sweet grass extracts is reported for the first time. Further investigations towards the structure elucidation of constituents responsible for the protection of the oil against oxidation will be studied in the near future.

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3. Antioxidant Activity of Extracts from Sweet Grass (*Hierochloe odorata*), Costmary (*Chrysanthemum balsamita*) and Horehound (*Marrubium vulgare*) Obtained By Different Extraction and Fractionation Procedures*

3.1. Introduction

In this chapter investigations for antioxidative activity on three herbs: sweet grass (*Hierochloe odorata*), costmary (*Chrysanthemum balsamita*) and horehound (*Marrubium vulgare*) are described.

Sweet grass (*Hierochloe odorata*) is a plant of the genus *Hierochloe*, family Gramineae. Its root and the aerial parts smell sweet. Dried sweet grass foliage is fragrant because of its coumarin content and it is used as incense and for making perfume. Sweet grass tea was used for coughs and sore throats, to treat chapping and windburn, and as an eyewash [1].

Costmary, *Chrysanthemum balsamita* L. (syn. *Balsamita major* L.) Asteraceae, is a large perennial plant of Asian origin with yellow flowers, grown in Europe and Asia since the Middle Ages [2]. The name costmary is derived from Costus (*Saussurea lappa* Clarke), an Oriental plant, the root of which is used as a spice and a preserve, and "Mary" in reference to Our Lady. The other name of the herb is alecost, because it was much used to give a spicy flavouring to ale. Fresh and dried leaves of costmary possess a strong mint-like aroma and an astringent taste. Leaves may be used with meats and poultry and as a tea.

Horehound (*Marrubium vulgare* L.), a member of the Labiatae, is native to North Africa, Central and Western Asia, and Southern Europe. It grows wild in dry sandy soils and wastelands. The species can be cultivated successfully in Lithuania and is harvested twice a year as a medicinal raw material [3]. Horehound serves also as raw material for herbal extracts and beverage industries. The plant has been used as a substitute for hop in beer-breweries and it can be used as an ingredient of cough pastilles.

Phytochemical investigations of horehound resulted in the isolation of the flavonoids apigenin and luteolin and their 7-glucosides together with quercetin and its 3-glucoside and 3-rhamnoglucoside [4]. Nawwar et al. [5] reported on the isolation and structural elucidation of the flavonoids luteolin, and apigenin 7-lactates together with their 2”-O-β-glucuronides and 2”-O-β-glucosides. In addition, several diterpenoids have been isolated and characterised, the main one being marrubiin [6-9].

*This chapter is mainly based on the papers: A. Pukalskas, P. R. Venskutonis, T. A. van Beek, S. Salido Ruiz. Isolation, identification and activity of natural antioxidants from horehound (Marrubium vulgare) cultivated in Lithuania. Molecules, in preparation (2009); A. Pukalskas, T. A. van Beek, P. R. Venskutonis, I. Dijkgraaf. Isolation, identification and activity of natural antioxidants from costmary (Chrysanthemum balsamita) cultivated in Lithuania. Molecules, in preparation (2009); and also includes some data published in K. G. C. Weel, P. R. Venskutonis, A. Pukalskas, D. Gruzdiene, J. P. H. Linssen. Antioxidant activity of horehound (Marrubium vulgare L.) grown in Lithuania. Fett/Lipid, Vol. 101 (10), 363 - 415, 1999.

Acetone extracts of these three herbs have been prepared and studied for their antioxidant properties. Deodorised acetone extracts (obtained from plant material after removal of the essential oil) of some plants are reported to have equal or even better antioxidant properties than the acetone extracts of the same material [10-12]. This raises the possibility to use the residue remaining after the steam distillation of the essential oil extraction as a valuable source of natural antioxidants.

Although acetone is one of the most used solvents, it extracts mainly apolar compounds. Most antioxidants are polyphenolics and many are rather polar due to the hydroxyl groups and attached sugars. So it is likely that acetone does not extract all antioxidants (especially glycosides), but only the ones of lower polarity.

Therefore, the isolation of antioxidants with polar nature will be carried out with extraction of a mixture mixture of methanol-water (8:2). One percent of acetic acid was added to decrease the amount of chlorophyll in the extract.

3.2 Materials and Methods

3.2.1. Materials

Sweet grass, costmary, horehound and sage plants were cultivated in the experimental garden of the Lithuanian Institute of Horticulture in Babtai, Lithuania, and harvested in August 1998.

Tween 40, *trans*-β-carotene, linoleic acid (purity *ca*. 99%), butylated hydroxytoluene (BHT), and DPPH radical were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) and rosmarinic acid from Fluka (Fluka AG, Buchs, Switzerland). Freshly manufactured rapeseed oil obtained from low erucic acid bearing seeds of *Brassica napus* L. was donated by the company Obeliu aliejus, Lithuania.

3.2.2. Methods

3.2.2.1. Isolation of essential oil (EO).

EO was hydrodistilled in a semi preparative Clevenger-type apparatus from 20 g of air-dried freshly ground leaves during 3 h using distilled water. A layer of 10 ml of a mixture of pentane and diethyl ether (1:1) on top of the water was used during distillation to separate the volatile oil from the water in the distillate collector tube of the apparatus. The solution with the distilled EO was concentrated under a stream of nitrogen to 0.5 ml.

3.2.2.2. Preparation of deodorised extracts.

The residue of the hydrodistillation was filtered sequentially through cotton wool and filtration paper, which resulted in a filtrate and a residue. The filtrate was spray-dried using a Büchi 190 mini spray dryer (inlet temperature 200 °C, outlet temperature 115 °C). This sample will be

referred to as deodorised water extract (DWE). The residue was dried in four days by squeezing the remaining water out of the sample through a fine sieve, followed by additional drying in a SPT 200 vacuum dryer (Horyzont, Poland) at 50 °C and 0.08 MPa. Acetone extraction of this sample resulted in the deodorised acetone extract (DAE).

3.2.2.3. Preparation of acetone extracts.

Acetone extracts were obtained by extracting an amount of freshly ground leaves (AE) or an amount of dried hydrodistillation residue (DAE) of approximately 15 g with 900 ml of acetone, during 4 h. A Soxhlet extraction apparatus was used for the extraction. The extracts were concentrated to 20 ml using a R114 rotary evaporator, a *B480* water bath (60 °C) and a B169 vacuum pump (Büchi Labortechnik AG, Flawil, Switzerland). The remaining acetone was evaporated to dryness by applying a stream of nitrogen, or by placing the samples in a SPT 200 vacuum dryer (Horyzont, Poland) at 50 °C and 0.08 MPa.

3.2.2.4. Preparation of methanol-water extracts.

Plant material was air dried in a Vasara ventilated oven (Utenos krosnys, Utena, Lithuania) at 30°C for about 48 h and ground before use. Dried and ground plant material (50 g) was extracted (2 × 1 L) with methanol - water - acetic acid (80:20:1) at room temperature for 24 h, under nitrogen. Solvent and plant material was constantly mixed with an Ikamag RTC basic magnetic stirrer (IKA Labortechnik, Staufen, Germany). The extract obtained was concentrated in a rotary evaporator at 40°C to about 150-200 mL.

3.2.2.5. Fractionation of acetone and methanol-water extracts.

The methanol water extract (MWE), or acetone extract (AE), remaining after evaporation was diluted to 500 ml with ultra pure water and then successively extracted with several 100 ml volumes of hexane, *tert*-butyl methyl ether and finally butanol. In total amounts of 500 – 600 ml of each solvent were used. The remaining aqueous phase was freeze-dried. The extraction scheme is shown in figure 3.1.

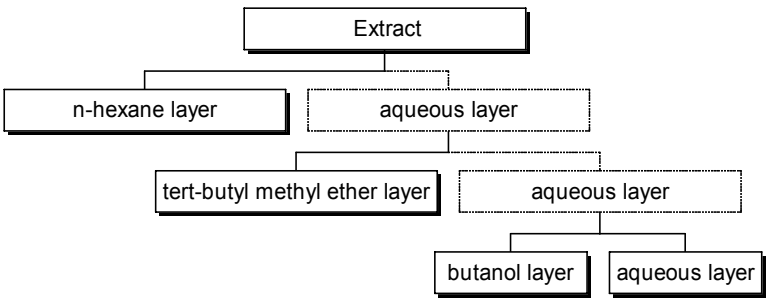

Figure 3.1. Fractionation scheme of acetone and methanol-water extracts

3.2.2.6. Evaluation of antioxidant activity in rapeseed oil.

The oxidative deterioration was monitored under *Schaal Oven Test* conditions. Extracts were dissolved in 25.00 ml of rapeseed oil at a concentration of 0.01% (w/w), in duplicate. The extracts were added directly to the oil and mixed on a magnetic mixer for 10 min at 50 °C. The samples were placed in open 150 ml beakers in a ventilated oven HS122A (ZPA, Hungary) and protected from light. Experiments were carried out at 40, 55 and 80 °C. A blank sample was prepared under the same conditions, without addition of any antioxidant. These samples were used for the evaluation of the antioxidant activity of the plant extracts, by determining the peroxide value, and by measuring the UV absorption.

3.2.2.7. Determination of peroxide value (PV).

An aliquot of the sample was weighed, to within 0.1 mg, into a dry 250 ml flask. 25 ml of chloroform/acetic acid (3:2) were added, immediately followed by 0.5 ml of a saturated potassium iodide solution. The sample was shaken for 1 min and then 25 ml of distilled water were added. The liberated iodine was titrated with 0.01 M sodium thiosulphate solution shaking vigorously, using a starch solution as indicator [13].

3.2.2.8. UV absorbance test.

An aliquot of the sample of approximately 0.02 g was weighed, to within 0.1 mg, into a 25 ml volumetric flask. First the test portion was dissolved in a few ml of hexane, and then hexane was added up to the mark. The sample was mixed thoroughly. The prepared solutions were measured in 1 cm long quartz cells, using a Varian Cary 219 spectrophotometer with hexane as a reference. The absorption was measured at wavelengths of 232 and 268 nm [13]. It should be explained that the IUPAC method suggests weighing an amount of sample such that the absorbance would be between 0.2 and 0.8, usually 0.2 g. However, when 0.2 g of sample was used the UV value was too high. To keep the absorbance between 0.2 and 0.8 the amount of oil was lowered to 0.02 g. This amount was optimal one to get reliable results at 80 °C. The absorbance $E_{1\%1cm}$ at various wavelengths is given by the formula: $E_{1\%1\ cm} = A_\lambda \times c^{-1}\ cm \times d^{-1}$ in which A_λ is the absorbance measured at wavelength λ; c is the concentration in g per 100 ml of sample in the test solution, and d is the length of the cell in cm.

3.2.2.9. *p*-Anisidine values

Measured using the official AOCS procedure Cd 18-90 [14].

3.2.2.10. Calculations of some oil oxidation parameters.

To compare the stability of the blank sample with the stabilities of the samples with additives, protection factors (PF) were calculated:

$$PF = \frac{IP_X}{IP_B}, \qquad (3.1)$$

where: IP_x – induction period of the sample with additive, h;

IP_B – induction period of the blank sample, h.

To compare the activities of natural additives with the activity of 0.02% BHT antioxidant, activity coefficients (AA) were calculated [15]:

$$AA = \frac{(IP_X - IP_B)}{(IP_S - IP_B)}, \qquad (3.2)$$

where: IP_B – induction period of the blank sample, h;

IP_x – induction period of the sample with additive, h;

IP_S – induction period of the sample with synthetic antioxidant BHT, h.

IP – the time when PV of the sample reaches 20 meq/kg.

The TOTOX value evaluates the total oxidation process in the oil:

$$TOTOX = 2PV + p\text{-AnV} \qquad (3.3)$$

where: PV – peroxide value, in meq/kg;

p-AnV – *p*-anisidine value.

3.2.2.11. *β*-Carotene bleaching test.

The AA's of herb extracts and reference antioxidants (BHT and rosmarinic acid) were determined using the method developed by Marco [16] and modified by Dapkevicius et al. [17]. Some other changes in the procedure were applied and are described below. *trans*-*β*-Carotene (1 mg) was dissolved in 5 ml of chloroform and 1.0 ml of this *trans*-*β*-carotene solution was transfered with a pipette into a 100 ml round bottom flask. Linoleic acid (25 µl) and Tween 40 (200 mg) were added to the *β*-carotene solution and the chloroform was evaporated under vacuum at 40 °C. Oxygenated ultra pure water (50 ml), obtained by sparging with air during 15 min, was added and the mixture was vigorously shaken by hand.

This emulsion was freshly prepared prior to each experiment. Stock solutions of reference antioxidants (BHT and rosmarinic acid, each 0.01%) and herb extracts (0.1%) were prepared in methanol. The *β*-carotene – linoleic acid emulsion (250 µl) was dosed into every well of the 96-well microtiter plates (Greiner Labortech, The Netherlands) and 30 µl of ethanol solutions of the antioxidants were added. An equal amount of methanol was used for the blank sample. Four replicates were prepared for every sample that was tested. The microtiter plates were incubated at 55 °C during 120 min. The absorbance of the samples was measured in an EAR 400 Microtiter

reader (SLT instruments, Austria) at 490 nm. Readings of all samples were performed immediately after preparation of the samples (t = 0 min) and at 15 min intervals during 120 min. The antioxidant activity coefficient (AAC) was calculated from the obtained data by the formula proposed by Chevolleau et al. [18]:

$$AAC = [(A_{A,120} - A_{B,120})/(A_{B,0} - A_{B,120})]\times 1000, \quad (3.4)$$

where $A_{A,120}$ and $A_{B,120}$ is the absorbance of the sample with added antioxidant and the blank sample respectively, at t = 120 min, and $A_{B,0}$ is the absorbance of a blank sample at t = 0 min.

3.2.2.12. DPPH Assay.

Radical scavenging activity of sweet grass extracts, BHT, and rosmarinic acid, against the stable radical DPPH$^{\bullet}$ was measured using the method of Von Gadow et al. [19], modified as described below. Methanolic solutions of DPPH$^{\bullet}$ (10^{-4} M) were mixed in a 1 cm path length disposable plastic half-micro cuvette (Greiner Labortech, Alphen a/d Rijn, The Netherlands) with sweet grass extracts and the reference compounds BHT and rosmarinic acid in such a way that the final mass ratio of the extract to DPPH$^{\bullet}$ was 3 to 1. The samples were kept for 15 min in the dark at room temperature and the decrease of absorbance at 515 nm was measured against methanol using a Lambda 18 spectrophotometer (Perkin - Elmer, Ueberlingen, Germany). The absorbance of a blank sample containing the same amount of methanol and DPPH$^{\bullet}$ solution was prepared and measured daily. DPPH$^{\bullet}$ solution was freshly prepared daily and kept in the dark at 4°C in between the measurements. All determinations were performed in triplicate. The radical scavenging activity of the tested samples, expressed as % inhibition, was calculated using the following formula [20]:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B]\times 100, \quad (3.5)$$

in which A_B is the absorbance of the blank sample (t = 0), and A_A is the absorbance of the sample with antioxidant after 15 min.

3.3. Results and Discussion.

3.3.1. Preliminary fractionation. Deodorisation.

Investigation of the acetone extracts of sweet grass, costmary and horehound showed that at least in the early stages (i.e. during the induction period) all investigated extracts slowed down oxidation processes in rapeseed oil. From the curves, obtained by measuring peroxide values (Figure 3.2) one can see that the highest antioxidative effect on rapeseed oil oxidation, relative to

the synthetic antioxidant BHT, is shown by the acetone extract isolated from sweet grass. This extract had an even higher activity than the sage acetone extract.

The acetone extracts of costmary and horehound do not show high antioxidant activity, the formation of peroxides in samples with these extracts did not differ from the blank sample.

To evaluate the influence of herb acetone extracts on the formation of peroxides in rapeseed oil, induction periods (time at which the peroxide value reaches 20 meq/kg) were determined graphically from the curves of the changes of the peroxide values. From the values obtained, protection factors (PF) were calculated as ratios of the induction periods of samples with additives and the blank sample (formula 3.1). The stability of oil samples with different additives was evaluated according to the scale [21]: 1-1.5 (very low); 1.5-2 (low); 2-2.5 (medium); 2.5-3 (high) and >3 (very high). To compare the activity of natural additives with that of the synthetic antioxidant BHT, antioxidant activity coefficients were calculated according to formula (3.2).

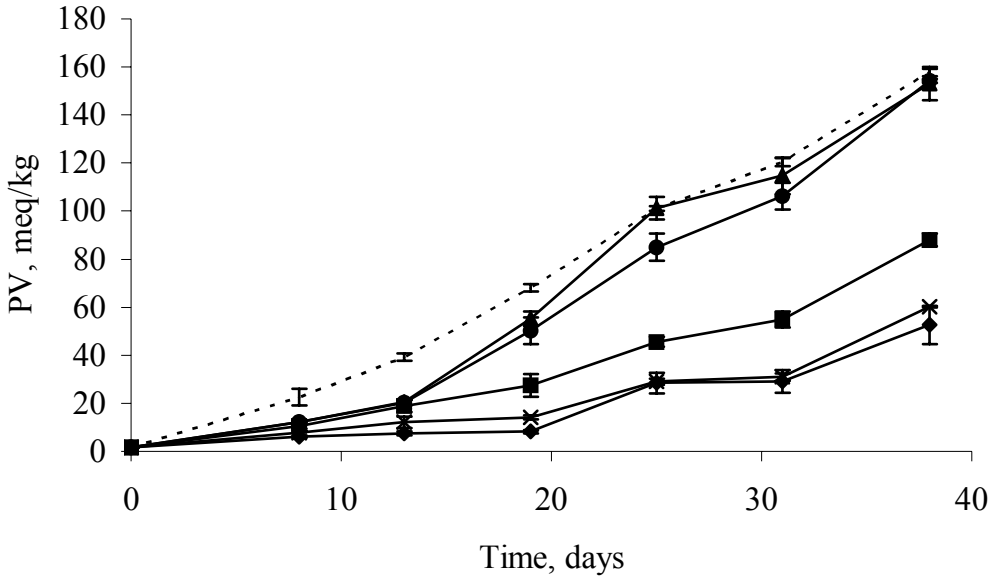

Figure 3.2. Peroxide accumulation in rapeseed oil at 40ºC in: --- blank sample; ■ oil with 0.02% BHT; × oil with 0.1% sage AE; ♦ oil with 0.1% sweet grass AE; ● oil with 0.1% costmary AE, ▲ oil with 0.1% horehound AE.

From the results presented in table 3.1 it can be seen that addition of 0.1% of sweet grass acetone extract increased the stability of rapeseed oil about 1.5 times more than addition of 0.02% of the synthetic antioxidant BHT. The addition of 0.1% of the acetone extracts of costmary and horehound had a slightly lower effect than BHT.

Table 3.1. The influence of plant extracts on the stability of rapeseed oil

Sample	IP, days	PF	AA	PF evaluation
blank	7.0	-	-	-
with 0.02% BHT	13.8	1.97	-	low
with 0.1% sage AE	21.0	3.00	2.06	high
with 0.1% sweet grass AE	22.5	3.21	2.28	high
with 0.1% costmary AE	13.1	1.87	0.90	low
with 0.1% horehound AE	13.0	1.86	0.88	low

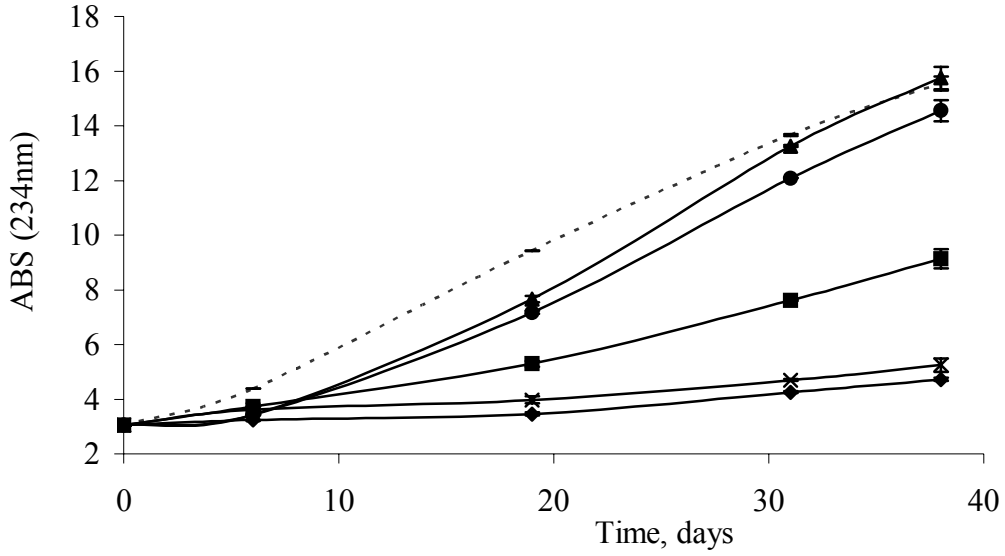

Figure 3.3. UV absorbance changes of rapeseed oil stored at 40 ºC, in: --- blank sample; ■ oil with 0.02% BHT; × oil with 0.1% sage AE; ♦ oil with 0.1% sweet grass AE; ● oil with 0.1% costmary AE, ▲ oil with 0.1% horehound AE.

A statistical analysis with the one tailed distribution equal sample variance Student T-test showed that peroxide formation in oil samples with 0.02% BHT, 0.1% sage and sweet grass acetone extracts differs ($P<0.05$) from the peroxide formation process in the blank sample. The same test showed that the addition of 0.1% of costmary and horehound extracts did not influence peroxide formation rates in rapeseed oil at 40 °C. So it could be concluded, that even if during the induction period all additives used had a stabilising effect on the oil, the amounts of antioxidant compounds in horehound and costmary acetone extracts were too low and were rapidly consumed. That is why peroxides in these samples form at a similar rate as in the blank sample.

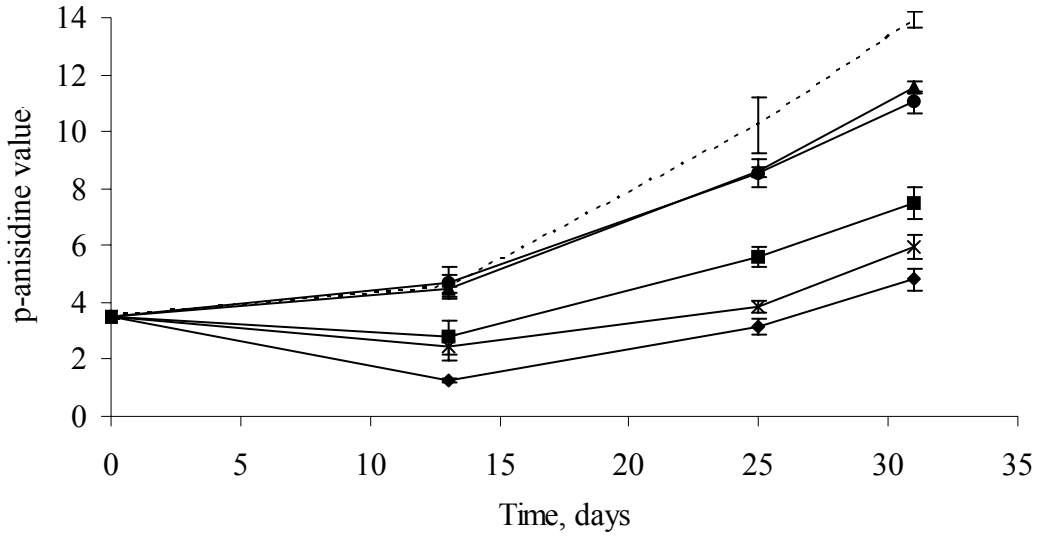

Figure 3.4. Accumulation of secondary oxidation products in rapeseed oil at 40 ºC in: --- blank sample; ■ oil with 0.02% BHT; × oil with 0.1% sage AE; ♦ oil with 0.1% sweet grass AE; ● oil with 0.1% costmary AE, ▲ oil with 0.1% horehound AE.

The formation of primary oxidation products in oil was also monitored by the UV absorbance test at λ=234 nm. The results obtained by this method (Figure 3.3) are in good agreement with the ones obtained by the peroxide value measurement. The same tendencies as in the PV test can be observed also in the UV absorbance curves.

The accumulation of secondary oxidation products in rapeseed oil was monitored by the p-anisidine test. As can be seen from figure 3.4 the tendency of formation of secondary oxidation products is the same as that of the primary ones in the corresponding samples.

Small differences in the formation of secondary oxidation products can be seen between samples with costmary and horehound extracts and the blank sample. However, statistically these samples still do not differ from the blank.

From the sum of the PV and *p*-anisidine values, the TOTOX value (3.3) was calculated. The TOTOX value is a measure of the overall oxidation of the oil. The results are shown in figure 3.5. Summarizing these results, it can be concluded that the highest activity is shown by the acetone extracts of sweet grass and sage. To obtain similar stabilization effects for the other extracts higher concentrations must be used.

Literature data indicate [10-12] that sometimes deodorised acetone extracts of herbs possess higher antioxidant activities than the corresponding acetone extracts. Therefore it was decided to carry out a fractionation of sweet grass, costmary and horehound acetone extracts. The last two

herbs were potentially the most interesting in this respect because their total acetone extracts did not have much effect on the oil oxidation processes.

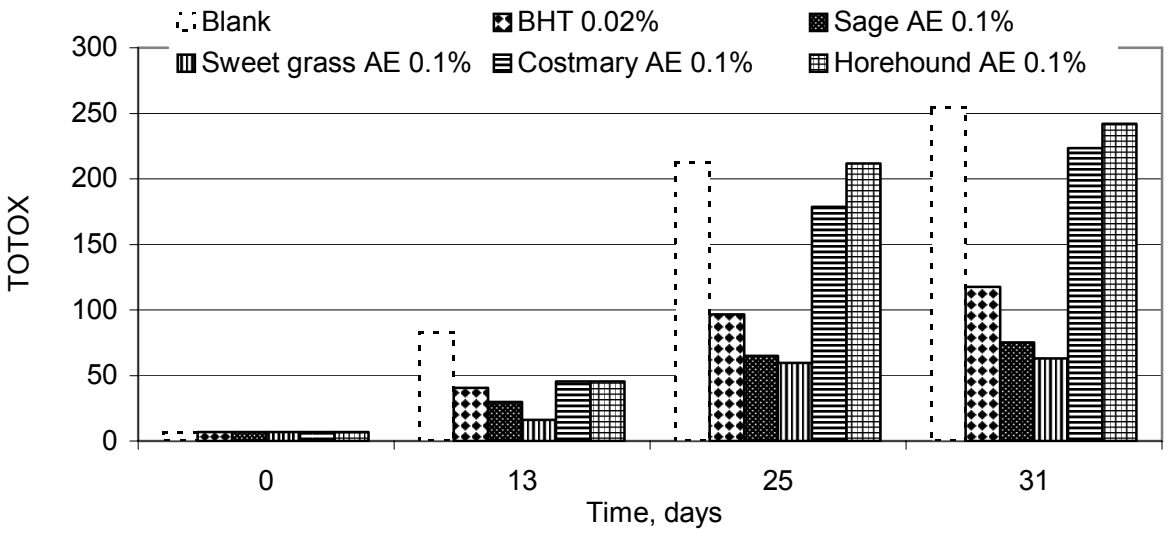

Figure 3.5. Accumulation of the total oxidation products (TOTOX) in rapeseed oil with herb extracts and BHT at 40 ºC.

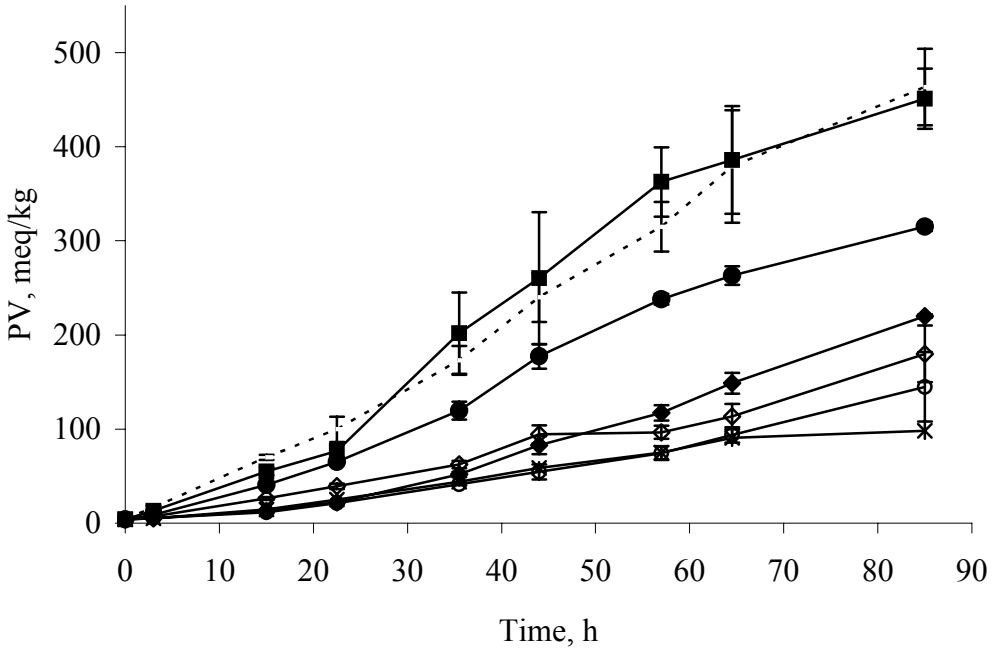

Figure 3.6. Accumulation of peroxides in rapeseed oil at 80 ºC: --- blank oil sample; ■ oil with 0.02% BHT; × oil with 0.1% sage AE; ♦ oil with 0.1% sweet grass AE; ● oil with 0.1% costmary AE; ◇ oil with 0.1% oil with 0.1% sweet grass DAE, ○ oil with 0.1% costmary DAE.

The comparison of the antioxidant activity of AE and DAE of sweet grass, costmary and horehound was investigated in rapeseed oil at 80°C. The accumulation of peroxides in oil samples with added sweet grass and costmary extracts is shown in figure 3.6. Deodorised extracts of both herbs retarded accumulation of peroxides in rapeseed oil better than the corresponding acetone extracts under the same conditions. This was especially obvious for costmary, while the activity of sweet grass extract increased only slightly after deodorisation. The increase of antioxidant activity of these herbs extracts could be caused by the removal of some compounds, present in the essential oil, which promote oxidation processes, or hydrolysis of some glycosides. Aglycones are usually more active antioxidants than their glycosides. The activities of DAE of costmary and sweet grass were comparable to that of the well-known natural antioxidant – sage AE.

To evaluate the oxidation processes in rapeseed oil, induction periods were determined graphically from the curves of peroxide formation, and protection factors were calculated according to formula (3.1). The results are presented in table 3.2.

Table 3.2. Evaluation of the influence of sweet grass and costmary extract additives on the formation of peroxides in rapeseed oil at 80 °C.

Sample	IP, h.	***PF***	PF evaluation
blank	3.8	-	-
with 0.02% BHT	4.9	1.29	very low
with 0.1% sage AE	19.1	5.03	very high
with 0.1% costmary AE	7.5	1.97	low
with 0.1% costmary DAE	21.7	5.71	very high
with 0.1% sweet grass AE	21.4	5.63	very high
with 0.1% sweet grass DAE	11.2	2.95	high

These results show that during the induction period the acetone extract of costmary retards the formation of peroxides, more than 1.5 times, relative to BHT. The low activity of BHT under these conditions can be explained by the volatility of this compound. After deodorisation costmary extract showed an even higher activity, however, this tendency is not observed in the samples with sweet grass extracts. The induction period of the sample with sweet grass AE is almost twice as long as that of the sample with sweet grass DAE. Although after about 50 hours the accumulation of peroxides in the sample with sweet grass DAE is a little lower than that in the sample with sweet grass AE, there was no significant difference ($P < 0.05$) between these two samples.

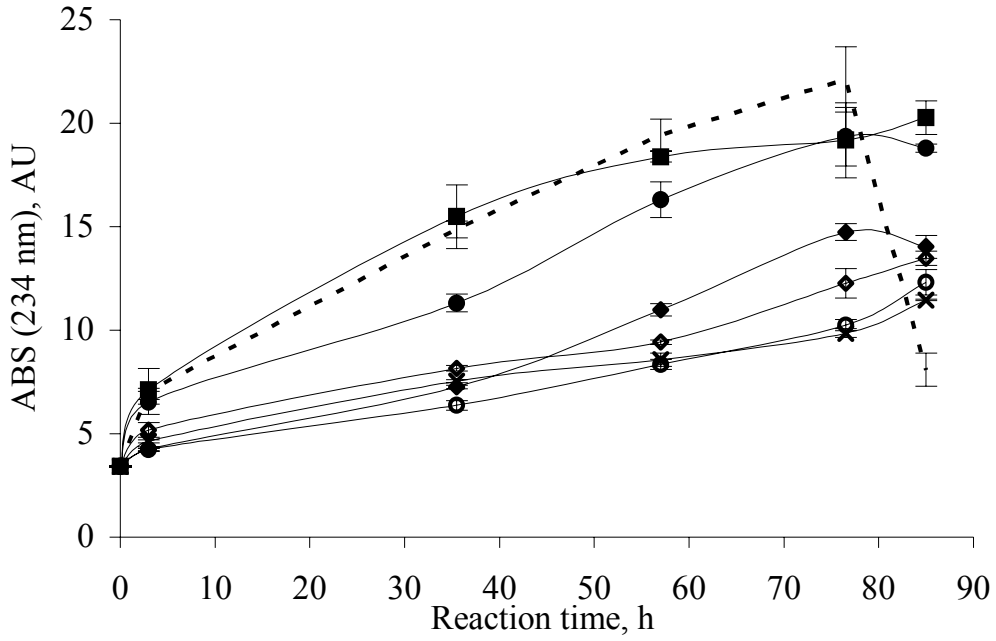

Figure 3.7. Accumulation of primary oxidation products in rapeseed oil at 80 ºC: --- blank oil sample; ■ oil with 0.02% BHT; × oil with 0.1% sage AE, ♦ oil with 0.1% sweet grass AE, ● oil with 0.1% costmary AE; ◇ oil with 0.1% sweet grass DAE, ○ oil with 0.1% costmary DAE.

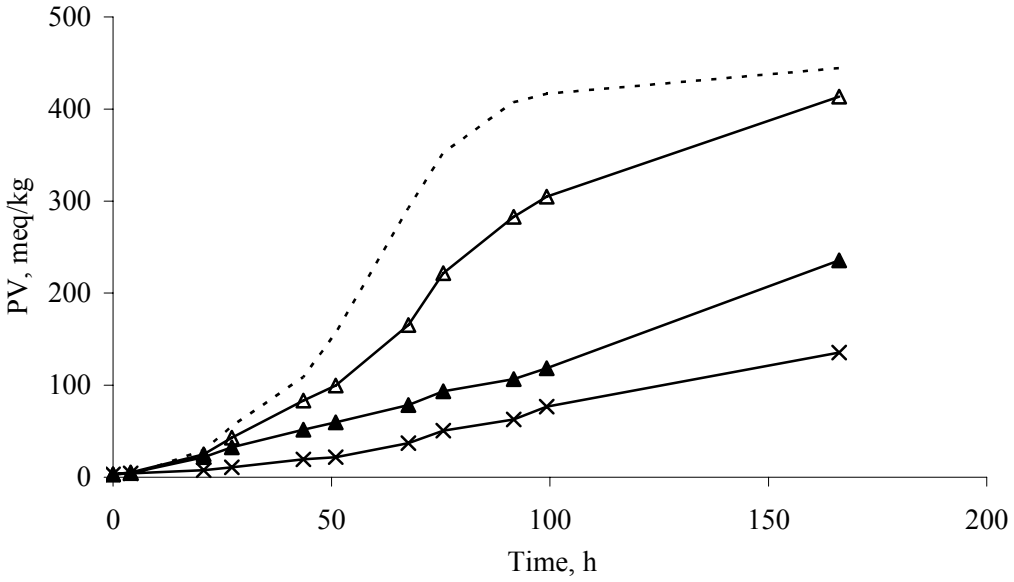

Figure 3.8. Accumulation of peroxides in rapeseed oil at 80 ºC: --- blank sample;
× oil with 0.1 % sage AE, ▲ oil with 0.1% horehound AE, Δ oil with 0.1% horehound DAE

The primary oil oxidation products were also determined by the UV absorbance at λ = 234 nm (Figure 3.7.). The results correspond well with the ones obtained by PV measurement. This test also confirmed that deodorisation of costmary acetone extract has a great influence on its activity and that deodorisation of sweet grass extract only slightly increases its activity.

Table 3.3. Evaluation of the influence of horehound extracts on the formation of peroxides in rapeseed oil at 80°C in comparison with sage AE.

Sample	IP, h	PF	PF evaluation
blank	15.0	-	-
with 0.1% sage AE	44.9	2.99	high
with 0.1% horehound AE	19.9	1.33	very low
with 0.1% horehound DAE	16.6	1.11	very low

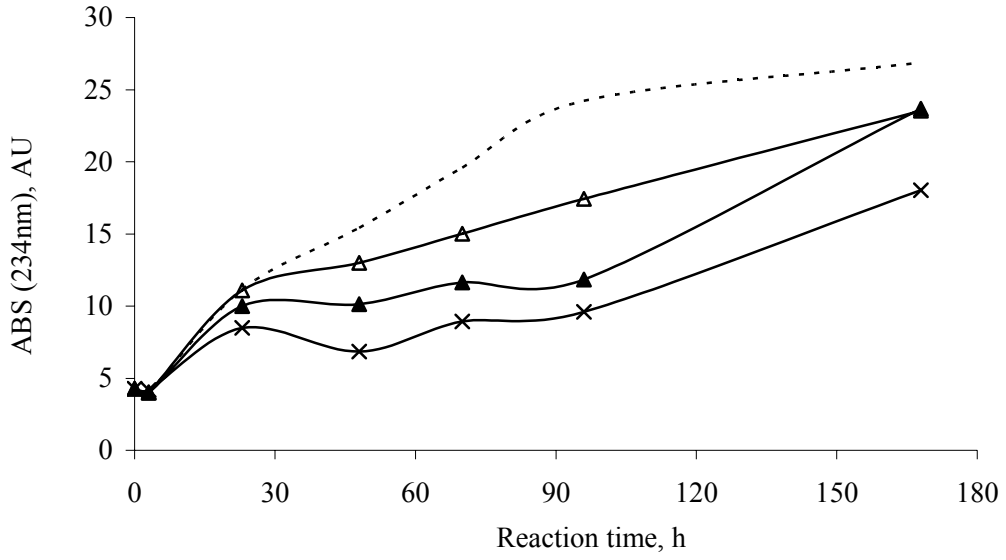

Figure 3.9. Accumulation of primary oxidation products in rapeseed oil at 80°C: --- blank sample; × oil with 0.1 % sage AE, ▲ oil with 0.1 % horehound AE, Δ oil with 0.1 % horehound DAE

The AE of horehound showed significant antioxidant activity in rapeseed oil at 80 ºC but less than that of sage AE (Figure 3.8.). Oxidation of an oil sample with the DAE of horehound did not differ significantly from the blank sample (P > 0.05). The deodorisation process clearly decreased the activity of horehound extract, this suggests the loss of some active compounds during the deodorisation process.

Induction periods, obtained graphically from PV curves and calculated protection factors show that both (AE and DAE) horehound extracts only slightly retard lipid oxidation during induction periods and that their effect is not comparable with that of sage AE.

Measurements of the oil UV absorbance at λ=234 nm (Figure 3.9) gave similar results to those obtained by the PV method, but the differences between horehound AE and DAE extracts were even smaller.

3.3.2. Activities of fractions obtained after partitioning.

The results of the *β*-carotene bleaching test showed that the highest activity in the linolenic acid model system was exhibited by the sweet grass (Antioxidant activity coefficient (AAC)=809) and the horehound (AAC=810) methanol-water extracts and the horehound butanol (AAC=818) and water (AAC=810) fractions obtained from the methanol water extract. All costmary fractions had lower AAC than rosmarinic acid or BHT (Table 3.4). The results show that fractions of higher polarity were the most active ones in this system.

A strange result was observed with the hexane fraction of the sweet grass methanol-water extract. The absorbance of the sample increased during the measurement. The only explanation for this is that some compounds are insoluble in the reaction medium and slowly precipitate.

In the DPPH assay the most effective radical scavengers were the horehound butanol fractions obtained from the acetone and methanol-water extracts, both the sweet grass *tert*-butyl methyl ether fractions and the crude acetone extract and the costmary crude methanol-water extract and its *tert*-butyl methyl ether and butanol fractions. The results from these two assays are not comparable, which shows that the assay type plays a most important role in antioxidant activity measurements.

All fractions from the methanol-water extracts were investigated in the accelerated rapeseed oil oxidation system. Fractions of methanol-water extracts of all herbs had little or no effect on the stability of rapeseed oil at 55 °C (table 3.5). Only sweet grass crude extract and its *tert*-butyl methyl ether fraction had a statistically significant effect on retarding the oxidation process in oil. Other fractions from the sweet grass methanol-water extract possessed no activity in this method. Horehound methanol-water extract and its butanol fraction had no effect on the oxidation of rapeseed oil. Other fractions showed some effect on the oxidation process, however only the water fraction retarded oil oxidation. Hexane and *tert*-butyl methyl ether fractions of horehound methanol-water extract had only negative effects on the oil oxidation process, i.e. they increased peroxide formation rates. A similar effect was observed with costmary extracts. Even if several

extracts had some effect on the oil oxidation process (table 3.5), only the water fraction of costmary possessed antioxidant activity.

Table 3.4. Yields and antioxidant activities of sweet grass, costmary and horehound methanol-water and acetone oleoresins fractions.

Herb	Extraction method	Fraction	Yield, %	AAC	DPPH scavenging, %
Sweet grass	MeOH 80%	crude	16.9	809	45.5±0.1
	water 19%	hexane	1.9	1390	13.9±0.5
	acetic acid	*tert*-butyl methyl ether	1.2	511	86.9±0.1
	1%	butanol	6.1	762	34.2±0.2
		water	7.7	763	5.7±0.2
	acetone	crude	9.3	597	84.1±0.1
		hexane	1.9	705	22.7±0.6
		tert-butyl methyl ether	2.1	597	81.8±0.1
		butanol	1.6	17	63.9±0.4
		water	0.5	259	19.7±0.2
Horehound	MeOH 80%	crude	20.1	810	58.2±0.7
	water 19%	hexane	0.95	774	6.1±0.2
	acetic acid	*tert*-butyl methyl ether	2.9	678	30.0±0.7
	1%	butanol	2.1	818	87.9±0.3
		water	10.5	810	22.2±0.8
	acetone	crude	10.9	657	19.5±0.2
	oleoresin	hexane	2.1	647	5.7±0.1
		tert-butyl methyl ether	1.2	778	48.5±0.5
		butanol	1.9	548	71.3±0.3
		water	0.3	643	32.7±0.3
Costmary	MeOH 80%	crude	31.9	752	87.0±0.1
	water 19%	hexane	2.1	440	10.2±0.8
	acetic acid	*tert*-butyl methyl ether	3.1	706	86.9±0.2
	1%	butanol	3.7	697	86.4±0.1
		water	12.9	702	56.2±0.9
	acetone	crude	10.0	564	23.9±0.4
	oleoresin	hexane	3.2	483	1.9±0.2
		tert-butyl methyl ether	3.2	770	45.4±0.6
		butanol	1.3	651	32.3±0.5
		water	0.6	728	13.6±0.2
Rosmarinic acid				882	88.6±0.2
BHT				870	68.9±0.6

Table 3.5. Accumulation of peroxides in rapeseed oil with different additives at 55 °C.

	PV values, (meq/kg)				P-value
Storage time, h	21	335	520	613.5	
Blank	1.9±0.2	159.1±11.8	448.9±22.5	745.5±24.6	
BHT 0.02%	2.3±0.3	88.9±1.0	176.3±25.6	514.8±28.5	0.0001*
Costmary crude extract 0.1% (MWE)	2.2±0.2	143.1±16.1	417.7±34.3	844.5±46.7	0.45
Costmary hexane fraction 0.1%	5.4±0.1	188.2±17.2	497.6±10.0	748.3±11.7	0.128
Costmary tert-Butyl methyl ether fraction 0.1%	4.4±0.3	162.2±25.8	452.7±70.7	861.9±130.6	0.096
Costmary butanol fraction 0.1%	2.2±0.6	149.5±12.2	500.0±16.4	866.9±28.8	0.068
Costmary water fraction 0.1%	2.8±0.3	123.0±6.4	303.6±30.0	729.7±32.0	0.0007*
5,8-dihydroxy coumarin 0.02%	3.1±0.5	27.0±2.3	32.4±6.2	113.4±14.8	0.0002*
5,8-dihydroxy coumarin 0.05%	3.5±0.5	23.0±3.9	63.4±11.3	59.5±7.0	0.0002*
Storage time, h	19.0	343.0	459.0	528.0	
Blank	1.7±0.5	97.3±21.9	159.4±20.6	303.2±10.8	
BHT 0.02%	2.1±0.2	58.3±8.9	101.3±17.8	107.6±5.9	0.0001*
Sweet grass crude extract 0.1% (MWE)	2.0±0.0	24.4±3.1	41.0±6.0	68.9±18.9	$1 \cdot 10^{-5}$*
Sweet grass hexane fraction 0.1%	3.1±0.3	130.2±16.4	164.9±8.2	265.0±22.5	0.1276
Sweet grass tert-butyl methyl ether fraction 0.1%	4.5±0.4	36.2±1.6	39.8±3.2	55.9±10.3	$4 \cdot 10^{-5}$*
Sweet grass butanol fraction 0.1%	3.5±0.6	131.8±8.4	164.9±9.1	257.1±7.2	0.3177
Sweet gras water fraction 0.1%	4.2±0.8	109.1±3.4	175.5±6.2	269.7±15.4	0.3871
Horehound crude ectract 0.1% (MWE)	2.0±0.2	113.1±3.9	154.0±9.0	218.3±27.2	0.0709
Horehound hexane fraction 0.1%	2.6±0.3	139.3±10.6	194.2±28.4	294.7±34.6	0.0003*
Horehound tert-butyl methyl ether fraction 0.1%	3.4±0.6	147.9±9.0	211.6±25.9	336.0±31.3	$5 \cdot 10^{-7}$*
Horehound butanol fraction 0.1%	2.6±0.3	112.4±2.4	172.5±19.9	234.1±38.9	0.248
Horehound water fraction 0.1%	3.2±0.7	110.3±28.3	132.3±19.1	181.4±23.9	0.0073*

* - sample showed statistically different activity from the blank.

There was a good correlation among the results obtained by the PV and the UV absorbance methods. The highest antioxidant activity among the tested fractions was shown by the sweet grass

crude methanol-water extract and by its *tert*-butyl methyl ether fraction. Their activity was comparable with that of synthetic antioxidant BHT. The activities of other fractions were considerably lower.

Extracts and fractions from horehound and costmary did not have significant activity under the conditions used. All these fractions were considerably less active than BHT. The water fractions of horehound and costmary were the most active among the other fractions of these herbs.

Table 3.6. Evaluation of the antioxidant activity of sweet grass, horehound and costmary methanol-water extracts and their fractions in rapeseed oil stored at 55 ºC

Sample		Induction period, h IP	Protection factor, PF	Evaluation of protection factor PF
Additive	Fraction			
Blank		173	-	-
BHT 0.02%		222	1.28	very low
Sweet grass	crude 0.1%	320	1.85	low
	hexane 0.1%	141	0.82	prooxidation
	t-but met ether 0.1%	267	1.54	low
	butanol 0.1%	182	1.05	very low
	water 0.1%	176	1.02	very low
Horehound	crude 0.1%	175	1.01	very low
	hexane 0.1%	112	0.65	prooxidation
	t-but met ether 0.1%	132	0.76	prooxidation
	butanol 0.1%	175	1.01	very low
	water 0.1%	189	1.09	very low
Blank		121	-	-
BHT 0.02%		152	1.26	very low
Costmary	crude 0.1%	131	1.08	very low
	hexane 0.1%	69	0.57	prooxidation
	t-but met ether 0.1%	97	0.80	prooxidation
	butanol 0.1%	108	0.89	prooxidation
	water 0.1%	136	1.12	very low

The effect on the stability of oil samples was calculated from the obtained PV changes and is presented in table 3.6. Only the sweet grass crude extract and its *tert*-butyl methyl ether fraction are active antioxidants under these conditions. The activity of all sweet grass fractions is lower than that of the crude extract. This suggests a synergistic effect between several compounds, which are later

distributed over different fractions, or decompose during fractionation. Activities of other fractions were insignificant, or showed prooxidative effects.

3.4. Conclusions

It is obvious that a deodorisation procedure is not suitable for all herb extracts. Only for costmary clearly positive results were obtained and the activity of the extract increased considerably. In other cases this procedure had no significant influence on the activity of the extract (sweet grass), or decreased the activity of the extract (horehound). These findings clearly demonstrate that isolation and preliminary purification processes are dependent on the type of compounds present in extracts and can be predicted only when the composition of extracts is known.

It also can be concluded that methanol – water extraction is not a suitable way to prepare extracts with antioxidant activity in oil. However, in general it is a good method for the isolation of a broad range of polar compounds, among which a lot of antioxidants can be present. As DPPH test results of different fractions show, the type of compounds having antioxidant activity differs depending on the herb and it is possible to choose the right extraction methods only after preliminary investigations.

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4. Isolation of Radical Scavengers from Sweet Grass, Costmary and Horehound*

4.1. Introduction

Antioxidants are widely used in foods, and also in cosmetics and pharmaceuticals [1]. Since the 1980's there has been an increased interest in research and application of natural antioxidants, instead of synthetic ones, caused by a consumer demand for natural food additives. Additionally for the latter the burden of proof of safety may be less rigorous than that required for synthetic antioxidants [2, 3]. The antioxidant activity of many plants has been investigated [4-8], however, to date, only rosemary and sage extracts are commercially available as flavourless, odourless, and colourless antioxidant extracts.

Sweet grass (*Hierochloe odorata* L.) belongs to the family Graminaceae. The root and the aerial parts of the herb possess a sweet smell. Sweet grass is a hardy aromatic perennial grass normally growing from Alaska to Newfoundland in rich, moist soil in the full sun. It is also native to northern Europe. There are few publications on sweet grass properties and chemical composition. Only the volatile compounds of this herb have been investigated [9]. No reports were found on the antioxidative activity of sweet grass. However, preliminary screening results of sweet grass showed that extracts of this herb retard lipid oxidation [10].

Costmary, *Chrysanthemum balsamita* L. (syn. *Balsamita major* L.) Asteraceae, is a large perennial plant from Asian origin with yellow flowers grown in Europe and Asia since the Middle Ages [11]. The name costmary is derived from costus (*Saussurea lappa* Clarke), an Oriental plant, the root of which is used as a spice and in preserves, and "Mary" in reference to Our Lady. The other name of the herb is alecost, because it was much used to give a spicy flavouring to ale. Fresh and dried leaves of costmary possess a strong mint-like aroma and an astringent taste. Leaves may be used with meats, poultry and tea. Costmary likes dry soils. Late in the season small yellow flowers appear on flower stalks about eighty centimetres in height. If planted in the shade, it will give many leaves, but no flowers.

Horehound (*Marrubium vulgare* L.), a member of the Labiatae, is native to North Africa, Central and Western Asia, and Southern Europe. It grows wildly in dry sandy soils and wastelands. The plant is used in traditional medicine, because of its stimulating action on the flow of bile and gastric actions, and it is a laxative, a purgative, and a cough soother. The species can be cultivated successfully in Lithuania and is harvested twice a year as a medicinal raw material [12]. It can be used as an ingredient in cough pastilles. Horehound serves as raw material for herbal extracts and

*This chapter based on the papers: Pukalskas, Audrius; Van Beek, Teris A.; Venskutonis, Petras Rimantas; Linssen, Jozef P.H.; Van Veldhuizen, Albertus; De Groot, Aede. Identification of radical scavengers in sweet grass (Hierochloe odorata). Journal of Agricultural and Food Chemistry. Vol. 50 (10) p. 2914-2919, 2002; A. Pukalskas, P. R. Venskutonis, T. A. van Beek, S. Salido Ruiz. Isolation, identification and activity of natural antioxidants from horehound (Marrubium vulgare) cultivated in Lithuania. Molecules, in preparation (2009); A. Pukalskas, T. A. van Beek, P. R. Venskutonis, I. Dijkgraaf. Isolation, identification and activity of natural antioxidants from costmary (Chrysanthemum balsamita) cultivated in Lithuania. Molecules, in preparation (2009);

beverage industries. The plant has also been used as a substitute for hop in beer-breweries. Phytochemical investigations of horehound resulted in the isolation of the flavonoids apigenin and luteolin and their 7-glucosides together with quercetin and its 3-glucoside and 3-rhamnoglucoside [13]. *Nawwar* et al. reported on the isolation and structural elucidation of several flavonoids: luteolin and apigenin 7-lactates together with their 2´´-O-β-glucuronides and 2´´-O-β-glucosides [14]. In addition, several diterpenoids have been isolated and characterised, the main one being marrubiin [15-18].

As part of an on-going investigation of Lithuanian herbs, the antioxidant properties and structures of isolated antioxidants of sweet grass, costmary and horehound are reported in this chapter.

4.2. Materials and Methods

4.2.1. Chemicals.

The following solvents were used for the extraction and fractionation: methanol, hexane, *tert*-butyl methyl ether, and butanol. All solvents were distilled prior to use. Solvents used for preparative chromatography and antioxidant activity testing were of analytical grade (Sigma Chemical, St. Louis, MO). For HPLC separations solvents of HPLC grade (Lab-Scan Analytical Sciences, Dublin, Ireland) were used. The following reagents were used in the antioxidant activity experiments: 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) (95%, Sigma-Aldrich Chemie, Steinheim, Germany), rosmarinic acid (Extrasynthese, Genay, France), 2,6-*di*-*tert*-butyl-hydroxytoluene (BHT) (Sigma-Aldrich Chemie, Steinheim, Germany), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Fluka Chemie, Buchs, Switzerland) and Trolox 97% (Sigma–Aldrich Chemie, Steinheim, Germany). Deuterated methanol, deuterated chloroform and deuterated dimethyl sulfoxide (Acros Organics, Geel, Belgium) were used to prepare solutions of compounds for NMR analysis.

4.2.2. Preparation of Plant Extracts.

Aerial parts of *Hierochloe odorata, Chrysanthemum balsamita* and *Marrubium vulgare* were obtained from the collection of Kaunas Botanical Garden in 1998, air dried in a Vasara ventilated oven (Utenos krosnys, Utena, Lithuania) at 30°C for about 48 h and ground to fine particles before use. Dried and ground plant material (50 g) was extracted (2 × 1 L) with methanol - water - acetic acid (79:20:1) at room temperature for 24 h. Solvent and plant material was constantly mixed on an Ikamag RTC basic magnetic stirrer (IKA Labortechnik, Staufen, Germany). The extract obtained

was concentrated in a rotary evaporator at 40°C to about 150-200 mL. The solution was diluted to 500 ml with ultra pure water and then extracted with hexane, *tert*-butyl methyl ether and finally butanol. Several successive extractions with every solvent were made, every time using 100 ml of solvent. Total amounts of 500 - 600 ml of each solvent were used. Organic solvents were removed with a rotary evaporator at 40°C. The remaining aqueous phase was freeze-dried.

4.2.3. DPPH Assay.

Radical scavenging activities of isolated pure compounds against the stable radical $DPPH^{\bullet}$ were measured using the method of Von Gadow et al. [19], as described in chapter 3.2.2, except that the concentrations of compounds tested and DPPH were taken on a molar basis, and the concentrations of compounds (EC_{50}, (mol/L antiox)/ (mol/L DPPH)) needed to scavenge 50% of the DPPH [20] were determined. For reasons of clarity the antiradical power (ARP) of antioxidants, as $1/EC_{50}$ were calculated. The higher the ARP, the more efficient the antioxidant.

4.2.4. ABTS Assay.

The $ABTS^{\bullet+}$ radical cation was produced by oxidising ABTS with potassium persulfate [21]. To prepare the stock solution, ABTS was dissolved at a 2 mM concentration in 50 mL of phosphate buffered saline (PBS) prepared from 8.18 g of NaCl, 0.27 g of KH_2PO_4, 1.42 g of Na_2HPO_4 and 0.15 g of KCl dissolved in 1 L of ultra pure water. If the pH was lower than 7.4, it was adjusted with NaOH. A 70 mM of $K_2S_4O_8$ solution in ultra pure water was prepared. The ABTS radical cation was produced by reacting 50 mL of ABTS stock solution with 200 µL of $K_2S_4O_8$ solution and allowing the mixture to stand in the dark at room temperature for 16-17 h before use. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of antioxidant compounds the $ABTS^{\bullet+}$ solution was diluted with PBS to an absorbance of 0.800 ± 0.030 AU at 734 nm. Stock solutions of the compounds in methanol were diluted with 10% methanol in PBS such that after introduction of a 10 µL aliquot of each dilution into the assay, they produced a 10-80 % decrease of the blank absorbance.

After addition of 990 µL of diluted $ABTS^{\bullet+}$ solution ($A_{734\ nm}$ = 0.800 ± 0.030) to 10 µL of antioxidant compounds or Trolox standards (final concentration 0 - 20 µM) in ethanol or PBS, the absorbance was read at ambient temperature exactly 1 and 6 min after the initial mixing. Appropriate solvent blanks were run in each assay. All determinations were carried out in triplicate. The percentage decrease of the absorbance at 734 nm was calculated and plotted as a function of the concentration of the antioxidants and of Trolox for the standard reference data. To calculate the Trolox equivalent antioxidant coefficient (TEAC), the slope of the plot of the percentage inhibition

of absorbance vs. concentration for the antioxidant was divided by the slope of the plot of Trolox. This gives the TEAC at the specific time point [21].

4.2.5. HPLC – DPPH Conditions and Instrumentation.

The on-line DPPH scavenging tests were performed using the method developed by Koleva et al. [22] and modified by Dapkevicius et al. [23] on an HPLC system equipped with a Waters 600E multisolvent delivery system (Millipore Corp., Waters Chromatography Division, Milford, MA), and an autosampling injector Model 231 (Gilson Medical Electronics, Middleton, WI). The linear binary gradient was formed at a constant flow rate of 0.8 mL/min. Solvent A was a 20% methanol solution in water, and solvent B 100% methanol. Separation of compounds was carried out on a 25 cm × 0.46 mm i.d. end-capped Alltima C18 analytical column (Alltech Associates, Deerfield, IL).

Sweet grass extracts were separated using the following conditions: an initial isocratic flow of 100% of solvent A for 8 min. was followed by an increase to 100% solvent B during 17 min, then isocratic conditions were maintained for 17 min. Finally the gradient was returned to its initial conditions in 3 min and the column was equilibrated during 5 min.

Costmary extracts were chromatographed using the following conditions: an initial isocratic flow of 75% of solvent A for 10 min. was followed by an increase to 55% of solvent B during 20 min., then increase to 100% of solvent B during 10 min, and isocratic conditions for 5 min. Finally the gradient was returned to its initial conditions in 5 min and the column was equilibrated during 5 min.

The linear binary gradient for horehound was formed at a constant flow rate of 0.8 mL/min. Solvent A was a 2% acetonitrile solution in water, and solvent B was 100% acetonitrile. Separation of the *tert*-butyl methyl ether fraction was performed as follows: initial isocratic conditions of 80% of solvent A for 5 min. were followed by an increase to 50% of solvent B during 20 min., then isocratic conditions for 5 min., followed by an increase of solvent B to 100% during 10 min. and holding the isocratic conditions for 5 min. Finally the gradient was returned to its initial conditions in 5 min and the column was equilibrated during 5 min.

Separation of the butanol fraction was performed as follows: initial isocratic conditions of 85% of solvent A for 35 min. were followed by an increase to 80% of solvent B during 10 min., then isocratic conditions for 5 min. Finally the gradient was returned to its initial conditions in 5 min and the column was equilibrated during 5 min.

Compounds eluted from the column were detected with a Waters 990 series Photodiode Array Detector (Millipore Corp. Waters Chromatography Division, Milford, MA) over the range 210-450 nm. Data were processed with Waters software, version LCA-6.22a. After the separation and detection a 10^{-4} M solution of DPPH in methanol was added with a 45 mL laboratory-made syringe

pump (Free University, Amsterdam, The Netherlands) at a flow rate of 0.70 mL/min. The mixture was continuously introduced into a 15 m reaction coil and the decrease in absorbance of a DPPH solution was measured at 517 nm with a 759A model absorbance detector (Applied Biosystems, Foster City, CA) equipped with a tungsten lamp.

4.2.6. Isolation of radical scavengers from sweet grass.

Fractionation conditions were determined using Silica gel 60 F_{254} TLC plates (5×10 cm) (Merck, Darmstadt, Germany). The fractionation of the *tert*-butyl methyl ether fraction (0.4 g) was performed on a 50 g silica gel column (40-63 μm, Fluka Chemie, Buchs, Switzerland) with ethyl acetate-hexane 1:1. A total of 100 fractions (10 mL each) were collected. Radical scavenging activity was determined by spotting fractions on a TLC plate and then spraying the TLC plate with 0.2% DPPH solution in methanol. The active fractions were 9 to 19. Active fractions were checked for purity on TLC. Fractions 11 to 19 were found to contain a single compound and they were combined and evaporated to dryness with a rotary evaporator. 141 mg of yellow crystalline material **1** (m.p. 216°C) was obtained. The yield of **1** was 0.44% based on the dry plant material.

0.8 g of the butanol fraction was separated with chloroform-methanol-water (60:22:4) on an 80 g silica gel column and 85 fractions were collected. Fractions 4-6 and 13-17 showed activity in the DPPH test. TLC showed that in fractions 4-6 the same active compound **1** was present that was previously isolated from the *tert*-butyl methyl ether fraction. The radical scavenging fractions 14-16 were combined and the solvent was evaporated with a rotary evaporator. The material was then dissolved in methanol and left overnight in a refrigerator. The white crystals (m.p. 197°C) that were obtained were separated from the solvent and dried. 62 mg of compound **2** were obtained in a 0.47% yield based on the dry plant material.

Hydrolysis of **2**. 10 mg of **2** was dissolved in a 20 ml of 0.1 M HCl and refluxed for 90 min. The solvent was removed in a rotary evaporator, 20 ml of water were added and evaporated again. 10 ml of water were added and extracted 3 times with 2 ml of *tert*-butyl methyl ether. The *tert*-butyl methyl ether fractions were combined and evaporated in a rotary evaporator and the solid material obtained (3 mg) was tested on HPLC using the same conditions as for the isolated compound **1.** According to the retention time, and the UV spectra, recorded with the DAD, the aglycone part of **2** is 5,8-dihydroxybenzopyranone. The aqueous solution was freeze-dried and a specific optical rotation $[\alpha]_{589/D}^{20}$ in water of the remaining material of +20° was obtained.

Instruments. ^{1}H NMR spectra were recorded on Bruker DPX 400 or Bruker AC-E 200 spectrometers (Bruker, Rheinstetten, Germany). ^{13}C spectra were recorded on a Bruker DPX 400 operating at 100 MHz. DEPT spectra and 2D experiments (COLOC and HMBC) and the

deuteration experiment were performed on a Bruker DPX 400. NMR spectra of compound **1** were recorded in a mixture of deuterated chloroform and deuterated methanol (4:1). For the deuteration experiment 4 drops of non-deuterated methanol were added, and the ^{13}C NMR spectrum was recorded. NMR spectra of compound **2** were recorded in deuterated DMSO. NMR assignments are shown in Table 4.2.

Mass spectra and accurate mass measurements were recorded on a Finnigan/MAT95 MS analyzer (Thermo Finnigan MAT, Bremen, Germany) in the EI mode.

UV spectra were recorded on a Lambda 18 spectrophotometer (Perkin - Elmer, Ueberlingen, Germany) and IR spectra on a Perkin Elmer, 1725 X FT-IR spectrometer). Optical rotation measurements were performed in a 10 cm 1 mL measuring cell on a Perkin Elmer 241 polarimeter (Perkin - Elmer, Ueberlingen, Germany) using a sodium lamp at 589 nm.

Melting points of compounds were measured on a Buchi 510 apparatus (Buchi, Flawil, Switzerland).

Spectral data of **1**: UV (MeOH) λ_{max} 267, 305 nm. and 363 nm. IR (KBr) 3397, 3221, 1690 (C=O), 1621, 1581, 1509, 1460, 1189 and 1037 cm^{-1}. EI-MS spectrum (70 eV) *m/z* 178 (M^+, 100%), 150 (11), 122 (20), 94 (19), 66 (10). Accurate mass measurements see text. ^1H and ^{13}C-NMR spectra see Table 4.2.

Spectral data of **2**: UV (MeOH) λ_{max} 260, 301 and 349 nm. IR (KBr) 3332, 2945, 2833, 1450, 1115 and 1027 cm^{-1}. FD-MS *m/z* 341 (79), 340 (M^+, 81%) 178 (74), 177 (31), 163 (13). $[\alpha]_{589/D}^{20} = -63°$ (c = 0.3, MeOH).

Spectral data of **6**: UV (MeOH) λ_{max} 251, 270 and 345 nm. EI-MS (70 eV) *m/z* 346 (M^+, 100%), 331 (38), 316 (19), 303 (17), 69 (32), 55 (20), 43 (28).

4.2.7. Isolation of Radical Scavengers from Costmary

4.2.7.1. Preparative MPLC separation.

About 57 g of RP-18 stationary phase (Baker Bond Phase C18 For Flash, Mallinckrodt Baker B.V., Deventer, Holland) were used to pack the column (46 x 2 cm i.d.). Approximately 0.4 g of the *tert*-butyl methyl ether (C2) fraction was loaded on this column. The pressure on the column was about 12 bar (Jobin Yvon axial compression system, I.S.A. Jobin Yvon d'Instruments S.A., Longjumeau, France). Fractions were collected automatically (Gilson 202 Fraction Collector and Gilson 201-202 Fraction Controller). The separation was started with a mixture of 25% of acetonitrile and 0.5% of formic acid in ultra pure water as the eluting solvent (Gilson 802C pump). During the separation, a step gradient elution of 25%, 35%, 45% and 55% of acetonitrile in water

acidified with 0.5% of formic acid (98%-100%, Merck, Darmstadt, Germany) was used. Detection of the separation was carried out with a Gilson 111 UV detector at 254 nm. The detector was connected to a recorder (Kipp & Zonen BD40, Delft BV, The Netherlands). The obtained fractions were tested on TLC and sprayed with 0.2% $DPPH^{\bullet}$ in methanol solution. Active fractions with the same composition were combined.

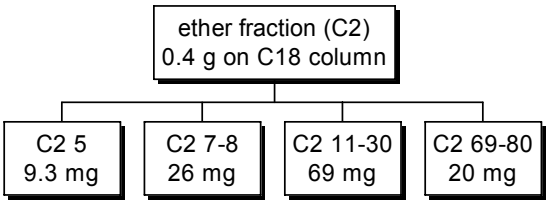


Figure 4.1. Fractionation scheme of the preparative MPLC separation

The fractions 5, 7-8, 11-30 and 69-80 were tested on HPLC. For all HPLC measurements made, the same equipment as described in the on-line HPLC-DPPH method was used.

Fraction 11-30 contained compound (**4**) and this fraction was used for separation on a semi-preparative C18 column (5 µm, 10.0 x 250.0 mm i.d., 300Å, Rainin Instrument Co., Inc., Emeryville, USA). A linear gradient elution was used as given in table 2.2. Samples were injected manually (injector module 480, Applied Biosystems, connected to 500 µL loop).

Table 4.1. Linear gradient of semi-preparative separation

Time (min)	Solvent A (%)	Solvent B (%)
0	85	15
35	85	15
40	0	100
45	85	15
50	85	15

Solvent A = 2% acetonitrile and 0.1% formic acid in water

Solvent B = 100% acetonitrile

Twelve mg of compound **4** were isolated and ^{1}H, ^{13}C, 2D COSY and HMBC spectra were recorded (Bruker AM-400). Also an LC-MS spectrum in direct infusion mode (ESI, negative mode, Finnigan MAT) was recorded. Fraction 69-80 contained two major compounds (**5** and **6**). However the amount of the fraction was too small for further separation.

Table 4.2. Elution mixtures for the separation on a silica column

ethyl acetate (%)	methanol (%)	water (%)	formic acid (%)
100	0	0	0
90	10	0	0
80	20	0	0.5
70	30	0	0.5
50	50	0	0.5
30	70	0	0.5
0	100	0	0.5
0	90	10	0.5

4.2.7.2. Separation on a silica column

Approximately 0.9 g of the *tert*-butyl methyl ether fraction was separated on a silica column (100 g, particle size 0.063-0.200 mm, Merck, Darmstadt, Germany) using step gradient elution. Elution was started with ethyl acetate: hexane (70:30). Subsequently the following solvents were used, table 4.2. Fractions were collected manually and tested on TLC (silica gel, layer thickness 0.2 mm; medium pore diameter 60 Å; Fluka Chemica, Buchs, Switzerland). Active fractions with the same composition were combined. The five fractions obtained 6-8, 9-19, 62-65, 66-96 and 99-132 were analyzed on HPLC. It appeared that the compound of interest was present in fractions 6-8 and 9-19. The fractions 6-8 and 9-19 were combined and loaded on a silica column. Fractions 62-65, 66-96 and 99-132 were re-chromatographed on an MPLC C18 column (described under preparative MPLC C18 separation). The fractions were collected automatically.

Fraction 62-65. The separation of this fraction was started with a mixture of 20% acetonitrile and 1% formic acid in water as mobile phase. The separation proceeded with 50% acetonitrile and 1% formic acid in water.

Fraction 66-96. This fractionation was started with a mixture of 2% acetonitrile and 1% formic acid in water as the elution solvent. Subsequently mixtures with 15%, 20% and 30% acetonitrile in water acidified with 1% formic acid were used as mobile phases.

Fraction 99-132. A mixture of 2% acetonitrile and 1% formic acid in water was used as the starting elution solvent. Then mixtures with 25%, 40% and 50% acetonitrile in water acidified with 1% formic acid were used. The fractionation ended with 1% formic acid in acetonitrile as elution solvent.

Fraction 6-19. For fractionation of this sample about 25 g of silica were used. The gradient elution, used for fractionation is given in table 4.3.

Table 4.3. Step gradient elution used for the separation of fraction 6-19

n-hexane (%)	ethyl acetate (%)	formic acid (%)
70	30	0
50	50	0
40	60	0
30	70	0
0	100	0
0	100	0.5

The column was washed with 50% ethyl acetate, 50% n-hexane and 0.5% formic acid. The fractionations made can be seen in figure 4.2.

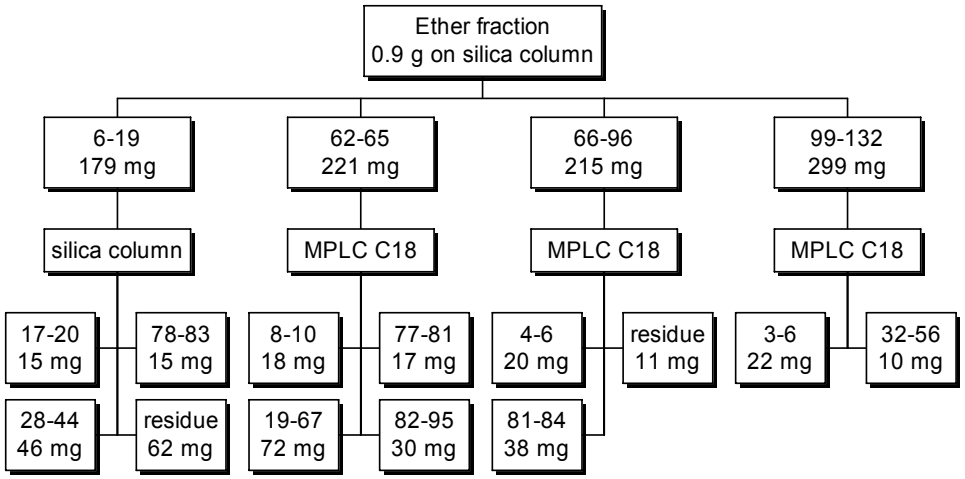


Fig. 4.2. Fractionation scheme of 0.9 g of the crude *tert*-butyl methyl ether fraction

On-line HPLC-DPPH measurements of all obtained fractions were made. It appeared that fraction 82-95 contained the same two compounds as fraction 69-80. Fraction 82-95 was separated on a semi-preparative C18 column (5 μm, 10.0 x 250.0 mm i.d., 300Å, Rainin Instrument Co., Inc., Emeryville, USA) using isocratic conditions with 75% solvent A and 25% solvent B. Solvent A = 2% acetonitrile in water and solvent B = 100% acetonitrile. Samples were injected manually (injector module 480, Applied Biosystems, connected to 500 μl loop). 1.5 mg of compound **5** and 0.9 mg of compound **6** were isolated. ^{1}H-NMR spectra of both compounds were recorded.

Fraction 28-44 contained the same two compounds as fraction 82-95 and was later separated on a Sephadex LH-20 column (Pharmacia, Sweden) with different concentrations of methanol in water as elution solvent. About 9 mg of compound **5** and 7 mg of compound **6** were isolated.

For isolation of compound **3** the butanol fraction was used, because this compound was present in higher concentration in the butanol fraction than in the *tert*-butyl methyl ether fraction.

Approximately 0.5 g of the butanol fraction was loaded on the same MPLC C18 column as described before under preparative MPLC separation. The separation was started with 5% acetonitrile and 0.1% formic acid in water as the eluting solvent. Then 50% acetonitrile and 0.1% formic acid in water was used. The column was washed with 50% acetonitrile and 0.1% formic acid in water. Two fractions were collected manually. To get more material again 0.4 g of butanol fraction was loaded on the MPLC C18 column. The first fraction was the fraction of interest. After testing on RP-18 TLC, it appeared that this fraction consisted of more than one compound. This fraction was again separated on the MPLC C18 column. Now the eluting solvent used was 0.5% formic acid in water. Several concentrations of acetonitrile in water with 0.1% formic acid were used. When 50% acetonitrile and 0.1% formic acid in water was used, one fraction, containing antioxidant compounds was obtained. When this fraction was tested on RP-18 TLC, it was apparent that it contained more than one compound. After its separation on a C18 column (10 g, Baker Bond Phase C18 For Flash, Mallinckrodt Baker B.V., Deventer, Holland), 11 mg of compound **3** were isolated.

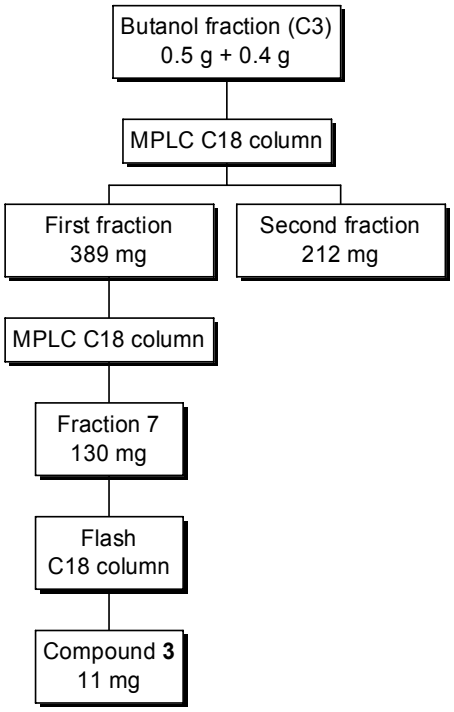


Fig. 4.3. Fractionation scheme of the butanol fraction

4.2.8. Isolation of Active Compounds from Horehound.

4.2.8.1. *tert*-Butyl methyl ether fraction.

There was one active compound detected with on-line DPPH radical scavenging in the *tert*-butyl methyl ether fraction of the methanol-water-acetic acid extract of horehound. The fractionation of this material was carried out on a 4 cm internal diameter 15 cm length column, packed with Sephadex LH-20. 0.48 g of the fraction was loaded on the column and pure methanol was used as the mobile phase. A total of 50 fractions (4-5 ml each) was collected. All fractions were checked for purity on TLC afterwards by spraying them with a 0.2 % DPPH solution. The active fractions 29 to 37 appeared to be identical, and they were combined and checked for purity with HPLC-DAD using the same gradient as for the *tert*-butyl methyl ether fraction. According to its HPLC profile and UV spectrum, fraction 29-37 appeared to be pure. It was evaporated until dryness with a rotary evaporator and 15 mg of dry compound **7** was obtained.

4.2.8.2. Butanol fraction.

This fraction was separated on a silica gel column. Two g of the material were loaded on 100 g of silica gel and eluted with a mixture of chloroform-methanol-water (60:22:4) till fraction 112; with a mixture of chloroform-methanol-water (6:4:1) till fraction 144; with methanol till fraction 163, and finally with a mixture of methanol-water (1:1). In total 192 fractions (15-20 ml each) were collected. After checking all fractions for purity on a silica gel TLC and detecting active compounds by spraying with DPPH, most of the factions were found to have radical scavenging activity. Similar active fractions were combined and collected. Fractions obtained after combining were evaporated till dryness in a rotary evaporator. The fractions were named 3; 4-5; 6; 7-9; 10-12; 13-14; 15-17; 18-24; 25-28; 29-35; 36-47; 48-59; 60-80; 81-90; 91-119; 120-128; 129-131; 132-148; 151-155; 164-170; 179-186.

Fraction 25-28 (59.7 mg) was chromatographed on a 2 cm i.d. 20 cm length column loaded with Sephadex LH-20. The material was eluted with a mobile phase of methanol-water (1:1). 26 fractions were collected in total. All the fractions were checked on HPLC (analysis conditions described in 4.2.5) for purity and presence of the wanted compound. Fractions 18 and 19 were combined, and the solvent was evaporated with a rotary evaporator at a temperature of 45°C. Five mg of compound **8** were obtained.

Fraction 36-47 (123 mg) was chromatographed on a 2 cm i.d. 20 cm length column loaded with Sephadex LH-20. The material was eluted with a mobile phase of methanol-water (1:1) and 18 fractions (6-8 ml each) were collected. After checking on HPLC (analysis conditions described in 6.2.5) fractions 11 and 12 were combined and evaporated to dryness in a rotary evaporator to yield 27 mg of material **9**.

Fraction 60-80 (286 mg) was chromatographed on a 2 cm i.d. 20 cm length column loaded with Sephadex LH-20. The material was eluted with a mobile phase of methanol-water (2:3) and 23 fractions were collected in total. After checking on HPLC (analysis conditions described in 4.2.5) fractions 5,6 and 8,9 were combined and evaporated to dryness in a rotary evaporator. From the combined fraction 5-6 compound **5** (9 mg) was obtained. Fraction 8-9 (88 mg) consisted of two compounds. One of the compounds was the previously isolated **9**. To separate the unknown compound the material was again loaded on a Sephadex column and eluted with a mobile phase of methanol-water (1:4). 16 Fractions were collected and then the column was washed with about 100 ml of methanol. All fractions obtained were checked on HPLC. The compound of interest was in fraction 12. This fraction was evaporated till dryness and 24 mg of compound **10** were obtained.

4.3. Results and Discussion

4.3.1. Sweet grass

4.3.1.1. DPPH Radical Scavenging.

The yields of various fractions and their DPPH radical scavenging data, calculated by formula (3.5) are presented in Table 4.4. The results show that the acetone extract was a more effective radical scavenger than the methanol–water extract. This finding suggests that the most active radical scavengers in sweet grass are rather non-polar compounds.

Table 4.4. The yields of sweet grass extracts and fractions (weight ratio extract - DPPH 3:1) and their DPPH scavenging %

Extraction method	**Fraction**	Yield, %	DPPH scavenging %
MeOH 79%	Crude extract	16.9	52.2 ± 0.8
water 20%	Hexane	1.9	13.5 ± 0.5
acetic acid 1%	*tert*-Butyl methyl ether	1.2	86.9 ± 0.1
	Butanol	6.1	34.2 ± 0.3
	Water	7.7	5.7 ± 1.1
Acetone	Crude extract	9.3	84.1 ± 0.1
extract	Hexane	1.9	22.3 ± 0.8
	tert-Butyl methyl ether	2.1	81.8 ± 0.1
	Butanol	1.6	63.5 ± 0.8
	Water	0.5	19.7 ± 0.2
Rosmarinic acid			88.6 ± 0.2

Similar results were obtained from the screening of the different fractions. The highest radical scavenging activity was shown by the fraction obtained with *tert*-butyl methyl ether. The activity of this fraction was comparable with that of rosmarinic acid (scavenging = 90%). The activity of the other fractions obtained from the methanol-water extract was considerably lower. Although the fractions from the acetone extract were more active than those of the methanol-water extract, their yields were considerably lower. This only shows that compounds of higher polarity had little or no effect on the antioxidant activity of sweet grass extracts, and that antioxidants of higher purity may be extracted with acetone.

4.3.1.2. Separation of Active Compounds.

Based on the DPPH screening results, the *tert*-butyl methyl ether fraction of the methanol-water-acetic acid extract was selected for further fractionation, separation and identification of radical scavengers. The HPLC separation of the *tert*-butyl methyl ether fraction was performed with on-line detection with DPPH solution. It can be observed (Figure 4.4) that there is only one compound possessing free radical scavenging activity. As this compound **1** was non-polar it was purified on silica gel, yielding 141 mg of pure compound. The structure of the compound was determined by NMR and mass spectral data.

The ^{1}H NMR spectrum of **1** (Table 4.5) showed four doublets in the low field ppm range. This indicated that the compound had four non-exchangeable hydrogen atoms in aromatic rings or in a conjugated system. The ^{13}C NMR spectrum showed nine carbon atoms. In combination with the HRMS data (M^+, 178.0273) a molecular formula of $C_9H_6O_4$ (calculated M^+ 178.0266) was determined for this compound. A signal of a carbonyl carbon at 162.3 ppm (most likely an ester) was present in the ^{13}C NMR spectrum.

From the data obtained it was concluded that the compound possessed a coumarin structure (benzopyranone) with two hydroxyl groups attached. The coupling constants of the H atoms suggested that there are two pairs of vicinal protons. As could be seen from the ^{1}H NMR spectrum (two pairs of doublets), there could not be any hydroxyl groups in the heteroaromatic ring. Thus, both hydroxyl groups were in the benzene ring and only three possible structures for **1** remained. The hydroxyls could be attached to carbons 5 and 6, 5 and 8, or 7 and 8 (Figure 4.5). On the basis of chemical shift evidence in the ^{13}C NMR spectrum, the known 7,8-dihydroxy isomer (daphnetin) could be excluded [24, 25]. To determine the exact position of the hydroxyl groups a long-range two-dimensional C-H NMR (COLOC) spectrum was recorded. From the cross peaks seen in the two-dimensional NMR (Figure 4.6) both the 5,6- and 5,8-substitution patterns were in accordance with the data obtained from this experiment.

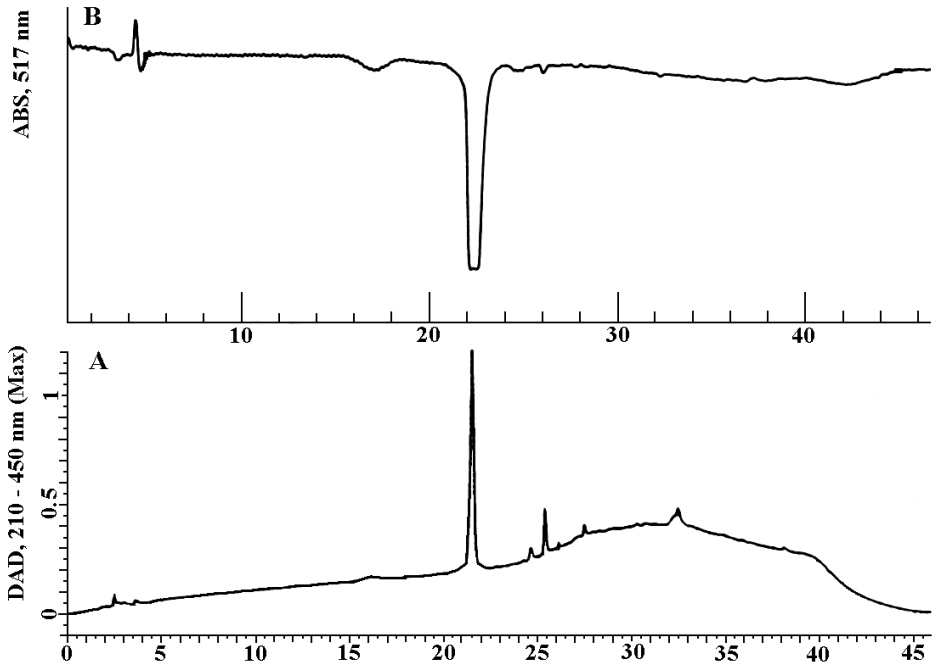


Figure 4.4. HPLC UV (A) and DPPH (B) on-line chromatograms of the *tert*-butyl methyl ether fraction

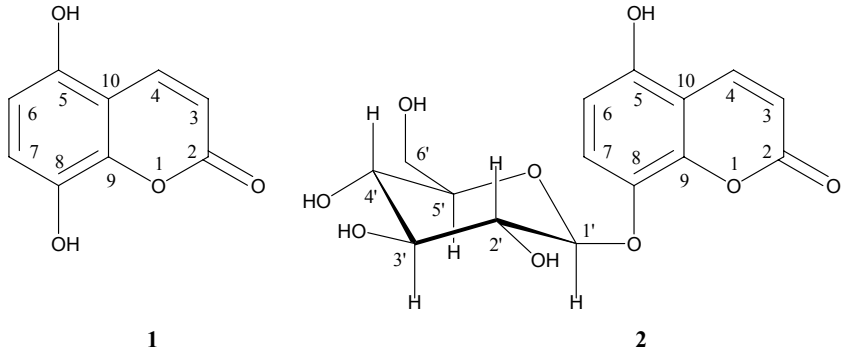


Figure 4.5. Radical scavengers isolated from *Hierochloe odorata*

To distinguish between the two remaining possibilities a deuteration experiment was performed [26]. The crux of this experiment is that carbons near a hydroxyl group appear as two peaks due to an isotope effect. Replacement of hydrogen by a deuterium atom causes a 0.15 ppm shift at the ipso carbon and a 0.05 ppm shift at the neighbouring carbons. Six carbon peaks were split due to an exchange of H and D at the hydroxyls (Figure 4.7). This establishes the structure with the hydroxyls at the 5,8 position, i.e. 5,8-dihydroxycoumarin **1**.

In the literature 5,8-dihydroxy-coumarin has once before been reported by Dopke et al. [27], as a compound obtained from its dimethoxy derivative after dealkylation. However, the ^{13}C NMR

data of the supposed 5,8-dimethoxycoumarin are not in agreement with solid, earlier reported ^{13}C NMR data on 5,8-dimethoxycoumarin [25].

Comparing the data provided in the above mentioned reports, it can be concluded that the initial compound, used by Dopke et al. [27] to obtain dihydroxycoumarin was in fact not 5,8-dimethoxycoumarin, but its 7,8-dimethoxy isomer. Thus to my knowledge this is the first time that 5,8-dihydroxycoumarin is reported as a natural product. The *para*-phenolic groups can readily explain the radical scavenging activity of **1**.

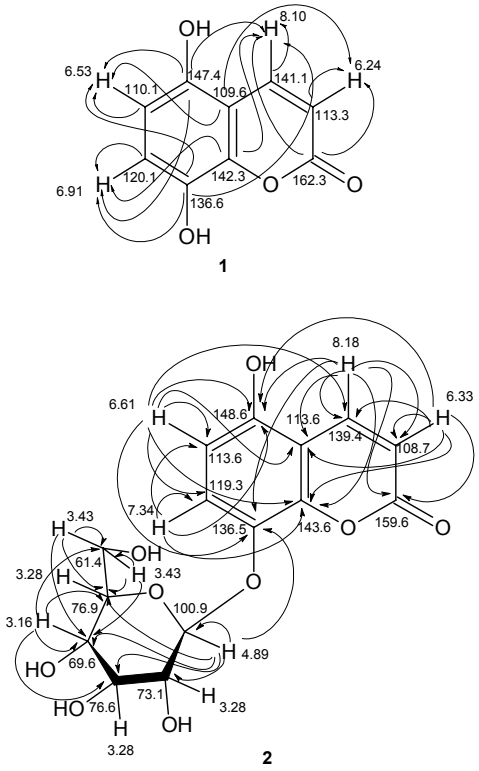


Figure 4.6. 2D NMR (COLOC) interactions of 5,8-dihydroxycoumarin (**1**) and HMBC interactions of 5-hydroxy-8-*O*-*β*-D-glucopyranosyl benzopyranone (**2**)

No other active fractions were found in the *tert*-butyl methyl ether fraction; therefore the next experimental step was the fractionation of the butanol fraction of the sweet grass methanol-water-acetic acid extract.

After separation, 62 mg of pure **2** were obtained and various spectroscopic data were recorded. The ^{1}H NMR spectrum (Table 4.5) of **2** was quite similar to that of 5,8-dihydroxycoumarin, except that there was a doublet at 4.89 ppm, two doublets at 3.43 and 3.46 ppm, a 3H multiplet at 3.3 ppm and a triplet at 3.16 ppm, suggesting a hexose residue. Based on HR-MS results (M^+ 340.0796) a molecular formula of $C_{15}H_{16}O_9$ (calculated M^+ 340.0794) was proposed for **2**. The *m/z* value of 340 corresponds to the molecular mass of a dihydroxycoumarin with a hexose attached. HR-MS confirmed the elemental composition of these two fragments.

Table 4.5. ^{1}H and ^{13}C NMR data of the compounds isolated from sweet grass.

Comp-ound	*Solvent*	Molecular formula	^{1}H-NMR, δ (ppm)	^{13}C-NMR, δ (ppm)
1	chloroform-d1*-methanol-d3	$C_9H_6O_4$	H-3 – 6.24 (d, *J* =9.7 Hz) H-4 – 8.10 (d, *J* =9.7 Hz) H-6 – 6.54 (d, *J* =8.8 Hz) H-7 – 6.92 (d, *J* =8.8 Hz)	C-2 – 162.3 C-3 – 113.3 C-4 – 141.1 C-5 – 147.4 C-6 – 110.1 C-7 – 120.1 C-8 – 137.0 C-9 – 142.7 C-10 – 109.6
2	DMSO-d6**	$C_{15}H_{16}O_9$	H-3 – 6.33 (d, *J* =9.7 Hz) H-4 – 8.18 (d, *J* =9.7 Hz) H-6 – 6.61 (d, *J* =8.9 Hz) H-7 – 7.34 (d, *J* =8.9 Hz) H-1′ – 4.89 (d, *J* =7.7 Hz) H-2′+3′+5′ – 3.28 (m) H-4′ – 3.16 (dd, *J* =8.7 Hz and 8.7 Hz) H-6a′ – 3.43 (m) H-6b′ – 3.46 (m)	C-2 – 159.6 C-3 – 108.7 C-4 – 139.4 C-5 – 148.6 C-6 – 113.6 C-7 – 119.3 C-8 – 136.5 C-9 – 143.6 C-10 – 113.6 C-1' – 100.9 C-2' – 73.1 C-3' – 76.6 C-4' – 69.6 C-5' – 76.9 C-6' – 61.4

* in 1H spectra calibrated on the residual $CHCl_3$ signal at 7.26 ppm; in ^{13}C – at 77.2 ppm

** in 1H spectra calibrated on the residual DMSO signal at 2.5 ppm; in ^{13}C – at 39.5 ppm

The accurate mass corresponding to the aglycone part was recorded at 178.0263 ($C_9H_6O_4$, calculated mass 178.0266). Based on the ^{1}H and ^{13}C NMR chemical shifts and the 7.7 Hz coupling between H-1′ and H-2′ (Table 4.5) the hexose was identified as *β*-glucopyranose. To determine

whether the glucose unit was attached to the C-5 or C-8 hydroxyl group an HMBC spectrum was recorded. HMBC correlations of compound **2** are presented in figure 4.3. Some interactions in the glucose moiety are not depicted due to the overlapping of several glucose protons in the ^{1}H NMR. A cross peak between C-8 of the aglycone and H-1´ of glucose was present. Hydrolysis of **2** gave as products **1** and *β*-D-glucopyranose providing further proof about the structure of compound **2**, which was finally identified as 5-hydroxy-8-*O*-*β*-D-glucopyranosyl-benzopyranone (Figure 4.5). The unsubstituted phenolic group must be responsible for the radical scavenging activity of **2**.

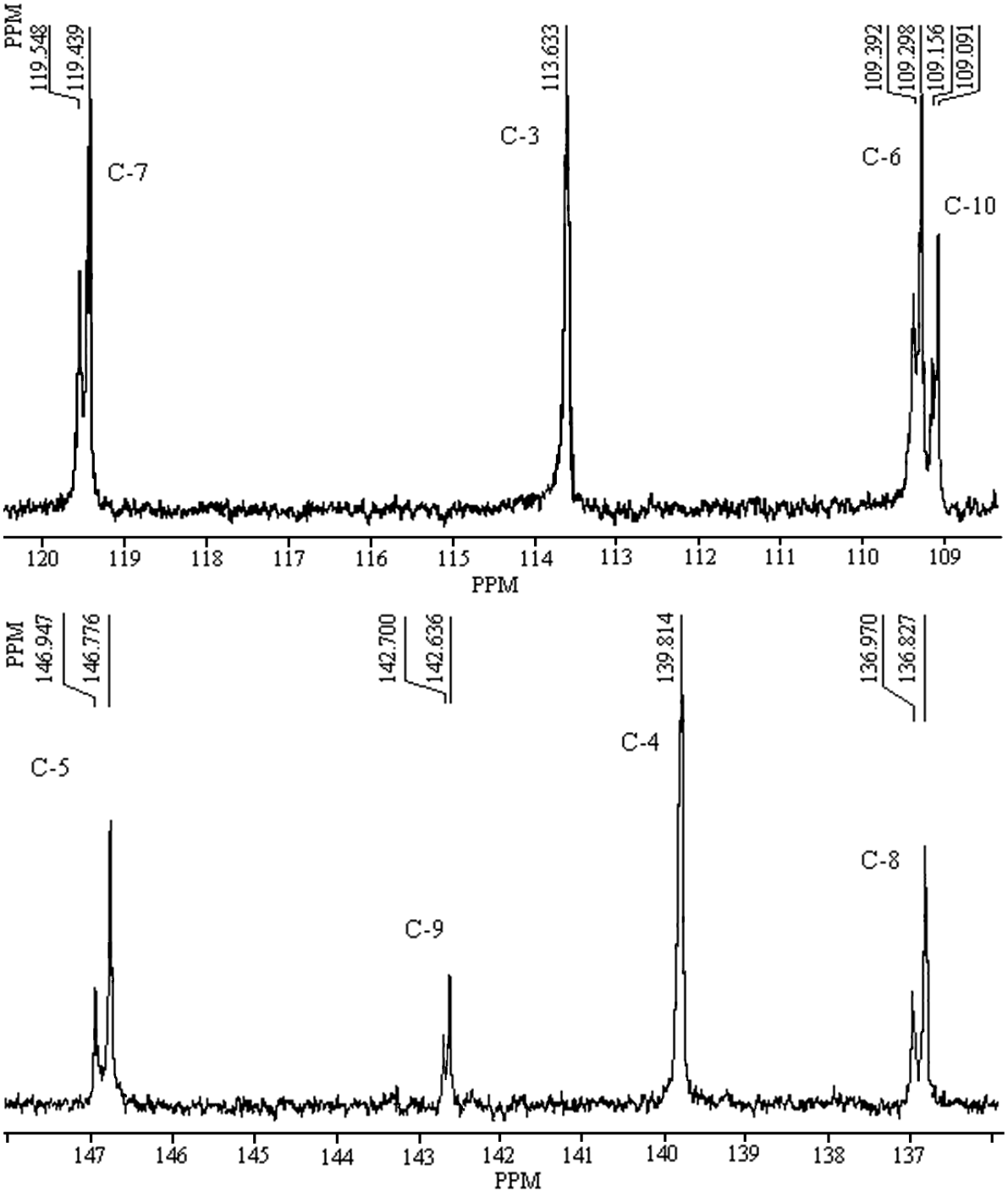


Figure 4.7. ^{13}C NMR results of the deuteration experiment in $CDCl_3$-CD_3OD-CH_3OH

4.3.1.3. Radical scavenging activities of isolated compounds.

Both isolated compounds were tested for ABTS and DPPH radical scavenging activity (Table 4.3). The ABTS method gives the radical scavenging activity by measuring the reduction of the radical cation as the percentage decrease of absorbance at 734 nm relative to a control. In this case the values were determined after 1 and 6 min from the start of the experiment. The activities of the tested compounds were compared with the activity of Trolox and expressed as TEAC values [21]. In the other method the percentage of DPPH scavenging as a function of the concentration of the test substance was determined. The concentration of compound in the reaction mixture, needed to decrease the initial DPPH concentration by 50%, was calculated and expressed as antiradical power. It was assumed that the reaction reaches the steady state after 30 min. It was proposed by Sanchez-Moreno et al. [28] that antioxidants having a reaction time with DPPH longer than 30 min are considered as slow reacting.

Table 4.6. ABTS and DPPH radical scavenging activity of isolated compounds in comparison to the known natural antioxidant rosmarinic acid

Compound	$TEAC_{1min}$	$TEAC_{6min}$	ARP_{30min}
5,8-dihydroxybenzopyranone	0.62	0.73	8.2
5-hydroxy-8-*O*-β-D-glucopyranosyl-benzopyranone	0.58	0.69	0.8
rosmarinic acid	1.49	1.54	6.9

The results show that the two compounds act differently in the two systems. In the ABTS system they possess similar activity and both are much less active (TEAC ≈ 0.7) than rosmarinic acid ($TEAC_{6min}$=1.54). In contrast, in the DPPH system the aglycone was 10 times more powerful as an antioxidant than the glycoside, and its antiradical power (8.2) is comparable to that of rosmarinic acid (6.9) [20]. This finding proves that the ability of the compound to act as an antioxidant is dependent on the test system used. It can be explained by the different polarity of the two compounds. The more polar glycoside reacts faster in the more polar media of the ABTS system. Further experiments in real food systems have to be carried out to obtain more information on their antioxidant properties. Since simple coumarins are reported to be of low toxicity [29], an application of sweet grass extracts (or isolated coumarins) as antioxidants in food may be possible.

4.3.2. Costmary

4.3.2.1. Structure elucidation

Three antioxidative compounds were isolated from the MeOH-water extract of the aerial parts of *Chrysanthemum balsamita*. One compound was isolated from the butanol fraction (**3**) and three were isolated from the *tert*-butyl methyl ether fraction (**4, 5** and **6**).

Table 4.7. NMR data of (**3**). ^{13}C values are derived from the HMBC spectrum (400 MHz, D_2O)

position	δ ^{1}H (ppm)	δ ^{13}C (ppm)
1	-	77.57
2	2.08, bm	39.04
3	4.15, m	71.33
4	3.78, dd, J_1 = 3.2 Hz, J_2 = 9.6 Hz	73.59
5	5.23, bm	71.84
6	1.93, bm	38.00
7	-	181.50
1'	-	127.72
2'	7.08, s	115.81
3'	-	144.98
4'	-	147.72
5'	6.84, d, J = 8.4 Hz	116.93
6'	7.02, d, J = 8.4 Hz	123.41
7'	7.54, dd, J_1 = 3.2 Hz, J_2 = 16 Hz	146.77
8'	6.28, dd, J_1 = 3.2 Hz, J_2 = 16 Hz	115.26
9'	-	167.87

bm, broad multiplet; dd, double doublet; s, singlet; d, doublet.

The 1D-NMR data of compound **3** are presented in table 4.7. The data, together with a molecular mass of 354 Da ($[M-H]^-$ at *m/z* 353) obtained with LC-MS operating in infusion mode, suggested the structure to be a caffeoyl quinic acid. The UV spectrum of **3** was typical for a caffeic acid moiety, showing maximal absorbance at 217, 245 (sh) and 326 nm. The caffeoyl moiety can be attached to carbon 3, 4, or 5 of quinic acid. These three compounds have different patterns in their ^{13}C-NMR spectra. If the caffeic acid is attached to C-3, it is called neochlorogenic acid (3-*O*-caffeoyl quinic acid). Literature values for the chemical shifts of C-3 and C-5 in 3-*O*-caffeoyl quinic

acid are reported as 73.0 ppm and 68.3 ppm, respectively. For 4-*O*-caffeoyl quinic acid the chemical shifts for C-3 and C-5 are 69.6 and 65.5 ppm [30]. In compound **3** the chemical shift values for C-3 and C-5 were 71.33 and 71.84 ppm respectively. Pauli et al. [31] reported chemical shifts for C-3 and C-5 of 5-O-caffeoyl quinic acid (chlorogenic acid) of 71.35 and 71.86 ppm respectively. This was in accordance with the data obtained for **3**, therefore this compound was identified as chlorogenic acid **3** (Figure 4.8).

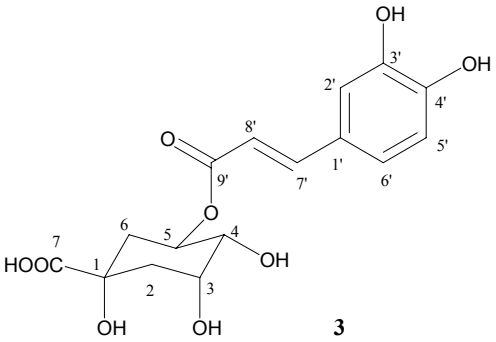


Fig. 4.8. Structure of 5-*O*-caffeoyl quinic acid (chlorogenic acid) (**3**).

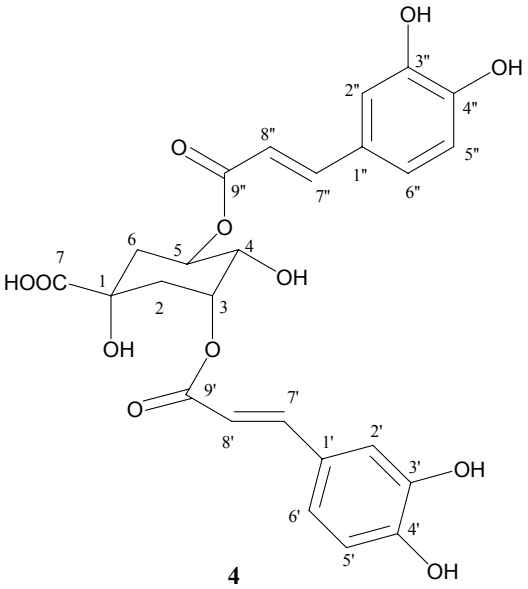


Fig. 4.9. Structure of 3,5-dicaffeoyl quinic acid

The HMBC spectrum of **3** showed a cross peak of a H-5/C-9' coupling which confirmed that the caffeic acid moiety was indeed attached to C-5.

Compound **4**, had a UV spectrum similar to **3**, with maxima at 218, 245 and 327 nm, which is characteristic for a caffeoyl moiety. The ESI-MS, recorded in infusion mode, showed a

pseudomolecular ion peak at *m/z* 515 $[M-H]^-$, which corresponds to the mass of quinic acid with two attached caffeoyl moieties. The NMR data of compound **4** are presented in table 4.8.

Table 4.8. NMR data of (**4**) (400 MHz, CD_3OD)

position	δ ^{1}H (ppm)	δ ^{13}C (ppm)
1	-	78.96
2	2.25 - 2.24, m	36.79
3	5.42, bm	71.13
4	4.00, dd J_1 = 3.3 Hz, J_2 = 7.6 Hz	71.67
5	5.45, bm	69.77
6	2.36 - 2.16, bm	35.08
7	-	176.66
1'	-	126.82
2'	7.09, d J = 2 Hz	114.27
3'	-	145.76
4' *	-	148.48
5'	6.81, d, J = 8.2 Hz	115.52
6'	6.98, m	122.12
7'	7.60, d, J = 16.0 Hz	146.33
8' #	6.29, d, J = 16.0 Hz	114.17
9'	-	167.50
1"	-	126.94
2" #	7.09, d, J = 2 Hz	114.10
3"	-	145.76
4" *	-	148.59
5"	6.81, d, J = 8.2 Hz	115.52
6"	7.00, m	122.05
7"	7.64, d, J = 16.0 Hz	146.11
8"	6.38, d, J = 16.0 Hz	114.74
9"	-	167.98

The ^{1}H chemical shift of 5" is 0.002 ppm higher than the ^{1}H chemical shift of 5'. # The ^{13}C chemical shifts of 2" and 8´ might be interchanged. * The ^{13}C chemical shifts of 4' and 4" might be interchanged. m, multiplet; bm, broad, multiplet; dd, double doublet; d, doublet.

The ^{1}H and ^{13}C NMR data showed the presence of two caffeoyl moieties and a quinic acid. These data are in accordance with the NMR data of 3,5-dicaffeoyl quinic acid published by Chuda et al. [32]. In the HMBC spectrum cross peaks for H-5/C-9" and H-3/C-9' were observed. This unequivocally proved the structure of 3,5-dicaffeoyl quinic acid (**4**) (Figure 4.9).

Table 4.9. NMR spectral data of compounds **5** and **6**.

	5,7,4'-trihydroxy-3',8-dimethoxy flavone (**5**)		5,7,3',4'-tetrahydroxy-3,8-dimethoxy flavonol (**6**)	
position	δ ^{1}H (ppm)	δ ^{13}C (ppm)	δ ^{1}H (ppm)	δ ^{13}C (ppm)
2	-	164.5	-	155.7
3	6.88, s	103.7	$3.86_{(OMe)}$, s	137.6
4	-	183.1	-	178.2
5	-	153.2	-	151.8
6	6.61, s	95.2	6.69, s	94.2
7	-	157.9	-	158.7
8	$3.75_{(OMe)}$, s	131.8/61.1	$3.77_{(OMe)}$, s	131.4
9 *	-	-	-	152.2
10	-	104.9	-	104.5
1'	-	122.3	-	120.8
2'	7.54, s	111.0	7.67, s	115.5
3'	$3.89_{(OMe)}$, s	149.0/56.8	-	145.3
4'	-	151.6	-	148.7
5'	6.93, d, J=8.5 Hz	116.7	7.04, d, J=8.5 Hz	115.9
6'	7.63 d, J=8.5 Hz	121.1	7.55, d, J=8.5 Hz	120.8

* The intensity of the quaternary C-9 was very low, because of the long relaxation time. s, singlet; d, doublet.

The UV spectrum of compound **5** showed maxima at 252, 269 and 346 nm which is typical for flavones. The ESI-MS in negative mode gave a pseudomolecular ion peak at *m/z* 329. The ^{1}H-NMR spectrum showed two singlets at 3.75 ppm and 3.89 ppm, each integrating for 3H; these

peaks were assigned to two methoxyl groups. Three singlets at 6.61 ppm, 6.88 ppm and 7.54 ppm integrating for 1H each corresponded with non-coupled aromatic protons. Two doublets (J = 8.5 Hz) at 6.93 ppm and 7.63 ppm, and a singlet at 7.54 ppm each integrating for 1H, corresponded to a trisubstituted benzene ring. These signals are in accordance with a flavone skeleton with a 1,3,4 trisubstituted B ring and one proton at C-6 in the A ring. Taking into account all ^{13}C NMR data, and 2D NMR (HMBC) results, compound **5** was deduced to be 5,7,4'-trihydroxy-3',8-dimethoxy flavone (Figure 4.10). The ^{1}H-NMR spectral data are in accordance with those reported in the literature [33]. The HMBC long range interactions obtained for compound **5** are presented in figure 4.11

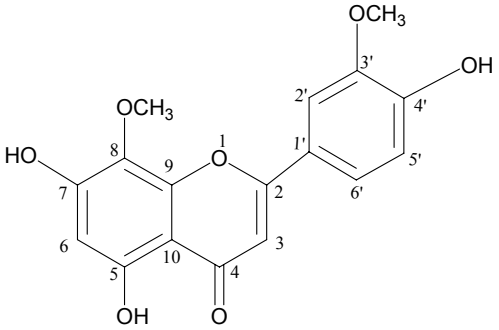


Figure 4.10. Structure of 5,7,4'-trihydroxy-3',8-dimethoxy flavone (**5**).

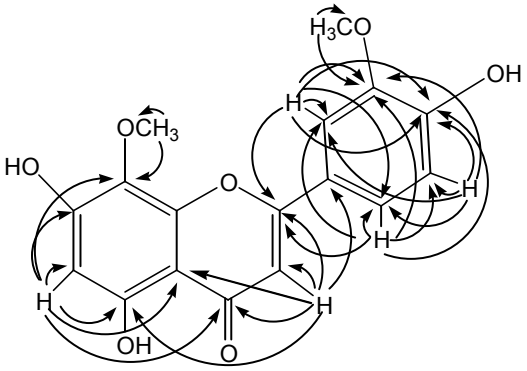


Figure 4.11. HMBC interactions of 5,7,4'-trihydroxy-3',8-dimethoxy flavone (**5**).

Compound **6** showed similar NMR spectrum as compound **5**, except that there was one singlet less in the aromatic area of the ^{1}H spectrum. After examining an HMBC spectrum of the compound (interactions shown in figure 4.13) the structure of 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavone was proposed for this compound. In EI-MS spectra of C-8 methoxylated flavonols the $[M-15]^{+}$ ion is the base peak [34]. In the MS of **6** a peak at *m/z* 331 = [M-15] is observed, which supports the presence of a methoxyl group at C-8 in the A ring.

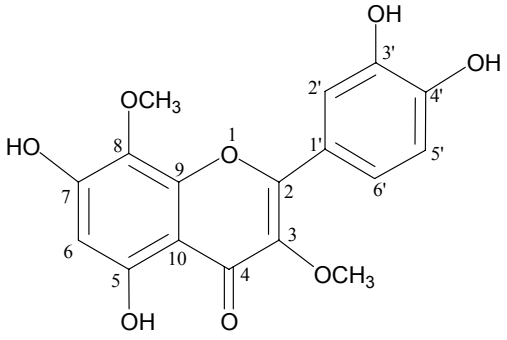


Fig. 4.12. Structure of 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol (**6**)

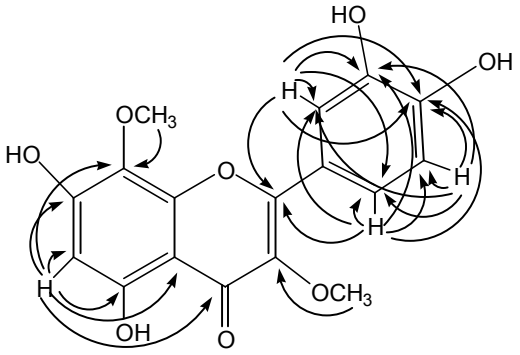


Fig. 4.13. HMBC couplings of 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol (**6**)

Compounds **3-6** were isolated as phenolic antioxidants from the aerial parts of *Chrysanthemum balsamita* L. Esters of hydroxycinnamic acids including 5-*O*-caffeoyl quinic acid and 3,5-dicaffeoyl quinic acid are widely distributed in the plant kingdom [35, 36]. High amounts of caffeoyl quinic acids and dicaffeoyl quinic acids are found in coffeebeans (6.57-9.04% of dried beans) [37]. 5-*O*-Caffeoyl quinic acid is found in fruits such as peaches (18.6 mg/100 g of fresh weight), apples (13.9 mg/100 g of fresh weight) and pears (13.4 mg/100 g of fresh weight) and in vegetables such as corn salad 101.6 mg/100 g of fresh weight), eggplant (57.5-63.2 mg/100 g of fresh weight and artichoke (43.3 mg/100 g of fresh weight) [36]. 3,5-Dicaffeoyl quinic acid is reported as one of the predominant phenolic antioxidants in the young leaves of garland (22.9 mg/g of dry weight) [38]. 5,7,4'-Trihydroxy-3',8-dimethoxyflavone was first found and identified in *Ambrosia dumosa* [33] and later in *Verbena littoralis* [39]. 5,7,3',4'-Tetrahydroxy-3,8-dimethoxyflavonol (**6**) has so far been found only in some Asteraceae [34, 40].

4.3.2.2. Radical scavenging activity as determined by means of the DPPH$^{\bullet}$ method

Concentrations of the compounds needed to scavenge 50% of the DPPH radical after 30 min were determined and ARP values for the compounds were calculated. Results are presented in table 4.10.

Table 4.10. EC_{50} and ARP values of the isolated compounds

No.	Compound	EC_{50} (mol/L antiox)/ (mol/L DPPH)	ARP
1	Chlorogenic acid (**3**)	0.259	3.85
2	3,5-Dicaffeoyl quinic acid (**4**)	0.160	6.25
3	5,7,4'-Trihydroxy-3',8-dimethoxyflavone (**5**)	33.333	0.03
4	5,7,3',4'-Tetrahydroxy-3,8-dimethoxyflavonol (**6**)	0.264	3.79

The presence of a second hydroxyl group in the ortho or para position increases the antioxidative activity due to additional resonance stabilization and o-quinone or p-quinone formation [41].

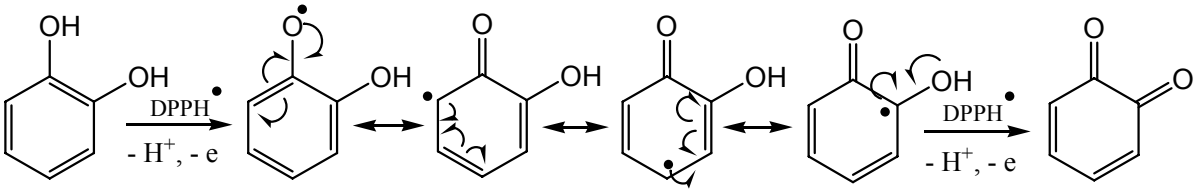


Fig. 4.14. Resonance stabilization and ortho-quinone formation

This explains why the ARP value of 3,5-dicaffeoyl quinic acid (6.25) is about twice as high as the ARP value of chlorogenic acid (3.85) and 3,8-dimethoxy gossypetin (3.79). 3,5-Dicaffeoyl quinic acid has two caffeic acid moieties and chlorogenic acid has only one caffeic acid moiety. Although 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol (**6**) contains four hydroxyl groups it has about the same ARP value as chlorogenic acid. The two ortho hydroxyl groups contribute more to the radical scavengers than the two hydroxyl groups in meta position. For example protocatechuic acid has two hydroxyl groups in the ortho position and an ARP value of 7.14 while gentisic acid, with two para hydroxyls has an ARP value of 11.1 [20]. 5,7,4'-Trihydroxy-3',8-dimethoxyflavone has a very low activity and this can also be explained by the fact that there are no ortho or para hydroxyls.

Caffeic acid has an ARP value of 9.1 [20], so 3,5-dicaffeoyl quinic acid and 5-*O*-caffeoyl quinic acid have a lower antioxidative activity. According to the results of Cuvelier [42], esterification of caffeic acid decreases its antioxidative activity. Rosmarinic acid has an ARP value of 6.90 [20] approximately the same value as 3,5-dicaffeoyl quinic acid. Both compounds possess two ortho-dihydroxybenzene moiety.

4.3.2.3. Radical scavenging activity in the $ABTS^{\bullet+}$ decoulorization assay

The TEAC values for chlorogenic acid, 3,5-dicaffeoyl quinic acid and 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol at 6 and 1 minute were calculated as described in 5.2.4 and are given in table 4.11.

Table 4.11. TEAC values of the isolated compounds after 6 and 1 min.

Compound	TEAC 6 min	TEAC 1 min
Chlorogenic acid	0.60	0.56
3,5-Dicaffeoyl quinic acid	1.16	1.09
5,7,3',4'-Tetrahydroxy-3,8-dimethoxyflavonol	1.50	1.29

The TEAC values of 3,5-dicaffeoyl quinic acid at 6 min (1.16) and 1 min (1.09) are also about twice as high as the TEAC values of chlorogenic acid at 6 min (0.60) and 1 min (0.56). Caffeic acid has a TEAC value at 1 min of 0.99 [21] and is a stronger radical scavenger than chlorogenic acid. The decrease of activity by esterification of caffeic acid with a sugar moiety is described in 4.3.2.2.

The TEAC value of 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol 1 min (1.29) is approximately the same as the TEAC value of luteolin at 1 min (1.29) [21]. This can be explained by the fact that both 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol and luteolin have two hydroxyl groups in ortho position and two hydroxyl groups in meta position. Quercetin is a more active radical scavenger (TEAC 6 min 3.1, TEAC 1 min 2.77) [21] due to the presence of one additional hydroxyl group in the C-ring. 5,7,3',4'-Tetrahydroxy-3,8-dimethoxyflavonol is more active than 3,5-dicaffeoyl quinic acid in the $ABTS^{\bullet+}$ assay, whereas 3,5-dicaffeoyl quinic acid is about twice as active as 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol in the $DPPH^{\bullet}$ assay. $ABTS^{\bullet+}$ is a less stable, more reactive radical. Probably in the $ABTS^{\bullet+}$ assay also other phenolic groups than those in an ortho position influence the radical scavenging activity.

4.3.3. Horehound

4.3.3.1. Structure elucidation

Compound **7,** having radical scavenging activity, was isolated from the *tert*-butyl methyl ether fraction of the methanol-water-acetic acid extract of *Marrubium vulgare*. The structure of this compound was determined by MS and NMR.

The ESI mass spectrum, recorded in positive mode, showed pseudomolecular ions at *m/z* 315 $[M+H]^+$ and at *m/z* 337 $[M+Na]^+$. In negative mode an $[M–H]^-$ peak at *m/z* 313 was observed. These data correspond to a molecular mass of 314 amu. The UV spectrum of **7** had three maxima at 217, 285 and 332 nm. The ^{1}H-NMR spectrum showed 2 singlets at 3.91 ppm and 3.99 ppm, integrating for 3H each which were assigned to two methoxyl groups. Two singlets at 6.69 ppm and 6.88 ppm integrating for 1H each correspond to two non-coupled aromatic protons. Two doublets (J = 8.9 Hz) at 7.13 ppm and 8.04 ppm, integrating for 2H each, are typical of a 1,4-disubstituted benzene ring and one singlet at 12.65 ppm, integrating for 1H, corresponds to a hydroxyl proton involved in hydrogen bonding were observed. These signals agree with a flavone skeleton with a 1,4-disubstituted B ring and hydroxyl groups at C-5 and C-8 [43]. After analysing the HMBC spectrum of this compound (interactions are shown in figure 4.16) and taking into account the ^{13}C-

NMR assignments found in the literature [44], it was concluded that compound **7** is 5,8-dihydroxy-7,4′-dimethoxyflavone (Figure 4.15). ^{1}H and ^{13}C NMR spectral assignments of **7** are given in table 4.12.

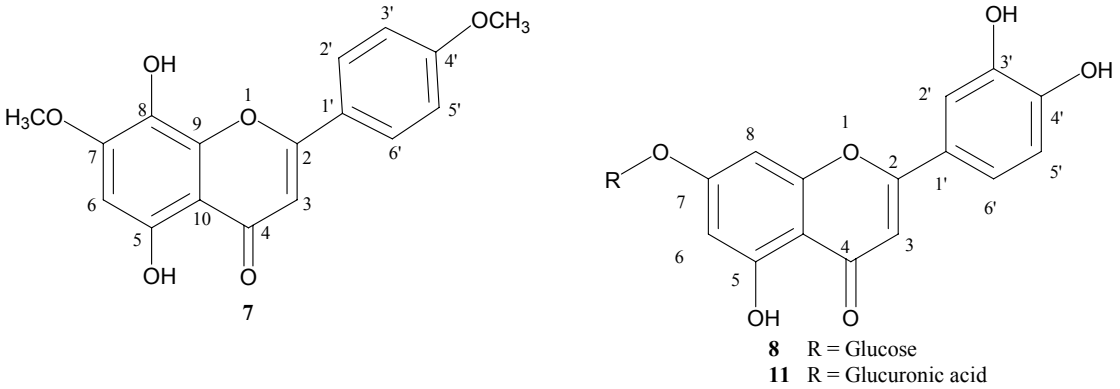
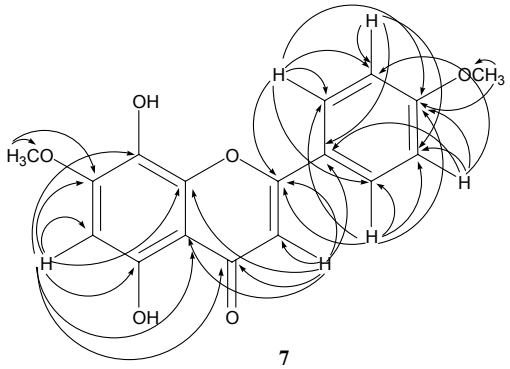


Figure 4.15. Flavonoids isolated from Horehound: 5,8-dihydroxy-7,4′-dimethoxy flavone (**7**); 7-*O*-*β*-glucopyranosyl luteolin (**8**); 7-*O*-*β*-glucuronyl luteolin (**11**)

Table 4.12. NMR spectral assignments of 5,8-dihydroxy-7,4′-dimethoxyflavone (**7**)

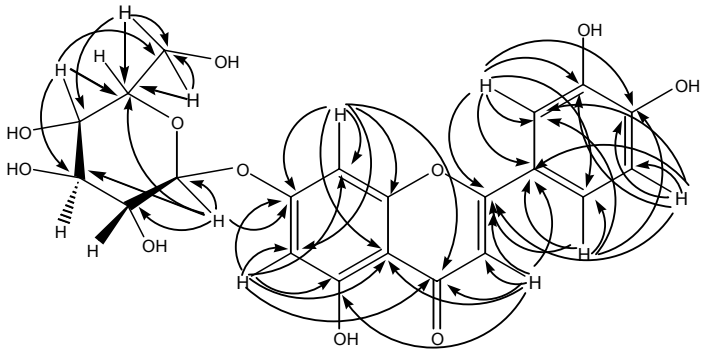
Position	^{1}H	^{13}C
2		165.2
3	6.70, s	104.2
4		184.0
5		151.6
6	6.88, s	92.0
7	$3.99_{(OMe)}$, s	155.4/58.0
8		131.5
9		147.9
10		106.7
1′		124.8
2′	8.04, d, J=8.9 Hz	129.3
3′	7.13, d, J=8.9 Hz	115.8
4′	$3.92_{(OMe)}$, s	164.3/56.5
5′	7.13, d, J=8.9 Hz	115.8
6′	8.04, d, J=8.9 Hz	129.3



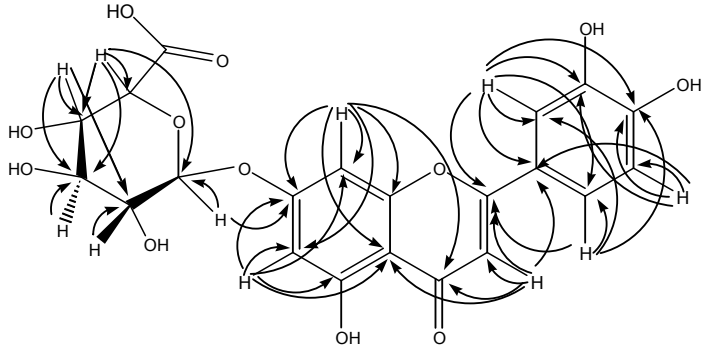
7

Figure 4.16. HMBC interactions of 5,8-dihydroxy-7,4′-dimethoxyflavone recorded in $(CD_3)_2CO$

Compound **8** was isolated from the butanol fraction of the methanol-water-acetic acid extract of horehound. The structure of this compound was elucidated using UV, 1D and 2D NMR techniques and mass spectrometric data. The UV spectrum of **8** showed maxima at 252, 266 (sh) and 342 nm. The profile of the spectrum was characteristic for that of a flavone. The ESI mass spectrum of compound **8** showed a pseudomolecular ion peak $[M-H]^-$ at m/z 447. The ^{1}H-NMR spectrum showed two singlets at 6.67, and 7.39 ppm integrating for 1H each, corresponding to non-coupled aromatic protons, two doublets at 6.43 and 6.76 ppm with J = 2 Hz, integrating for 1H each, probably corresponding to two protons in meta position, and two doublets at 6.89 and 7.40 ppm (J = 8.6 Hz), integrating 1H each and corresponding to two protons in ortho position. A doublet at 5.02 ppm suggested the anomeric proton (H-1) of a sugar unit. According to the ^{1}H and ^{13}C spectral data (Table 4.13), the sugar is glucopyranose. The large coupling (7.3 Hz) indicated the β configuration of the sugar moiety. To determine the exact structure of the aglycone part of the molecule an HMBC spectum was recorded. The HMBC assignments are shown in figure 4.17. The HMBC spectral data in combination with the ^{1}H and ^{13}C spectra revealed a luteolin moiety. An interaction between the sugar H-1 and the flavone C-7 shows that the sugar is attached to the carbon at the seven position. On the basis of the above data 7-*O*-*β*-glucopyranosyl luteolin was proposed as the structure for **8** (Figure 4.17). This compound has been reported from numerous plants [45-48]. The ^{13}C NMR spectral data obtained for **8** are in agreement with previously published ones [46].



8



11

Figure 4.17. Characteristic HMBC interactions of 7-O-*β*-glucopyranosyl luteolin (**8**) recorded in DMSO-d6 and 7-*O*-*β*-glucuronyl luteolin (**11**) recorded in D_2O

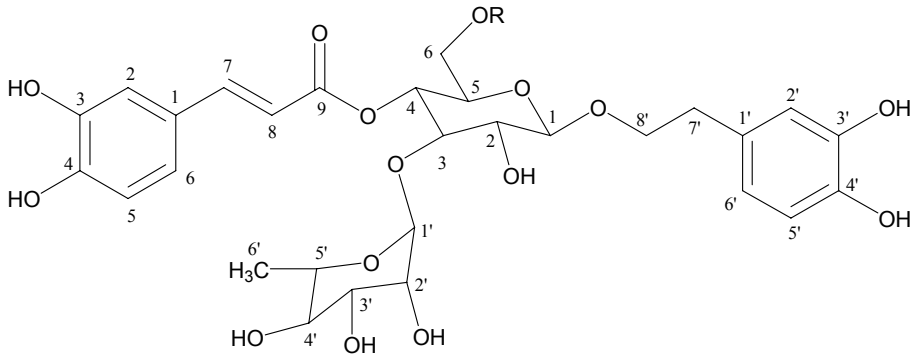
Compound **11**, isolated from the butanol fraction of horehound, had a similar UV spectrum as **8**, with maxima at 255, 266 (sh) and 349 nm. The ESI-MS spectrum recorded in negative mode showed a pseudomolecular ion peak $[M-H]^-$ at *m/z* 461, i.e. 14 Da higher than that of **8**. This can be explained by the presence of an additional CH_2 group or by the replacement of CH_2OH by COOH. The aglycone part of its ^1H NMR spectrum was identical to that of 7-O-*β*-glucopyranosyl luteolin. There were some interchanges in peak assignments according to the HMBC spectrum, but this could be explained by solvent effects, or by the presence of a different sugar. The peak at 175.5 ppm in the ^{13}C NMR spectrum indicated that the sugar must be an uronic acid. The large coupling of H-1 proved the *β* configuration and excluded the possibility of mannuronic acid. H-5 indicated that the sugar cannot be galacturonic acid. Consequently the uronic acid must be glucuronic acid. There was a cross peak between H-1 of glucuronic acid and C-7 of the aglycone part, so 7-*O*-*β*-glucuronyl luteolin was proposed as the final structure. The ^1H and ^{13}C spectral data of the compound are presented in table 4.13. and the HMBC interactions are shown in figure 4.17.

Table 4.13. NMR spectral assignments of 7-*O*-*β*-glucopyranosyl luteolin (**8**) and 7-*O*-*β*-glucuronyl luteolin (**11**)

Nr.	7-*O*-*β*-glucopyranosyl luteolin (**8**) (in DMSO-d6)		7-*O*-*β*-glucuronyl luteolin (**11**) (in D_2O)	
	^1H	^{13}C	^1H	^{13}C
2		65.2		164.2
3	6.67, s	103.8	6.12, s	102.2
4		182.6		182.5
5		161.6		159.3
6	6.43, d, *J*=2 Hz	100.1	6.26, d, *J*=2 Hz	99.9
7		163.6		161.9
8	6.76, d, *J*=2 Hz	95.4	6.00, d, *J*=2 Hz	95.0
9		157.7		156.2
10		106.0		105.3
1′		122.2		121.1
2′	7.39, s	113.9	6.68, s	112.7
3′		146.2		144.0
4′		150.3		151.1
5′	6.89, d, *J*=8.6 Hz	116.5	6.45, d, *J*=7.9 Hz	115.4
6′	7.40, d, *J*=8.6 Hz	119.9	6.74, d, *J*=7.9 Hz	119.8
Sugar				
1′	5.02, d, *J*=7.3 Hz	100.6	5.03, d, *J*=7.1 Hz	99.0
2′	3.35, m	73.7	3.66, m	72.9
3′	3.35, m	76.8	3.66, m	72.0
4′	3.20, dd, J_1=9.0 Hz, J_2=8.9 Hz	70.1	3.70, dd, J_1=9.3 Hz, J_2=9.1 Hz	75.1
5′	3.44, m	77.7	3.95, d, *J*=9.3 Hz	76.4
6′a	3.72, d, *J*=10.8 Hz	61.2		175.7
6′b	3.52, dd, J_1=10.8 Hz, J_2=3.8 Hz			-

Compound **9**, isolated from the butanol fraction of horehound had a UV spectrum similar to that of caffeic acid with maxima at 219, 242 (sh), 302 (sh) and 331 nm. The ESI mass spectrum

showed a pseudomolecular ion peak $[M–H]^-$ at *m/z* 623. The ^1H NMR showed signals in the low field part of the spectrum in close agreement with those given by caffeic acid [49], and its derivatives [50]. Another part of the spectrum was attributable to phenyl ethanol. A doublet with a small coupling constant at 4.78 ppm was recognized as an anomeric proton and a doublet at 0.96 ppm as the methyl group of rhamnose. The small coupling constant of the anomeric proton indicated the α configuration for rhamnopyranose. The anomeric proton at 4.37 belongs to glucose. The large coupling constant (J = 7.9 Hz) is in accordance with the β configuration. In the spectrum of compound **9**, a triplet at 4.65 ppm, attributed to H-4 of glucose, was observed. For glucobioses normally only the anomeric proton can be observed above 4 ppm. Thus the signal at 4.65 ppm reflects the esterification with caffeic acid of the 4-OH of glucose. To confirm all links between the sugars and the aglycone part of the molecule an HMBC spectrum was recorded. Cross peaks between glucose H-4 and caffeic acid C-9, glucose H-3 and rhamnose C-1, and H-8′ of the aglycone and C-1 of the glucose confirm that compound **9** is verbascoside (Figure 4.18). All ^1H and ^{13}C NMR spectral assignments are presented in table 4.14. The spectral data are in agreement with those presented in the literature [51].



9 R = H - Verbascoside
10 R = Apiose - Forsythoside B

Figure 4.18. Structures of verbascoside (**9**) and forsythoside B (**10**)

The UV spectrum of compound **10** was similar to that of caffeic acid with maxima at 221, 245 (sh), 301 (sh) and 330 nm. The ESI mass spectrum, recorded in negative mode, showed a quasi-molecular ion peak at m/z 755. The NMR spectral data were very similar to those recorded for verbascoside. An anomeric proton signal at 4.94 ppm must belong to an additional sugar unit. Taking into account all the NMR ^1H and ^{13}C data, the additional sugar was identified as β-apiose. In the HMBC spectrum a cross peak between H-1 of apiose and C-6 of glucose was present. This confirms that compound **10** is forsythoside B. The structure of the compound is presented in figure

4.18 and the NMR spectral assignments are given in table 4.14. All NMR spectral data are in agreement with the literature data reported for this compound [52].

Table 4.14. NMR spectral assignments of verbascoside (**9**) and forsythoside B (**10**)

Atom number		Verbascoside		Forsythoside B	
		^{1}H	^{13}C	^{1}H	^{13}C
Caffeic	1		126.0		126.8
acid	2	7.03, d, *J*=2 Hz	115.3	7.15, d, *J*=2 Hz	113.8
	3		146.5		145.6
	4		149.6		148.5
	5	6.75, d, *J*=8.3 Hz	116.1	6.87, d, *J*=8.3 Hz	116.0
	6	6.98, dd, J_1=2 Hz, J_2=8.3 Hz	122.1	7.05, dd, J_1=2 Hz, J_2=8.3 Hz	122.8
	7	7.45, d, *J*=16 Hz	146.5	7.65, d, *J*=15.9 Hz	145.4
	8	6.20, d, *J*=16 Hz	114.0	6.35, d, *J*=15.9 Hz	114.8
	9		167.0		167.9
Phenyl	1′		129.9		131.0
ethanol	2′	6.63, d, *J*=2 Hz	117.0	6.78, d, *J*=2 Hz	115.9
	3′		145.5		144.7
	4′		144.2		143.3
	5′	6.62, d, *J*=8 Hz	116.1	6.77, d, *J*=7.9 Hz	116.5
	6′	6.49, dd, J_1=2 Hz, J_2=8 Hz	120.1	6.66, dd, J_1=2 Hz, J_2=8.1 Hz	120.9
	7′	2.68, m	35.8	2.83, t, *J*=8 Hz	35.3
	8′	3.85, m	72.0	3.99, m	72.5
		3.57, m		3.64, m	
Gluc.	1	4.37, d, *J*=7.9 Hz	103.0	4.46, d, *J*=8.1 Hz	102.9
	2	*	74.0	3.44, dd, J_1=8.1Hz, J_2=9.2 Hz	76.7
	3	3.71, t, *J*=8.3 Hz	79.7	3.82, t, *J*=9.2 Hz	81.2
	4	4.65, t, *J*=8.3 Hz	69.1	4.99, t, *J*=9.2 Hz	71.1
	5	3.26, m	75.0	3.6-3.5*	74.8
	6	*	63.7	3.8-3.9*	69.8
Rha.	1′	4.78, d, *J*=2.8 Hz	109.8	5.14, d, *J*=1.6 Hz	102.2
	2′	*	70.8	3.94, bs	73.1
	3′	3.28, dd, J_1=2.5 Hz, J_2=9.3 Hz	70.8	3.58, d, *J*=2.4 Hz	71.6
	4′	3.09, t, *J*=9.3 Hz	49.0	3.33, t, *J*=9.6 Hz	74.1
	5′	*	69.1	3.32, m	70.8
	6′	0.96, d, *J*=6 Hz	18.7	1.07, d, *J*=6.2 Hz	17.4
Api.	1′		-	4.94, d, *J*=2.6 Hz	109.8
	2′		-	3.91, d, *J*=2.6 Hz	77.2
	3′		-	-	79.8
	4′		-	3.95, d, *J*=10 Hz	76.4
				3.71, d, *J*=10 Hz	
	5′		-	3.52, s	64.3

* - signals unclear due to overlapping.

4.3.3.2. Radical scavenging activities of isolated compounds

All isolated compounds were tested for radical scavenging activity in DPPH and ABTS assays. Activities of the compounds are presented in table 4.15. Comparing the activities of the compounds isolated from horehound with the well known natural antioxidant rosmarinic acid, showed that in polar media (ABTS assay) the activities of verbascoside (**9**) and forsythoside B (**10**) are very similar to those of rosmarinic acid. This could be explained by similarities in the aglycone parts of these compounds. However, in the DPPH assay rosmarinic acid was about three times more active. Comparing the activities of luteolin glycosides, it should be noted that in the ABTS assay they have similar activities. This is not surprising, as the flavonoid part of these compounds is identical. However in the DPPH assay the activity of 7-*O*-*β*-glucuronyl luteolin is about twice as high as its glucopyranosyl analogue. This finding shows that the sugar substituent can substantially affect the ability of compound to scavenge free radicals. No literature data about this phenomenon was found.

Table. 4.15. Activities of compounds isolated from horehound in DPPH and ABTS assays

No.	Compound	$TEAC_{1min}$	$TEAC_{6min}$	ARP_{30min}
1	5,8-Dihydroxy-7,4′-dimethoxy flavone	1.07	1.31	2.45
2	7-*O*-*β*-Glucopyranosyl luteolin	0.94	1.48	1.85
3	Verbascoside	1.43	1.65	2.76
4	Forsythoside B	1.47	1.66	2.47
5	7-*O*-*β*-Glucuronyl luteolin	0.95	1.25	5.22
6	Rosmarinic acid	1.49	1.54	6.9

4.4. Conclusions.

DPPH tests showed that extracts of sweet grass are strong radical scavengers. Further fractionation of the extracts and screening of the fractions by on-line HPLC-DPPH revealed that the main activity is concentrated in the *tert*-butyl methyl ether fraction and is caused by a single active compound. MS, UV, IR and NMR data enabled to identify this compound as 5,8-dihydroxy-coumarin (**1**), which, to my knowledge, has not been reported previously as a natural product.

5-Hydroxy-8-*O*-β-glucopyranosyl benzopyranone (**2**), was isolated from the butanol fraction and it also possessed radical scavenging activity in the ABTS and DPPH assays. However its content in the fraction was much smaller and the activity was lower than that of its aglycone.

Four radical scavenging compounds were isolated from the aerial parts of *Chrysanthemum balsamita* L. 5-*O*-Caffeoyl quinic acid (chlorogenic acid) (**3**) was isolated from the butanol fraction. 3,5-Dicaffeoyl quinic acid (**4**), 5,7,4'-trihydroxy-3',8-dimethoxy flavone (**5**) and 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol (**6**) were isolated from the *tert*-butyl methyl ether fraction. On-line HPLC-DPPH was used for the screening of the fractions and this significantly speeded up the detection of radical scavenging compounds in the plant extracts.

The radical scavenging activity in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ systems was compared with literature data. The presence of a second hydroxyl group in the ortho or para position increases the antioxidative activity due to additional resonance stabilization and o-quinone or p-quinone formation [41].

Five compounds having radical scavenging activity were isolated from horehound using bioassay-guided fractionation. All isolated compounds were identified using 1D and 2D NMR and MS techniques. The compounds were mainly glycosides and as such they were rather polar. Activity tests of isolated compounds in two different assays gave positive results. All compounds were active DPPH and ABTS radical scavengers.

Due to their high polarity it is not expected that the isolated glycosides can have any practical application in bulk oils. However, possibly the glycosides may find an application in more polar food systems, such as lipid emulsions. 5,8-Dihydroxy-7,4′-dimethoxy flavone (**7**) – a compound isolated from the *tert*-butyl methyl ether fraction of horehound – is expected to be somewhat soluble in non-polar media such as bulk oils and may be applied in fat-containing foods.

4.5. References

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5. Development of a triple hyphenated HPLC-radical scavenging detection-DAD-SPE-NMR system for the rapid identification of antioxidants in complex plant extracts*

5.1. Introduction

To retard the oxidation process and prolong the shelf life of food containing (multiple) unsaturated fats, antioxidants are frequently added. Purified extracts from rosemary leaves having antioxidative properties are widespread and commercially applied as food additives [1-12]. In some foods rosemary extracts give effects similar to those of synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole BHA [13]. Crude rosemary extracts possess a green colour and a rather strong odour. Therefore extracts are usually processed to obtain a neutral extract with regard to colour, taste and smell. In order to avoid losing antioxidant activity during the processing step, knowledge of the properties and identity of individual antioxidants is essential. Thus much work has been carried out to reveal the chemical composition of rosemary extracts [14-21]. Compounds responsible for the antioxidant properties are rosmarinic acid and phenolic diterpenes, such as carnosol, carnosic acid, rosmanol, epirosmanol and isorosmanol [6, 20].

Investigations on the antioxidant activity and identity of individual constituents in complex plant extracts are usually time-consuming. Each individual compound needs to be purified to homogeneity and only then its activity and structure can be determined with off-line methods. Recently a technique has become available to measure the radical scavenging activity of individual compounds on-line when they elute from an HPLC column [22-24]. As all of the more powerful natural antioxidants are also radical scavengers, this technique makes it possible to directly identify active constituents. On-line spectroscopic methods like LC-UV and LC-MS are sensitive and useful methods but for the exact identification of more complex natural products NMR is frequently necessary. Therefore LC-NMR is a logical development, which is gaining rapidly popularity, even though sensitivity remains a problem [25-27]. To gain sensitivity, a solid phase (SPE) extraction technique is available for trapping and introducing the sample to the NMR [28, 29]. Also, a cryogenic flow probe, coupled with an SPE unit can be applied [30]. In this chapter a LC-DAD-radical scavenging detection (RSD), coupled with LC-SPE-NMR is described. This hyphenated set-up provides retention times, radical scavenging activity, UV data and NMR data of individual peaks in a single run.

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5.2. Experimental

5.2.1. Materials and chemicals

Rosemary extract RBT 255 (Robertet, Grasse, France) was used as sample. All solvents used for chromatography were of HPLC grade (Lab-Scan Analytical Sciences Ltd., Dublin, Ireland). Ultra pure water (0.05 μS cm^{-1}) was obtained from a combined Seradest LFM 20 and Seralpur Pro 90 C apparatus (Seral, Ransbach-Baumbach, Germany). The following reagents and compounds were used: 2,2′-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$, 95%), (trimethylsilyl)-diazomethane (2.0 M solution in hexane), carnosic acid, deuterated methanol and deuterated chloroform from Sigma-Aldrich Chemie (Steinheim, Germany) and ammonium acetate from Fluka Chemie (Buchs, Switzerland).

5.2.2. Sample preparation

The rosemary extract (0.5 g) was dissolved in 25 mL of hexane and successively extracted with five 10 mL portions of methanol - water (9:1). The five aqueous methanolic layers were combined and the solvent was removed in vacuum with a rotary evaporator which yielded 0.058 g of dry material. This was dissolved in methanol at a concentration of 4% (w/v).

5.2.3. HPLC-RSD-DAD-SPE-NMR conditions and instrumental setup

Separation, radical scavenging detection (RSD), UV detection and recording of NMR spectra were carried out with the system schematically represented in Fig. 5.1. The linear binary gradient was formed with an LC 22 pump equipped with an LC 225 gradient former (Bruker BioSpin GmbH, Rheinstetten, Germany), at a constant flow rate of 0.8 mL min^{-1}. Solvent A = 0.1% TFA, 1.0% acetonitrile, 98.9% water; solvent B = acetonitrile. At t = 0 min A = 70%, t = 15 min, A = 50%, t = 50 min A = 45%, t = 55 min A = 35%, t = 75 min A = 10%, t = 80 min A = 0%, t = 85 min A = 0%, t = 90 min A = 70%. Analytes were injected with a Rheodyne model 7125 manual injector (Rheodyne, Rohnert Park, CA) equipped with a 100 μL injection loop (4 mg injected on column) and separated on an Alltima C18 5 μm analytical column (15 cm × 4.6 mm i.d. Alltech, Deerfield, IL). The compounds eluted from the column were split into two streams using an adjustable high-pressure stream splitter (Supelco Port, Bellefonte, PA). One part (0.6 mL min^{-1}) went to a Bruker DAD detector (Bruker, Rheinstetten, Germany) operating λ = 235 and 280 nm. After the detector, make-up water with 0.1% TFA at a flow rate of 0.4 mL min^{-1} was added to the eluent stream with a Knauer K-120 pump (Knauer, Berlin, Germany). This combined stream entered the Prospekt 2 SPE unit (Spark Holland, Emmen, The Netherlands), where compounds, detected on the DAD, were collected on 10 x 2 mm cartridges with HySphere Resin SH 15-25 μm sorbent (Spark Holland, Emmen, The Netherlands). The other part of the column flow (0.2 mL min^{-1}) was used for the

radical scavenging detection (RSD). For this purpose 10^{-4} M DPPH solution in methanol, buffered with ammonium acetate (5×10^{-3} M) was added at a flow rate of 0.4 mL min^{-1} with a 50 mL syringe pump (laboratory made; Free University, Amsterdam, The Netherlands). After the addition of DPPH solution, the mixture passed a reaction coil made of peek tubing (4.4 m × 0.25 mm). The decrease of absorbance after the reaction was monitored with a 759A model visible light detector (Applied Biosystems, Foster City, CA) equipped with a tungsten lamp. Compounds having radical scavenging activities were detected as negative peaks in the RSD chromatogram. All SPE cartridges with trapped compounds were subsequently dried with nitrogen (30 min, at 0.5 MPa, at room temp.). Afterwards compounds having radical scavenging activity were transferred from the Prospekt 2 system to a Bruker DPX 400 spectrometer equipped with a 120 µL NMR flow probe (Bruker, Rheinstetten, Germany) with approximately 550 µL of deuterated chloroform or methanol.

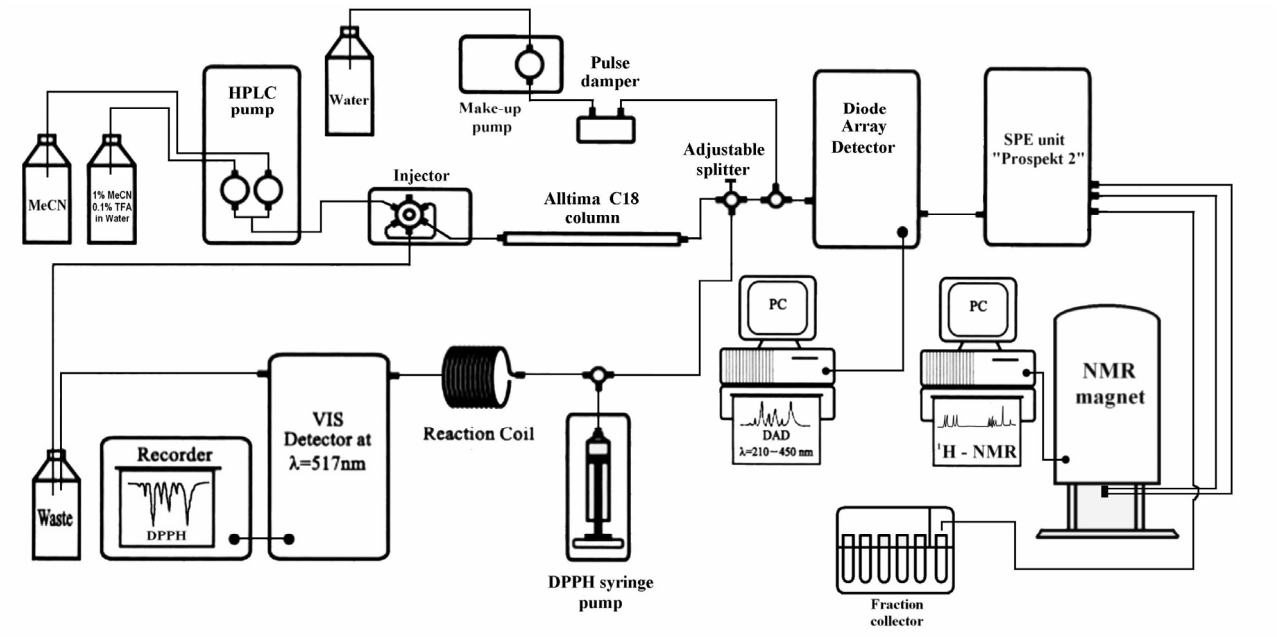

Figure 5.1. Schematic HPLC-RSD-DAD-SPE-NMR instrumental set-up.

NMR spectra were recorded at a probe temperature of 25 °C. Chemical shifts were expressed in ppm relative to internal methanol: 3.34 ppm for ^{1}H (or chloroform: 7.26 ppm for ^{1}H and 77.1 ppm for ^{13}C). The 1D ^{1}H proton spectra were recorded at 400.13 MHz. For the 2D HMBC spectrum a standard gradient enhanced 2D-HMQC pulse sequence delivered by Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 53 ms. For the HMBC experiment 1024 experiments of 2048 data points were performed with 128 scans per increment.

A 759A model UV detector (Applied Biosystems, Foster City, CA) was used to monitor the rosmarinic acid content coming out of the SPE cartridges during method optimization. The in-line pulse damper (toroid mixer) connected to the make-up water pump was from Scientific Systems (State College, PA).

5.3. Results and discussion

The first parameters that were optimised were the different flows through the system. The total flow rate through the SPE unit should preferably not exceed 1.5 mL min^{-1}. Higher flow rates cause high back pressures in SPE cartridges and increase the possibility of damaging the UV cell. As the ratio between the make-up water flow rate necessary to reduce the eluent strength and the flow coming from the HPLC column is suggested as 4 to 1, frequently 2.1 mm i.d. columns and a flow rate of 0.2 mL min^{-1} of HPLC eluent are used. Then, the total flow going through the cartridge is 1 mL min^{-1}.

Since in our set-up (Fig. 5.1) part of the eluent was directed to the RSD reaction coil, it was necessary to increase the total amount of loaded sample and therefore a 4.6 mm i.d. column was used. The compound separation conditions were optimised for a flow rate of 0.8 mL min^{-1} [22]. The greater part of the flow was directed to the SPE unit, since RSD is much more sensitive than NMR detection. Under these conditions it was impossible to add make-up water at the suggested 4:1 ratio as the total flow rate had to be kept under 1.5 mL min^{-1}. So it was attempted to lower the flow rate of make-up water used for trapping the analytes.

The trapping abilities of the cartridges filled with highly non-polar polymeric stationary phase were tested at several different flow ratios using rosmarinic acid as a model antioxidant. This compound was chosen because of its relatively high polarity. The more polar the compound, the more difficult it is to trap. As HPLC eluent 20% acetonitrile in water was used. The flow rate from the HPLC system was set to 0.6 mL min^{-1} and several make-up water flow rates (0.2, 0.4 and 0.8 mL min^{-1}) were chosen. A UV detector connected to the outlet of the cartridges was used to monitor any rosmarinic acid breakthrough. A make-up water flow rate of 0.4 mL min^{-1} was found to be sufficient for trapping rosmarinic acid into a cartridge for about 2 minutes and gave an acceptable backpressure. Since the make-up water pump gave relatively high pulsations disturbing the baseline in both the UV and RSD chromatograms, a pulse damper was connected to the make-up water stream.

Every SPE cartridge gives a slightly different backpressure, causing changes in flow rates and split ratios during the peak trapping process. When a frequently used cartridge was in line, the flow

through the cartridge decreased and more eluent from the HPLC column was passing through the DPPH reaction coil. Because of this, DPPH solution at the moment of trapping was diluted and baseline stability was disturbed. Sometimes, because of the lower flow rates through the cartridge, not enough compound was trapped for recording an NMR spectrum. To ensure stable flow rates during the entire separation and compound collection process, an additional backpressure consisting of a piece of 13.5 m x 0.25 mm i.d. peek tubing was connected to the exit of the SPE unit. This significantly reduced base line disturbances and trapping problems during the time the cartridge was connected to the system.

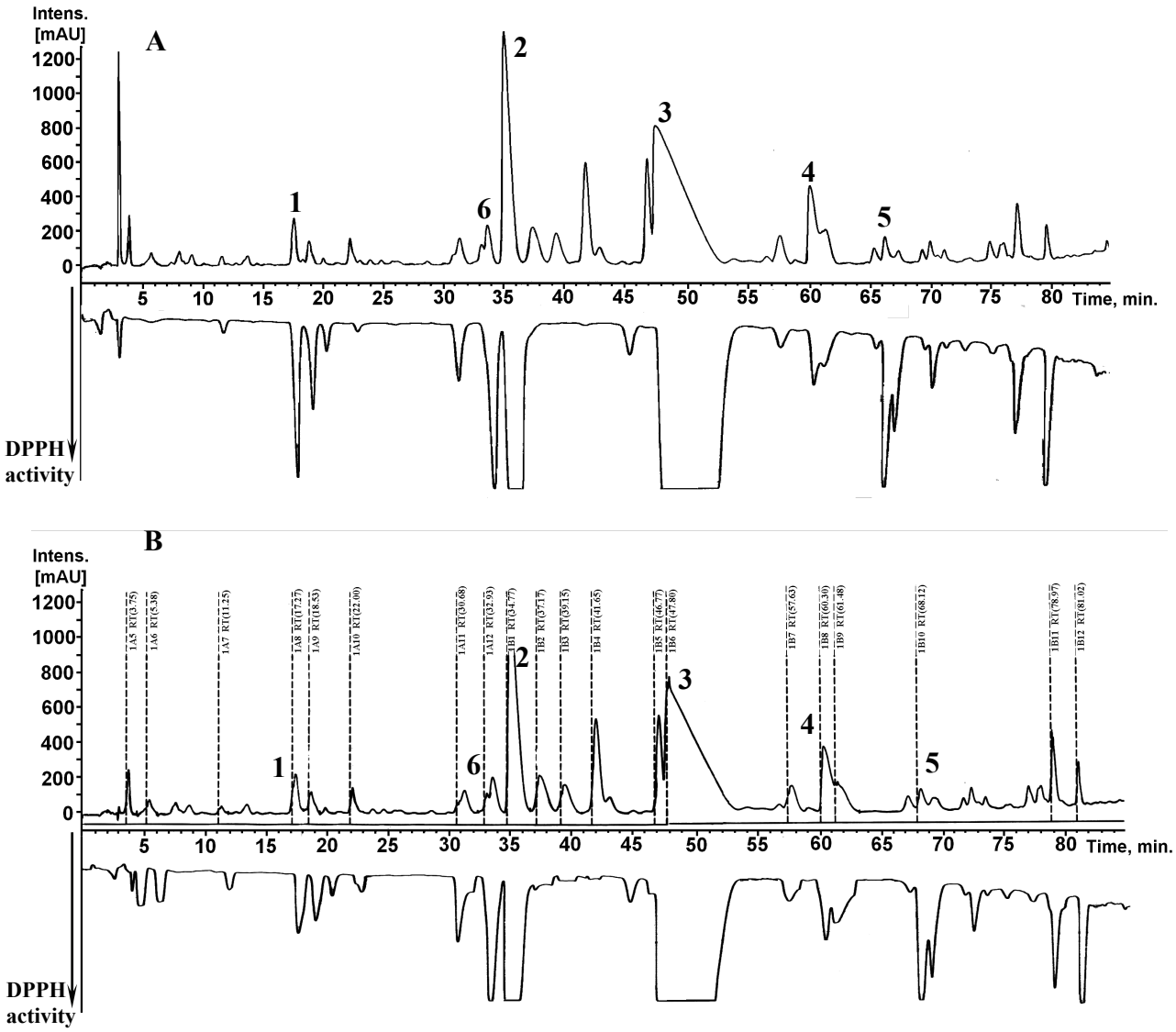

Figure 5.2. HPLC UV and RSD profiles of rosemary extract: A – no trapping performed; B- compounds are trapped on cartridges.

UV and RSD chromatograms are shown in Fig. 5.2. Fig. 5.2A and 5.2B are identical except for the trapping effect of the trapping procedure on the baseline. In Fig. 5.2A no peaks were trapped. It shows that the trapping can be performed without markedly affecting peak resolution or peak intensity of both the UV and RSD signal. Only in the very beginning of the trapping chromatogram (Fig. 5.2B) two artificial peaks at 4 and 6 min respectively can be observed. All major (marked in Fig. 5.2B by the retention time and cartridge number) peaks appearing in the UV chromatogram were collected on SPE cartridges. After drying with nitrogen, compounds with radical scavenging activity were delivered to a 120 μL LC-NMR flow cell with fully deuterated solvents and ^{1}H-NMR spectra were recorded. Because of the large sample size (4 mg) needed for LC-NMR, the major compound in the extract is clearly overloaded without however adversely affecting the separation of the other constituents.

Sufficient amounts of compounds were trapped into cartridges 1A8 (**1**), 1B1 (**2**), 1B6 (**3**), 1B8 (**4**), 1B10 (**5**), and 1A12 (**6**), to allow the recording of enough NMR data for structure elucidation. In some cases 2-dimensional proton NMR data (TOCSY and COSY) could be collected and one injection was sufficient to record an HMBC spectrum of the major compound.

The NMR spectrum of the most polar radical scavenging compound identified (trap 1A8) showed two methyl singlets at 0.90 and 1.02 ppm, a doublet integrating for 6 protons at 1.21 ppm, a 1H septet at 3.28 ppm and a 1H singlet at 6.77 ppm. This combination of signals is characteristic of a carnosic acid type diterpene. After pumping the pure compound out of the LC-NMR probe, evaporating the deuterated solvent and redissolving in methanol, infusion ESI-MS measurements in negative mode showed a pseudomolecular ion $[M–H]^-$ at *m/z* 345. This corresponded to a MW of 346 amu, i.e. a rosmanol isomer. The UV absorption maximum at 288 nm and a peak shoulder at about 225 nm were in accordance with the data presented by Cuvelier [4]. A doublet of doublets at 4.30 ppm (J_1 = 4.3 Hz, J_2 = 4.2 Hz) coupled with a doublet at 5.13 ppm (J = 4.3 Hz), and a doublet at 1.38 ppm (J = 4.2 Hz) indicated that the compound was epiisorosmanol **1** [20]. In the case of rosmanol and epirosmanol no couplings between H-5 and H-6 can be observed because of the near 90° angle between them [20]. The completely assigned ^{1}H-NMR spectrum of epiisorosmanol **1** is presented in Table 5.1.

Similar to **1**, compound **2** (trap 1B1) showed signals characteristic of a carnosic acid type skeleton (Fig. 5.3A). However in contrast to rosmanol type of compounds and carnosic acid, only one signal between 4 and 5.5 ppm was present. A comparison with literature NMR data [18, 31] allowed the identification of **2** as carnosol, after carnosic acid the second most important antioxidant in rosemary extracts. Its ^{1}H-NMR spectral data can be found in Table 5.1. All couplings in the COSY spectrum were in accordance with the carnosol structure. Mass spectral (pseudomolecular ions at *m/z* 331 $[M+H]^+$ and *m/z* 329 $[M–H]^-$ in positive ion (PI) and negative ion (NI) mode infusion ESI-MS respectively) and UV data (λ_{max} 283 nm) further confirmed the assignment.

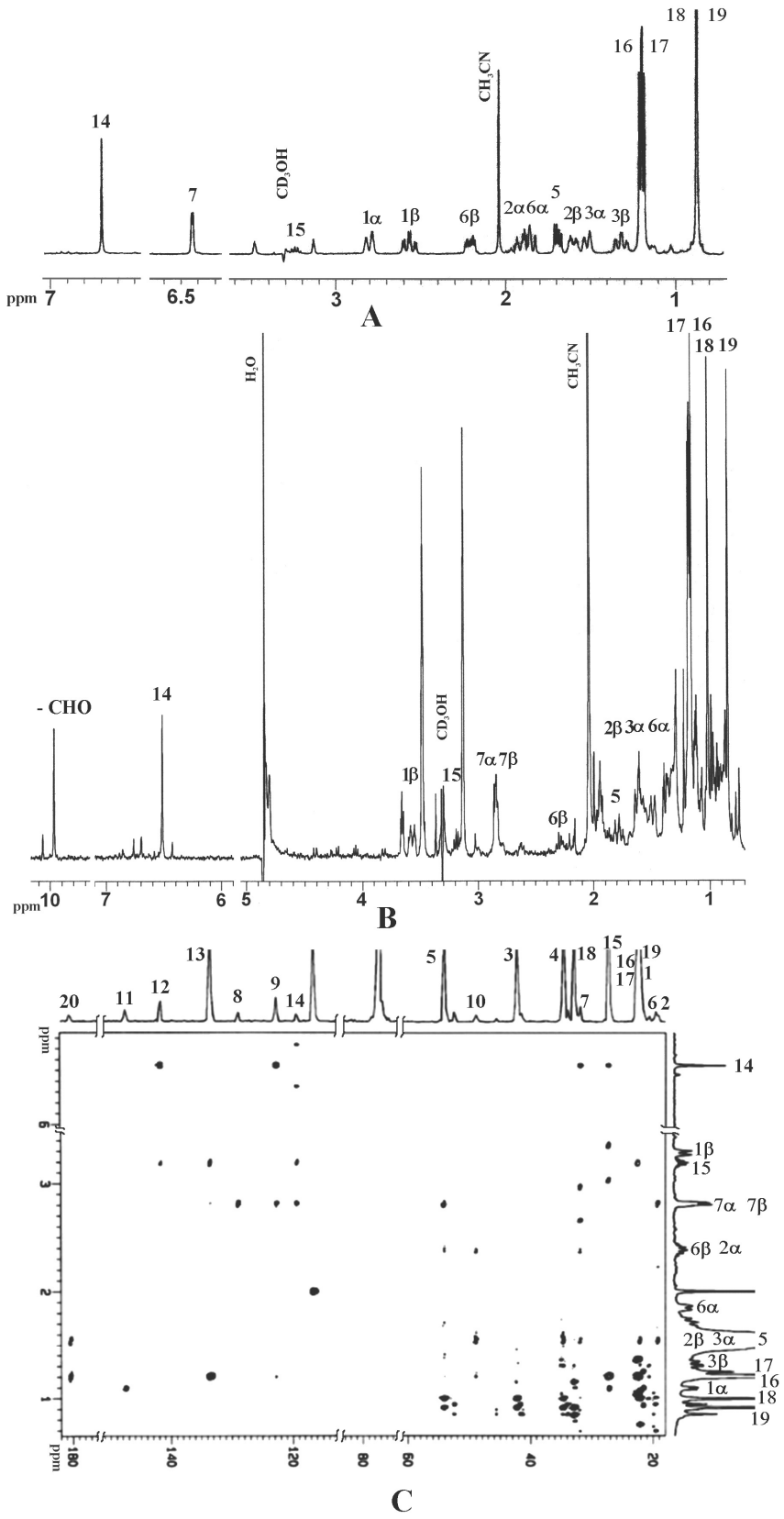

Figure 5.3. A: ^{1}H-NMR spectrum of carnosol **2**; B: ^{1}H-NMR spectrum of carnosaldehyde **5**; C: HMBC spectrum of carnosic acid **3**

Table 5.1. ^{1}H-NMR spectral data of identified compounds.

H #	Epiisorosmanol **1** mult., (J, Hz)	Carnosol **2** mult., (J, Hz)	Carnosic acid **3** mult., (J, Hz)	12-Methoxy-carnosic acid **4** mult., (J, Hz)	Carnosaldehyde **5** mult., (J, Hz)
1 α	2.78 d (14.4)	2.80 d (14.3)	1.12 m	1.10 ddd (12.9, 12.2, 4.4)	1.11 m*
1 β	2.57 ddd (4.5, 13.3)	2.57 ddd (4.4, 14.1)	3.53 ddd (13.8, 3.4, 3.4)	3.64 m	3.57 d (13.4)
2 α	1.87 m	1.89 m	2.08 d (13.0)	2.27 m	~ 1.95 - 2.05 m*
2 β	1.59 m	1.62 dt (13.7, 4.9)	1.5-1.6 m	1.53 m	1.45-1.65 m
3 α	1.50 d (12.3)	1.51 d (13.1)	1.5-1.6 m	1.53 m	1.45-1.65 m
3 β	1.31 m	1.32 ddd (13.5, 13.3, 3.1)	1.33 ddd (13.1, 13.4, 4.3)	1.32 m	~ 1.22 - 1.45 m*
5	1.38 d (4.2)	1.69 dd (10.6, 5.7)	1.5-1.6 m	1.53 m	1.80 d (13.4)
6 α	-	1.84 m	1.82 bd (13.3)	1.82 bd (12.0)	~1.45-1.65 m*
6 β	4.30 dd (4.3, 4.2)	2.20 m	2.37 m	2.27 m	2.29 m
7 α	5.13 d (4.3)	5.43 d (2.8)	2.78 m	2.80 m	2.85 m
7 β	-	-	2.78 m	2.80 m	2.85 m
14	6.77 s	6.69 s	6.45 s	6.47 s	6.52 s
15	3.28 m	3.25 m	3.18 m	3.18 m	3.19 m
16	1.21 d (6.7)	1.20 d (6.7)	1.16 d (7.0)	1.17 d*, (7.1)	1.17 d (7.0)
17	1.20 d (6.8)	1.19 d (6.6)	1.18 d (7.3)	1.19 d*, (7.3)	1.18 d (6.8)
18	1.02 s	0.87 s	0.99 s	0.99 s	1.02 s
19	0.90 s	0.87 s	0.92 s	0.91 s	0.85 s
20	-	-	-	3.66 s	9.97 s
Solvent			CD_3OD		
NS	982	104	40	144	704

* Exact peak positions not clear due to overlapping.

NS – number of scans used for recording spectrum.

The overloaded compound in cartridge 1B6 was suspected to be carnosic acid, the main antioxidative compound in rosemary extracts. The ^{1}H-NMR spectral data of **3** corresponded well with the literature NMR data of carnosic acid [16, 31]. Enough of **3** was collected to record an HMBC spectrum. Although not all H-C interactions were present in the spectrum, the 2-dimensional spectrum clearly substantiated the structure of carnosic acid. The ^{13}C NMR shifts, obtained from the HMBC correlated well with the ones found in the literature [5]. For instance the characteristic multiplet at 3.18 ppm assigned to H-15 had a correlation with the carbon signal at 27.2 ppm, which in turn showed cross peaks with the H-16 and H-17 methyl groups. This indicates an isopropyl side chain. Its position was proven by couplings between H-15 & C-14, and H-14 & C-15. Couplings of H-18 and H-19 with C-3, 4 and 5 confirmed that these methyl groups are both attached to C-4. Cross peaks of H-1 and H-2 with the carbonyl carbon also corresponded with the structure of carnosic acid. However a mismatch of the shift for C-1 with the literature data was observed. A single bond coupling of H-1 with C-1 clearly indicated that C-1 was at 22.1 ppm instead of 34-36 ppm as given in the literature [5, 16]. The ^{13}C NMR shifts obtained from the HMBC spectrum of **3** were as follows: 19.2 (C-2); 19.5 (C-6); 22.1 (C-1, 19); 22.5 (C-16, 17); 27.2 (C-15); 31.9 (C-7); 33.0 (C-18); 34.5 (C-4); 42.4 (C-3); 48.6 (C-10); 54.1 (C-5); 119.5 (C-14); 122.8 (C-9); 129.1 (C-8); 133.4 (C-13); 142.2 (C-12); 147.5 (C-11); 180.5 (C-20). The HMBC spectrum of carnosic acid is given in Fig. 5.3 C. Couplings obtained from the COSY spectrum also corresponded with all proton assignments. Finally the ESI-MS measurements in negative mode (MW 332 amu) and the UV spectrum (λ_{max} 284 nm [31]) fully confirmed the identification based on the NMR spectrum.

The ^{1}H-NMR shifts of compound **4** (Table 5.1) were very similar to those of carnosic acid **3**, i.e. no signals between 4 - 5.5 ppm. However an additional singlet at 3.66 ppm, integrating for three protons, suggested the presence of a methoxy group. ESI-MS confirmed this, as measurements in both NI and PI mode gave pseudomolecular ions $[M-H]^-$ at *m/z* 345 and $[M+H]^+$ at *m/z* 347 corresponding with the MW of 346 amu of methylated carnosic acid. The UV spectrum (λ_{max} at 225 and 282 nm) was identical with the literature values of carnosic acid methyl ester [31]. However, it was not possible to determine if the compound was really carnosic acid methyl ester, based only on molecular mass and UV data. To confirm that **4** was carnosic acid methyl ester methylation of carnosic acid was performed, as described by Hashimoto et al. [32]. The obtained methyl carnosate was investigated with LC-MS with the same gradient as described for LC-NMR. Although the retention time of carnosic acid methyl ester was very close to that of **4** present in the extract, the mass spectrum was different. The major fragment in positive mode for carnosic acid methyl ester was the same as for carnosic acid (*m/z* = 287), but the major fragment in the mass spectrum of **4**

was 301 amu. This means that the methyl group is not split off with the loss of formic acid, so it should be attached to one of the hydroxyls. The chemical shift of the methoxy group in the NMR spectrum, recorded for **4** was identical to that of 12-methoxycarnosic acid [5, 33]. Also the chemical shift changes of 1β, 2α and 6α of **4,** compared with those in carnosic acid were identical to the literature data [5]. Taking this into account compound **4** was identified as 12-methoxycarnosic acid. It should be noted, that the assignments of the 6α and 6β protons found in the literature [5] are not correct. Since the 6β proton has three large diaxial couplings, it is impossible that it appears as a broad doublet.

Also the NMR spectrum of compound **5** (Fig. 5.3B) was almost identical to the one of carnosic acid **3**, except for a 1H singlet at 9.96 ppm, which can be explained by the presence of an aldehyde group. ESI-MS in negative mode suggested a MW of 316 amu, which corresponds with the replacement of the carboxylic acid group of carnosic acid by an aldehyde group. This compound has not yet been described in the literature. Closely related aldehydic compounds are euphracal (11,12,15-trihydroxyabieta-8,11,13-trien-20-al), the aldehydic proton of which has a shift of 9.79 ppm in $CDCl_3$ [34] and 11,12,16-trihydroxyabieta-8,11,13-trien-20-al which has its aldehydic proton at 9.92 ppm in $CDCl_3$ [35]. The UV data were in correspondence with the ones described for euphracal (λ_{max} 230 and 271 nm). Thus compound **5** was identified as carnosaldehyde.

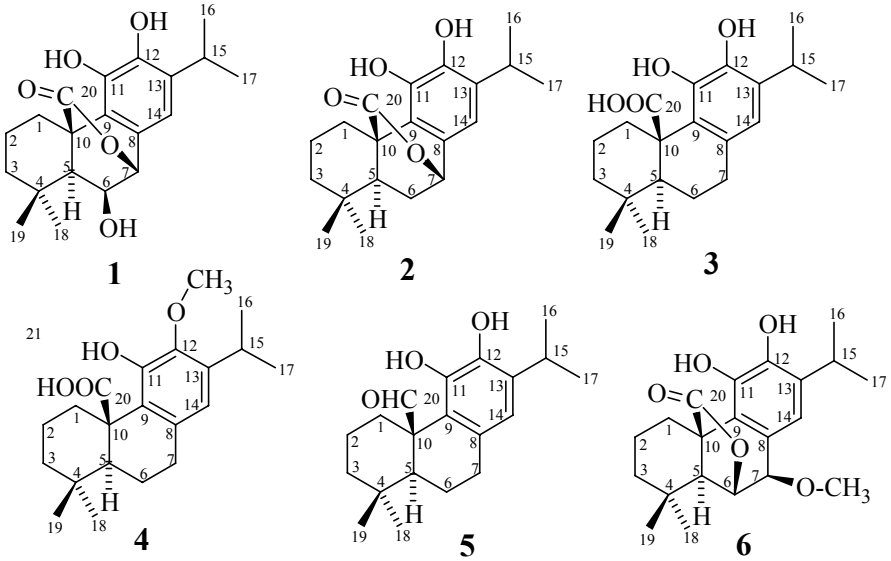

Figure 5.4. Structural formulas of compounds **1 – 6**.

The ^{1}H-NMR spectrum of compound **6** (trapped on cartridge 1A12) showed two methyl singlets at 0.88 and 0.93 ppm, and a 3H double doublet at 1.18 ppm. Together with an aromatic

proton signal at 6.77 ppm, these are characteristic for an aromatic diterpene skeleton. A signal at 3.66 ppm showed the presence of a methoxy group. Unfortunately, due to the small amount present, the S/N ratio of the spectrum was rather low and not all signals could be clearly observed. However, a doublet (J = 3.2 Hz) at 4.28 ppm was clearly visible. The ESI-MS gave a pseudomolecular ion peak at *m/z* 359 in negative mode (i.e. MW = 360) and a major fragment at *m/z* 283. This suggested the structure of epirosmanol methyl ether for compound **6** [4] but in that case an additional doublet at 4.81 ppm [16] coupled with the signal at 4.28 ppm should be present. Unfortunately the residual water signal overlapped this characteristic signal at 4.8 ppm. Taking into account all collected data compound **6** was tentatively identified as epirosmanol methyl ether. It cannot be excluded that this compound is an artefact formed from epirosmanol and methanol which was used for dissolving the extract.

The injection of rosemary extract was repeated four times and each time the same compounds were trapped. In each case the NMR measurements gave the same spectrum, so it can be stated that the method is reproducible enough to perform simultaneous detection and identification of radical scavenging compounds in rather complex extracts.

5.4. Conclusions

A triple hyphenated HPLC – radical scavenging detection – DAD – SPE – NMR system was developed for the rapid identification of antioxidants in complex plant extracts. The SPE unit allowed temporary peak parking without peak broadening. Thus it was possible to first assess which peaks possessed radical scavenging and then at a later stage to measure with NMR the compounds that showed activity. Additional advantages of on-line SPE-NMR relative to stop-flow or loop-storage LC-NMR is that normal non-deuterated solvents can be used for the HPLC separation and that spectra of trapped compounds can be recorded in fully deuterated solvents. Thus less solvent suppression techniques are necessary and spectra are easier to compare with literature NMR data.

Analysis of a commercial rosemary extract with this set-up showed that it was possible to identify a significant number of constituents without having access to reference compounds and without prior isolation. The NMR spectra were decisive for the correct identification of some closely related compounds with the same mass, e.g. in the case of epiisorosmanol. Molecular weight information could be simply obtained by infusion ESI-MS of the pure LC-NMR samples. Thus this method greatly improves and speeds up the identification of antioxidants, since it eliminates compound purification and activity assays of individual compounds, normally a very laborious task. Also, using this method, problems of compound degradation, occurring with intrinsically labile

compounds such as antioxidants, and the introduction of impurities prior to the NMR measurements, are avoided. This method could be useful for monitoring the quality of antioxidative extracts, since it shows not only changes in antioxidant activity, but also in chemical composition of the extract. In the case of chemical degradation, the data should be able to shed light on the type of degradation (e.g. oxidation, hydrolysis).

In spite of the fact that it was feasible to record an HMBC spectrum of the main constituent, a disadvantage of the method remains the relatively poor sensitivity. For instance it was not possible to record an intelligible NMR spectrum of the peak at 81 min that shows potent radical scavenging activity. To further increase the sensitivity of the NMR part of this technique several options are available: (1) changing the NMR flow cell from 120 μL to 30 μL, because in the SPE unit all trapped compounds are dried and then eluted with the first 40 μL of deuterated solvent; (2) using stronger magnets or a cryoprobe system; (3) using multiple trapping of peaks on the same cartridge after repeated injections. As the presented method is well reproducible this can certainly be realized albeit at the expense of time; (4) using the LC-NMR option without simultaneous radical scavenging detection, which effectively means that 30% more compound ends up in the LC-NMR probe; (5) prior enrichment of minor compounds of interest, e.g. by removing carnosic acid by partitioning or preparative HPLC. Several of these possibilities are currently investigated.

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6. Influence of extraction solvents on the yield of antioxidants from sweet grass, costmary and horehound*

6.1. Introduction

Antioxidants need to satisfy some requirements, prior to their application in food. In particular safety aspects are important. Therefore the solvents used for extraction of these compounds must be chosen according to certain safety requirements. Hexane, acetone and ethanol are the allowed organic solvents in food manufacturing if no detectable amount of them is left in the final product. Hexane is a non-polar solvent, so it is not considered as a suitable solvent for extraction of antioxidants; however it is used for removing unwanted compounds from plant material prior to the main extraction. This must be done because antioxidants should not only be safe, but should also have a minimal effect on food quality parameters, such as colour, taste or odour. These secondary qualities can be achieved by removing chlorophyll and essential oil. Hexane is a suitable solvent for this purpose.

6.2. Materials and methods

6.2.1. Materials

All solvents used for extraction were of analytical grade, obtained from PLIVA-Lachema (Brno, Czech Republic). Food grade 96.5% ethanol was obtained from "Stumbras" (Kaunas, Lithuania). BHT (3,5-di-*tert*-butyl-4-hydroxytoluene), potassium iodide and sodium thiosulfate were purchased from Sigma-Aldrich (Taufkirchen, Germany). HPLC solvents methanol and acetonitrile were of HPLC grade (Merck KGaA, Darmstadt, Germany). Commercial refined deodourized rapeseed oil was produced by „Obeliu Aliejus" (Obeliai, Lithuania). The initial PV of the oil was 1.1 meq/kg.

Horehound (*Marrubium vulgare*), sweet grass (*Hierochloe odorata*) and costmary (*Chrysanthemum balsamita*) were harvested in June of 2001 from the collection of the Lithuanian Institute of Horticulture. Plant material was dried at ambient temperature in the shade.

6.2.2. Preparation of extracts

Dried plant material was ground with a TEFAL/SEB 8100 mill (SEB, Dijon, France). Ground plant material (25 g of each herb) was twice extracted with 250 ml (total volume 500 ml) of hexane, acetone and ethanol, according to the scheme in Figure 6.1. Residual plant material after extraction with hexane was left in a fume cupboard to dry and then subsequently extracted with acetone or

ethanol (2 × 250 ml). Extractions were performed by shaking samples at 150 rpm for 24 h at ambient temperature under nitrogen.

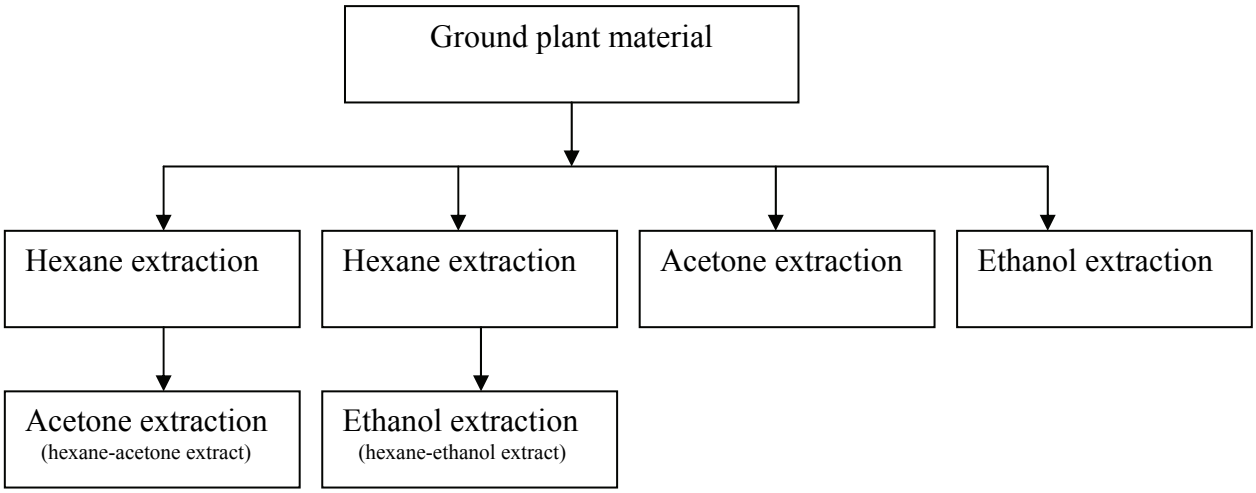

Figure 6.1 Extraction scheme.

The obtained extracts were filtered through filter paper (grade 289, Sartorius AG, Goettingen, Germany), under vacuum and concentrated in a rotary evaporator. Yields of extracts are presented in table 6.1.

Table 6.1. Yields (%) of extracts obtained from sweet grass, costmary and horehound.

Herb	Yields, %				
	Hexane	Acetone	Ethanol	Acetone, after hexane extraction	Ethanol, after hexane extraction
Sweet grass (*Hierochloe odorata*)	7.89	4.52	15.28	2.34	12.12
Costmary (*Chrysanthemum balsamita)*	7.60	10.28	12.67	7.36	12.13
Horehound (*Marrubium vulgare)*	6.85	6.98	12.35	4.49	10.12

6.2.3. HPLC separation of extracts.

Extracts were investigated on an HPLC consisting of an Agilent 1100 series quaternary pump, an Agilent 1100 series vacuum degasser and a Hitachi L-7400 UV detector, set at 254 nm. Compounds were separated on a 250 × 4.6 mm Synergy MAX-RP 4 μm C12 column (Phenomenex, Torrance, CA). Gradients for each particular herb were the same as described earlier in chapter 4.2.5.

6.2.4. Oil oxidation analysis

Oil oxidation was carried out in the oven at 55 ºC. Extracts were dissolved in oil by ultrasonification for 30 minutes. All samples were prepared in triplicate as described in chapter 3.2.2.

Weight gain test: The weight of the samples increases due to binding of atmospheric oxygen by unsaturated fatty acids [1]. The increase of sample weight was measured as a function of time. For evaluation of the antioxidant activities of the extracts, the induction periods (IP) of oil samples were determined and compared with the IP's of a blank and an oil with the synthetic antioxidant BHT. The IP was chosen as the time when the weight had increased 0.05%.

6.3. Results and discussion

6.3.1. Determination of the amount of phenolic compounds in sweet grass, costmary and horehound extracts by HPLC

Using an HPLC method it was determined which extraction solvent yielded the highest amounts of phenolic compounds from the investigated herbs. Only one known antioxidant (5,8-dihydroxybenzopyranone **1**) was monitored in the sweet grass extracts. The other compound, also known to possess antioxidant activity, 5-hydroxy-8-*O*-*β*-D-glucopyranosyl benzopyranone (**2**), could not be detected in extracts probably due to its too high polarity. Ethanol, acetone, and especially hexane are too low polarity solvents to extract this compound. The highest amount of compound **1** was extracted with acetone. Initial extraction with hexane did not help to increase the amount of this compound in subsequent acetone and ethanol extracts (fig. 6.2). Amounts of antioxidant compound **1** were not higher after a hexane preextraction (using hexane-acetone it was even much lower than with only acetone). The reason for this may be some residual hexane in the plant material, which could decrease the solubility of 5,8-dihydroxy benzopyranone (**1**) in acetone or ethanol. It is known that this compound is poorly soluble in non-polar solvents, so even small amounts of hexane could decrease the efficiency of its extraction.

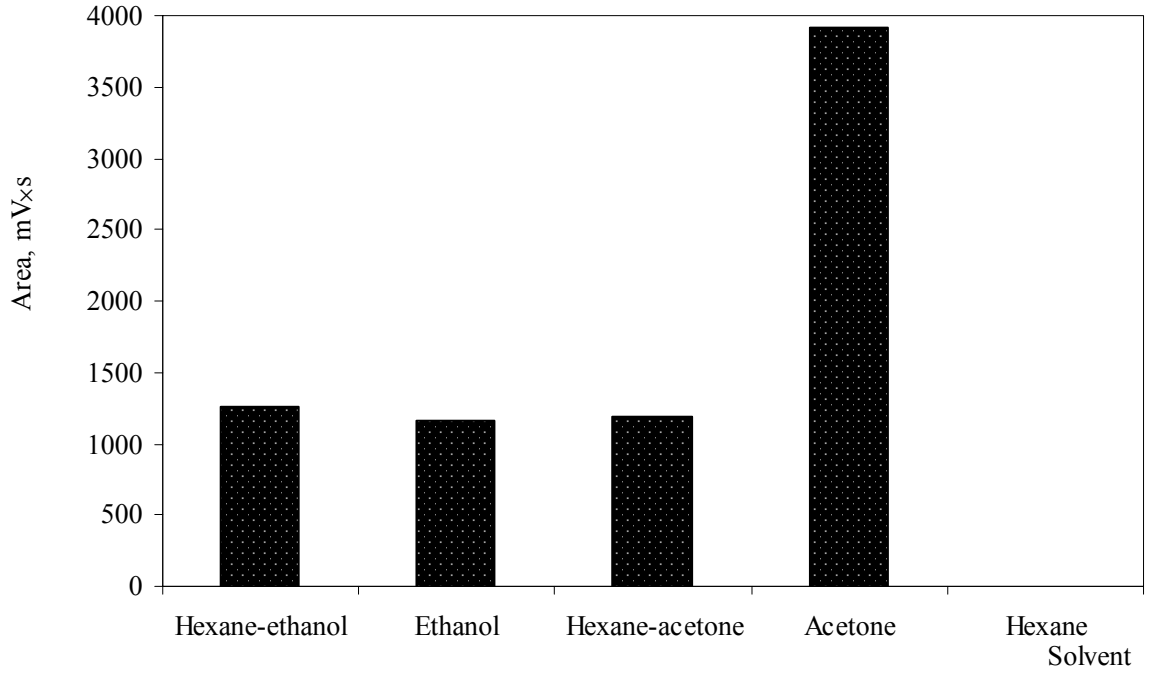

Figure 6.2. Relative amount of 5,8-dihydroxybenzopyranone (**1**) in different sweet grass extracts.

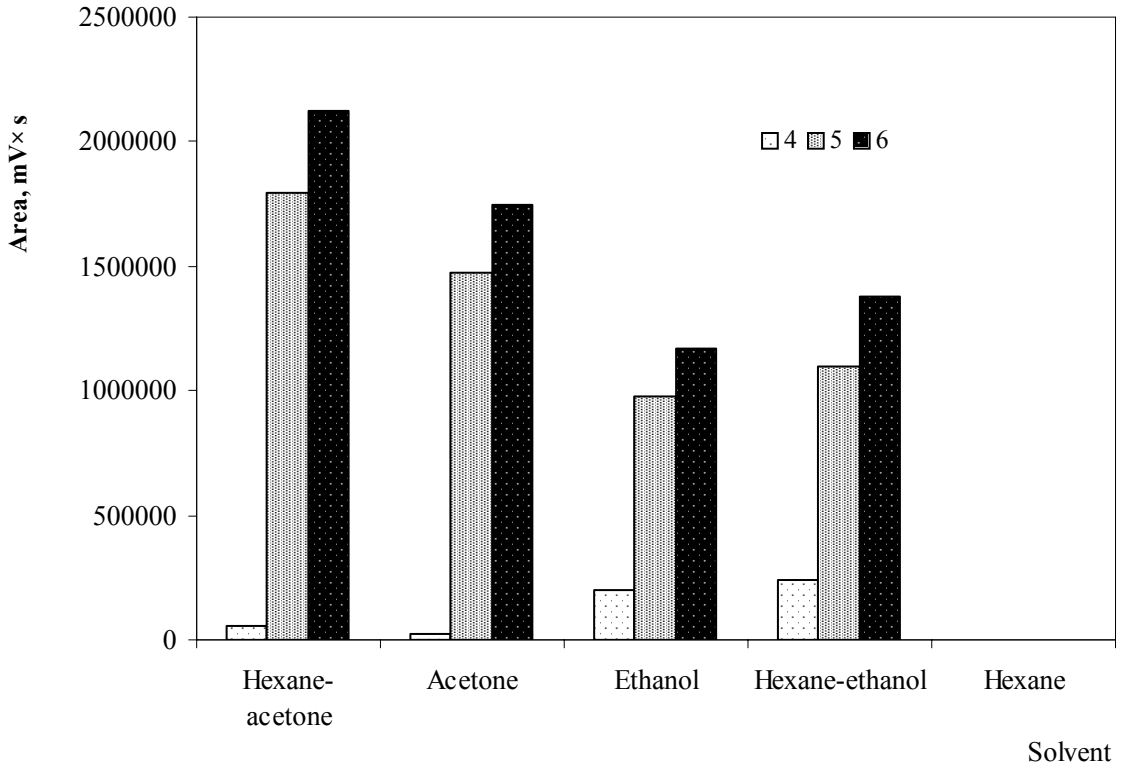

Figure 6.3. Peak areas of 5,7,4'-Trihydroxy-8,3′-dimethoxyflavanone (**5**), 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavone (**6**) and the more polar 3,5-dicaffeoylquinic acid (**4**) in different costmary extracts.

5,7,4'-Trihydroxy-8,3′-dimethoxyflavanone (**5**), 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavone (**6**) and the more polar 3,5-dicaffeoylquinic acid (**4**) were detected in costmary extracts obtained with the above solvents (fig. 6.3.). The most polar antioxidant found in costmary – chlorogenic acid (**3**) was not extracted with the used solvents, probably because it is too polar. As shown in figure 6.3, costmary antioxidants are better extracted after a defatting step with hexane. This is true for all analysed compounds in this herb.

Since no detectable amounts of the three analysed compounds were found in hexane extracts, a preliminary extraction with this solvent could be considered as a suitable pretreatment step for obtaining antioxidant extracts with less matrix substances, especially chlorophylls, fats and waxes.

Earlier five antioxidants were identified in horehound (see 4.3.3.1). In this experiment 5,8-dihydroxy-7,4′-dimethoxyflavanone (**7**) (fig. 6.4), was the major compound extracted with all solvents used. Other – more polar compounds: 7-O-β-glucopyranosyl-luteolin (**8**), verbascoside (**9**) and forsythoside B (**10**) were extracted in much smaller amounts. 7-O-Glucuronyl-luteolin (**11**) was not extracted with any of the solvents, because it was too polar. Since there were no antioxidant compounds in the hexane extract, hexane is a suitable solvent for removing unwanted compounds from horehound prior to the main extraction process. Of additional benefit are the higher concentrations of antioxidants in extracts obtained after initial extraction with hexane.

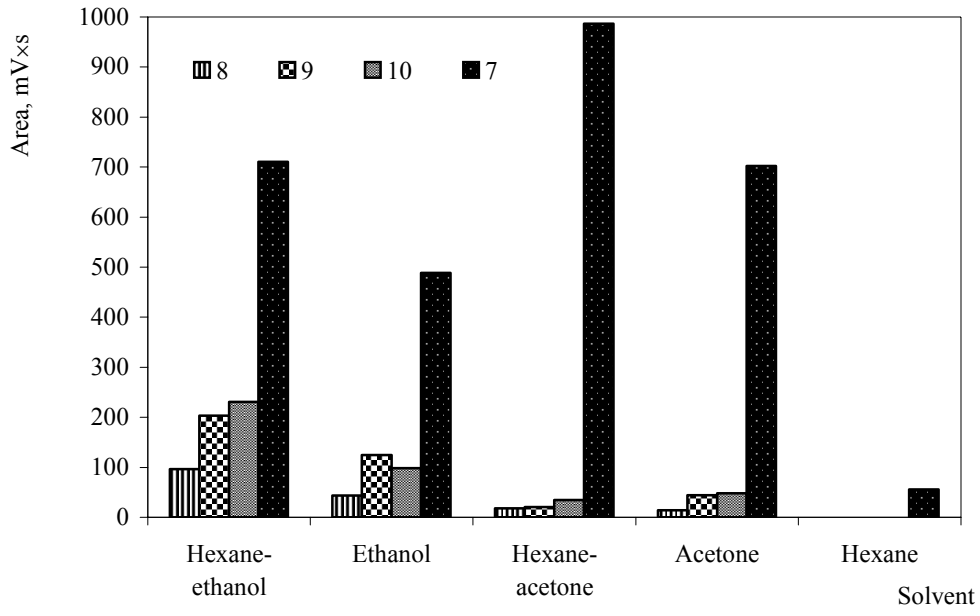

Figure 6.4. Peak areas of 5,8-dihydroxy-7,4′-dimethoxyflavanone (**7**), 7-O-β-glucopyranosyl-luteolin (**8**), verbascoside (**9**) and forsythoside B (**10**) in different horehound extracts.

6.3.2. Influence of sweet grass, costmary and horehound extracts on the oxidation of rapeseed oil

When oxygen reacts with unsaturated fatty acids, hydroperoxides are formed and the weight of the fat increases. Using a weight gain method as described in 6.2.4, the induction periods of rapeseed oil samples with extract additives were determined, and extract activities were compared with the synthetic antioxidant BHT. All extracts were used at concentrations of 0.1%, and BHT at the highest allowable concentration of 0.02%. The experiment was carried out at 55° C. For determining the induction periods a weight increase of 0.05% was chosen and taken from the profiles of the weight gain curves. Graphically determined induction periods are shown in table 6.2 for all oil samples.

Table 6.2. Induction periods of oil samples stored at 55° C

Extraction method	IP values of oil samples, h		
	with 0.1% sweet grass extract	with 0.1% costmary extract	with 0.1% horehound extract
Hexane	230±13	240±9	99±15
Acetone	685±2	311±12	350±9
Ethanol	244±50	248±16	293±11
Hexane-acetone	582±52	248±24	248±26
Hexane-ethanol	544±6	323±24	427±2
BHT 0.02%		570±21	
Blank sample		345±6	

Preliminary oxidation experiments had already indicated that the hexane extracts of all herbs did not contain appreciable amounts of antioxidants. The induction periods (IP) of oil samples with hexane extracts were even lower than that of a blank sample. This means that hexane extracted constituents are potentially able to increase the oxidation rate (i.e. they are prooxidants).

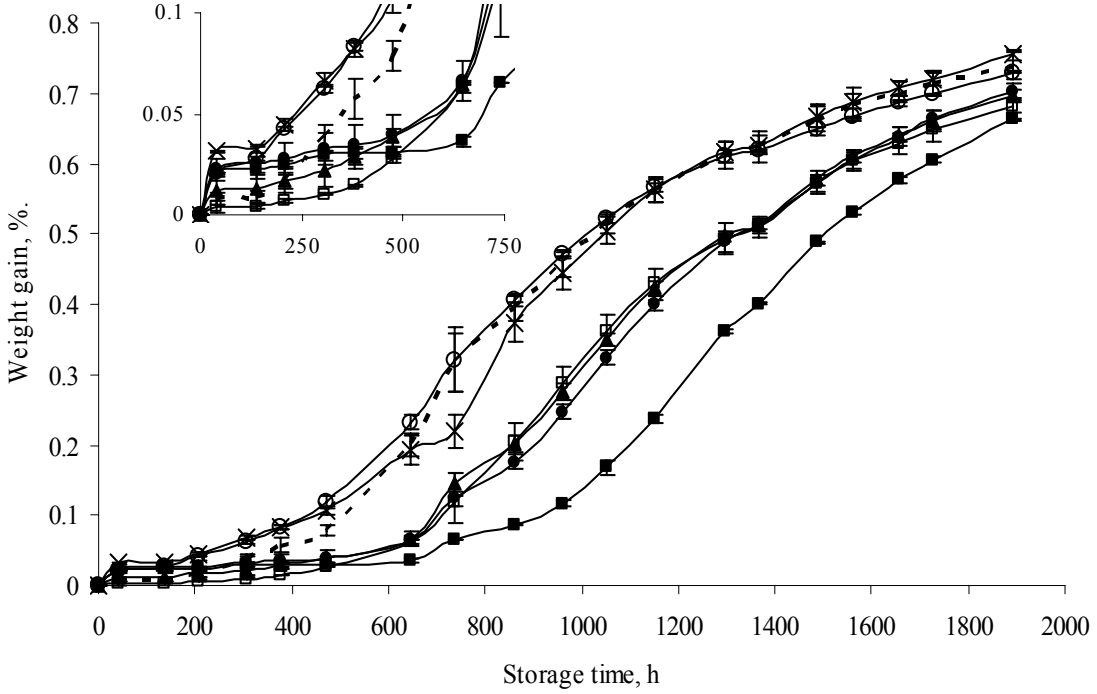

Figure 6.5. Weight gain of rapeseed oil stored at 55 ºC: blank (---), with 0.02 % BHT (▲), with 0.1% sweet grass hexane extract (×), ethanol extract (○), hexane-ethanol extract (●), hexane-acetone extract (□), and acetone extract (■).

Sweet grass hexane-acetone, hexane-ethanol and ethanol extracts retarded oxidation of rapeseed oil. This was not surprising, since 5,8-dihydroxybenzopyranone **1** was found in all these extracts and this is the main radical scavenger in sweet grass. The acetone extract, containing the highest amount of **1**, exhibited even higher activity than BHT. The sweet grass hexane extract did not contain 5,8-dihydroxybenzopyranone, so it did not show any antioxidant activity. A surprising result was the absence of activity in the ethanol extract. According to HPLC results (fig. 6.3) the sweet grass ethanol extract contained approximately the same amount of **1**, as the hexane-acetone, or hexane-ethanol extracts, however its activity was considerably lower. This shows that other compounds beside radical scavengers also influence the total antioxidant activity of the extracts.

Extracts from costmary had little effect on the oil oxidation. The antioxidant activities of acetone, hexane-acetone, and hexane-ethanol extracts were not significantly different from the blank. Other costmary extracts (ethanol, and hexane) exhibited prooxidant activity. These extracts contained the lowest amounts of 5,7,3′,4′-tetrahydroxy-3,8-dimethoxyflavone (**6**) and 5,7,4′-trihydroxy-8,3′-dimethoxyflavanone (**5**) – compounds which are expected to be the main antioxidants in vegetable oils, as dicaffeoyl quinic acid is too polar to dissolve in oils. All costmary extracts showed considerably lower antioxidant activity than BHT.

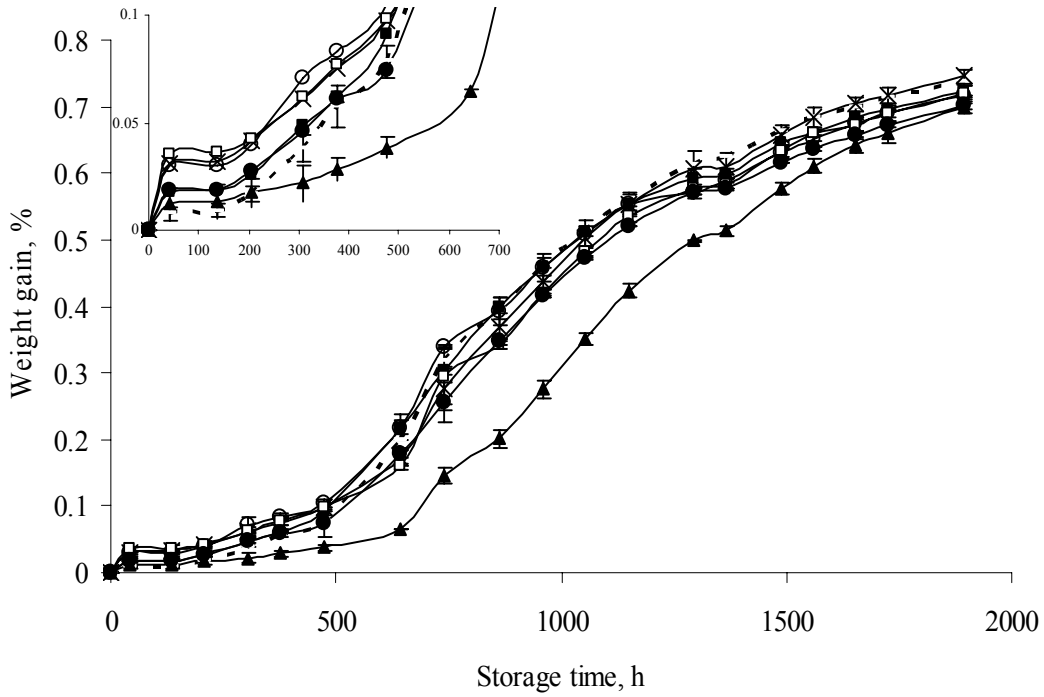

Figure 6.6. Weight gain of rapeseed oil stored at 55 ºC: blank (---), with 0.02 % BHT (▲), with 0.1% costmary hexane extract (×), ethanol extract (○), hexane-ethanol extract (●), hexane-acetone extract (■) and acetone extract (□).

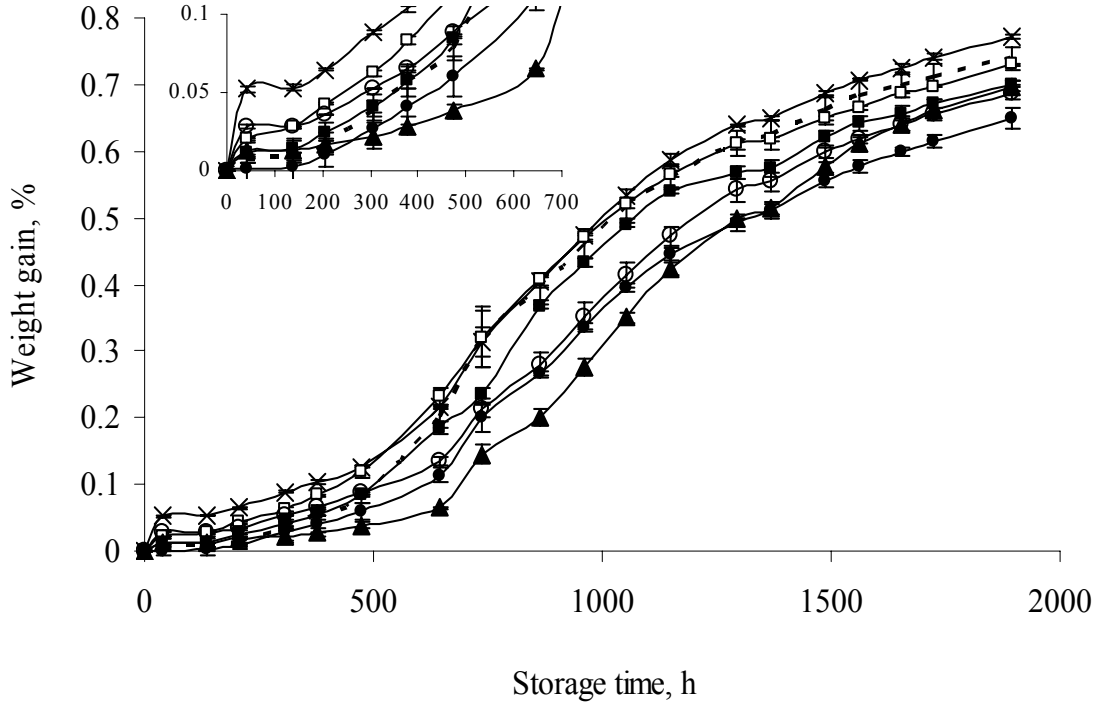

Figure 6.7. Weight gain of rapeseed oil stored at 55 ºC: blank (---), with 0.02 % BHT (▲), with 0.1% horehound hexane extract (×), ethanol extract (○), hexane-ethanol extract (●), hexane-acetone extract (■) and acetone extract (□).

Hexane-ethanol and ethanol extracts showed the highest activities among all horehound extracts. Some activity was also observed for the hexane-acetone extract from horehound. An increased activity of hexane-acetone and hexane-ethanol extracts, compared to their acetone and ethanol analogues, can be clearly observed. Taking into account the changes in amounts of all compounds monitored by HPLC, 5,8-dihydroxy-7,4′-dimethoxyflavanone (**7**), had the largest influence on the antioxidant activities of horehound extracts in oil. However, the hexane-ethanol extract possessed the highest activity among all horehound extracts. So probably 7-O-*β*-glucopyranosyl luteolin (**8**), verbascoside (**9**), and forsythoside B (**10**), that are most abundant in the hexane-ethanol extract, also had some influence on oil oxidation.

6.4. Conclusions

HPLC analyses showed that only few antioxidants were extracted with hexane, so hexane can be used to remove non-antioxidant compounds from costmary and horehound. In the case of sweet grass hexane had some negative effect on the yield of antioxidants, especially when used in combination with acetone.

Acetone and hexane-acetone extracts of sweet grass showed the highest activity in rapeseed oil stored at 55 °C. Ethanol and hexane-ethanol extracts of this herb showed lower activities. Ethanol and hexane-ethanol extracts from horehound had little influence on retarding oil oxidation, other extracts from this herb and all extracts from costmary did not show any activity in this system.

Summarizing the results it could be noted, that there is no single ideal extraction method available for every plant material. Each plant poses a different matrix containing different compounds, which are best extracted with different solvents or combinations thereof.

6.5. References

1. J. Pokorny, H. T. T. Nguyen and J. Korczak, *Antioxidant activities of rosemary and sage extracts in sunflower oil*, Nahrung/Food, **41**, 176-177 (1997)

7. General discussion

Nowadays everybody consumes processed foods, and as buyers expect a certain shelf life, their manufacture requires the usage of additives such as antioxidants. Since synthetic antioxidants are believed to have side effects on human health, more and more attention is paid to compounds of natural origin. The negative feelings about synthetic antioxidants are not really deserved, because they have been thoroughly tested and found suitable for use in foods. When looking for natural sources of antioxidants, attention is often focused on isolates of aromatic and medicinal plants. Although these herbs have been in use as spices in cooking, food additives at home or folk medicine for a long time, their use as a commercial food additive should be considered with care. Testing for adverse effects is also necessary for natural antioxidants, especially when they will be applied on an industrial scale.

In this study we have focused on herbs from the Baltic area like sweet grass (*Hierochloe odorata*), costmary (*Chrysanthemum balsamita*) and horehound (*Marrubium vulgare*) as a potential source of natural antioxidants (aims 1 and 2). The quantities and structural diversity of antioxidants in these herbs is unique for each herb and therefore it is sensible to investigate first the optimal conditions for extraction, fractionation and screening of the antioxidants that may be present.

Two different extraction and fractionation procedures were evaluated and the antioxidant activity of the obtained fractions was determined with several assays (aim 3). These experiments showed the distribution of the antioxidants in fractions of different polarity. The results led to some suggestions for certain extraction procedures, which could be used for the isolation of most of the antioxidants. The fractions are suitable for retarding oxidation of bulk oils.

Fractionation and screening of the fractions not only gave data about the properties of the antioxidants, but also provided antioxidant-enriched material, which was later used for the isolation and identification of individual antioxidants (aim 4).

So far there were no literature data available on antioxidants from sweet grass and costmary. The antioxidant activity of horehound is mostly ascribed to the flavonoids luteolin, apigenin and their glycosides.

In this study two previously unknown compounds were isolated from sweet grass. Their structures, physical and antioxidant properties were published for the first time. One of these compounds (5,8-dihydroxybenzopyranone) is not only the main radical scavenging compound in sweet grass extracts but also has suitable properties for acting as antioxidant in bulk oils. Toxicological testing and the development of large scale isolation technology will have to be

investigated but after a favourable outcome it may be possible to use this compound as antioxidant in lipid-rich foods.

The four compounds that were isolated from costmary were known to possess radical scavenging activity, but none of them had been previously reported from costmary. The main compound was chlorogenic acid, a compound present in coffee beans. Because of its physical properties, this polar compound is not suitable for use as an antioxidant in bulk oils; however other costmary compounds can be used for this purpose. Due to the big differences in polarity it is possible to obtain extracts (e.g. by acetone extraction) with substantial antioxidant activity in bulk oils. Of all the antioxidants that were identified in horehound only 7-O-β-glucopyranosyl-luteolin had been previously isolated from horehound. The isolated compounds were mainly flavonoid glycosides, which resulted in highly polar extracts making them unsuitable as additives in bulk oils. Only 5,8-dihydroxy-7,4′-dimethoxyflavanone is relatively non-polar, but it is present in horehound in very low amounts. The application of horehound extracts as antioxidants is also restricted by their high content of diterpenes; the main diterpene is marubiin. These diterpenes should be removed if horehound extracts are to be used as antioxidants.

The last aim of this research was to develop a method, which would enable the structure elucidation of antioxidant compounds without their previous isolation. The isolation of pure compounds is a laborious and time consuming procedure. Moreover, these processes often lead to degradation of the genuine antioxidative compounds due to their intrinsically labile nature. Recently an on-line HPLC method was developed that can measure the radical scavenging activity of individual compounds in complex mixtures without their prior isolation [1-3]. Coupling of one of these methods (HPLC-DAD-DPPH) to SPE-NMR enabled the direct identification of six compounds present in a commercial rosemary extract. With a slight modification of the chromatographic conditions, the method could also be applied to other complex plant extracts.

The industrial use of pure natural antioxidant compounds is very limited, due to their high production costs. Usually extracts are used for retarding oxidation processes in foods. However, crude extracts have a characteristic colour, odour and taste, which limit their application. Therefore, the production of purified extracts is desirable for the industry. We have made an attempt to produce purified extracts, suitable for retarding oxidation processes in edible oil. Based on the obtained data about the antioxidants in the investigated herbs, it was decided to extract the plant material first with hexane, to remove most of the chlorophyll and odour (essential oil compounds). This initial step, when applied before the main extraction procedure, in most cases, but not always, increased the antioxidant activities of the extracts. This proved again that for every plant material a unique extraction and purification procedure has to be developed.

Even if some of the investigated extracts are expected to be suitable for application in foods, still additional research will remain necessary. The main concern is the toxicity of extracts, or pure compounds. Natural origin does not automatically imply that they are safe. Toxicological analysis of these extracts (or pure compounds) is necessary before application in foods can be considered.

7.1. References

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Summary

Food products are susceptible to oxidation processes. Oxidation of lipids (fat) is the major cause of food deterioration however also proteins and carbohydrates can be affected. To prevent or retard this process, antioxidants are used. With regard to foods, more and more natural products are preferred by consumers so new natural sources of compounds able to retard oxidation processes and prevent spoilage of food products are continuously investigated.

The research described in this study aims at the evaluation of several herbs as possible sources of food antioxidants. Lipid oxidation processes, natural sources of antioxidants and methods for evaluation of antioxidants are briefly described in chapter 1.

An initial screening of extracts from roman camomile, tansy, sweet grass, costmary, sea-buckthorn and sage for antioxidant activity in rapeseed oil has been performed (chapter 2). Sweet grass and sage acetone extracts retarded oxidation processes in oil best. However, the total phenolics content in sweet grass acetone extract was about twice lower than in sage, and lower than in some other herbs having lower antioxidant activities. This finding showed that the content of total phenolics in herbs is not a reliable indicator of their antioxidant activity. The structures of the individual constituents need to be elucidated and assessed in order to obtain more precise results and information.

Sweet grass was chosen for further investigation as the herb having the highest antioxidant activity. Two other herbs, namely horehound and costmary were further selected for the evaluation of their antioxidant activity in different assays. At first preliminary fractionation of the selected herb extracts was performed. Fractions were tested in three different assays: β-carotene oxidation, $DPPH^{\bullet}$ reduction and rapeseed oil oxidation (chapter 3). The experiments did not provide any straightforward answers, about the fractions with the most active antioxidants. Different assays gave different results. More polar fractions were more active in model systems like $DPPH^{\bullet}$, while in the edible oil assay these fractions acted as weak antioxidants, and some even exhibited a prooxidation effect. Acetone extracts performed better than methanol-water extracts in retarding oil oxidation, probably due to their higher compatibility with the medium or the more non-polar nature of the contained analytes.

In further steps the structures of the radical scavenging compounds present in extracts of sweet grass, horehound and costmary were elucidated (chapter 4). Two compounds, namely 5,8-dihydroxybenzopyranone and 5-hydroxy-8-*O*-β-D-glucopyranosyl-benzopyranone were isolated and identified from sweet grass extract. Both compounds were identified for the first time as natural

products. Four compounds, namely 5-*O*-caffeoylquinic acid (chlorogenic acid), 3,5-dicaffeoylquinic acid, 5,7,4'-trihydroxy-3',8-dimethoxyflavone and 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol were identified in costmary extracts. These compounds are quite common in the plant kingdom. However, they have not previously been isolated from costmary. Five compounds, namely 5,8-dihydroxy-7,4′-dimethoxyflavone, 7-*O*-*β*-glucopyranosyl-luteolin, 7-*O*-*β*-glucuronyl-luteolin, verbascoside and forsythoside B were isolated from horehound extracts. These compounds are common in the Labiatae family. Their radical scavenging activity was measured using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays and compared with the activity of rosmarinic acid and Trolox. 5,8-Dihydroxybenzopyranone, 3,5-dicaffeoylquinic acid and 7-*O*-*β*-glucuronyl-luteolin had similar or higher activity in the $DPPH^{\bullet}$ assay than rosmarinic acid. Other compounds were much less active than rosmarinic acid. The highest activity in the $ABTS^{\bullet+}$ assay was shown by 5,8-dihydroxy-7,4′-dimethoxyflavone, 7-*O*-*β*-glucopyranosyl-luteolin, 7-*O*-*β*-glucuronyl-luteolin, verbascoside and forsythoside B. Activities of these compounds were higher than that of Trolox, and comparable to that of rosmarinic acid. 3,5-Dicaffeoylquinic acid, isolated from costmary also possessed higher activity than Trolox in $ABTS^{\bullet+}$ assay.

The isolation and identification of compounds was carried out using bioassay-guided fractionation. An on-line HPLC-DPPH method was very helpful in obtaining information about the individual compounds with radical scavenging activity. However, this fractionation procedure is laborious and time consuming. More labile compounds may be lost during the fractionation. Therefore, an on-line HPLC-DPPH-DAD-NMR system for the rapid identification of compounds in complex mixtures was developed (Chapter 5). The developed system was tested on commercial rosemary extract and six compounds were identified without the need to isolate them. This proved that simultaneous detection and identification of radical scavengers is possible.

Pure compounds are usually too expensive to be used in food products, because of the costs of the purification process. The use of crude extracts is more likely. The extracts need to satisfy some requirements prior to their application in food products. One of these requirements is the safety of the product. Therefore the solvents not only have to extract the desirable compounds, but they should not occur in the final product. Several solvents, namely hexane, acetone ant ethanol and their combinations were tested for their suitability to extract all known radical scavengers from sweet grass, costmary and horehound (chapter 6). The most polar antioxidants (7-*O*-*β*-glucuronyl-luteolin and chlorogenic acid) were not extracted with any of used solvents. It was further found that initial extraction of the plant material with hexane increased the concentrations of radical scavengers in the following extraction stages. The highest activity in rapeseed oil stored at 55° C was shown by

direct acetone and acetone extracts of sweet grass after a preextraction with hexane; direct ethanol and ethanol extracts of this herb after a preextraction with hexane showed lower activities. Ethanol and ethanol extracts from horehound after a preextraction with hexane had little influence on retarding oil oxidation. Other extracts from this herb and all extracts from costmary did not possess any significant activity in rapeseed oil.

An overall discussion and concluding remarks of the current study are presented in chapter 7. It is concluded, that only the sweet grass extracts could be a potential source of antioxidants in lipophilic food products. Extracts of the other two herbs – costmary and horehound proved to be more effective in more polar media, because mainly polar compounds are responsible for the radical scavenging activity of these extracts.

Samenvatting

Voedsel is onderhevig aan oxidatie. Oxidatie van vetten is de voornaamste oorzaak van voedselbederf maar ook eiwitten en koolhydraten kunnen bederven. We gebruiken antioxidanten om deze processen te voorkomen of te vertragen. De consument verlangt meer en meer natuurlijke producten in zijn voedsel en daarom zoeken we naar natuurlijke bronnen voor antioxidanten die bederf van voedsel kunnen voorkomen.

In dit onderzoek zijn een aantal kruiden onderzocht als mogelijke bron van antioxidanten voor voedsel. Oxidatieprocessen in voedsel, natuurlijke bronnen voor antioxidanten en methoden voor de evaluatie van die antioxidanten zijn kort beschreven in hoofdstuk 1.

Een eerste onderzoek van extracten van Roomse kamille, boerenwormkruid, veenreukgras, balsemwormkruid, duindoorn en salie op antioxidant activiteit in raapzaad olie, staat beschreven in hoofdstuk 2. De aceton extracten van veenreukgras en salie vertragen de oxidatie in raapzaad olie het beste. De totale hoeveelheid fenolische stoffen in het veenreukgras extract was echter tweemaal zo klein als in salie en ook lager dan in andere kruiden. Dat gegeven toont aan dat het totale gehalte aan fenolische stoffen in kruiden niet een betrouwbare maat is voor hun antioxidant activiteit. Voor een juiste beoordeling van deze gegevens is het noodzakelijk de structuren van de afzonderlijke verbindingen op te helderen en hun antioxidatieve activiteit te bepalen.

We hebben in de eerste plaats veenreukgras onderzocht omdat dit kruid de hoogste antioxidant activiteit vertoonde. Daarnaast zijn ook malrove en balsemwormkruid uitgekozen voor verder onderzoek. Om te beginnen zijn de extracten van die drie kruiden globaal gescheiden in meerdere fracties die alle getest zijn op hun antioxidant activiteit met drie verschillende methoden: de β-caroteen oxidatie, de DPPH$^{\bullet}$ reductie en de raapzaad olie oxidatie test (hoofdstuk 3).

Deze experimenten gaven geen duidelijke antwoorden op de vraag in welke fracties de meest actieve antioxidanten zaten. De drie testen gaven verschillende resultaten. De meer polaire fracties waren actiever in de DPPH$^{\bullet}$ test, terwijl dezelfde fracties maar zwakke antioxidanten waren in de raapzaad olie oxidatie, en soms zelfs oxidatie bevorderende effecten vertoonden. De aceton extracten gaven betere antioxidant resultaten dan de methanol extracten. Dit zou te wijten kunnen zijn aan het beter oplossen in het test medium door de meer apolaire aard van de actieve verbindingen in de betreffende fracties.

Daarna zijn de structuren van de actieve antioxidanten in de extracten van de drie kruiden opgehelderd (hoofdstuk 4). Uit het extract van veenreukgras zijn twee verbindingen geïsoleerd en geïdentificeerd: 5,8-dihydroxybenzopyranon en 5-hydroxy-8-*O*-β-D-glucopyranosyl-benzopyranon.

Dit is de eerste keer dat deze twee verbindingen zijn gevonden in de natuur. In het extract van balsemwormkruid zijn vier verbindingen gevonden: 5-*O*-caffeoylkininezuur (chlorogeenzuur), 3,5-dicaffeoylkininezuur, 5,7,4'-trihydroxy-3',8-dimethoxyflavon en 5,7,3',4'-tetrahydroxy-3,8-dimethoxyflavonol. Deze stoffen komen veel voor in de natuur maar ze zijn niet eerder gevonden in balsemwormkruid. In de malrove extracten zijn vijf verbindingen gevonden: 5,8-dihydroxy-7,4′-dimethoxyflavon, 7-*O*-*β*-glucopyranosyl-luteoline, 7-*O*-*β*-glucuronyl-luteoline, verbascoside en forsythoside B. Dit zijn normaal voorkomende verbindingen in de lipbloemenfamilie.

Hun antioxidatieve vermogen is getest met $DPPH^{\bullet}$ en $ABTS^{\bullet+}$ radicalen en vergeleken met die van rozemarijnzuur en Trolox. De activiteit van 5,8-dihydroxybenzopyranon, 3,5-dicaffeoylkininezuur en 7-*O*-*β*-glucuronyl-luteoline was gelijk of hoger dan die van rozemarijnzuur in de $DPPH^{\bullet}$ test. De andere verbindingen waren veel minder actief dan rozemarijnzuur. 5,8-Dihydroxy-7,4′-dimethoxyflavon, 7-*O*-*β*-glucopyranosyl-luteoline, 7-*O*-*β*-glucuronyl-luteoline, verbascoside and forsythoside B gaven de hoogste activiteit in de $ABTS^{\bullet+}$ test. De activiteit van deze verbindingen was hoger dan die van Trolox en vergelijkbaar met die van rozemarijnzuur. 3,5-Dicaffeoylkininezuur uit balsemwormkruid had ook een hogere activiteit dan Trolox in de $ABTS^{\bullet+}$ test.

De isolatie en identificatie van bovenstaande verbindingen is uitgevoerd met behulp van antioxidant testen. Een on-line HPLC-DPPH methode was handig bij het bepalen van de antioxidatieve activiteit van afzonderlijke verbindingen. De fractionering is echter bewerkelijk en tijdrovend en instabiele verbindingen kunnen verloren gaan tijdens de fractionering. Om deze bezwaren op te vangen is een on-line HPLC-DPPH-DAD-NMR systeem ontwikkeld voor snelle identificatie van verbindingen in complexe mengsels (hoofdstuk 5). Dit systeem is uitgetest op een commercieel extract van rozemarijn. Hierin zijn zes verbindingen geïdentificeerd zonder voorafgaande isolatie. Op deze manier is bewezen dat gelijktijdige detectie en identificatie van radicaal afvangende verbindingen mogelijk is.

Zuivere verbindingen zijn meestal te duur om in voedsel te gebruiken, de zuivering van verbindingen kost nu eenmaal geld. Het gebruik van ruwe extracten ligt daarom meer voor de hand. Deze extracten moeten echter wel aan een aantal voorwaarden voldoen voordat we ze in voedsel kunnen toepassen en veiligheid is natuurlijk een eerste eis. Dit betekent ook dat we maar een beperkt aantal oplosmiddelen kunnen gebruiken. De actieve antioxidanten moeten goed in dit oplosmiddel oplossen maar het oplosmiddel mag niet meer in het uiteindelijke extract aanwezig zijn.

De oplosmiddelen hexaan, aceton, ethanol en combinaties van deze drie zijn onderzocht op hun geschiktheid voor de extractie van antioxidanten uit veenreukgras, balsemwormkruid en

malrove (hoofdstuk 6). Echter geen van deze oplosmiddelen kon de meer polaire antioxidanten (7-*O*-*β*-glucuronyl-luteoline en chlorogeenzuur) uit het plantenmateriaal extraheren. Hogere antioxidant concentraties in fracties van daaropvolgende extracties konden worden verkregen na een voorafgaande extractie van het plantenmateriaal met hexaan. Een extract van veenreukgras, verkregen door directe extractie met aceton of door extractie met aceton na een voorafgaande extractie met hexaan, gaf de hoogste activiteit in de raapzaad olie test bij 55 °C. Een extract van malrove, verkregen na extractie met ethanol of door extractie met ethanol na een voorafgaande extractie met hexaan, vertoonde weinig activiteit. Extracten van malrove die op andere manieren waren verkregen en alle extracten van balsemwormkruid hadden geen significante activiteit in de raapzaad olie test.

In hoofdstuk 7 staat een discussie en noemen we een aantal conclusies van dit onderzoek. De voornaamste conclusie is dat alleen veenreukgras extracten toegepast zouden kunnen worden als antioxidant in vetachtige voedselproducten. Extracten van de andere twee kruiden, balsemwormkruid en malrove, zijn actiever in meer polaire producten, omdat vooral polaire verbindingen in deze extracten verantwoordelijk zijn voor de antioxidatieve activiteit.

Santrauka

Maisto produktai yra linkę oksiduotis. Pagrindinė maisto produktų gedimo priežastis yra lipidų (riebalų) oksidacija, tačiau gali oksiduotis ir baltymai bei angliavandeniai. Kad sustabdyti ar sulėtinti oksidacinius procesus maisto produktuose, yra naudojami antioksidantai. Vartotojai vis daugiau linksta į natūralius produktus, todėl pastaruoju metu pastoviai atliekami tyrimai ieškant naujų natūralių antioksidantų šaltinių.

Šiame darbe aprašytais tyrimais siekiama įvertinti keletą augalų kaip potencialių antioksidantų šaltinių. Pirmame skyriuje trumpai aprašomi lipidų oksidacijos procesai, natūralūs antioksidantų šaltiniai ir antioksidantų tyrimo metodai.

Antrame skyriuje pateikiami pradiniai tauriųjų bobramunių, paprastųjų bitkrėslių, stumbražolių, balzamitų, šaltalankių ir šalavijų ekstraktų antioksidacinio aktyvumo tyrimai rapsų aliejuje. Geriausiai oksidacijos procesus stabdė stumbražolių ir šalavijų ekstraktai. Tačiau bendras fenolinių junginių kiekis stumbražolių acetono ekstrakte buvo apie du kartus mažesnis nei šalavijo ekstrakte ir netgi mažesnis nei kai kurių kitų augalų, turėjusių mažesnį antioksidacinį aktyvumą, ekstraktuose. Šis atradimas parodė, kad bendras fenolinių junginių kiekis nėra patikimas augalo antioksidacinio aktyvumo indikatorius. Tam kad gauti tikslesnius ir patikimesnius rezultatus, turi būti nustatytos atskirų junginių struktūros ir įvertintas jų aktyvumas.

Tolesniam tyrimui buvo pasirinktos stumbražolės, kaip augalas turėjęs didžiausią antioksidacinį aktyvumą. Taip pat kiti du augalai, balzamitos ir šantros, buvo pasirinkti kad įvertinti jų aktyvumą skirtingais metodais. Pirmiausiai buvo atliktas pirminis pasirinktų augalų ekstraktų frakcionavimas. Gautos frakcijos buvo ištirtos trimis skirtingais metodais: β-karotino blukinimo, $DPPH^{\bullet}$ sujungimo ir rapsų aliejaus oksidacijos (3 skyrius). Šie eksperimentai nesuteikė tikslių atsakymų apie tai, kokiose frakcijose pasiskirstę aktyviausi antioksidantai. Skirtingi metodai davė skirtingus rezultatus. Modelinėse sistemose, tokiose kaip $DPPH^{\bullet}$, aktyvesnės buvo poliškesnės frakcijos, tuo tarpu rapsų aliejuje šios frakcijos veikė kaip silpni antioksidantai arba net turėjo prooksidacinį aktyvumą. Acetono ekstraktai geriau stabdė aliejaus oksidaciją nei metanolio-vandens ekstraktai, galbūt dėl geresnio jų suderinamumo su terpe, arba mažesnio juose esančių junginių poliškumo.

Tolesniame darbe buvo nustatytos radikalus sujungiančių junginių, esančių stumbražolių, balzamitų ir šantrų ekstraktuose, struktūros (4 skyrius). Iš stumbražolių ekstrakto buvo išskirti ir identifikuoti du junginiai: 5,8-dihidroksibenzopiranonas ir 5-hidroksi-8-*O*-β-D-gliukopiranozil-benzopiranonas. Abu šie junginiai buvo pirmą kartą identifikuoti kaip natūralūs produktai. Iš

balzamitų ekstrakto buvo išskirti ir identifikuoti keturi junginiai: 5-*O*-cafeoilchino rūgštis (chlorogeno rūgštis), 3,5-dikafeoilchino rūgštis, 5,7,4'-trihidroksi-3',8-dimetoksiflavonas ir 5,7,3',4'-tetrahidroksi-3,8-dimetoksiflavonolis. Šie junginiai gana plačiai paplitę augaluose, tačiau ankščiau jie niekada nebuvo išskirti iš balzamitų. Iš šantrų ekstraktų buvo išskirti penki junginiai: 5,8-dihidroksi-7,4′-dimetoksiflavonas, 7-*O*-*β*-gliukopiranozil-luteolinas, 7-*O*-*β*-gliukuronil-luteolinas, verbaskozidas ir forsitozidas B. Šie junginiai yra dažnai aptinkami Labiatae šeimoje. Visų išskirtų junginių aktyvumai buvo išmatuoti naudojant $DPPH^{\bullet}$ ir $ABTS^{\bullet+}$ radikalų sujungimo metodus ir palyginti su rozmarinų rūgšties ir Trolokso aktyvumais. $DPPH^{\bullet}$ sujungimo metode 5,8-dihidroksibenzopiranonas, 3,5-dikafeoilchino rūgštis ir 7-*O*-*β*-gliukuronil-luteolinas pasižymėjo didesniu aktyvumu nei rozmarinų rūgštis. Kiti junginiai buvo žymiai mažiau aktyvūs nei rozmarinų rūgštis. $ABTS^{\bullet+}$ sujungimo metode didziausiu aktyvumu pasižymėjo 5,8-dihiroksi-7,4′-dimetoksiflavonas, 7-*O*-*β*-gliukopiranozil-luteolinas, 7-*O*-*β*-gliukuronil-luteolinas, verbaskozidas ir forsitozidas B. Šių junginių aktyvumai buvo didesni nei Trolokso ir panašūs į rozmarinų rūgšties aktyvumą. Iš balzamitų išskirta 3,5-dikafeoilchino rūgštis $ABTS^{\bullet+}$ sujungimo metode taip pat pasižymėjo didesniu aktyvumu nei Troloksas.

Junginių išskyrimas ir gryninimas buvo atliekamas biotestavimo pagalba. Labai daug informacijos apie atskirų junginių antiradikalinį aktyvumą davė kombinuotas HPLC-DPPH metodas. Tačiau naudota frakcionavimo procedūra reikalauja daug laiko ir darbo. Labilesni junginiai gali netgi suskilti frakcionavimo metu. Todėl buvo sukurtas HPLC-DPPH-DAD-BMR metodas greitam junginių identifikavimui sudėtinguose mišiniuose (5 skyrius). Sukurtas metodas buvo patikrintas tiriant komercinį rozmarinų ekstraktą ir be jokių papildomų išskyrimo procedūrų šiame ekstrakte buvo identifikuoti šeši junginiai. Šis tyrimas įrodė, kad yra įmanoma vienu metu atlikti ir radikalų sujungėjų aptikimą ir jų identifikavimą.

Dažniausiai gryni junginiai yra per brangūs, kad juos naudoti maisto produktuose, kadangi labai brangūs yra gryninimo procesai. Todėl labiau tikėtina, kad maiste bus naudojami negryninti ekstraktai. Ekstraktai turi atitikti tam tikrus reikalavimus, kad juos galima būtų panaudoti maiste. Vienas iš tokių reikalavimų yra produkto saugumas. Dėl šios priežasties ekstrakcijai naudojami tirpikliai turi ne tik išekstrahuoti pageidaujamą junginį, bet jų taip pat turi nelikti galutiniame produkte. Buvo tiriamas keleto tirpiklių (heksano, acetono, etanolio) ir jų kombinacijų tinkamumas išekstrahuoti žinomus antioksidantus iš stumbražolių, balzamitų ir šantrų (6 skyrius). Poliškiausi antioksidantai (7-*O*-*β*-gliukuronil-luteolinas ir chlorogeno rūgštis) neišsiekstrahavo nei su vienu iš naudotų tirpiklių. Buvo nustatyta, kad pradinė augalinės žaliavos ekstrakcija heksanu padidino antioksidantų koncentracijas galutiniuose ekstraktuose. Didžiausiu antioksidaciniu aktyvumu rapsų

aliejuje, laikomame 55° C temperatūroje, pasižymėjo stumbražolių acetono ekstraktas ir acetono ektraktas gautas po pradinės ekstrakcijos heksanu. Šio augalo etanolio ekstraktai turėjo mažesnį aktyvumą. Šantrų etanolio ir etanolio po pirminės ekstrakcijos ekstraktai turėjo mažai įtakos rapsų aliejaus oksidacijai. Acetoniniai šantrų ekstraktai ir visi balzamitų ekstraktai neturėjo įtakos oksidacijos procesams rapsų aliejuje.

Bendras šio darbo aptarimas ir išvados yra pateikiamos 7 skyriuje. Galima padaryti išvadą, kad tik stumbražolių ekstraktai gali būti potencialūs antioksidantų, tinkamų lipofiliniams maisto produktams, šaltiniai. Kitų dviejų augalų, balzamitų ir šantrų ekstraktai buvo efektyvesni labiau polinėse terpėse, kadangi už jų antiradikalinį aktyvumą daugiausiai atsakingi gana poliniai junginiai.

Abbreviations

AA – antioxidant activity

AAC – antioxidant activity coefficient

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

AE – acetone extract

AOM – active oxygen method

ARP – antiradical power

AU – absorbance unit

BHA – butylated hydroxyanisole

BHT – butylated hydroxytoluene

CE – costmary extract

COLOC – correlation via long-range coupling

COSY - correlated spectroscopy

DAD – diode array detector

DAE – deodorized acetone extract

DMSO - dimethylsulfoxide

DPPH – 2,2-diphenyl-1-picrylhydrazyl hydrate

DWE – deodorized water extract

EC – effective concentration

EI – electron impact

EO – essential oil

ESI – electrospray ionization

GAE – gallic acid equivalents

GRAS – generally recognized as safe

HMBC – heteronuclear multiple bond coherence

HPLC – high performance liquid chromatography

HR-MS – high resolution mass spectroscopy

IP – induction period

LC – liquid chromatography

MS – mass spectroscopy

NMR – nuclear magnetic resonance

MeOH – methanol

MPLC – medium pressure liquid chromatography

MWE – methanol-water extract

PF – protection factor

PV – peroxide value

RCE – roman chamomile extract

RA – rosmarinic acid

RP – reversed phase

RSD – radical scavenging detection

SBE – sea buckthorn extract

SE – sage extract

SGE – sweet grass extract

SPE – solid phase extraction

TBHQ – tertiary butylhydroquinone

TE – tansy extract

TEAC – Trolox equivalent antioxidant capacity

TFA – trifluoroacetic acid

TLC – thin layer chromatography

TOCSY – total correlation spectroscopy

TOTOX – total oxidation value

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Curriculum Vitae

Audrius Pukalskas was born on June 26, 1973 in Kaunas, Lithuania. During 1980 - 1991 he attended 22nd Secondary school in Kaunas, Lithuania.

In 1991 he started studies at Kaunas University of Technology (KTU), Faculty of Chemical Technology. In 1995 he obtained a bachelor degree in Chemical engineering. During 1995 – 1997 he continued his studies at Kaunas University of Technology and in 1997 obtained his M.Sc. in Food Products Technology (specialization – fermentation technology).

In 1997 he started his Ph.D. studies in KTU, department of Food Technology. In 1999 he was admitted as a Ph.D. candidate for a so-called "sandwich" research project, between the depertment of Food Technology of KTU and two laboratories (Laboratory of Organic chemistry and Laboratory of Product Design and Quality Management) in the department of Agrotechnology and Food Sciences of Wageningen University, the Netherlands. His research area covered the investigation of biologically active substances (antioxidants) from some medicinal and aromatic plants and development of methods for investigation of antioxidants.

At present he works as a lecturer at the Department of Food Technology of Kaunas University of Technology.

List of publications

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