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Variability and biologically active components of some Lamiaceae species

Ph.D. Thesis

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Chemical Composition and Antimicrobial Activities of Essential Oils of Four Lines of *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart Grown in Hungary
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CONTENTS

Abbreviations

GC	–	gas chromatography
GC/MS	–	gas chromatography/mass spectrometry
TLC	–	thin-layer chromatography
SPE	–	solid-phase extraction
LPO	–	lipid peroxidation
MIC	–	minimal inhibitory concentration
SSD	–	statistically significant difference
RA	–	rosmarinic acid
OA	–	oleanolic acid
UA	–	ursolic acid
CA	–	cholesteryl acetate
EtOH	–	ethanol
MeOH	–	methanol

1. INTRODUCTION

Numerous species of the family Lamiaceae which are rich in volatile oils are of great importance as herbs and spices. As regards their morphology and chemical compositions, a substantial proportion of these plants are polymorphic. Such diversity raises important theoretical and practical issues. It can be challenging to classify the species, basic research for the purposes of variety breeding is incomplete, and quality assurance is also often problematic. Consistent with these features, the variability in essential oil composition may alter the gastronomic value of the spices and modify the physiological effects of the herbs in the event of medicinal use. Therefore, it is easy to understand why complex explorative pharmacological studies of the active constituents are playing an increasingly important role over and above chemical and analytical evaluations.

Practical evaluations of numerous Lamiaceae species have brought various classes of constituents other than essential oils into focus. These include hydroxycinnamic acid derivatives (mainly rosmarinic acid) as representatives of natural antioxidants, and also triterpene carboxylic acids, characterized by physiological activity.

In this context, complex research into the family Lamiaceae was begun some decades ago as a cooperative program in which several Hungarian research centers are engaged in the evaluation of medicinal plants. Participating in this research, I have focused on hyssop and origanum, two herbs of diverse appearance and chemical constitution, in order to study their variability and constituent characteristics. The initial results of this research activity were discussed in detail in my thesis for the degree of University Doctor[1], while those of the continued studies are presented in this Ph.D. thesis.

As regards my experiments with both species, I participated in the basic research preceding variety breeding, and working together with researchers at the Department of Medicinal and Aromatic Plants of Corvinus University (the former University of Horticulture), focusing on variety breeding; I was mainly engaged in chemical analyses.

Hyssopus officinalis and *Origanum vulgare* are considered differently in terms of international and Hungarian research activities regarding medicinal and spice plants. Whereas hyssop is little studied and has a rather scanty literature, origanum is at the focus of international interest.

2. AIMS OF THE STUDY

1. The hyssop cultivar registered in Hungary as the variety Kékvirágú is far from being uniform in terms of the colour of the corolla and the chemical composition of its volatile oil. This issue gave rise to further studies of variety breeding. The basis of selection was the composition of the uniform blue colour of the corolla, a high volatile oil content and a definite essential oil composition. The primary aim of our study was a thorough evaluation of the strain and progeny lines originating from different hyssop populations, and a follow-up of the selected characteristics in progeny generations.
2. As regards origanum, no cultivar of this plant is available in Hungary, making it an up-to-date issue to come up with basic research on different origanum populations to achieve results with the potential of utilization for variety breeding. We have focused on the following areas:
    - determining the amount and composition of the essential oil fraction,
    - completing a stock evaluation for the selection of favourable chemotypes,
    - evaluating the characteristics of the Mediterranean subspecies *O. vulgare* subsp. *hirtum* rich in essential oil, grown under the climate of Hungary,
    - performing stability studies on the essential oils.
3. Exploring the relationship between the dynamics of accumulation of rosmarinic acid (RA) and the phenological stage, for both species.
4. Elaborating methodology for parallel determination of the oleanolic acid (OA) and ursolic acid (UA) contents of Lamiaceae species and drawing chemotaxonomic conclusions from the results.
5. Studying biological, e.g. antimicrobial and antioxidant activities.

3. LITERATURE OVERVIEW

3.1. LAMIACEAE FAMILY

3.1.1. Taxonomy of the family Lamiaceae

The family Lamiaceae is one of the largest, most distinctive and most diversified families of flowering plants (Angiosperms), with about 220 genera and almost 4000 species worldwide. This family has an almost cosmopolitan distribution.[2,3] Erdtman (1945) divided

the family Lamiaceae into two subfamilies on the basis of the pollen morphology: the subfamilies Lamioideae and Nepetoideae.[4]

The plants investigated, *Hyssopus officinalis* and *Origanum vulgare,* belong in the tribe *Mentheae* of the subfamily *Nepetoideae* of the family *Lamiaceae* of the order *Lamiales*.[5,6]

3.1.2. Chemical constituents of the family Lamiaceae

The chemical characteristics of the subfamilies Nepetoideae and Lamioideae sensu Erdtman are very different, e.g. the contents of essential oils, RA and caffeic acid, phenylethanoid glycosides and betaines.[3,7–12]

40 per cent of the species of Lamiaceae family are thought to contain compounds that possess aromatic properties.[2] Most studies have focused on their essential oil components.

Flavour and fragrance preparations incorporated into various food, perfumery and cosmetic products are of substantial value on the world market. The economic interest in the fragrance components of aromatic plants direct the selection of commercially cultivated species to focus on both the amount and the quality of the volatile substances. The essential oil content may vary considerably within a single species from one growth season to another, affected by climatic parameters and agrotechnical factors, such as fertilization, water supply, and harvesting, and especially the phase of plant development at the time of harvest.[13,14] Many plants have various phenotypes that differ in their appearance, and both quantitative and qualitative diversity is often detected in the composition of essential oils obtained, e.g. in the cases of hyssop[15] and oregano.[16]

The phenolic acids, caffeic acid and RA, are common constituents of the family Lamiaceae. Caffeic acid plays a central role in the biochemistry of this family. RA is an excellent chemotaxonomic marker, present in the subfamily Nepetoideae, but absent in the subfamily Lamioideae in Erdtmann's (1945) two-subfamily system.[7]

OA and UA are common constituents of plants. These triterpenes may occur as aglycones of saponins or as free acids. Reports on their wide-ranging occurrence in the family Lamiaceae usually also describe the isolation of free OA and UA, besides other compounds.[17-19] Unfortunately, insufficient information is available concerning the distributions of OA and UA in the family Lamiaceae, because published quantitative investigations generally related to only a few species, and mainly qualitative data are to be found on the presence of these compounds.

3.1.3. Activity of compounds

The antibacterial activity of essential oils and their main constituents was recognized long ago.[20,21]

The antimicrobial activities of essential oils against important human pathogenic microorganisms have been examined in detail.[22] The inhibitory effects of the essential oils or their main components on microorganisms that cause food spoilage have also been studied.[23] Interest in the application of essential oils for the control of plant pathogens has recently increased.[24]

It seems possible that the known biological properties of volatile oil compounds[25,26] could justify some of the uses of plants containing essential oil in traditional therapies.

The constituent RA has antibacterial, antiviral, antioxidant and anti-inflammatory properties.[26,27]

Both OA and UA exert many important pharmacological effects, which are rather similar because of the close relationship of their chemical structures. The literature furnishes numerous data on their anti-inflammatory,[28,29] hepatoprotective,[30] antitumour,[31] anti-HIV,[32] antimicrobial,[33] antifungal,[34] gastroprotective,[35] hypoglycemic[36] and antihyperlipidemic[37] properties. They are relatively non-toxic and have been used in cosmetics and health products.[30] Both OA and UA have antioxidant activities.[38] Balanehru and Nagarajan identified OA as a strong, and UA as a mild protector against adriamycin-induced lipid peroxidation.[39]

3.2. HYSSOPUS

3.2.1. Botany of *Hyssopus officinalis* L.

The genus Hyssopus comprises aromatic perennial herbs or subshrubs. It is mainly cultivated, but can also be found in the wild. The inflorescence is 20-25-cm long, false spike-like, composed of 4-10 flowered pseudoverticils in the terminal. The calyx is tubular and 15-veined, with 5 equal teeth. The corolla is infundibuliform and 2-lipped. The upper lip is erect and emarginated, while the lower one is patent and 3-lobed, the middle lobe being the largest.

The root of *H. officinalis* is a strongly branching, multi-headed tap root. It has a stalk dividing into many woody stems. The stems are 0.5-0.7 m in height, erect or decumbent. It is lignified and brownish near the ground, and above it soft and green. The leaves are opposite, shiny dark-green, entire-edged, and lanceolate or oblong, obtuse to acuminate. The well-developed leaves are 2-4 cm long and 0.5-1 cm wide. Both sides of the leaf are densely

covered with embedded oil glands. The basal leaves petiolate and are upper sessile. The bracts are linear, acuminate, and not aristate or with an arista measuring 1-3 mm. The calyx is glabrous or puberolent, the tube is 3-5 mm, and the teeth are 1-3 mm, acuminate, aristate or not. The corolla is blue, violet or rarely white. The fruit is dull, dark or blackish-brown, and oblong, with an acute nutlet on one side.[40]

Four subspecies have been recognized within *H. officinalis* L.: subsp. *canescens*, subsp. *montanus*, subsp. *aristatus* and subsp. *officinalis*.[40] Among these subspecies, subsp. *officinalis* and subsp. *canescens* are considered significant in Central Europe.[13,14]

In its natural habitat, it is found on dry, rocky, calcareous soils.

Hyssop flowers in August in the first year. From the second year on, it flowers in July, and after being cut flowers for a second time in September under good weather conditions. The second flowering in September takes place only if the weather is rainy in August and September. The plant's life-span under cultivation is 7-9 years.[13,14]

3.2.2. Chemistry of *Hyssopus officinalis* L.

H. officinalis contains 0.3-1.0% essential oil.[41] Hyssop oil is well known, and its composition has been examined and reported by various researchers.[2,42,43] Essential oils of plants of the genus Hyssopus (Lamiaceae) have been studied from different subspecies,[44] chemotypes[2] and phenotypes[15] of *H. officinalis* with reference to the parts used (leaves, stems, roots, aerial parts, flowering tops or only flowers, which may be blue, red, white or of mixed colour[45]), to the stages of vegetation of wild-growing or cultivated plants at the time of the harvest, and also considering their different origins.[46–48]

Numerous studies in the literature have reported the compositions of essential oils isolated from *H. officinalis;* the major constituents were reported to be pinocamphone, isopinocamphone, *β*-pinene, 1,8-cineole and pinocarvone.[48-54] These studies have revealed that the composition of the essential oil varies according to genotype, location and climate conditions.

The chemical compositions of the essential oils vary considerably between oils obtained from the same type of hyssop, although the presence of bicyclic monoterpene ketones, pinocamphone and isopinocamphone remains peculiar to these products. In fact, there are oils with high levels of pinocamphone (up to 80%) or isopinocamphone (up to 50%), although the ISO 9841 Standard (1991 E) recommends 5.5-17.5% for pinocamphone and 34.5-50% for

isopinocamphone.[55,56] There are also oils in which other substances may predominate; for example, a *H. officinalis* from Spain had a cineol-rich oil (52.9%)[47] and one from Montenegro a methyl eugenol-rich oil (38.3%).[46] Thus, these oils do not meet the recommended ISO standards.

The volatile compounds of the phenotypes or forms of *H. officinalis*, differentiated by the colour of the corolla, have also been investigated.[15,53,57] Three phenotypes of hyssop cultivated in the Tashkent region, with either red, blue, or white flowers, analyzed by Khodzhimatov and Ramazanova, were found to differ in their chemical composition.[57] Chalchat *et al.* examined three forms of *H. officinalis* cultivated in Yugoslavia. They observed that these differed in the yield of oils, but not in their chemical composition.[53] Kerrola studied the intensity of odour of the four phenotypes by sensory methods. The blue-flowered phenotype was assessed to be more intense in odour than the others.[15]

Besides the essential oils, the plant contains phenolics, di- and triterpenes, etc.[41]

3.2.3. Biological activity and medicinal use of *Hyssopus officinalis* L.

Essential oils of hyssop have been tested for their inhibitory effects against 9 strains of Gram-negative and 6 strains of Gram-positive bacteria. The data obtained indicated that hyssop oil has a general bacteriostatic activity.[58]

Oils of *H. officinalis* subsp. *officinalis* displayed antifungal activity against 13 strains of phytopathogenic fungi; the oils extracted from plants grown at 1000 m above sea level exhibited higher activity.[54]

The antifungal effects of hyssop (*H. officinalis*) oil and its individual components were also studied in a series of *in vitro* and *in vivo* experiments by Letessier. Hyssop oil and its components, used individually, completely inhibited the growth of plant pathogenic fungi.[59]

The essential oil of hyssop may be used as an expectorant and antiseptic. Hyssop-based phytopharmaceuticals are traditionally administered by the oral route for acute benign bronchial disease, and locally to relieve nasal congestion in the common cold.[41]

3.3. ORIGANUM

3.3.1. Botany of the investigated Origanum species

Origanum is used throughout the world as a very popular spice, under the vernacular names oregano or origanum. Oregano, one of the most important Mediterranean spices,

exhibits rather high diversity from both taxonomic and chemical aspects.[60] A large number of species referred to as “Oregano” belong in different botanical families and genera. Four main groups commonly used for culinary purposes can be distinguished, i.e. Greek oregano (*O. vulgare* subsp. *hirtum* (Link) Ietswaart); Spanish oregano (*Coridohymus capitatus* (L.) Hoffmanns & Link); Turkish oregano (*O. onites* L.); and Mexican oregano (*Lippia graveolens* HBK).[61]

In Europe and, in general, all over the world, the most common oregano species belong in the botanical genus Origanum. The genus Origanum (Lamiaceae) is characterized by a large morphological and chemical diversity.

Several efforts have been made to classify the genus Origanum properly. According to Flora Europaea, the genus is divided into three sections, i.e. Origanum, Majorana and Amaracus.[40] All three sections were revised by Ietswaart (1980), who identified a total of 38 species and 17 taxa of hybrid origin.[60] The morphological variation within the genus results in the distinction of 10 sections consisting of 42 species or 49 taxa (species, subspecies and varieties).[62] In Ietswaart's classification (1980), Section IX is Origanum. It is a monospecific section that contains only the species *O. vulgare*, widely distributed in Eurasia and North Africa. Introduced by humans, this species has also been encountered in North America.[62] The plants of *O. vulgare* have dense spikes, and tubular 5-toothed calyces, never becoming turbinate in fruit. Six subspecies have been recognized within *O. vulgare* based on differences in the indumentum, the number of sessile glands on leaves, bracts and calyces, and in the size and colour of the bracts and flowers. The southernmost range of *O. vulgare* is occupied by the three subspecies rich in essential oils, whereas those poor in essential oils are found toward the northern most range of distribution.

1.	*O. vulgare* L. subsp. *vulgare*	Europe, Iran, India, China
2.	*O. vulgare* L. subsp. *glandulosum* (Desfontaines) Ietswaart	Algeria, Tunisia
3.	*O. vulgare* L. subsp. *gracile* (Koch) Ietswaart	Afghanistan, Iran, Turkey, former USSR
4.	*O. vulgare* L. subsp. *hirtum* (Link) Ietswaart	Albania, Croatia, Greece, Turkey
5.	*O. vulgare* L. subsp. *viridulum* (Martrin- Donos) Nyman	Afghanistan, China, Croatia, France, Greece, India, Iran, Italy, Pakistan
6.	*O. vulgare* L. subsp. *virens* (Hoffmannsegg & Link) Ietswaart	Azores, Balearic Is., Canary Is., Madeira, Morocco, Portugal, Spain

Until 1980, there was no satisfactory classification of the Origanum genus, but since then Ietswaart's system has become widely accepted.

O. vulgare subsp. *hirtum* (Link) Ietswaart is native to Albania, Greece and Turkey, but alien to Hungary. Ecologically, these species prefer warm, sunny habitats, and loose, often rocky, calcareous soils, with low moisture content.[63]

3.3.2. Chemistry of the investigated Origanum species

Unfortunately, much confusion exists about the correct taxonomic classification of oregano, despite the taxonomic revision of the genus by Ietswaart in 1980.

It is not surprising that many analytical investigations of oregano and its essential oils did not discriminate the numerous subspecies that exhibit subtle morphological and chemical differences.[64] The yield of essential oil is the only character that demonstrates relative stability, thus being useful for the identification of subspecies.[65]

The essential oils of Origanum species vary in respect of their total amount per plant and also their qualitative composition.

On the basis of their essential oil content, the different taxa of the genus can be divided into three main groups:

1. Taxa poor in essential oils, with an essential oil content of less than 0.5%, e.g. the Greek endemic *O. laevigatum* and *O. vulgare* subsp. *vulgare*.
2. Taxa with an essential oil content between 0.5 and 2%, e.g. the Cretan endemic taxon *O. microphyllum* known as Cretan marjoram.
3. Taxa rich in essential oils, with an essential oil content of more than 2%, as for example the two commercially best-known 'oregano' plants, *O. vulgare* subsp. *hirtum* (Greek oregano) and *O. onites* (Turkish oregano).[66–69]

As concerns their essential oil composition, Origanum species may be characterized by the dominant occurrence of the following compounds:

- Linalool, terpinen-4-ol and sabinene hydrate, e.g. the essential oils of *O. majorana* (syn. *Majorana hortensis* Moench.).[70]
- Phenolic compounds, carvacrol and/or thymol, e.g. the essential oils of *O. vulgare* subsp. *hirtum* and *O. onites*,[67,68,71]
- Sesquiterpenes, e.g. the essential oils of *O. vulgare* susbp. *vulgare*.[61,72,73]

A number of studies have shown that variation in chemical features may occur within a single Origanum species. Furthermore, it has been found that the pattern of variation of a single species follows its geographical distribution or depends on the season of plant collection.[63]

The variation in the essential oil content of *O. vulgare* plants grown all over the species range in Greece has been studied. Plants grown in the Mediterranean zones are rich in essential oils (subsp. *hirtum*), whereas those under a continental climate are poor in essential oils (subsp. *viridulum* and subsp. *vulgare*).[67,74]

A survey of the literature reveals that *O. vulgare* subsp. *vulgare* has an extremely low essential oil yield, and the essential oils of *O. vulgare* subsp. *vulgare* have been found to be rich in acyclic compounds and sesquiterpenoids.[75,76] Its principal constituents are sabinene, (*Z*)-*β*-ocimene, *β*-caryophyllene and germacrene D, while the phenols thymol and carvacrol are absent. By contrast, *O. vulgare* subsp. *hirtum* has a high essential oil yield, whose principal components are phenols, *p*-cymene and *γ*-terpinene.[65]

Essential oils of *O. vulgare* subsp. *vulgare* from Turkey were studied by Sezik *et al.* and Sahin *et al.* In the former case, the essential oil was rich in terpinen-4-ol + *β*-caryophyllene (21%) and germacrene D (17.8%),[76] while Sahin *et al.* found that the main constituents of the essential oil of *O. vulgare* subsp. *vulgare* were *β*-caryophyllene and spathulenol, followed by germacrene D and *α*-terpineol.[77]

Chalchat and Pasquier demonstrated the chemical diversity of the plant grown in an experimental field in Italy: 6 chemical groups were discerned, in which hydrocarbons predominated.[78] Melegari investigated the essential oils of the inflorescences and found 4 chemotypes*:* *p*-cymene, terpinen-4-ol, thymol and *β*-caryophyllene.[79] Mockute *et al.* confirmed these results in a study of Lithuanian samples (*β*-caryophyllene, 10.8–15.4%; germacrene D, 10.0–16.9%; sabinene, 6.4–14.2%; (*Z*)-*β*-ocimene, 6.2–11.0%; and (*E*)-*β*-ocimene, 7.0–11.5%).[80] An Indian oil analyzed by Pande and Mathela unusually contained *γ*-muurolene (62.2%).[81]

The essential oils of 9 species and subspecies of oregano were studied by Figuérédo *et al*. *O. vulgare* subsp. *vulgare* was particularly rich in the terpene hydrocarbons sabinene (16.3%), *p*-cymene (2.3%) and (*Z*)-*β*-ocimene (1.5%) and the sesquiterpene hydrocarbons germacrene D (13.3%), *β*-caryophyllene (10.7%) and *β*-bourbonene (1.9%). These hydrocarbons were accompanied by the functionalized terpenes linalool (4.0%) and terpinen-4-ol (4.8%).[66]

As this subspecies grows native in an extremely wide area, various compositions have been found in studies of its oils. These results reveal a high degree of chemical variability.

The essential oils of separate parts of *O. vulgare* L. subsp. *vulgare* were investigated by Mockute. The mean amounts of the constituents in the investigated samples of the essential oil isolated from the inflorescences differed from those of the oil isolated from the aerial parts of the plants. The essential oils of the inflorescences and the leaves plus stems contained different quantities of major constituents (%): *β*-ocimene (13.3–18.4 and 20.6–25.3), sabinene (10.5–15.8 and 6.7–9.8), germacrene D (9.5–15.9 and 12.7–15.7) and *β*-caryophyllene (10.2-14.5 and 9.3–13.7). The essential oils of both origins were of the *β*-ocimene chemotype, but the amount of *β*-ocimene was lower in the inflorescence oil than in the oil isolated from the aerial parts of the plants.[82]

A number of studies have shown that *O. vulgare* subsp. *hirtum* (Link) Ietswaart (syn.: *O. hirturn L., O. heracleoticum* auct. non L.) is a very variable taxon in both morphological and chemical terms. In particular, it has been found that the essential oil content of this taxon in Greece ranges from 1.1 to 8.2%. Furthermore, it has been reported that the essential oil composition of the wild-growing plants is characterized either by the predominant presence of carvacrol or thymol or by almost equal amounts of these phenols. In all cases, the monoterpene hydrocarbons *γ*-terpinene and *p*-cymene were consistently present in all the essential oils analyzed, but always in lower amounts than those of the two phenols.[71,74,83,84] *γ*-Terpinene and *p*-cymene are biosynthetic precursors of the isomeric phenols carvacrol and thymol.[85,86]

O. vulgare subsp. *hirtum* was studied by Pasquier, and several main groups of plants were identified on the basis of their essential oil content, the first group being the most common one:

1. Thymol group: rich in thymol (>55%) and poor in carvacrol (< 6%).
2. Carvacrol group: rich in carvacrol (>50%) and poor in thymol (< 2.5%).
3. Thymol-carvacrol group: balanced presence of thymol and carvacrol (30-45%) for both components.
4. γ-Terpinene group: rich in γ-terpinene (>45%), very poor in thymol (<1%) and poor in carvacrol (13-15%).[16]

Twenty-four steam-distilled samples of the essential oil isolated from the inflorescences of *O. vulgare* subsp. *hirtum* (Link) Ietswaart growing wild in Calabria, southern Italy, were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

Four chemotypes were identified on the basis of the phenolic content, i.e. thymol, carvacrol, thymol/carvacrol, and carvacrol/thymol chemotypes, the first of these being the most frequent chemotype. A significant variability of composition was observed that possibly correlated with the individual genotypes.[65]

Other studies in the literature suggested that *O. vulgare* subsp. *hirtum* is more commonly rich in carvacrol and less commonly rich in thymol.[71,87-89]

Steam-distilled essential oils of 23 collections of *O. vulgare* subsp. *hirtum* from the Aegean region of Turkey were analyzed by GC and GC/MS. Forty-eight compounds, which amounted to 95.1-99.5% of all components detected, were identified. The carvacrol content in the oils varied between 23.43 and 78.73%.[90]

Essential oils of *O. vulgare* subsp. *hirtum* from 23 localities throughout Greece were analyzed in order to determine their quantitative and qualitative features. The total oil contents of the plants and the percentage contributions of the major oil constituents carvacrol, thymol, γ-terpinene and *p*-cymene varied markedly between localities. The contents of carvacrol and thymol displayed a significant inverse correlation. Altitude seemed to be the most important environmental factor influencing the essential oil content; high values were recorded at low altitudes, coinciding with Mediterranean-type ecosystems. The sum of the 4 major oil constituents, representing the 'phenol pathway', seemed to be influenced by the thermal efficiency of the climate. The hotter the climate, the higher their total concentration.[68]

The season of collection may also strongly affect the essential oil yields of the plants and the concentrations of their main components.[91] Differences were found between summer and autumn plants in the total essential oil content of *O. vulgare* subsp. *hirtum* and in the amounts of each of the 4 main oil components. The essential oil content was much lower in the autumn plants, ranging from 1.0 to 3.1%, as compared with those collected from the same areas in summer (4.8–8.2%). The most impressive difference was the increased amount of *p*-cymene in autumn, which ranged from 17.3–26.9% of the total oil content in plants from the South Peloponnese and Crete (versus 4.0–9.5% for the summer plants) to 37.1–51.3% of the oil content in plants from the Athos peninsula (versus 12.0–12.2% in the summer).[83]

The essential oils of *O. vulgare* subsp. *hirtum* plants collected in late autumn from 6 localities of 3 distinct geographic areas were studied. A high quantitative variation was found in the amounts of the 4 main components; γ-terpinene ranged from 0.6 to 3.6% of the total essential oil content, *p*-cymene from 17.3 to 51.3%, thymol from 0.2 to 42.8%, and carvacrol

from 1.7 to 69.6%. Plants collected from the northern part of Greece were rich in thymol (30.3–42.8% of the total oil), whereas those from the southern part of the country were rich in carvacrol (57.4–69.6% of the total oil). Furthermore, a comparison with the essential oils obtained from plants collected from the same localities in mid-summer demonstrated noticeable differences in total oil content and in the concentrations of the 4 main oil components.[67,68,71,74,83]

In spite of the striking quantitative differences in the major oil components, their sum (*γ*-terpinene + *p*-cymene + thymol + carvacrol) was almost equal in the essential oils of plants of different geographical origin or those collected in different seasons, ranging from 85.0 to 96.8%. These results suggest that the essential oils of *O. vulgare* subsp. *hirtum* are characterized by stability, irrespective of the season of plant collection.[20]

Besides the essential oils, the plant contains phenolics, di- and triterpenes, etc.[92,93]

3.3.3. Biological activity and use of the investigated Origanum species

The antibacterial and fungicidal activities of oregano have been reported in many works.[94,95] Its oil exerts bactericidal effects against many microorganisms.[96-98] Its inhibitory effect has also been described on the growth of some plant and animal pathogens, and organisms causing food spoilage.[99-101] As regards honeybee (*Apis mellifera*) pathogens, bactericidal and fungicidal effects of oregano extracts have been reported for *Bacillus larvae* (causing American foulbrood), *Ascosphaera apis* (causing chalkbrood) and *Bacillus alvei* (a secondary pathogen involved in European foulbrood). These findings, confirmed by Calderone *et al.*, highlight the important role of oregano in the management of honeybee diseases.[102]

The essential oil of *O. vulgare* is active against the growth of fungi and bacteria[103-105] and against *Clostridium botulinum*.[106] The high carvacrol content of the oil was found to play a major role in this activity.[107]

Essential oils from *O. vulgare* were tested for their inhibitory effect against 9 strains of Gram-negative and 6 strains of Gram-positive bacteria. The essential oils of *O. vulgare* appeared to be bactericidal at concentrations above 400 ppm, probably because of the high contents of phenolic compounds. The bacteriostatic activity was more marked against Gram-positive bacteria; in contrast, the bactericidal activity was greatest against Gram-negative bacteria.[58]

The essential oils of *O. vulgare* subsp. *hirtum* exhibited antifungal properties against human pathogens, and at a dilution of 1/50 000 caused a 95% reduction in the number of metabolically active cells within 6 h of exposure. Of the 4 main components of the oil, carvacrol and thymol displayed the highest levels of antifungal activity. The essential oils mentioned above did not exhibit any mutagenic activity in the Ames test.[108]

The study by Burt and Reinders indicated that the essential oils of *O. vulgare* possess significant *in vitro* colicidal and colistatic properties in a broad temperature range, which are amplified by the addition of agar as stabilizer. This could be an area of further research for application in the food sector to improve food safety by the partial or total elimination of *E. coli* O157:H7.[109]

The antimicrobial test results showed that the essential oils of *O. vulgare* subsp. *vulgare* had a high antimicrobial activity against all 10 bacteria and 15 fungi and yeast species tested. In contrast, the MeOH extract of the aerial parts of *O. vulgare* plant showed no antimicrobial effect.[77]

O. vulgare additionally has antiviral activity. Ethanolic extracts of *O. vulgare* proved to be active against ECHO9 Hill virus in cultures of monkey kidney cells, by inducing the production of a substance with interferon-like activity.[110]

The antioxidant activity of Origanum species, has been investigated.[111,112] Lagouri *et al.* demonstrated the antioxidant activities of *O. vulgare* subsp. *hirtum*, *O. onites*, *Coridothymus capitatus* and *Satureja thymbra*.[113] The essential oils of *O. vulgare* subsp. *hirtum,* and its individual compounds carvacrol and thymol, were found to possess antioxidant activity when tested on TLC plates.[113] Results from these studies indicated that the antioxidant effect may be related to the presence of carvacrol and thymol in the essential oils. Other chemical compounds extracted from the leaves, such as terpenoids, may also contribute to the antioxidant activity.[114-116]

The antioxidant activities of the MeOH extract and the essential oils of *O. vulgare* subsp. *vulgare* were measured. The results suggested that the extract behaved as a strong free radical scavenger, providing IC_{50} at even 9.9 μg ml^{-1}, whereas the oil showed weaker activity, with IC_{50} at 8.9 mg ml^{-1}.[77]

O. vulgare L. subsp. *hirtum* (Link) Ietswaart has less impressive properties; however, it is the most widely used spice throughout Greece. It is known as rigani, and records of its utilization date back to Theophrastus and Dioscorides.[117] The plant parts used are the leaves

and flowers, collected during the flowering period in summer. Literature sources have revealed the following uses of the essential oil: inhalation in chronic pneumonia, the relief of toothache, externally against rheumatism, and the preparation of soaps with antiseptic properties. Its infusion is used against colds, coughs and diarrhea.[117]

4. MATERIALS AND METHODS

4.1. PLANT MATERIAL

The plant material was gathered from the perennial populations of *H. officinalis* L., *O. vulgare* subsp. *vulgare* L. and *O. vulgare* subsp. *hirtum* (Link) Ietswaart cultivated at the Experimental Station of the Faculty of Horticultural Science of Corvinus University of Budapest at Soroksár and at the experimental field of the Institute of Ecology and Botany of the Hungarian Academy of Sciences at Vácrátót, Hungary. The original seeds had been obtained from various botanical gardens abroad and the origin of the populations was the gene-bank stock of the mentioned departments.

Sampling was performed according to a well-defined program, using both raw and dried plant material. Stock evaluation was carried out with mass selection, without isolation. In our studies for the selection of individual hyssop plants, the growth intensity, the blue colour of the corolla, the essential oil content and composition, and the RA content were the aspects of selection. This was the case for both the parent plants and their manifold selected and propagated offspring in each plant population. For origanum, the essential oil content and composition, and the RA content were the aspects of selection.

4.2. MORPHOLOGICAL INVESTIGATION

Morphological investigations were carried out: the height of the plants, length of the internodes, the length and width of the leaves, the compactness of the shrubs, the strength of the ramifications, and the colours of the leaves and petals were studied in the full flowering period. Twenty-five individuals of each strain were investigated.

4.3. CHEMICAL ANALYSIS

The RA and essential oil contents of the plants were investigated during the entire vegetation period (from April until September). The samples were taken to represent different phenological stages (Table 1).

Table 1. Samples and the corresponding phenological stages

No.	Stage
1	shoots
2	vegetative stage
3	floral buds
4	start of flowering
5	flowers
6	end of flowering
7	end of flowering - start of ripening
8	ripe fruit
9	second growth
10	reflorescence

4.3.1. Essential oils

The essential oils were gained by steam distillation for 2 h in a Hungarian Pharmacopoeia distillation apparatus,[118] dried over anhydrous sodium sulfate, filtered and stored at –18 ºC until tested and analyzed.

The oils were analyzed by GC and GC/MS techniques.

GC analysis was carried out with an HP 5890 Series II gas chromatograph (FID) (Hewlett Packard/now Agilent Technologies/, USA), using a 30 m x 0.32 mm x 0.25 μm HP-5 fused silica capillary column (J&W Scientific, USA). The temperature program was from 60 °C to 210 °C at 3 °C min^{-1}, and from 210 °C to 250 °C (2-min hold) at 5 °C min^{1}. The detector and injector temperature was 250 °C and the carrier gas was N_2, with split sample introduction.

GC/MS analysis was performed with a FINNIGAN MAT GCQ ion trap bench-top mass spectrometer (USA). All conditions were as above except that the carrier gas was He at a linear velocity of 31.9 cm s^{-1} and two capillary columns were used with different stationary phases: 1., DB-5MS (30 m x 0.25 mm x 0.25 μm; J&W Scientific, USA),

2. SolGel-WAX (30 m x 0.25 mm x 0.25 μm; SGE, Australia).

The positive ion electron ionization mode was used, with a mass range of 40–400 amu. Identification of the compounds was based on comparisons with published MS data[119] and a computer library search (the database was delivered together with the instrument) and also on comparisons of their Kovats retention indices[120] with those of authentic references and with literature values. The identification was confirmed with the aid of authentic samples. Kovats retention indices were calculated mainly from the GC/MS analysis results.

For the stability studies air-dried plants and the distilled oil were stored for 1 year at room temperature and at -18 °C, respectively.

Solvents and other chemicals used were of high purity (analytical grade).

4.3.2. Rosmarinic acid

RA contents of whole plants and of separated plant organs in several phenological phases were measured by TLC-densitometry.

The powdered samples (0.5 g each) were extracted with 70% aqueous methanol (7 ml) in an ultrasonic extractor (SONOMATIC® 375, 40 kHz) for 10 min. This process was repeated 3 times and the extracts were combined and made up to 25 ml. The extracts (5 μl each) were chromatographed with authentic samples of RA (1, 2, 4, 8 μl 0.05% of RA in MeOH) on Kieselgel 60 layers (10×20 cm glass plate, Merck, Germany). The developing system was toluene - ethyl acetate - formic acid (5:4:1). After development, chromatograms were dried at room temperature for 20 min and exposed to light for 20 min. The densitograms of RA were obtained by using a Shimadzu CS-9301PC densitometer (Japan) or LABCHROM densitometer (Hungary). The parameters of the densitometric determination were as follows: photo mode: fluorescence; scan mode: linear; wavelength: 325 nm; emission filter: I.[121]

4.3.3. Oleanolic and ursolic acids

Powdered samples (0.8 g) were extracted with MeOH (90 ml) for 6 h in a Soxhlet apparatus. Finally, the solutions obtained were diluted to 100 ml (stock solutions). For the clean-up procedure, solid-phase extraction (SPE), 4 ml of water was added to 1 ml of each stock solution to make 5 ml samples of each of the 20% aqueous MeOH mixtures. These solutions were transferred to Chromabond SB cartridges. For silylation, the SPE-cleaned extracts dried at 80 °C were dissolved in 200 μl of dry pyridine (Fluka, Germany) in vessels with a volume of 1.5 ml and derivatized by adding 400 µl of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (Supelco, USA). To achieve silylation, the samples were heated at 80 °C for 2 h.

GC was performed with an HP 5890 Series II instrument equipped with an FID detector. Other measurement parameters were as follows: column: HP-5 (30 m x 0.32 mm x 0.25 μm); split ratio: 1:10; carrier gas: nitrogen; injector, detector and column temperature: 300 °C; head pressure: 8 psi; injected volume: 1.0 μl; analysis time per sample: 22 min.

4.4. MEASUREMENTS OF BIOLOGICAL ACTIVITY

4.4.1. Bacteriostatic and fungistatic activities

The antibacterial activities of various oils and authentic individual oil components (*α*-pinene, sabinene, *β*-pinene, *α*-terpinene, *p*-cymene, limonene, *γ*-terpinene, borneol, thymol, carvacrol and *β*-caryophyllene; Extrasynthese, France) were tested on Gram-positive and Gram-negative (the proton pump-deleted mutant of *E. coli* and its wild type) bacterial strains, and on 2 *Saccharomyces* and two *Candida albicans* strains.

Bacterial and fungal strains: *E. coli* K12 LE140, *Staphylococcus epidermidis* (clinical isolate), *E. coli* AG100, *E. coli* AG100A, *Saccharomyces cerevisiae* 0425 δ/1, *S. cerevisiae* 0425 52C, *C. albicans* ATCC 10231 and *C. albicans* ATCC 14053.

Bacteriostatic and fungistatic activities were determined with an agar-well diffusion method. The antimicrobial activity was evaluated by measuring the diameter of the inhibitionzone observed.

MICs (minimal inhibitory concentrations) were also determined with the broth dilution assay. The MIC was defined as the lowest concentration of the test sample that resulted in complete inhibition of visible bacterial growth in the broth. DMSO was used as negative control, while penicillin, gentamycin and fluconazole were used as positive controls.

4.4.2. Antioxidant activity

The air-dried, crushed and powdered plant materials (5 g) were extracted with 70% MeOH in an ultrasonic extractor (SONOMATIC® 375, 40 kHz) for 10 min. The antioxidant effects of aqueous MeOH extracts were investigated in enzyme-dependent and enzyme- independent lipid peroxidation (LPO) systems. The enzyme-independent LPO was assayed on a standard ox-brain homogenate, and the enzyme-dependent LPO was assayed on rat liver microsomes.[122,123] *In vitro* experiments were conducted in duplicate and means were calculated. No error was computed; the differences between the two samples were within approximately 1%. Saturation curves were fitted to the measurement data and IC_{50} values (the concentration at which 50% of the maximal LPO inhibition is exerted) were calculated by means of the computer program GraphPad Prism 2.01.

5. RESULTS AND DISCUSSION

5.1. PRELIMINARY STUDIES

The first steps in our studies were the evaluation of available plant stocks and screening of inhomogenous and selected plant populations grown in Hungary as a result of international seed exchange from various botanical gardens (Table 2).*

Differences in genotypes, corolla colour, and essential oil content and composition were considered crucial.

Table 2. Populations screened

No	Name (origin)	Corolla colour
	Hyssopus officinalis subsp. *officinalis*	
1	Magyar Kékvirágú (Medicinal Plant Research Institute, Budakalász)	blue, pink, white
2	Német hyssop, agricultural plant (Müggenburg GmbH, gene bank stock)	blue
3	Bolgár hyssop, selected type (Pharmaplant)	blue, white, pink
4	f. cyaneus (Salaspils)	blue
5	f. albus (Salaspils)	blue, white
6	f. rubra (Wroclaw)	pink, white
7	f. cyaneus (Frankfurt)	pink
8	Antwerpen (Antwerp)	blue, white, pink
9	St Gallen (St Gallen)	blue, white, pink
10.	Zürich (Zürich)	blue, white, pink
11.	Latvia (Latvia)	blue, white, pink
12.	Halle (Halle)	blue, white, pink
13.	Montrea (Montreal)	blue, white, pink
14	*Origanum vulgare L.*	blue
15	*Origanum vulgare* subsp. *vulgare*	blue
16	*Origanum vulgare* subsp. *hirtum*	white

In contrast with the conclusions of Kerrola *et al*. and Chalchat *et al.*, our screening studies of hyssop did not reveal unequivocal correlations between the genotype, corolla colour and essential oil content and composition.[15,53]

* Varga E, Hajdú Zs, **Veres K,** Máthé I, Németh É, Pluhár Zs, Bernáth J: *Acta Pharm. Hung.* 1998; **68:** 183-188

For our further experiments, defined in the study plan, we used the following selected taxons:

- *H. officinalis* subsp. *officinalis*
  - Magyar Kékvirágú (No. 1), and its offspring selected on the basis of the blue colour of the corolla
  - Német hyssop, agricultural plant (No. 2)
  - Bolgár hyssop (No. 3)
- *O. vulgare* L. (No. 14)
  - subsp. *vulgare* (No. 15)
  - subsp. *hirtum* (No. 16), selected progeny line
  - subsp. *hirtum* (domestic plant population grown in Hungary as a result of international seed exchange)

Stocks of the perennial species of various ages studied were available from the experimental fields in Vácrátót and Soroksár. Re-planting and cultivation of generations of offspring were accomplished by our fellow-workers at the Department of Medicinal and Aromatic Plants of Corvinus University.

5.2. HYSSOP

5.2.1. Morphological studies

Variety breeding started in Soroksár in 1993 and included multiple selections of hyssop populations of various origins and their offspring, resulting in the appearance of promising basic material. The scheme of morphological studies of selected progeny lines of variety breeding is summarized in Table 3.

Table 3. Scheme of the hyssop studies

1998				**1999**	**2000**
Parent species		**F1 generation 1-year-old**		**F2 generation 1-year-old**	**F2 generation 2-year-old**
Német, 2-year-old	→	143	→	143	143
		144	→	144	144
Magyar, 2-year-old	→	K 17	→	K17	K17
				K 17 st.	K 17 st.
	→	K 19	→	K 19	K 19
				K 19 st.	K 19 st.

Our extensive morphological studies of hyssop starting in 1998 were followed by the screening of plant height, inflorescence length, corolla colour and earliness of flowering of the offspring during the next 2 years. Comparison of the data on selected stocks of the parent plants (Table 4) and those of the offspring (Tables 5/A and 5/B) revealed that the selected characteristics of the 2- and 3-year-old offspring were as good as or even better than those of the parent plant. The appearance of the purple colour of the corolla was consistent, whereas the earliness of flowering was only modest among the offspring.

Table 4. Characteristics of the 4-year-old parent plants of hyssop (2000)

Type	Parent plant no.	Plant height (cm)	Inflorescence length (cm)	Essential oil content (ml/100 g)	Earliness*	Corolla colour
Német hyssop	143	70.00	18.20	0.65	early	purple
	144	66.00	15.40	0.68	early	purple
Magyar hyssop	K17	59.00	17.30	1.14	late	purple
	K19	61.00	15.60	1.26	modest	purple

*start of flowering

Table 5/A. Characteristics of F1 progeny generations (1999→2-year-old, 2000→3-year-old)

Type	Progeny plant no.	Plant height (cm)		Inflorescence length (cm)		Essential oil content (ml/100 g)	
		1999	2000	1999	2000	1999	2000
Német hyssop offspring	143	40.40	62.20	11.56	14.20	0.74	1.06
	144	37.80	62.50	11.32	11.75	0.43	1.08
Magyar hyssop offspring	K17	46.40	67.80	17.80	18.24	0.63	1.32
	K17st	37.60	58.60	19.82	17.12	0.76	1.75
	K19	37.80	62.75	12.00	14.28	0.84	1.30
	K19st	38.40	49.75	14.28	13.58	1.24	1.66
Non-selected basic stocks	Német hyssop	42.80	64.60	11.20	13.92	0.57	0.80
	Magyar hyssop	38.00	59.40	10.16	9.96	0.81	1.39

Table 5/B. Characteristics of F1 progeny generations (1999→2-year-old, 2000→3-year-old)

Type	Progeny plant no.	Earliness*		Corolla colour	
		1999	2000	1999	2000
Német hyssop offspring	143	modest	early	purple	dark-purple
	144	early	early	light-purple	purple
Magyar hyssop offspring	K17	modest	modest	purple	purple
	K17st	early	late	purple	purple
	K19	modest	early	purple	purple
	K19st	early	early	purple	purple
Non-selected basic stocks	Német hyssop	modest	early	purple	purple
	Magyar hyssop	late	late	purple	purple

*start of flowering

Comparison of the basic stocks of domestic Magyar and Német hyssop types suggests that the different patterns of the beginning of flowering is a phenological mark (Tables 4 and 5/B). During the 3 years of our experiments (1998–2000), the Német hyssop type was observed to flower 1 or 2 weeks earlier than the Magyar type. This characteristic changed in the progeny generations. It seems that, unlike the blue colour of the corolla, the earliness of flowering is more difficult to inherit.

5.2.2. Studies of the essential oils

5.2.2.1. Quantitative studies

For the accumulation of essential oils, the proportional weights of the leaves, the stem and the inflorescence are crucial factors. Therefore, besides measuring the total weight, we also measured the proportional weight of each plant organ in the various phenological states studied. As the stem, which constitutes a substantial percentage of the total weight, contains no essential oil, its precise proportion within the drug is of particular importance. In 1999 and in 2000, almost identical results were gained. The correlations revealed are summarized in Table 6.

Table 6. Correlations between the proportional weights of the plant organs and the essential oil content of hyssop (full flowering)

Plant organ	Proportional weight (%)		Essential oil content (ml/100 g dry plant organ)	
	1999	2000	1999	2000
Leaves	27	25	0.41	0.63
Stem	44	40	0.00	0.00
Inflorescence	30	35	1.32	1.78
Total sprout	100	100	0.57	0.74

Hyssop stocks used for the measurements of the essential oil content are shown in Table 7.

Table 7. Hyssop stocks used for the measurements of the essential oil content

1998	1999	2000	2001	2002
Magyar hyssop, 2-year-old	Magyar hyssop, 3-year-old	Magyar hyssop, 4-year-old	Magyar hyssop, F2 (K 17) 3-year-old	Magyar hyssop, F3 (K 17) 2-year-old
Német hyssop, 2-year-old	Német hyssop, 3-year-old	Német hyssop, 4-year-old	Német hyssop, F2 (144) 3-year-old	
	Bolgár hyssop, 3-year-old	Bolgár hyssop, 4-year-old		

The series of studies (1998–2000) using average samples of hyssop revealed the variations in essential oil content as a function of the phenological stage (Figure 1). In contrast with the common view that the essential oil content reaches its maximum at full flowering, our results showed that this maximum appears as early as at the beginning of the differentiation of the inflorescences. This holds true for all the 3 hyssop types examined. The essential oil content decreases dramatically in the ripe fruit, whereas it increases again in the plants of the second growth. In the event of reflorescence, the essential oil content of the plant reached the level measured at the time of the first florescence. From a comparison of the 3 types of hyssop studied (Magyar, Német, Bolgár), it can be concluded that the Magyar type contains the highest amount of essential oils. As concerns the generations, the plants in 1998–1999 had a low essential oil content, while a significant increase was observed in 2000–2001 (Figure 1). As hyssop is a xerophyte species that prefers a dry and hot habitat, the low essential oil content can be related to the higher rainfall in 1998–1999.[13,14] According to the literature, the effect of the water supply on the essential oil accumulation results from the combination of numerous processes. In general, rainy periods have an indirect effect via less sunlight and a lower temperature, which that result in a lower level of essential oil accumulation. However,

the optimal level of water supply for a particular plant is basically dependent on the ecological demands of the particular species. The amount of essential oil is also modified by the loss induced by the wash-out effect of the rainfall, as the species of the family Lamiaceae mainly contain essential oils in their surface oil glands, making the plant susceptible to oil loss.[124]

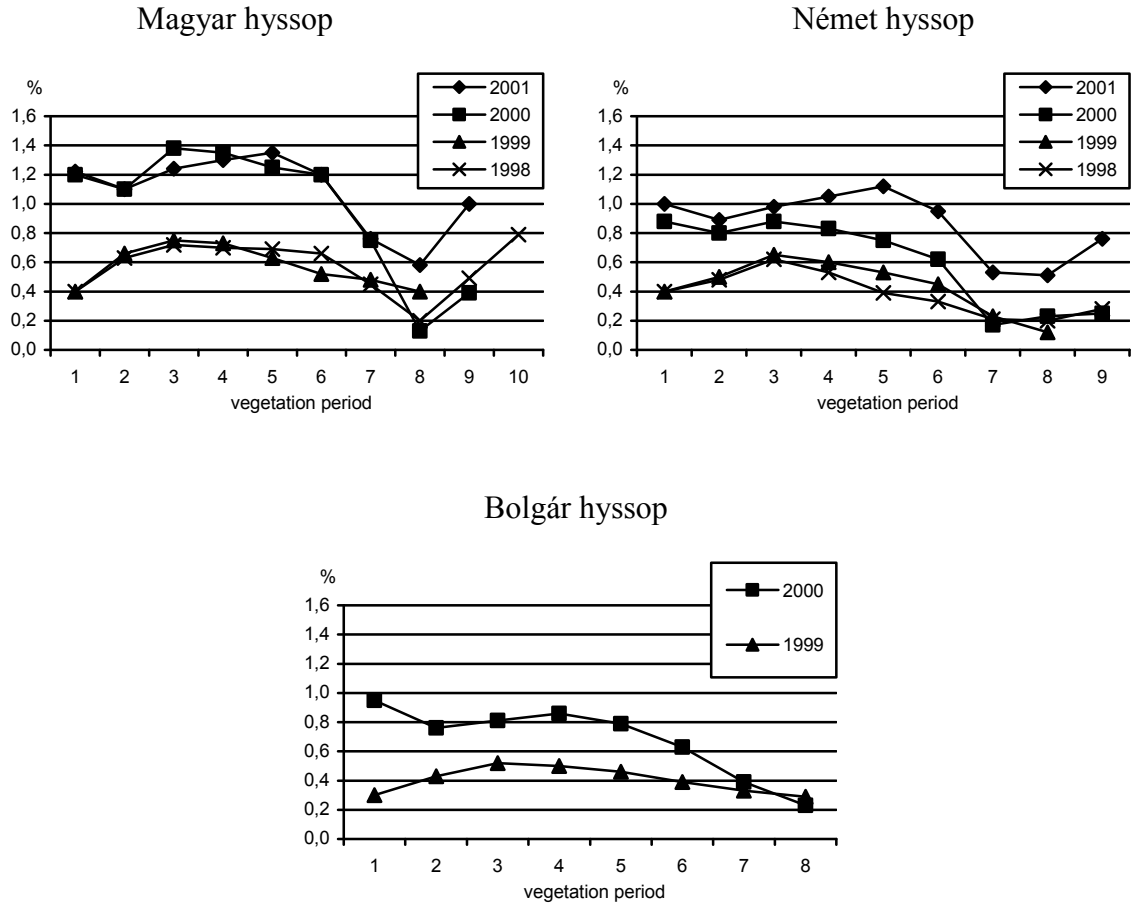


Figure 1. Alterations in the essential oil content of hyssop in the vegetation period

We extended our measurements to the progeny generations of variety breedings. In 2002, we examined the 2-year-old F3 progeny generation of the selected progeny line of Magyar hyssop and compared it with the 3-year-old F2 progeny generation of the same hyssop type (Figure 2). The tendency of the essential oil to accumulate did not change. Additionally, we measured the essential oil content of the average sample collected at the time of full flowering. From the appearance of the floral buds until the end of flowering, the essential oil content ranged from 1.55 to 2%. The essential oil content of the average sample was 1.96% in 2002 (versus 1.35% in 2001). The slight increase in the essential oil content between 2001 and 2002 presumably results from year-specific effects, but may also be related to the age of

the plant-stocks examined. According to the literature the essential oil content of hyssop may vary by as much as 50% from year to year.[124]

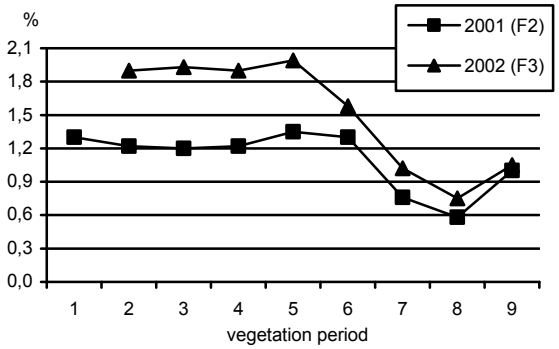


Figure 2. Alterations in the essential oil content of hyssop in the progeny generations.

5.2.2.2. Essential oil composition

The qualitative composition of the essential oil content is a significant chemical hallmark. Besides the uniform blue colour of the corolla and the high essential oil content, the qualitative composition of the essential oil was another important criterion of selection. Our previous studies identifies 4 characteristic chemotypes on the basis of the qualitative variations of the main constituents of the essential oils of hyssop: chemotype A, isopinocamphone >50%; B, pinocamphone >50%; A+B, isopinocamphone and pinocamphone 20–50% each; E, any other component (e.g. limonene) present in a substantial quantity (>30%) (Figure 3).† The presence of limonene as one of the major constituents is a novum literature finding.

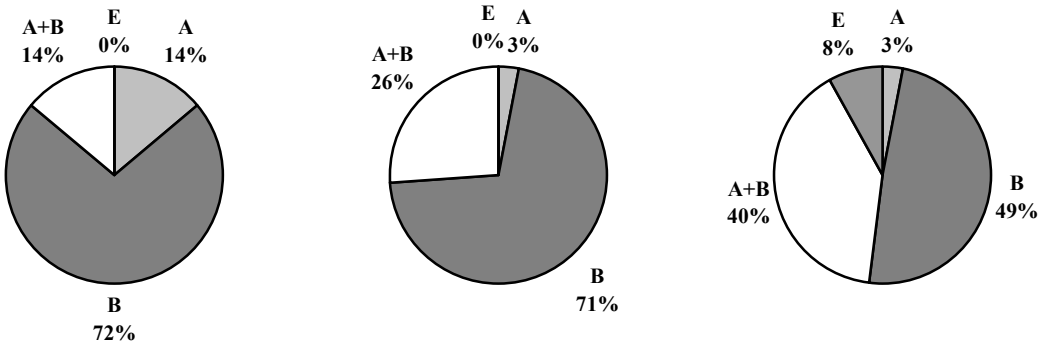
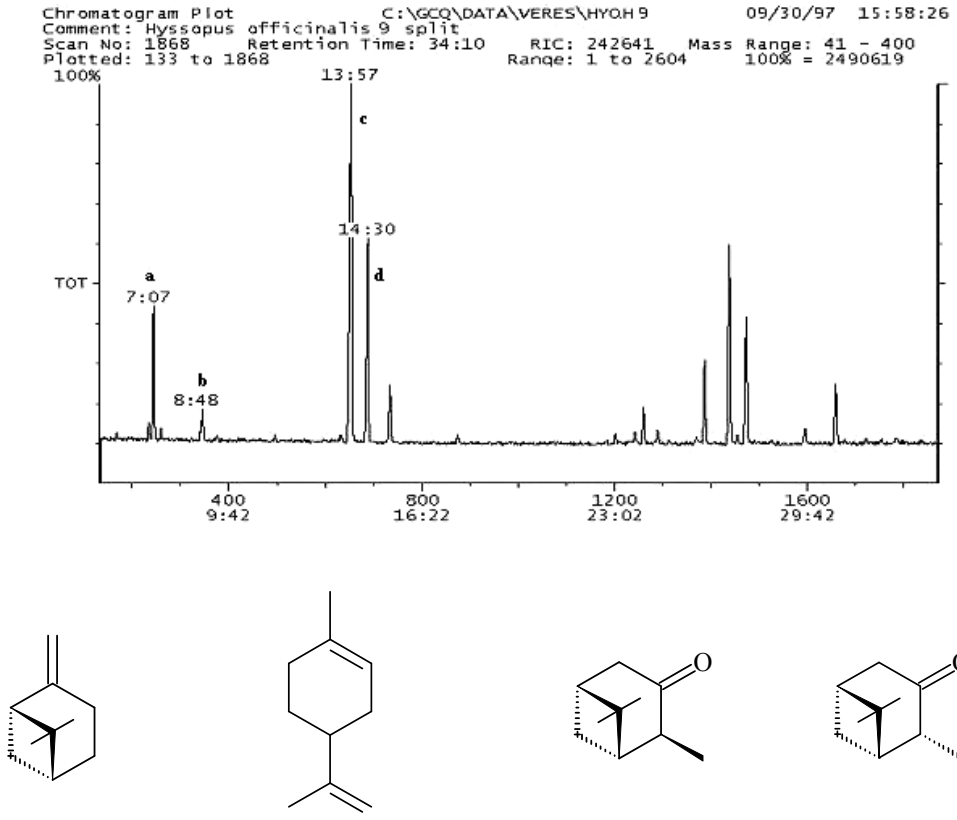


Figure 3. Proportions of the major constituents in several hyssop strains

† **Veres K,** Varga E, Dobos Á, Hajdú Zs, Máthé I, Pluhár Zs, Bernáth J: Investigation on Essential Oils of *Hyssopus officinalis* L. populations In: Franz Ch, Máthé Á, Buchbaner G, eds. *Essential Oils: Basic and Applied Research* Allured Publishing Comparation, 1997, pp 217-220.

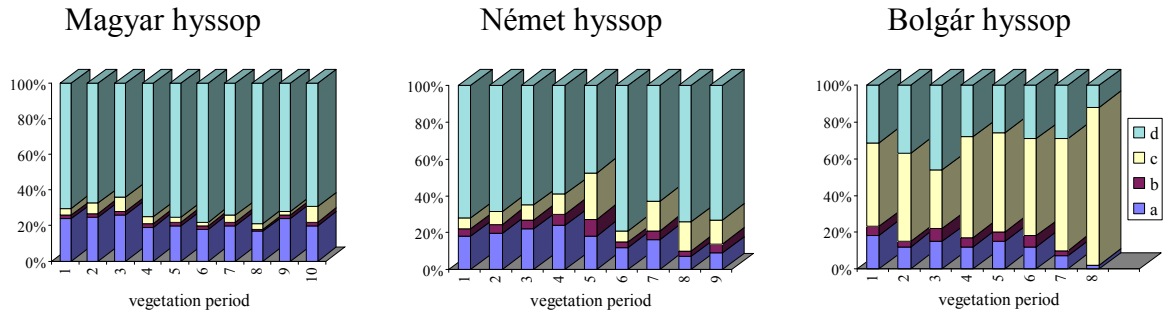
With regard to the information mentioned above, the ISO standard and the former Hungarian standards, we consider isopinocamphone and pinocamphone (50% together) and *β*-pinene to be significant constituents of the essential oil. Limonene is considered another characteristic hallmark (Figure 4, Annex I and Annex II). Thus, for control of the qualitative composition of the essential oil, we examined the presence of these 4 constituents and measured their absolute and relative quantities.



a: β-pinen **b**: limonen **c**: pinocamphone **d**: isopinocamphone

Figure 4. Total ion chromatogram of the essential oil isolated from hyssop No. **9** (Table 2)

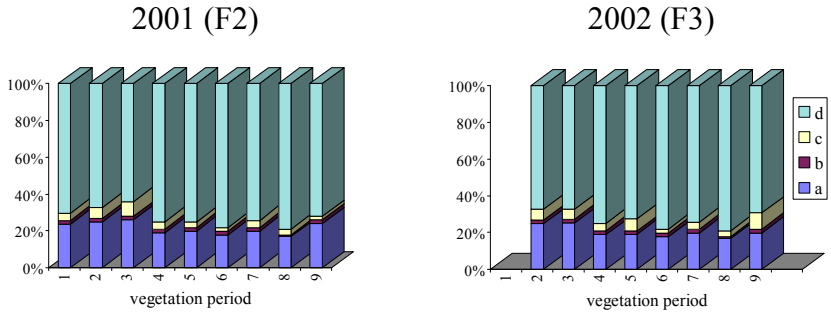
We found isopinocamphone to be the major constituent of the Magyar hyssop sample throughout the 3 years of our experiments (1998–2000), and its amount showed no substantial alterations in the vegetation period. In the Német hyssop isopinocamphone, whereas in the Bolgár hyssop pinocamphone was the main component. The sum of the quantities of isopinocamphone and pinocamphone met the standard requirements in all cases (Figure 5).



a: β-pinene; **b**: limonene; **c**: pinocamphone; **d**: isopinocamphone

Figure 5. The relative proportions of the main components in the essential oil isolated from hyssop in the vegetation period (1999)

The composition of the essential oil content as regards the 4 main components (isopinocamphone, pinocamphone, β-pinene and limonene) considered as characteristic chemical hallmarks was the same in the progeny generation as in the parental generation described above. From the comparision of the 2-year-old F3 progeny generation and the 3-year-old F2 progeny generation of the Magyar hyssop, it is unequivocal that the characteristics chosen for selection (stable essential oil composition and dynamics of essential oil accumulation) remain stable in the F3 progeny generation (Figure 6).



a: β-pinene; **b**: limonene; **c**: pinocamphone; **d**: isopinocamphone

Figure 6. The relative proportions of the main components in the essential oil isolated from the progeny generations of the selected Magyar hyssop in the vegetation period

Table 8. Composition of the essential oil isolated from *Hyssopus officinalis* (average samples from 2001 and 2003; the essential oils were distilled from fresh plant material)

RI*	Component	%	
		2001	2003
931	*α*-thujene	0.31	0.25
939	*α*-pinene	1.05	0.62
953	camphene	0.14	0.13
978	sabinene	2.08	2.01
980	***β*-pinene**	**21.81**	**11.91**
1005	*α*-phellandrene	1.67	1.74
1018	*α*-terpinene	0.05	0.11
1031	**limonene**	**1.65**	**4.28**
1031	*β*-felladrene	0.21	
1050	*E*-*β*-ocimene	1.17	
1062	*γ*-tepinene	0.20	1.11
1068	*E*-sabinene hydrate	0.08	0.25
1088	terpinolene	0.08	0.62
1098	linalool	0.76	0.68
1102	*α*-thujone	0.04	0.26
1160	**pinocamphone**	**26.06**	**39.20**
1173	**isopinocamphone**	**26.93**	**27.67**
1189	*α*-terpineol	0.25	0.26
1194	myrtenol	3.11	2.84
1384	*β*-bourbonene	0.18	0.52
1401	methyl eugenol	0.25	0.25
1418	*β*-caryophyllene	2.32	0.39
1460	seychellene	0.84	0.38
1480	germacrene D	2.27	1.13
1500	*α*-chamigrene	1.93	
1513	*γ*-cadinene	0.12	
1556	germacrene B	0.65	
1576	spathulenol	0.11	0.25
1581	caryophyllene oxide	0.16	0.25
1630	*γ*-eudesmol	0.18	
1640	epi-*α*-cadinol	0.29	0.27
1652	*α*-eudesmol	0.15	
	Total:	**97.10**	**97.38**

*Kovats retention indices measured by our team

The comparison of these 3 types of hyssop furnishes evidence of a differing chemosyndrome line. We can conclude that the chemical fingerprints determined at the same periods of time reflect different genetics manifesting as different phenotypes. This is supported by the fact that the experimental samples of all three types of hyssop were collected from plants harvested in parallel to one another. Thus, the factors inducing or influencing

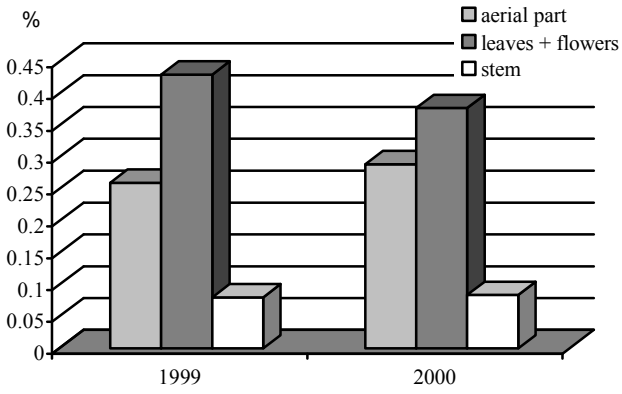
chemosyndrome lines were the same for all the plants studied. The fingerprint of the Magyar hyssop is uniform in the selected plants and in their offspring, and no phenological correlations were revealed. All samples had isopinocamphone as the main constituent. In contrast, the ratio of pinocamphone/isopinocamphone changed over time in the vegetation period in the Német hyssop. The Bolgár hyssop is characterized by a predominance of pinocamphone. A novel finding of our team is that the increase in pinocamphone content is accompanied by a decrease in a relative amount of isopinocamphone during vegetation. This change is most pronounced in those plants that have pinocamphone as the main constituent. In our case this was the most marked in the Bolgár hyssop sample (Figures 5 and 6).

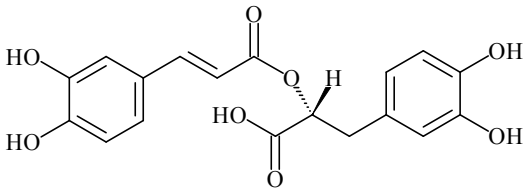
As concerns the harvests in the 2 years, the proportions of the main constituents of the average sample changed somewhat (Table 8). However, the presence of one of the main constituents, isopinocamphone, was relatively stable, and the sum of the proportions of isopinocamphone and pinocamphone was 50%, meeting the quality requirements of the ISO standard.

5.2.3. Quantitative determination of rosmarinic acid

As for the essential oil content, differentiation according to plant organs is a substantial factor for the determination of RA content.

Our measurements of the RA content in different plant organs revealed a significant accumulation of this component in the leaves and the flowers together (0.3–0.4%). In contrast, the stem had a very low RA content (<0.1%) (Figure 7).





Rosmarinic acid

Figure 7. RA content in various parts of the hyssop plant during full flowering

We extended our studies to the whole vegetation period of the plants to determine when the RA content reaches its maximum. Our measurements between 1998 and 2000 did not answer this question unequivocally. The dynamics of accumulation described in the first 3 years of our experiments indicates a trend toward the RA content reaching its non-outstanding maximum by the time of flowering. An occasional increase was also detected in the spring and autumn sprouts.

Our 3-year experience required us to further specify our experiments and findings in 2001. Besides examining average samples, we gathered individual plants in the main phenological stages to investigate a potential individual variance of the RA content. These results supported our previous findings.

Table 9 lists the average RA content per type and phenological stage. Comparison of the RA content of Magyar hyssop at the beginning and at the end of flowering yielded a statistically significant difference (SSD), which disappeared when we compared the stage at the end of flowering and that at ripening.

Table 9. RA contents of average hyssop samples (2001)

Phenological stage	**Magyar hyssop**	**Német hyssop**
Undeveloped, vegetative stage	0.20	0.20
Developed sprout, pre- budding	0.18	0.18
Start of flowering - budding	0.18	0.18
Start of flowering	0.19	0.22
Full flowering	0.17	0.21
End of flowering - July	0.18	0.15
End of flowering - August	0.12	0.10
Ripening	0.10	0.06
Second growth	0.23	0.26
Total average	**0.17**	**0.17**
SD	**0.04**	**0.06**
SSD 5%	**0.05**	**0.06**

Table 10. Studies for the selection of individual hyssop plants according to RA content (2001)

Magyar hyssop Phenological stage	**Individual plants**					**Average of individual values**	**SD**	**SSD 5%**
	1	**2**	**3**	**4**	**5**			
Start of flowering - budding	0.23	0.15	0.17	0.19	0.19	**0.18**	**0.03**	**0.01**
Start of flowering	0.17	0.30	0.11			**0.19**	**0.10**	**0.02**
Full flowering	0.17	0.23	0.16	0.18	0.08	**0.17**	**0.05**	**0.02**
End of flowering - July	0.13	0.10	0.23	0.13	0.30	**0.18**	**0.08**	**0.01**
Német hyssop Phenological stage	**Individual plants**					**Average of individual values**	**SD**	**SSD 5%**
	1	**2**	**3**	**4**	**5**			
Start of flowering - budding	0.26	0.15	0.14			**0.18**	**0.07**	**0.01**
Start of flowering	0.18	0.24	0.24	0.21		**0.22**	**0.03**	**0.01**
Full flowering	0.36	0.15	0.23	0.18	0.11	**0.21**	**0.10**	**0.01**
End of flowering - July	0.12	0.15	0.15	0.22	0.10	**0.15**	**0.04**	**0.01**

Table 10 outlines the differences between the individual plants in the phenological stages examined. When the RA contents of 3–5 plants were compared the differences reached statistical significance in almost every case, and thus there is a SSD between individual plants in every phenological stage. It seems reasonable to replicate these studies with a larger numbers of plants.

Similarly to our previous findings (1998–2000), we detected a slight increase in RA content in the spring and autumn sprouts of the progeny generations (Figure 8).

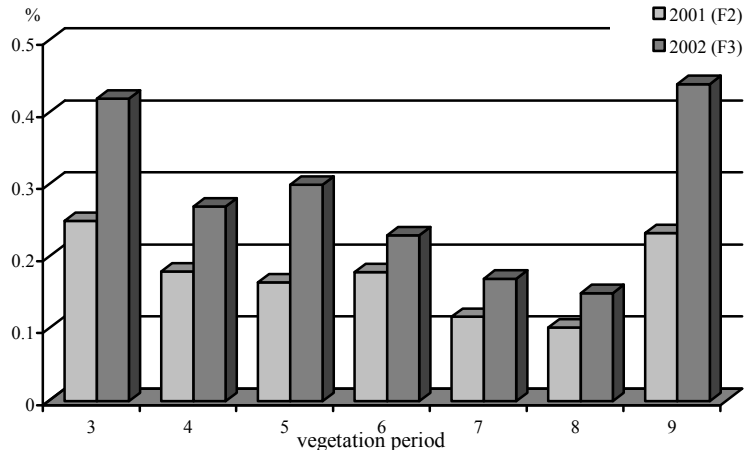


Figure 8. Changes in RA content of hyssop throughout ontogenesis in the progeny generations

5.3. ORIGANUM

For Origanum, the morphological evaluations and botanical classifications of various heterogenous stocks were carried out at the Department of Medicinal and Aromatic Plants at Corvinus University.[125] This is not discussed in details here. Evaluation of the selected populations and the inhomogenous stocks was at the focus of my chemical and analytical studies.

5.3.1. Studies of the essential oils

5.3.1.1. Quantitative studies

The two subspecies of *Origanum* studied, namely *O. vulgare* subsp. *vulgare* and *O. vulgare* subsp. *hirtum,* exhibited a striking difference in essential oil content. While *O. vulgare* subsp. *vulgare* had an essential oil content of only approximately 0.2%, the selected *O. vulgare* subsp. *hirtum* displayed a high essential oil content throughout the period of our measurements (4.3% in 2000, and 5.3% in 2001) (Figure 9). These findings are consistent with the literature data.[63,66] This indicates that selection of the genotype of *O. vulgare* subsp. *hirtum* and introduction of its cultivation in Hungary would be successful, as the high essential oil content of the Mediterranean plant remains characteristic even under the circumstances present in Hungary.

Unlike the hyssop, the origanum populations demonstrated their maximum essential oil content at the time of full flowering, though its minimum was also observed in ripe fruits.

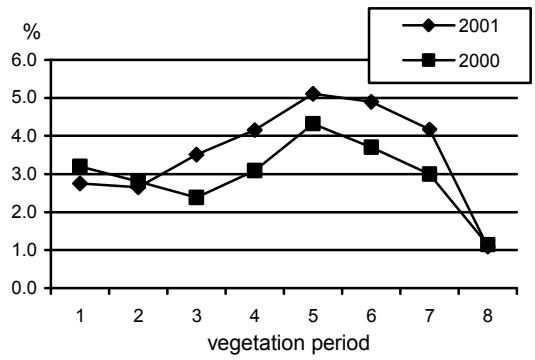


Figure 9. Changes in the essential oil content of *O. vulgare* subsp. *hirtum* in the vegetation period

5.3.1.2. Essential oil composition

The composition of the essential oil isolated from *O. vulgare* subsp. *vulgare* differed from that isolated from the subspecies *hirtum*. While *p*-cymene (approximately 13–21% of the total essential oil content) and the sesquiterpenes (approximately 22–24% of the total essential oil content) predominated in the former case, the subspecies *hirtum* proved to be a carvacrol-type subspecies[16] with 64–76% carvacrol content (Figures 10 and 11, Annex III). For the subspecies *hirtum,* we found a change in the essential oil composition as a function of the phenological stage only at the time of ripening. Consistent with the literature, not only quantitative, but also qualitative changes were detected, with the carvacrol content decreasing dramatically.[83]

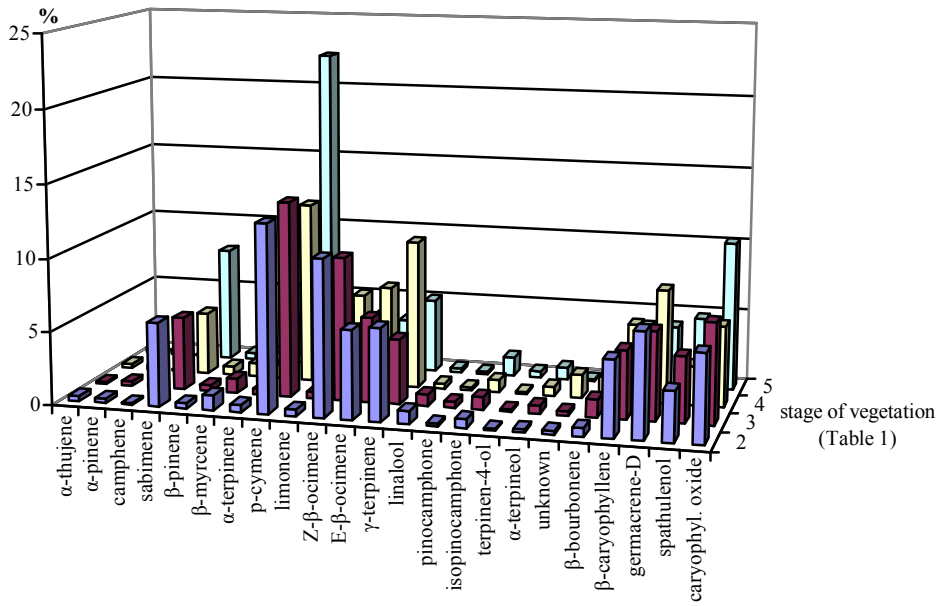


Figure 10. Essential oil composition of *Origanum vulgare* subsp. *vulgare*

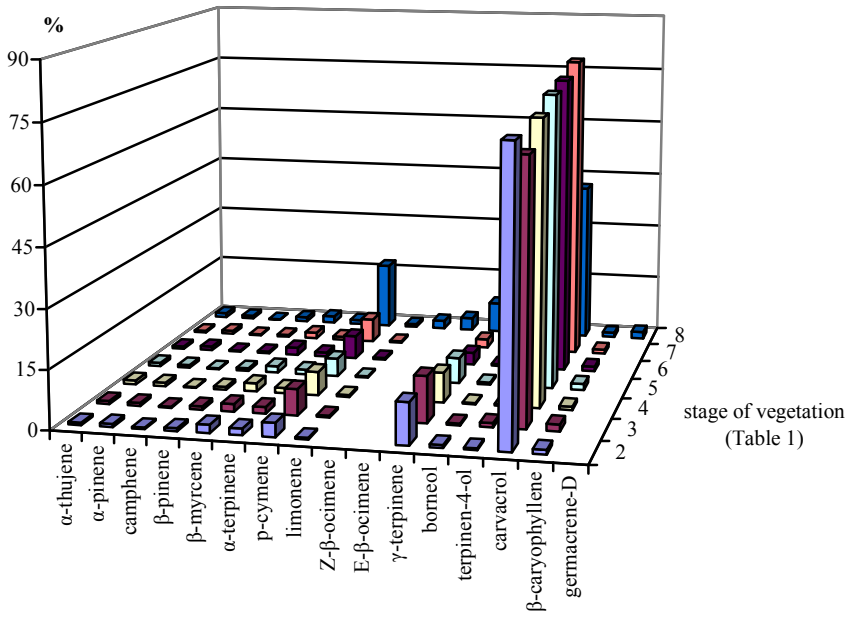


Figure 11. Essential oil composition of *Origanum vulgare* subsp. *hirtum*

5.3.1.3. Stability studies of the essential oils

As in many other areas of everyday life, quality aspects (and thus issues of stability) are of increasing concern in the case of herbal remedies. In examinations of the essential oil content, it is crucial to consider the artificial effects that may modify the quantitative and/or qualitative composition of the essential oils, such as those resulting from improper temperature and improper storage. Thus, we studied the compositions of various forms of the essential oils isolated from *O. vulgare* subsp. *vulgare*: oils distilled from the fresh drug, oils distilled from the plant after 1 year of storage at room temperature, and oils distilled from the fresh plant and stored frozen for 1 year.

In comparison with the essential oil distilled from the fresh drug, the essential oil distilled from the plant after 1 year of storage at room temperature was more stable than the oil distilled from the fresh plant and stored frozen for 1 year. The phase of monoterpenes showed a significant change in the latter case, while the composition of the sesquiterpene phase remained relatively stable (Table 11). This information is of practical importance for the determination of essential oil quality.[‡]

[‡] **Veres K,** Varga E, Dobos Á, Hajdú Zs, Máthé I, Németh É, Szabó K: *Chromatographia* 2003; **57**: 95-98

Table 11. Comparison of various forms of the essential oils of *Origanum vulgare* subsp. *vulgare*

			A	B	C
RI*	***RI*** **	**Compounds**	**%**	**%**	**%**
931	926-930	*α*-thujene	0.28	0.82	-
939	935-938	*α*-pinene	0.28	1.77	-
953	950-951	camphene	0.09	0.30	-
976	973-974	sabinene	4.33	5.14	0.69
980	978-980	*β*-pinene	0.60	1.03	1.90
991	987-989	*β*-myrcene	0.94	0.49	0.29
1018	1015-1018	*α*-terpinene	0.90	0.78	-
1026	1024-1025	*p*-cymene	12.41	25.33	6.62
1031	1030-1033	limonene	0.55	0.59	0.45
1040	1037-1038	*Z*-*β*-ocimene	6.27	0.71	0.24
1050	1047-1049	*E*-*β*-ocimene	6.88	1.51	0.10
1062	1058-1060	*γ*-terpinene	10.18	4.53	-
1098	1097-1102	linalool	0.36	0.60	0.80
1160	1161-1162	pinocamphone	0.22	-	2.19
1167	1168-1169	borneol	-	0.28	0.37
1173	1174-1175	isopinocamphone	0.86	-	4.23
1177	1178-1180	terpin-4-ol	0.15	0.81	1.24
1189	1187-1190	*α*-terpineol	0.63	0.68	1.18
1242	1238-1241	carvacrol methyl ether	-	0.32	0.41
	1285-1289	unknown	1.59	-	-
1290	1291-1293	thymol	0.34	0.90	2.14
1298	1297-1302	carvacrol	-	0.12	0.13
1384	1380-1384	*β*-bourbonene	1.60	1.75	3.07
1418	1417-1420	*β*-caryophyllene	5.28	2.53	5.62
1480	1477-1480	germacrene D	7.79	2.16	1.23
1576	1576	spathulenol	2.69	5.98	7.06
1581	1579-1582	caryophyllene oxide	5.53	12.83	14.30

A: Essential oil distilled from air-dried plants
B: Essential oil distilled from dried plants stored at room temperature for 1 year
C: Essential oil stored at –18 ºC for 1 year
RI* : Kovats retention indices according to literature data[119]
RI**: Kovats retention indices measured by our team

5.3.2. Quantitative studies of rosmarinic acid

Both origanum subspecies had their highest RA content at the time of flowering, with a significant decrease at the time of ripening (Table 12).

Table 12. Rosmarinic acid content in 2001

Origanum vulgare		
Phenological stage	**subsp. *hirtum***	**subsp. *vulgare***
Vegetative phase	0.36	
Green + white buds	0.66	
Budding	0.58	
Full flowering	0.62	0.77
End of flowering	0.57	
Defloration	0.59	0.56
Start of ripening	0.24	
Ripe fruit	0.29	0.28

Similarly as for hyssop, we examined the correlations between the RA content and the differentiation according to the plant organs of origanum. The proportional RA contents of the organs studied (aerial parts, leaves and stem) were found to be similar to those of hyssop. The most pronounced accumulation of this component was characteristic of the leaves, while the stem had a very low RA content.

5.3.3. Stock evaluation for selection of chemotypes

Within the scope of our basic studies serving variety breeding, we screened the essential oil and RA contents of individual plants of a population of *O. vulgare* grown in Hungary as a result of seed exchange from a gene bank and some previously unstudied inhomogenous stocks of *O. vulgare* subsp. *hirtum*. The large-scale selection of 50 and 10 individual plants, respectively, was performed according to the aspects described in the Materials and Methods section.

Individual plants of *O. vulgare* gathered in the same time frame had a low essential oil content, ranging from 0.07 to 0.3%. The RA content ranged from 0.3 to 0.95%. Individual plants of *O. vulgare* subsp. *hirtum* had a substantially higher essential oil content, ranging

from 1.42 to 6.35%, and their RA content ranged from 1.41 to 2.06% (Tables 13 and 14). The individual plants differed significantly in terms of essential oil and RA contents.

Table 13. Essential oil and RA contents (%) of samples of *Origanum vulgare* L.

Sample No.	Essential oil content	RA content	Sample No.	Essential oil content	RA content
1/1	0.27	0.93	**2/2**	0.12	0.39
1/2	0.13	0.62	**2/3**	0.19	0.55
1/3	0.16	0.74	**2/4**	0.04	0.64
1/4	0.17	0.62	**2/5**	0.10	0.85
1/5	0.20	0.78	**2/8**	0.09	0.58
1/6	0.23	0.44	**2/10**	0.07	0.80
1/7	0.16	0.66	**2/13**	0.13	0.84
1/8	0.07	0.31	**2/15**	0.27	0.92
1/10	0.14	0.94	**2/17**	0.18	0.52
1/11	0.13	0.74	**2/18**	0.22	0.78
1/12	0.27	0.89	**2/19**	0.10	0.63
1/16	0.11	0.6	**2/20**	0.14	0.47
1/19	0.28	0.42	**2/21**	0.13	0.72
1/20	0.11	0.88	**3/13**	0.17	0.49
1/21	0.21	0.43	**3/14**	0.33	0.65
1/22	0.19	0.61	**3/15**	0.06	0.81
1/23	0.13	0.95	**4/12**	0.14	0.65
1/24	0.23	0.47	**4/13**	0.30	0.69
1/25	0.28	0.72	**4/15**	0.30	0.48
1/26	0.10	0.86	**4/19**	0.25	0.81
1/27	0.13	0.61	**4/21**	0.21	0.47
1/29	0.07	0.72	**5/12**	0.14	0.95
1/30	0.16	0.84	**5/14**	0.16	0.54
1/31	0.08	0.77	**5/16**	0.15	0.34
1/32	0.07	0.91	**5/17**	0.24	0.64

	Essential oil content	RA content
Average	**0.17**	**0.67**
SD	**0.07**	**0.17**

Table 14. Essential oil and RA contents (%) of samples of *Origanum vulgare* subsp. *hirtum*

Sample No.	Essential oil content	Rosmarinic acid content
1/1	3.43	1.41
1/2	1.42	1.97
1/3	5.09	1.86
1/7	4.05	1.50
1/8	2.58	2.06
1/12	4.39	1.52
1/22	6.35	1.76
2/9	5.14	1.46
2/11	5.14	1.41
2/18	5.22	1.98
average	**4.28**	**0.69**
SD	**1.46**	**0.26**

The data on the RA contents of the individual plants of *O. vulgare* throughout the 3-year period of our experiments provided an interesting picture (Figure 13). In the second year (2003), the RA content was substantially higher in almost every plant examined, which is presumably a result of year-specific effects. Such a relationship was not observed for the essential oil content in the same period.

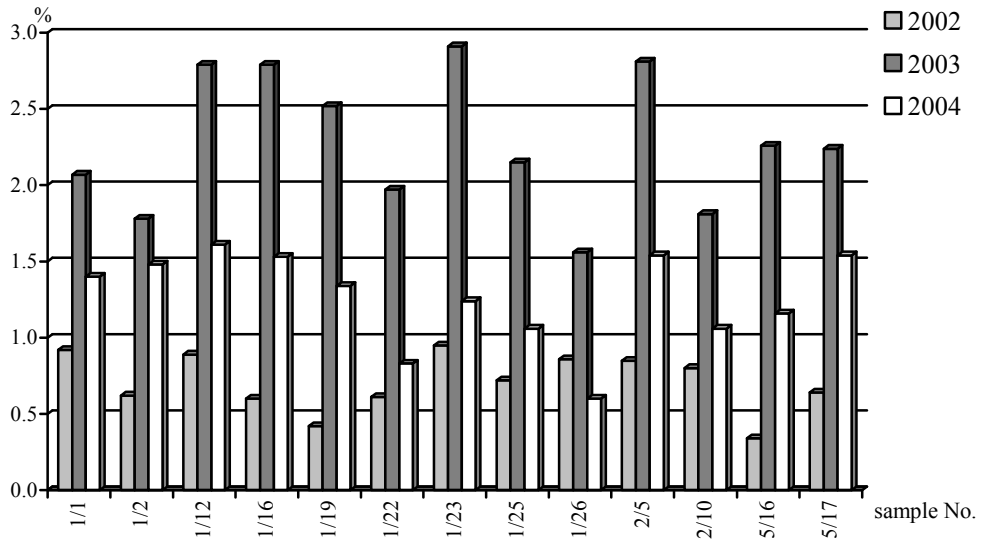


Figure 13. RA contents of several samples of *Origanum vulgare* L. (2002-2004)

As regards the composition of the essential oils isolated, all the individual plants of *O. vulgare* subsp. *hirtum* were characterized by a predominance of carvacrol, which belongs in the chemical group described by Pasquier[16] (Figure 14). The contents of carvacrol (low,

intermediate or high) and of other major constituents (*p*-cymene and *γ*-terpinene) were considered as criteria for selection (Figure 15, Annex IV and Annex V).

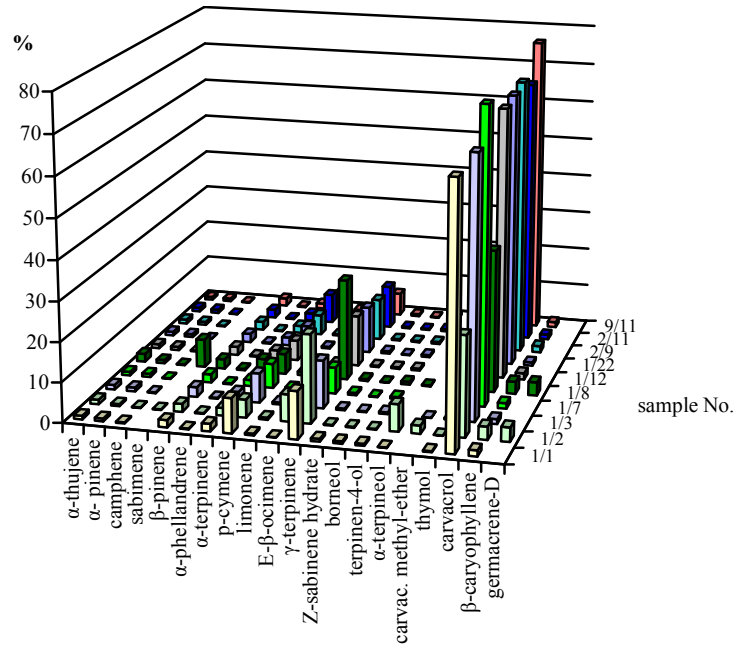


Figure 14. Compositions of the essential oils isolated from samples of *Origanum vulgare* subsp. *hirtum* (2002)

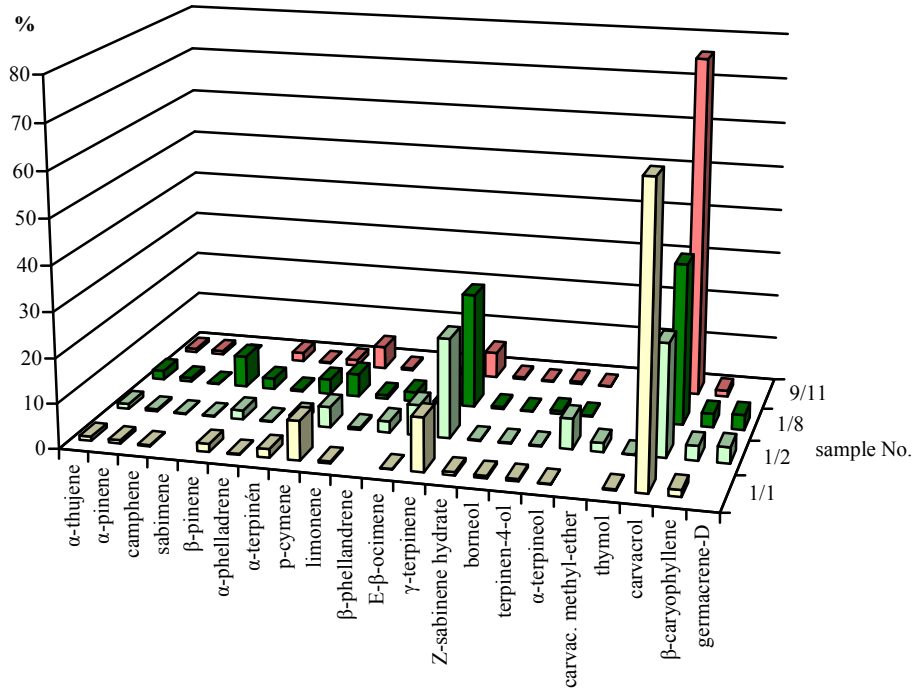


Figure 15. Compositions of the essential oils isolated from 4 selected samples of *Origanum vulgare* subsp. *hirtum* (2002)

The compositions of the essential oils isolated from the plants selected in 2002 only partially met our expectations in the following 2 years. Although the predominance of the main components remained consistent, the proportional quantities of the individual components did not meet the expectations on several occasions (Figure 16, Annex VI). This deviation was especially striking for the essential oils isolated from plants with a low carvacrol content. In contrast with what we had expected, the carvacrol content rose in these cases, while the amount of *γ*-terpinene (its precursor) decreased. This phenomenon may reflect the changes in the climatic conditions.

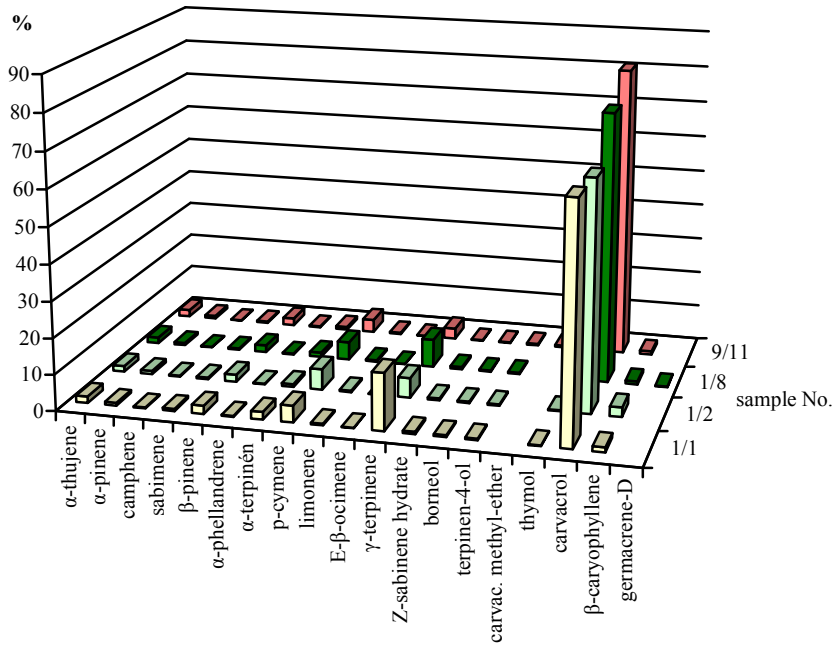


Figure 16. Compositions of the essential oils isolated from 4 selected samples of *Origanum vulgare* subsp. *hirtum* (2003)

Similarly as in the case of *O. vulgare* subsp. *hirtum*, we strived to select individual plants of *O. vulgare* with as diversified an essential oil composition as possible for microbiological studies. Criteria of selection were the contents of sabinene, *p*-cymene, *β*-phellandrene, *Z*-*β*-ocimene, *γ*-terpinene, caryophyllene oxide and germacrene D in the essential oil isolated from the individual plants (Figure 17, Annex VII).

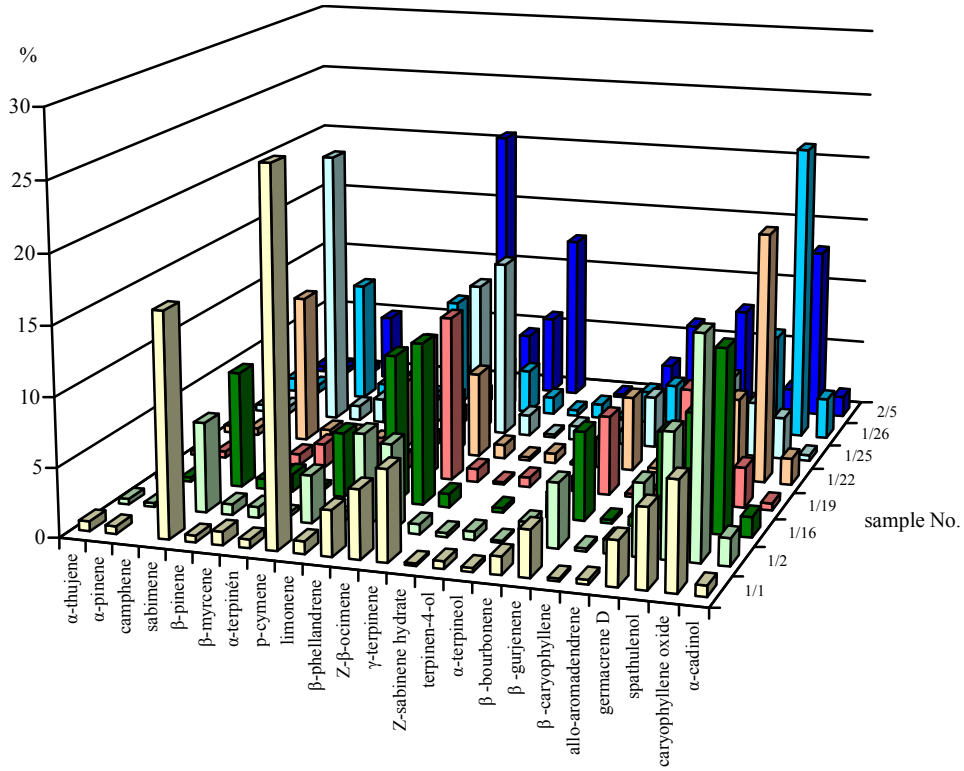


Figure 17. Compositions of the essential oils isolated from several samples of *Origanum vulgare* (2002)

5.4. ELABORATING A METHOD FOR THE PARALLEL MEASUREMENT OF OLEANOLIC ACID/URSOLIC ACID CONTENTS

UA and OA are present in most of the genera belonging in the subfamily Nepetoideae. However, there is a scarcity of literature data on their typical quantities or their quantities relative to each other. As these triterpene carboxylic acids exert valuable physiological effects, filling this gap is a priority. In view of their similar chemical structures, they can not be measured by TLC-densitometry. We therefore elaborated a novel method to measure the UA and OA contents in parallel. After the preparation of derivatives of the original compounds, GC analysis with an internal standard allows the selective measurement of each compound (Figure 18, Annex VIII).[§] We screened a large number of samples by using this method, including measurement of the OA/UA contents of samples of the *Hyssopus* and *Origanum* species discussed in the present thesis (Table 15).

[§] Janicsák G, **Veres K**, Kállai M, Máthé I: *Chromatographia* 2003; **58**: 295-299

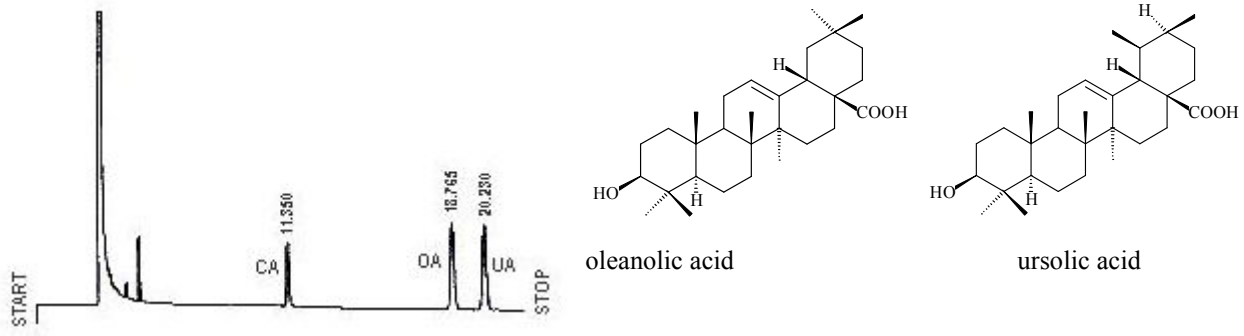


Figure 18. Example of a GC chromatogram for the measurement of OA and UA. Internal standard: CA

Table 15. Quantities of the triterpene carboxylic acids studied (%)

Sample	Oleanolic acid	Ursolic acid
H. officinalis L.	0.16	0.39
H. officinalis L. f. ruber	0.15	0.35
H. officinalis L. f. cyanus	0.15	0.38
H. officinalis L. f. albus	0.20	0.55
O. vulgare subsp. *hirtum*	0.04	0.11
O. heracleiticum L.	0.06	0.13

Both hyssop and origanum samples typically contain UA in an amount much higher than that of OA. A substantial difference between the two species is that hyssop contains higher amounts of both triterpene carboxylic acids. Studies of numerous *Lamiaceae* species revealed that these two triterpenoid acids are present in all of the taxa investigated. However, there are significant quantitative differences between the two subfamilies. The subfamily Lamioideae appears to be poorer in both OA and UA than the subfamily Nepetoideae. Mean values for the Lamioideae were OA: 0.012% and UA: 0.023%, while those for the Nepetoideae were OA: 0.263% and UA: 0.638%.[**]

[**] Janicsák G, **Veres K**, Kakasy AZ, Máthé I: *Biochem. Syst. Ecol.* 2006; **34**: 392-396

5.5. STUDIES OF PHYSIOLOGICAL EFFECTS

5.5.1. Correlations between essential oil composition and antimicrobial activity

Four samples of essential oils of different composition isolated from *O. vulgare* subsp. *hirtum* in 2004 were used for microbiological studies.[††] Approximately 97.26–99.35% of the constituents of these 4 samples were identified. The components present in the highest concentrations were carvacrol (61.72–84.21%) γ-terpinene (1.35–15.98%) and *p*-cymene (4.17–9.50%). The essential oil of sample **1/8** also contained an appreciable quantity of thymol (3.11%) (Table 16).

Table 16. Compositions of the essential oils isolated from several samples of *Origanum vulgare* subsp. *hirtum* (2004)

Compound[a]	%				KI[b]	KI[c]
	1/1	1/2	1/8	9/11		
α-thujene	1.75	1.79	1.65	1.49	930	1018
α-pinene	0.87	0.90	0.80	0.75	938	1015
camphene	0.26	0.30	0.31	0.16	951	1048
sabinene	0.50	0.44	0.13	0.29	974	1114
β-pinene	2.13	2.14	2.13	1.41	980	1102
α-phellandrene	0.14	0.20	0.21	0.14	1006	1162
α-terpinene	0.89	0.96	2.06	0.36	1018	1170
***p*-cymene**	**9.50**	**6.30**	**6.12**	**4.17**	1025	1259
limonene	0.40	0.41	0.41	0.32	1030	1191
γ-terpinene	**7.18**	**5.49**	**15.98**	**1.35**	1060	1236
Z-sabinene hydrate	0.52	0.52	0.42	0.16	1071	1451
terpinolene	0.25	0.33	0.20	0.25	1087	1282
borneol	0.35	0.46	0.55	0.20	1168	1679
terpin-4-ol	0.34	0.38	0.23	0.31	1178	1615
carvacrol methyl ether	-	-	-	0.72	1242	
thymol	0.27	0.33	**3.11**	0.27	1291	2154
carvacrol	**70.77**	**73.68**	**61.72**	**84.21**	1297	2180
β-caryophyllene	1.36	2.63	3.32	1.52	1420	1583
germacren D	0.27	-	-	-	1480	1703
Total	**97.75**	**97.26**	**99.35**	**98.08**		

[a]In the order of elution using a DB-5 MS column.
[b]Kovats retention indices (using a DB-5 MS column)
[c]Kovats retention indices (using a SolGel-WAX column)

Antimicrobial activity was tested on Gram-positive and Gram-negative (proton pump-positive and negative) bacterial strains and *C. albicans* strains, using antibiotics as positive controls. At the same time, we separately analyzed the antimicrobial activities of the isolated conpounds (α-pinene, sabinene, β-pinene, α-terpinene, *p*-cymene, limonene,

[††] **Veres K**, Varga E, Schelz Zs, Molnár J, Bernáth J, Máthé I: *Natural Product Communications* ***in press***

γ-terpinene, borneol, thymol, carvacrol and β-caryophyllene). All these experiments were carried out at the Department of Medical Microbiology and Immunobiology at the University of Szeged.

With an agar diffusion method and a positive control, the MIC values revealed that the presence of carvacrol is always essential for the antimicrobial effect, although other compounds also exerted minor antimicrobial activity (Tables 17 and 18). As regards bacteria, the sensitivity of various strains of *E. coli* should be emphasized. An outstanding sensitivity of the proton pump-deleted mutant of *E. coli* suggests that the antimicrobial effect of the essential oils studied is mediated via a proton pump-dependent mechanism.

The low essential oil contents of selected samples of *Origanum vulgare* L. did not allow us to study their antimicrobial properties with the methods described above. A future solution to this problem could be the use of other methods, such as bioautography or increase of the essential oil content via the vegetative propagation of selected plants.

Table 17. Bacteriostatic and fungistatic activities of various plant oils

Samples	**Conc. (v/v%)**	***E. coli* F'lac**	***E. coli* AG100**	***E. coli* AG100A**	***S. epidermidis***	***S .cerevisiae* δ/1**	***S. cerevisiae* 52C**	***C. albicans* 10231**	***C. albicans* 14053**
1/1	5	36	28	44	25	40	35	38	35
1/2	5	30	27	46	23	42	38	35	32
1/8	5	35	25	45	17	40	43	30	36
9/11	5	45	31	58	27	42	45	40	43
Penicillin	1 mg/ml	24	24	24	23	-	-	-	-
Gentamycin	1 mg/ml	32	32	32	33	-	-	-	-
Fluconazole	2 mg/ml	-	-	-	-	19	27	31	40
DMSO	100	0	0	0	0	0	0	0	0

Zones of growth inhibition (in mm) of microorganisms on nutrient agar plates

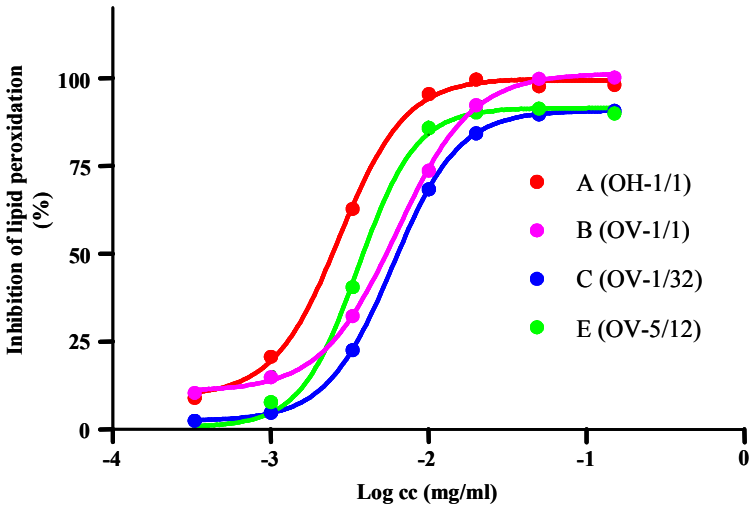
Table 18. IC determination of selected plant oils and authentic/individual oil components on various bacterial and fungal strains

Samples	***E. coli* F'lac**	***E. coli* AG100**	***E. coli* AG100A**	***S. epidermidis***	***S. cerevisiae* δ/1**	***S. cerevisiae* 52C**	***C. albicans* 10231**	***C. albicans* 14053**
1/1[a]	0.20	0.20	0.12	< 0.04	0.12	0.12	0.16	0.20
1/2[a]	0.16	0.20	0.08	0.08	0.12	0.12	0.16	0.16
1/8[a]	0.16	0.24	0.08	< 0.04	0.12	0.12	0.16	0.20
9/11[a]	0.16	0.20	0.08	0.08	0.08	0.12	0.16	0.16
α-pinene[a]	0.62	0.62	0.31	1.25	0.16	0.16	1.25	0.63
sabinene[a]	2.50	> 5	1.25	2.50	0.31	0.16	2.50	2.50
β-pinene[a]	1.25	3.75	0.62	1.25	0.31	0.08	0.62	0.62
α-terpinene[a]	2.50	3.75	1.25	1.25	0.62	1.25	> 5	5.00
p-cymene[a]	20.0	7.50	5.00	2.50	0.62	1.25	2.50	2.50
limonene[a]	0.62	0.62	0.31	1.25	0.16	0.16	1.25	0.62
γ-terpinene[a]	10.0	> 20	7.50	5.00	1.25	5.00	15.0	8.75
borneol[a]	5.00	2.50	1.25	25.0	2.50	5.00	2.50	5.00
thymol[a]	1.25	2.50	0.33	0.31	0.16	0.08	0.08	0.16
carvacrol[a]	0.16	0.16	0.16	2.50	0.31	0.31	1.25	1.25
β-caryophyllene[a]	> 20	20.0	1.25	20.0	> 20	> 20	> 20	> 20
Penicillin[b]	8.0	8.0	1.6	4.0	-	-	-	-
Gentamycin[b]	1.2	4.0	2.0	0.8	-	-	-	-
Fluconazole[b]	-	-	-	-	56.0	64.0	16.0	0.8
DMSO	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20

The tabulated numbers indicated MICs as the volumes of oils and components resulting in complete inhibition [a] μl/ml, [b] μg/ml

5.5.2. Correlations between rosmarinic acid content and antioxidant activity

Using TLC and a DPPH reagent to detect the extracts of hyssop and origanum mass samples, we found that the extracts had antioxidant properties. After some preliminary experiments, we carried out pharmacological studies in cooperation with the Department of Pharmacodynamics and Biopharmacy at the University of Szeged, using 70% MeOH extracts of several hyssop and origanum plants to evaluate the relationship between their RA content and antioxidant activity. All extracts of origanum inhibited lipid peroxidation in a concentration-dependent manner. Sample No. 1/1 of *O. vulgare* subsp. *hirtum* with the highest RA content was found to exert the highest activity against lipid peroxidation (Figure 19).



*OH: *O. vulgare* subsp. *hirtum*; OV: *O. vulgare* subsp. *vulgare*

Figure 19. Antioxidant activities of several samples of *Origanum* species

To depict the efficiency of the extracts, we plotted the RA content against the reciprocal of IC_{50} (Figure 20). The correlation coefficient strongly suggests that RA contributes substantially to the antioxidant activity of the extracts studied.

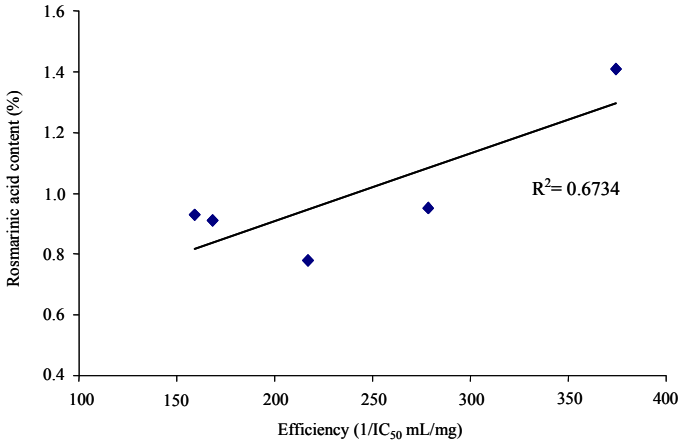


Figure 20. Relationship between RA content and antioxidant activity

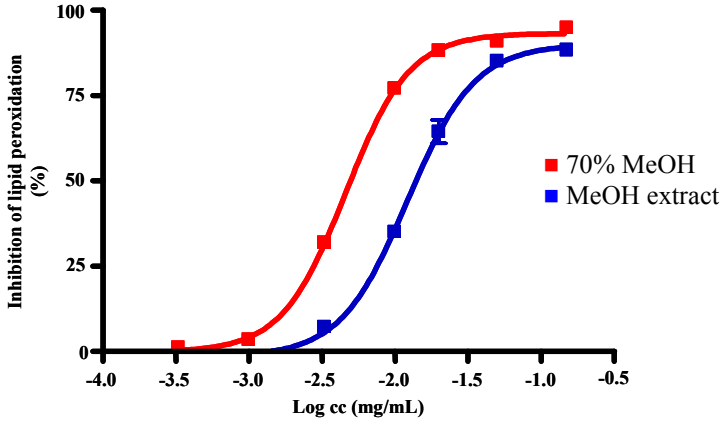


Figure 21. Antioxidant activities of extracts of hyssop samples

Despite its substantially lower RA content (0.3–0.4%), the antioxidant activity of the hyssop extract is similar to that of the origanum extract, which contains a relatively high amount of RA (0.93–1.41%) (Figure 21). This suggests that, besides RA, other constituents of hyssop also contribute to the antioxidant activity. A possible explanation could be the higher OA/UA content of hyssop, as the literature data support the antioxidant properties of these two compounds.[38,39] It is also obvious that the 70% MeOH extracts of hyssop are more efficient than those prepared with concentrated MeOH (Figure 21). This suggests that other, presumably more polar compounds may also contribute to the antioxidant activity.

6. SUMMARY

Hyssop and origanum, discussed in the present thesis, are members of the family Lamiaceae and are gaining increasing importance as herbs and spices. Both species share the characteristics of diverse appearance and chemical constitution, and both have numerous intraspecific taxons. Thus, in addition to the difficulties of classification, ensuring a drug quality that meets the increasingly strict requirements is often challenging. These issues furnished the background for the experiments on the variety breeding of both species and for the associated basic studies.

Studies of selected progeny generations of hyssop populations made it clear that the predisposition to earlier flowering is more difficult to inherit than the colour of the corolla as selection progresses. The typical main components of hyssop essential oil are isopinocamphone, pinocamphone, *β*-pinene and limonene among other constituents. Based on the quantitative variation in these components, we described 4 characteristic chemovariants. The chemotype containing a substantial amount (>30%) of limonene is a novum in the literature. Another novel finding of our team is that the pinocamphone content increases throughout ontogenesis, while the relative amount of isopinocamphone decreases. This change is most marked in plants that have pinocamphone as the main constituent. As a result of this complex selection research, we have a candidate variant of *Hyssopus officinalis* that has a uniform appearance and essential oil content, and additionally has a uniquely high essential oil production among Europe plants.

We also studied the characteristics of *Origanum* species and subspecies cultivated in Hungary. Our experiments demonstrated that the cultivated Mediterranean plant *O. vulgare* subsp. *hirtum* retained its original characteristics (essential oil content and composition) under temperate climate conditions. We have selected several individual plants from inhomogenous populations of origanum that have an essential oil yield and composition, and also a RA content that make them promising candidates for variety breeding.

Studies of whether the RA content depends on the stage of vegetation revealed only slight changes, without striking maxima. Year-specific differences were observed for the RA contents of both species studied.

Our stability studies provide information of value for quality assurance. As compared with the distilled oil, the composition of the essential oils of these species remains stable in the drug form for a longer period of time.

We have elaborated a novel method to measure the UA and OA contents of Lamiaceae species in parallel. On the basis of these results, we have drawn chemotaxonomic conclusions concerning the subfamilies Nepetoideae and Lamioideae.

Studies of physiological effects revealed that in our samples of Origanum species carvacrol played an essential role in the antimicrobial activity of the volatile oils, irrespective of the presence and quantities of the other constituents.

Despite a difference in their RA contents, hyssop and origanum exerted almost identical antioxidant effects. Apart from the crucial role of RA other compounds also contribute to this antioxidant activity, and these need to be explored in future complex studies.

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APPENDIX

The thesis is based on the following publications referred to in the text:

I. **Veres K,** Varga E, Dobos Á, Hajdú Zs, Máthé I, Pluhár Zs, Bernáth J:
Investigation on Essential Oils of *Hyssopus officinalis* L. populations
In: Franz Ch, Máthé Á, Buchbaner G, eds. *Essential Oils: Basic and Applied Research*
Allured Publishing Comparation, 1997, pp 217-220.

II. Varga E, Hajdú Zs, **Veres K,** Máthé I, Németh É, Pluhár Zs, Bernáth J:
Hyssopus officinalis L. produkcióbiológiai és kémiai változékonyságának tanulmányozása
Acta Pharm. Hung. 1998; **68**: 183-188

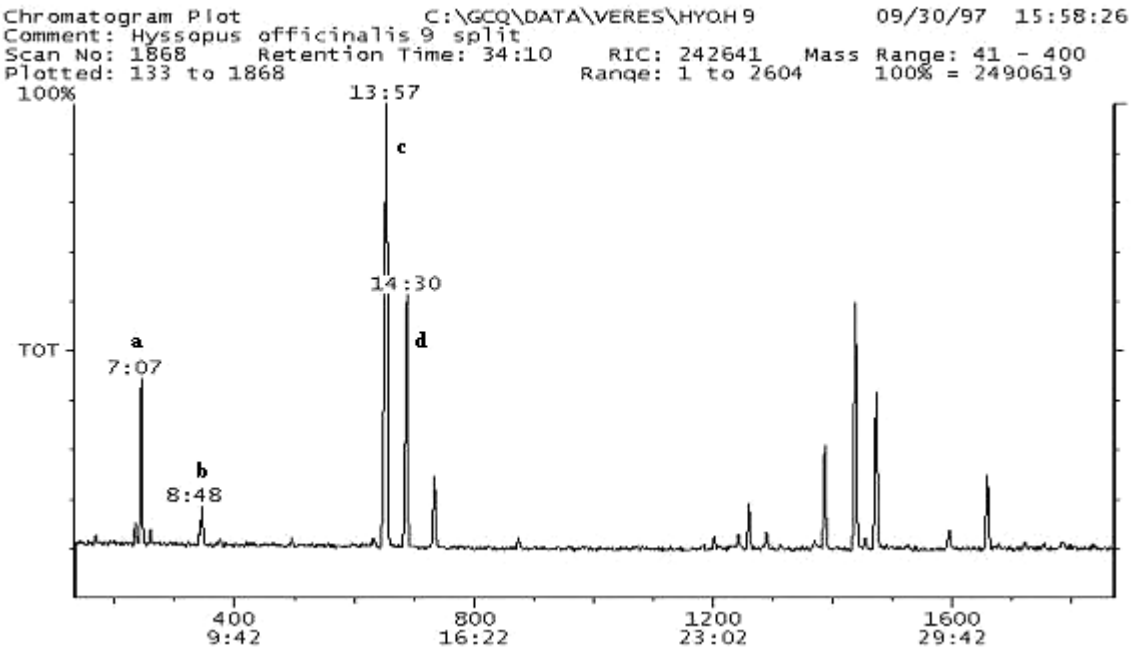
III. **Veres K,** Varga E, Dobos Á, Hajdú Zs, Máthé I, Németh É, Szabó K:
Investigation of the Content and Stability of Essential Oils of *Origanum vulgare* ssp. *vulgare* L. and *O. vulgare* ssp. *hirtum* (Link) Ietswaart
Chromatographia 2003; **57**: 95-98

IV. Janicsák G, **Veres K**, Kállai M, Máthé I:
Gas chromatographic method for routine determination of oleanolic and ursolic acids in medicinal plants
Chromatographia 2003; **58**: 295-299

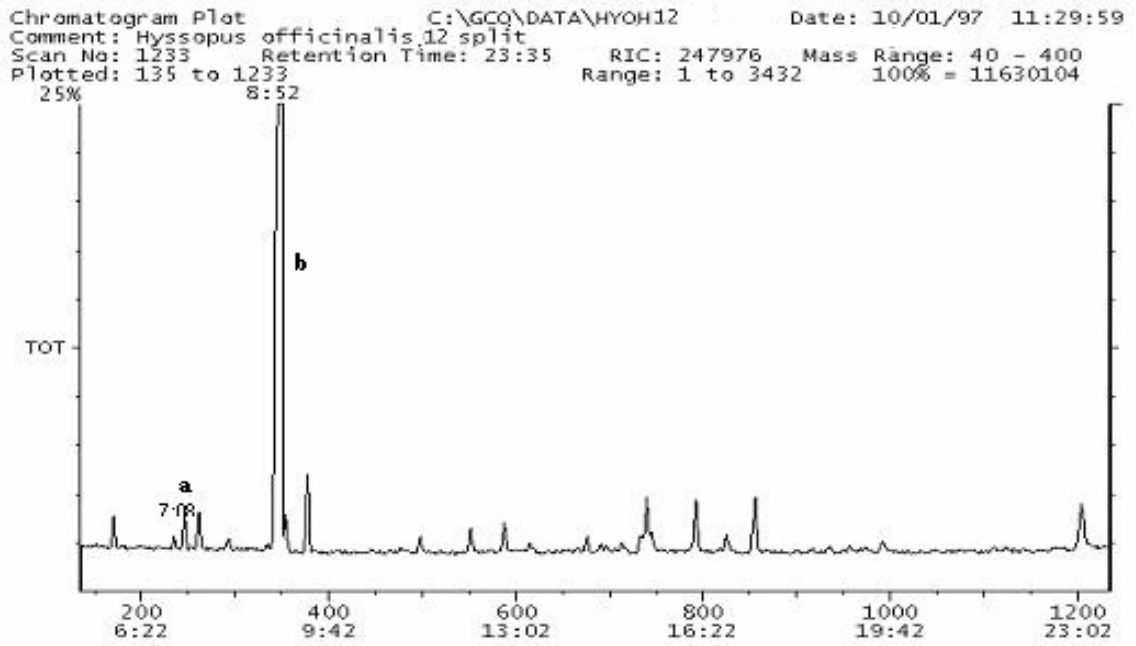
V. Janicsák G, **Veres K,** Kakasy AZ, Máthé I:
Study of the oleanolic and ursolic acid contents of some species of the Lamiaceae
Biochem. Syst. Ecol. 2006; **34**: 392-396

VI. **Veres K**, Varga E, Schelz Zs, Molnár J, Bernáth J, Máthé I:
Chemical Composition and Antimicrobial Activities of Essential Oils of Four Lines of *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart Grown in Hungary
Natural Product Communications 2007; **2**: 1155-1158

Annex I.

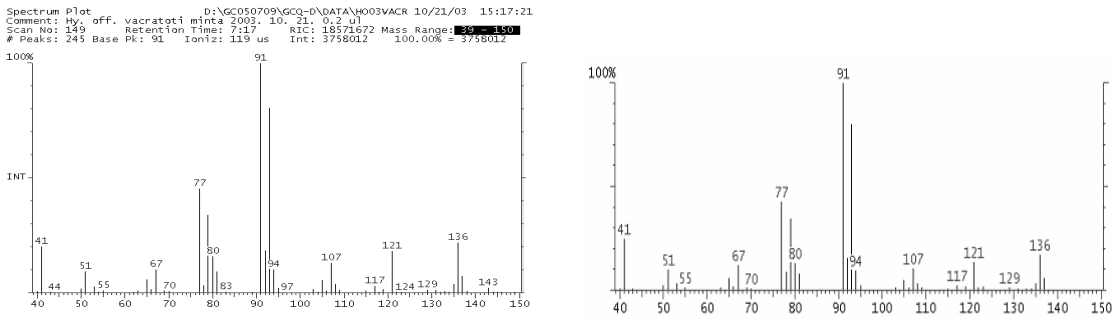


Total ion chromatogram of the essential oil isolated from hyssop No **9**
(a: β-pinene, **b:** limonene, **c:** pinocamphone, **d:** isopinocamphone)

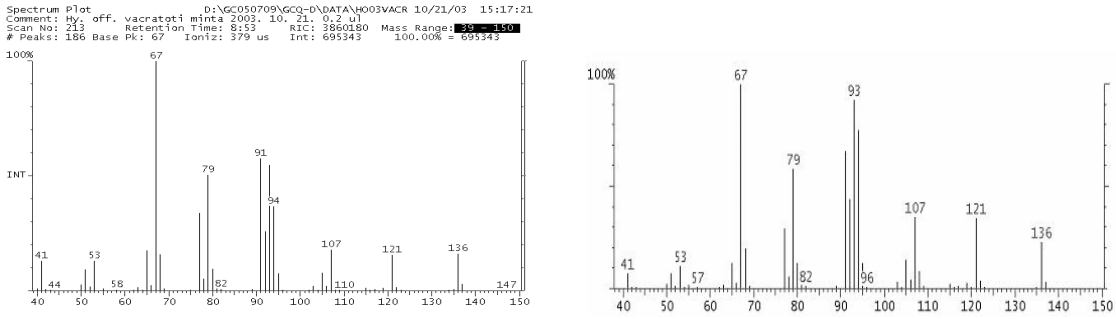


Total ion chromatogram of the essential oil isolated from hyssop No **12**
(a: β-pinene, **b:** limonene)

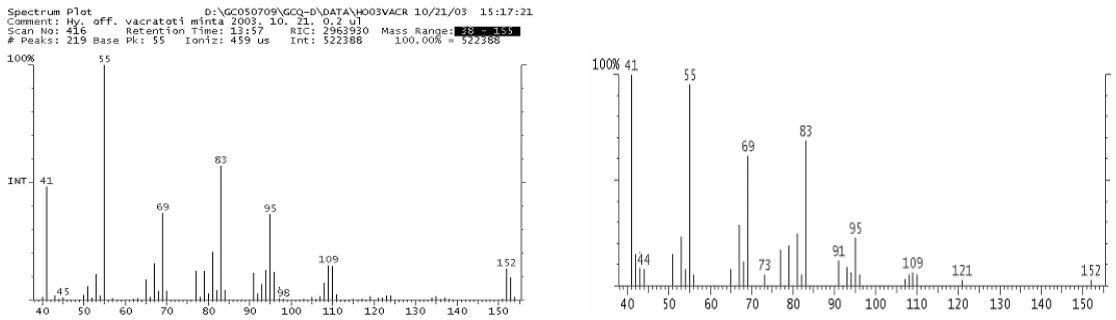
Annex II.



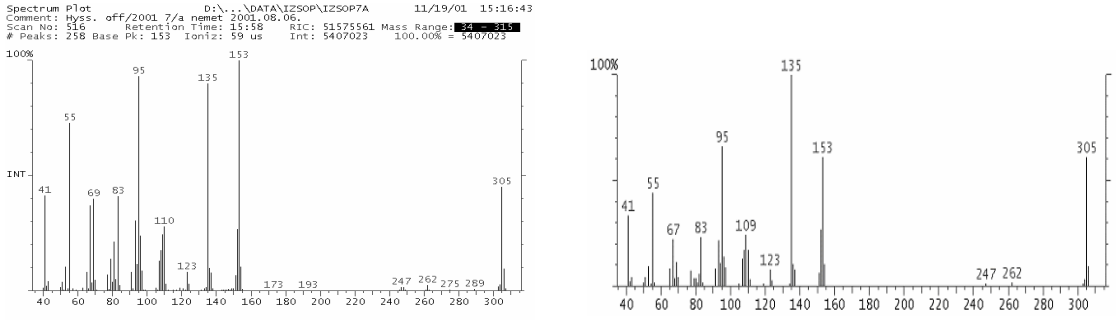
MS spectra of **β-pinene** (**A:** in sample; **B:** in own database)



MS spectra of **limonene** (**A:** in sample; **B:** in own database)



MS spectra of **pinocamphone** (**A:** in sample; **B:** in database)



MS spectra of **isopinocamphone** (**A:** in sample; **B:** in database)

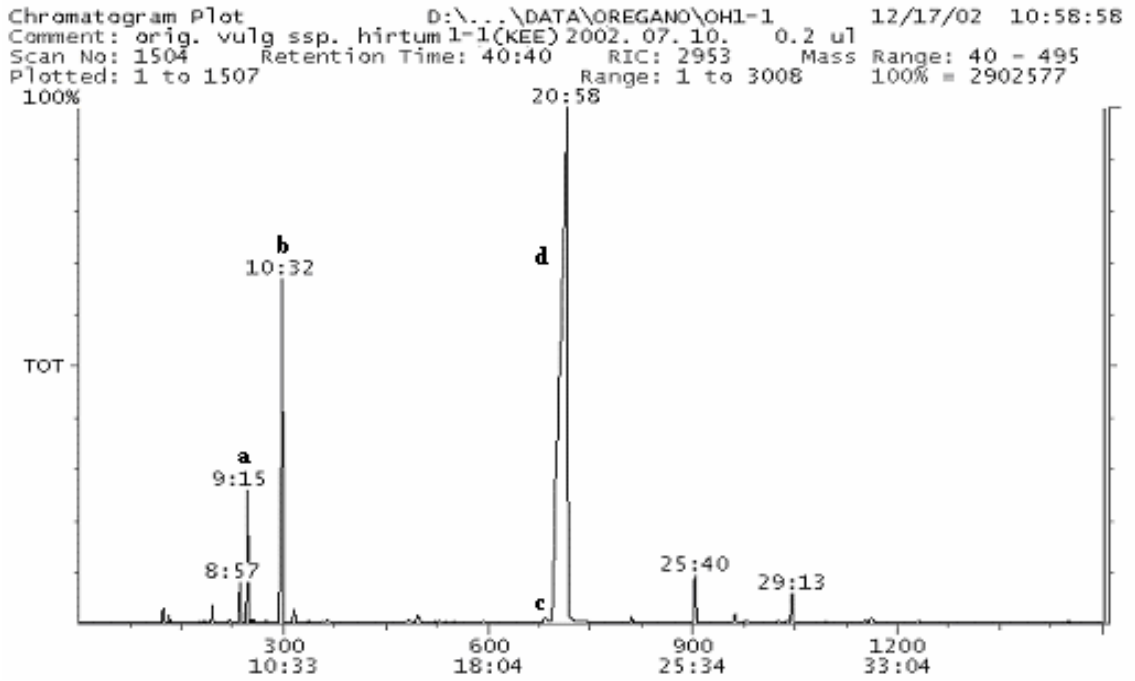
Annex III.

Composition of the essential oils of Origanum subspecies examined
(oils were distilled from flowering plants)

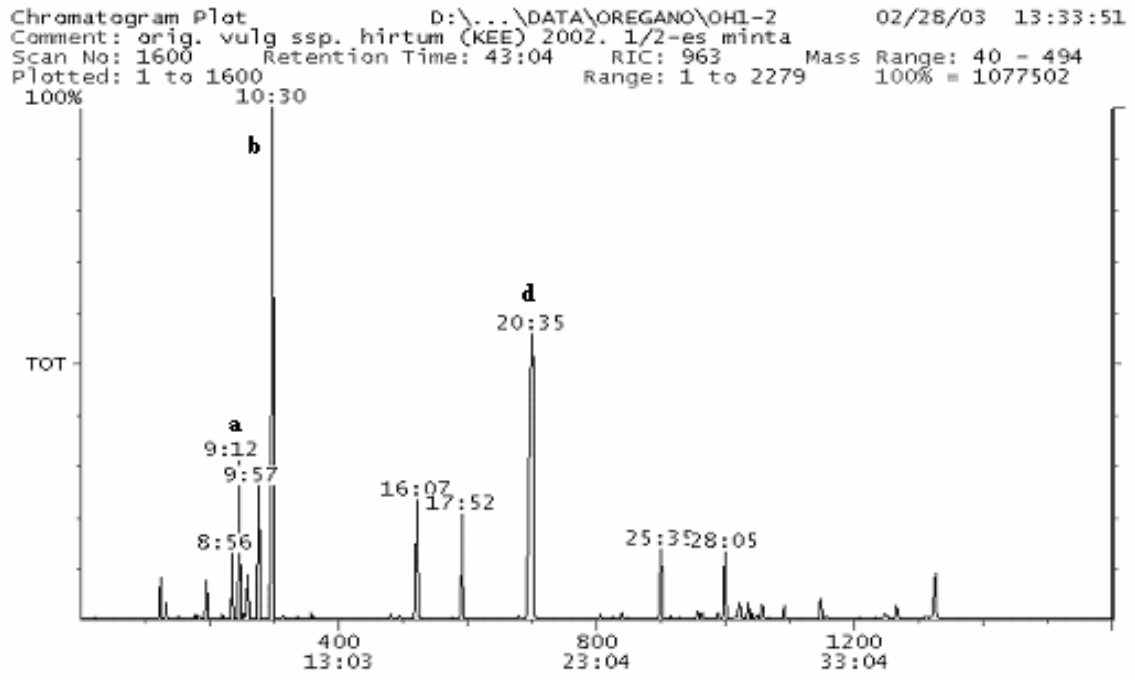
			O. vulgare* subsp. *hirtum	***O. vulgare* subsp. *vulgare***
RI*	***RI******	***Compounds***	***%***	***%***
931	926-930	α-thujene	0.86	0.40
939	935-938	α-pinene	0.72	0.31
953	950-951	camphene	0.27	0.04
976	973-974	sabinene	-	7.93
980	978-980	β-pinene	0.69	0.45
991	987-989	β-myrcene	1.73	0.61
1018	1015-1018	α-terpinene	1.24	0.63
1026	1024-1025	*p*-cymene	4.67	**22.31**
1031	1030-1033	limonene	0.39	0.62
1040	1037-1038	*Z*-β-ocimene	-	3.76
1050	1047-1049	*E*-β-ocimene	-	3.56
1062	1058-1060	γ-terpinene	6.63	5.11
1098	1097-1102	linalool	-	0.28
1160	1161-1162	pinocamphone	-	0.18
1167	1168-1169	borneol	0.57	-
1173	1174-1175	isopinocamphone	-	1.32
1177	1178-1180	terpin-4-ol	0.49	0.44
1189	1187-1190	α-terpineol	-	0.83
	1285-1289	unknown	-	0.13
1290	1291-1293	thymol	0.30	0.34
1298	1297-1302	carvacrol	**76.44**	-
1384	1380-1384	β-bourbonene	-	1.44
1418	1417-1420	β-caryophyllene	1.60	4.09
1480	1477-1480	germacrene D	-	4.18
1576	1576	spathulenol	-	4.86
1581	1579-1582	caryophyllene oxide	-	10.26

RI* : Kovats retention indices according to literature data
RI**: Kovats retention indices mesured by our team

Annex IV.

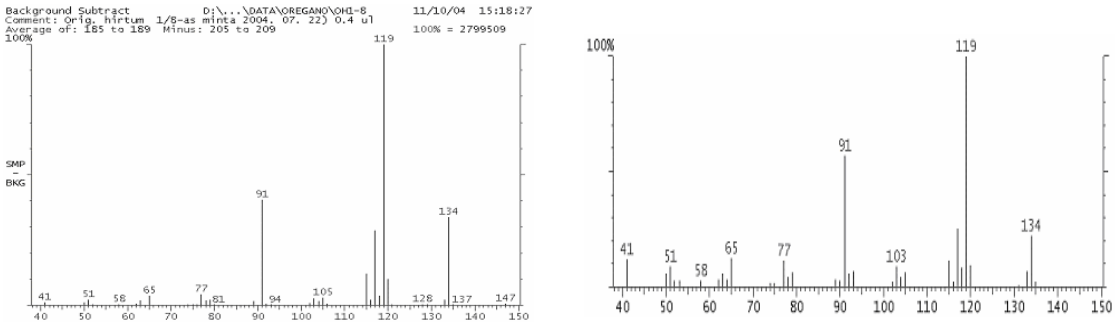


Total ion chromatogram of the essential of *O. vulgare* subsp. *hirtum* No **1/1** (2002)
(a: p-cymene, **b:** γ-terpinene, **c:** thymol, **d:** carvacrol)

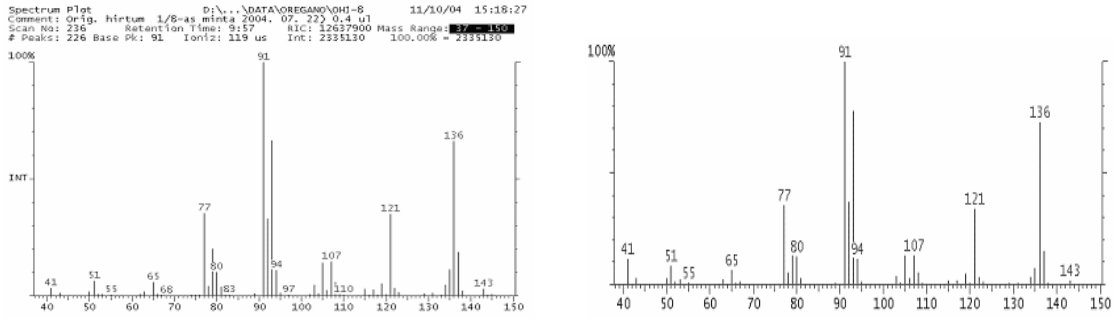


Total ion chromatogram of the essential of *O. vulgare* subsp. *hirtum* No **1/2** (2002)
(a: p-cymene, **b:** γ-terpinene, **d:** carvacrol)

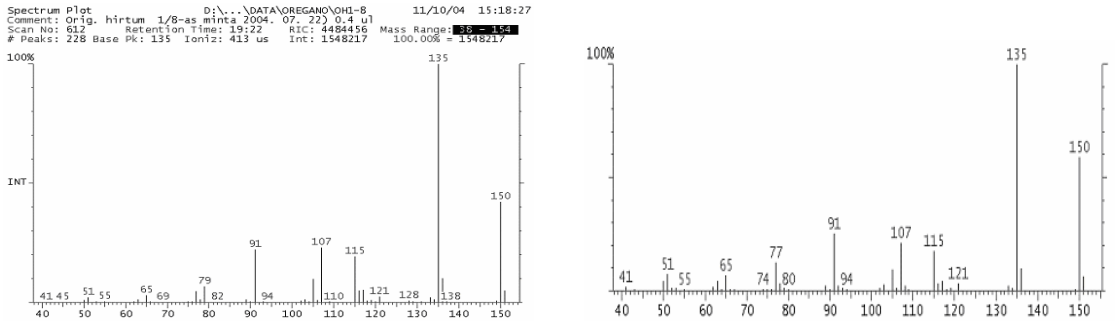
Annex V.



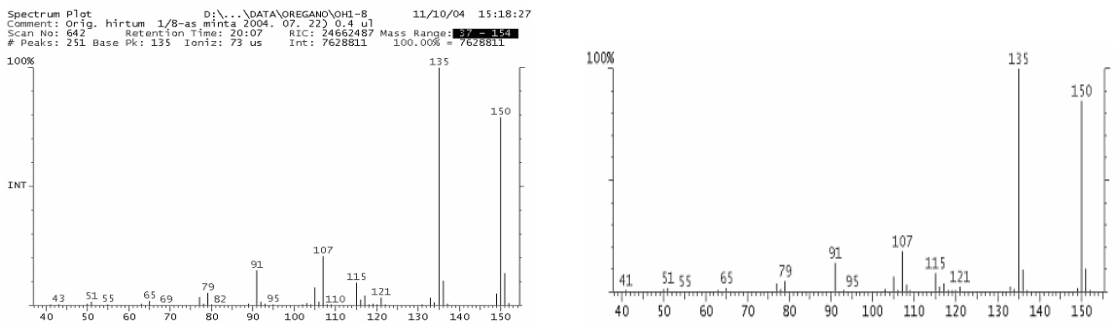
MS spectra of ***p*-cymene** (**A:** in sample; **B:** in database)



MS spectra of **γ-terpinene** (**A:** in sample; **B:** in own database)

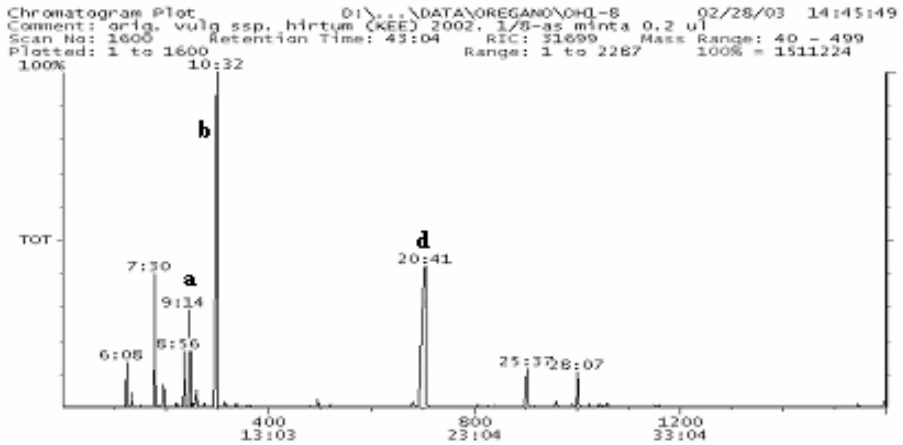


MS spectra of **thymol** (**A:** in sample; **B:** in own database)

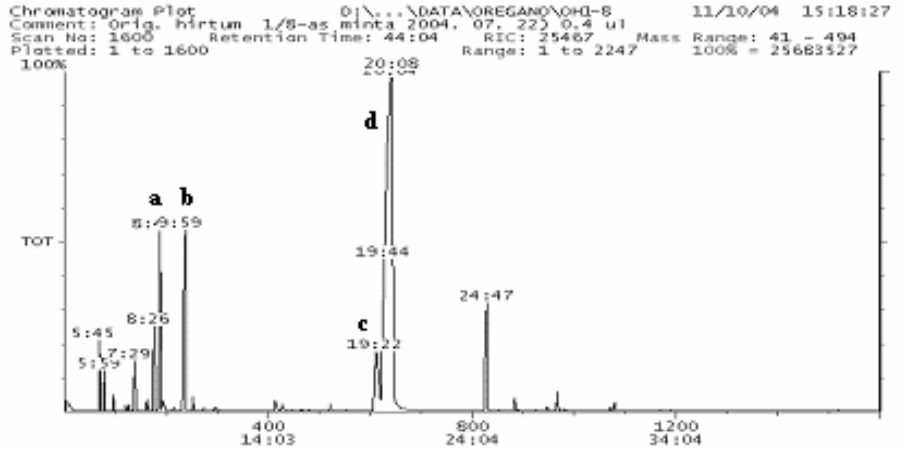


MS spectra of **carvacrol** (**A:** in sample; **B:** in own database)

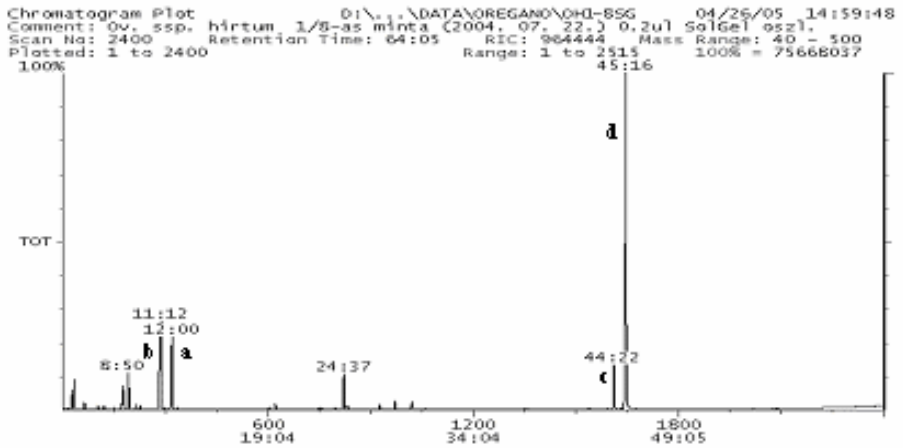
Annex VI.



1. Total ion chromatogram of the essential of *O. vulgare* subsp. *hirtum* No **1/8** (2002)



2. Total ion chromatogram of the essential of *O. vulgare* subsp. *hirtum* No **1/8** (2004)

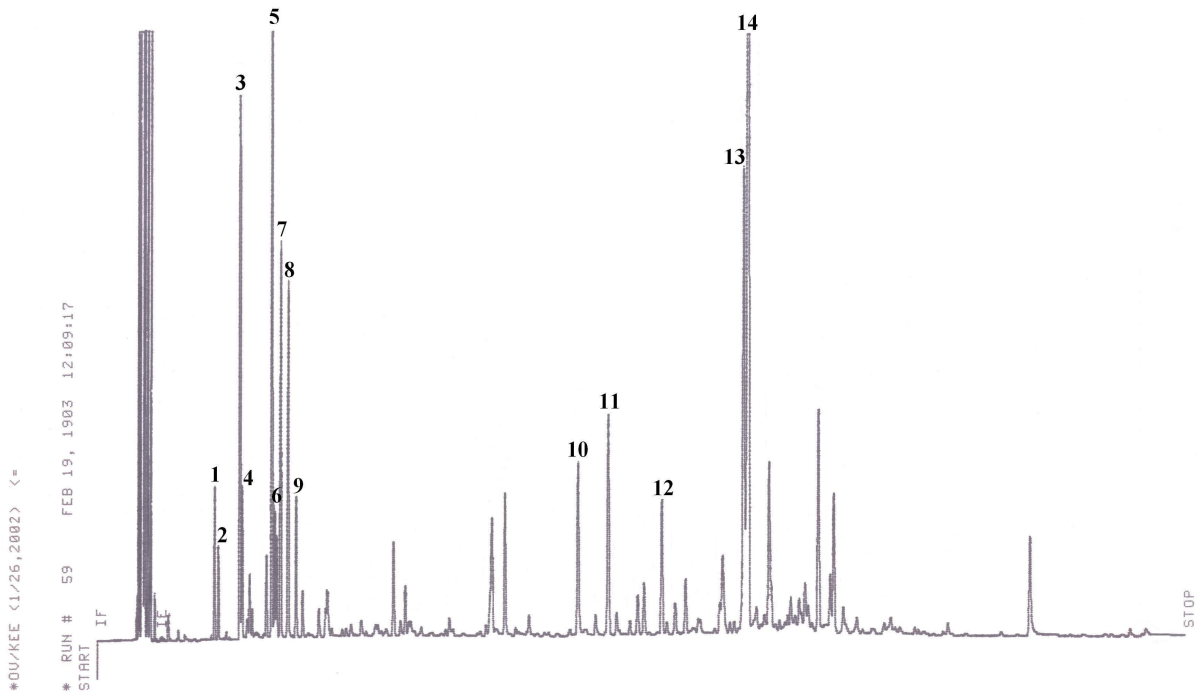


3. Total ion chromatogram of the essential of *O. vulgare* subsp. *hirtum* No **1/8** (2004)

(a: p-cymene, **b:** γ-terpinene, **c:** thymol, **d:** carvacrol)

(**1** and **2**: use DB-5 MS and, **3**: SolGel-WAX column)

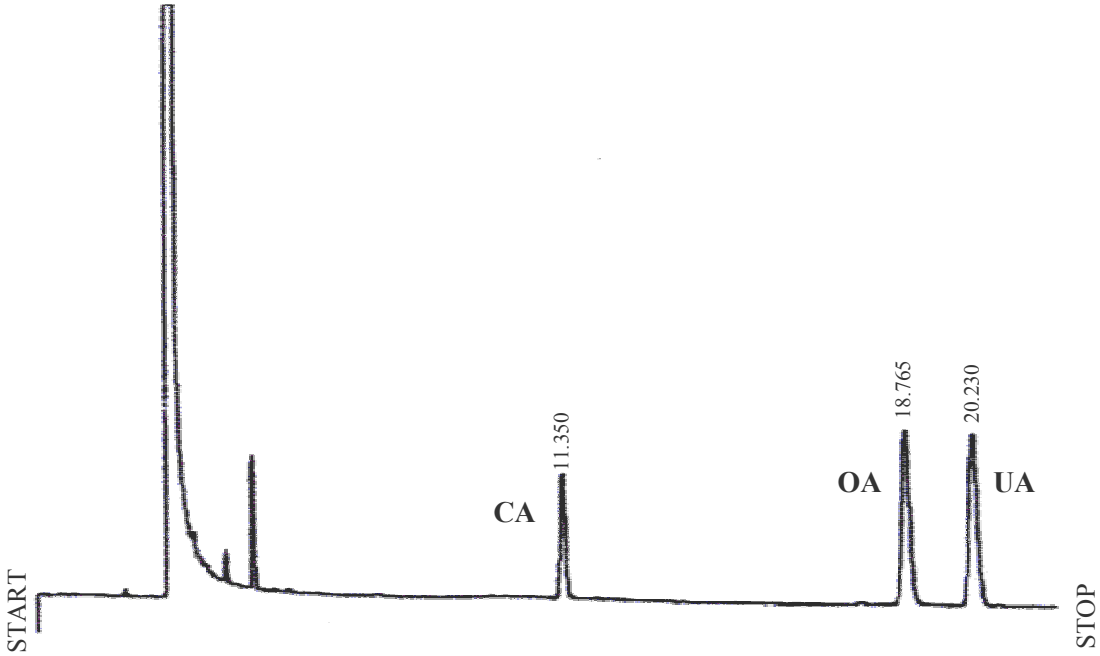
Annex VII.



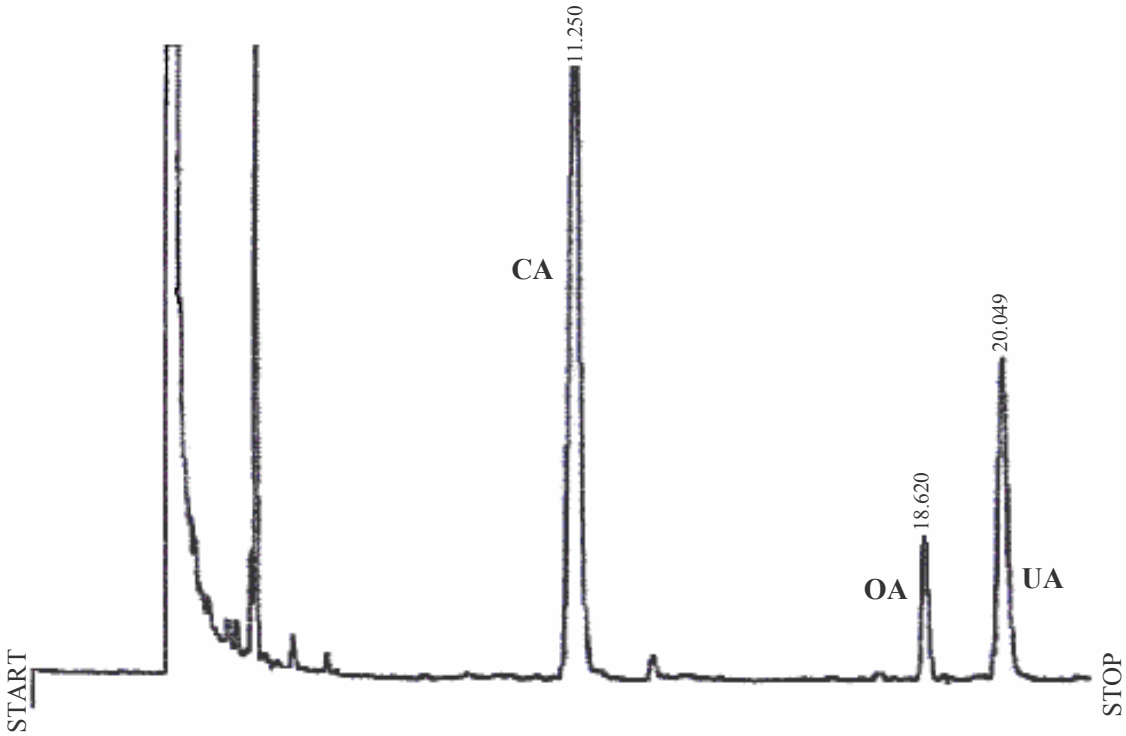
Gas-chromatogram of *Origanum vulgare* ssp. *vulgare* oil

Peaks: 1 = α-thujene 2 = α-pinene, 3 = sabinene, 4 = β-pinene 5 = *p*-cymene, 6 = limonene 7 = β-phellandrene 8 = (*Z*)-β-ocimene 9 = γ-terpinene, 10 = β-bourbonene 11 = β-caryophyllene, 12 = germacrene D, 13 = spathulenol, 14 = caryophyllene oxide

Annex VIII.



Gas chromatogram of a mixture of authentic compounds



Gas chromatogram of a mixture of a *Hyssopus officinalis* L. f. albus sample

(legends: **CA**: cholesteryl acetate , **OA:** oleanolic acid, **UA**: ursolic acid

INVESTIGATION OF THE COMPOSITION OF ESSENTIAL OILS OF *HYSSOPUS OFFICINALIS L.* POPULATIONS

Veres, K[1]., Varga, E[1]., Dobos, Á[1]., Hajdú, Zs[1]., Máthé, I[1,2].Pluhár, Zs[3], Németh, É[3]., Bernáth, J[3].

[1]Albert Szent-Györgyi Medical University, Department of Pharmacognosy, Szeged,
[2]Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót
[3]University of Horticulture and Food Industry, Department of Medicinal Plant Production, Budapest, Hungary

INTRODUCTION

Hyssopus officinalis L. (*Lamiaceae*) is a well-known spice, the flowering shoots and essential oil being used. It is also an excellent plant for attracting bees and is a decorative garden ornamental. The plant native to the Mediterranean has long been cultivated in Central Europe. In many countries, including Hungary, this species is used as a folk medicine against certain respiratory diseases, e.g. bronchitis due to its spasmolytic activity. However, little is known as concerns the variations in its oil content and composition under Central European, climatic conditions.

The cultivated populations of hyssop are characterised by a significant heterogeneity. The main aims in its breeding are flower colour uniformity (e.g. the blue form) and an increased oil content. As knowledge on the variation or genetic fixation of the oil composition is incomplete, we decided to study this question.

This paper deals with the study of the essential oils of *Hysssopus officinalis* of different origins, grown under the same climatic conditions in Hungary.

We studied how the oil composition depends upon the origin and also the time when the samples were harvested. The compositions of essential oil samples obtained by Supercritical Fluid Extraction (SFE) with CO_2 and by Water Steam Distillation (WSD) were compared. The oils were analysed by GC and GC/MS techniques.

MATERIAL AND METHODS

Different populations of *Hyssopus officinalis* grown from seeds and the offspring of the plants individually selected according to the colour of the flowers obtained from them served as starting material for our investigations. The original seeds had been acquired from various botanical gardens abroad.

Table 1. Identity and origin of populations of hyssop grown in Vácrátót

Samples	Origin of seed
I*	St Gallen
II*	Zürich
III*	Frankfurt
IV*	Latvia
V*	Halle
VI*	Montreal
VII**	Antverpen
VIII**	Salaspils
IX**	Wroclaw

* Date of sowing 10. 04. 1991
** Date of sowing 11. 11. 1993.

The essential oils were obtained by means of WSD and SFE in a small-scale CO_2 extractor.

The fresh plant material was subjected to WSD for 2 h in a Hungarian Pharmacopoeia distillation apparatus [1].

SFE was performed on air-dried herbs, using an Isco SFX 2-10 instrument at 40 °C and at 50 °C and at increasing pressures (80 to 500 bar). The extraction time was 30 min. and the flow rate was 0.6 ml/min.

The oils were analysed by GC and GC/MS techniques. Analytical GC was carried out on a HP 5890 SERIES II gas chromatograph (FID), using a 30 m HP-5 fused silica capillary column which was programmed from 60 °C (2 min. hold)

to 220 °C (2 min. hold) at 5 °C/min. Other important parameters were injector temp.: 250 °C; carrier gas: N_2; split sample introduction. GC/MS was performed on a FINNIGAN GCQ mass spectrometer. All conditions were as above, except that the carrier gas was He.

RESULTS AND DISCUSSION

The main observations were as follows:

The GC study of the oils demonstrated three main components in the oil: β-pinene (**a**), pinocamphone (**c**) and isopinocamphone (**d**) (Fig. 1). In addition to these substances and in contrast with literature data [2-5] limonene (**b**) was one of the main components (about 38 %) in some samples (Fig. 2). Such a chemotype has not been described previously. Accordingly, the inves-tigation of the proportions of the four compounds was considered interesting.

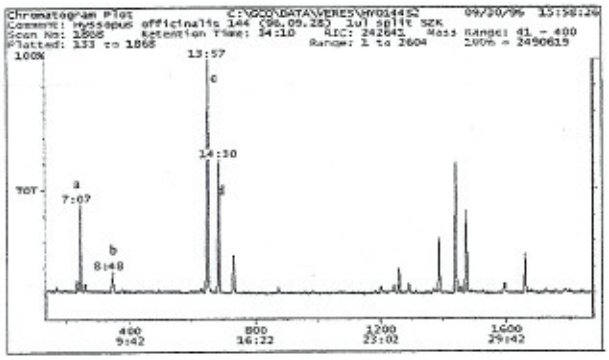


Fig. 1. Total ion chromatogram of the essential oil of *Hyssopus officinalis* L.

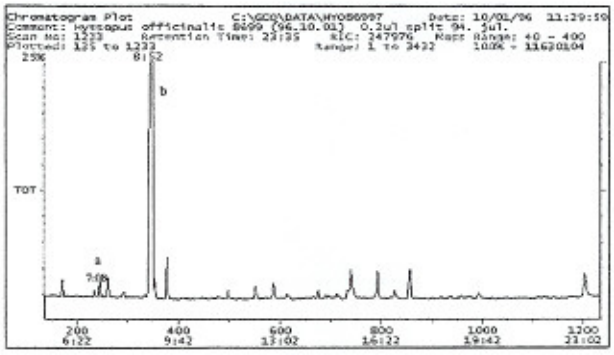


Fig. 2. Total ion chromatogram of the essential oil of *Hyssopus officinalis* L.

The heterogeneity in the oil composition of the various populations was found to be independent of the botanical gardens supplying the seed (Fig. 3).

Fig. 3. Proportions of main components in the volatile oil of *Hyssopus officinalis* L. of different origins (samples harvested in October, 1994)

On SFE extraction, the oil yields were in general higher that those obtained by WSD (Fig. 4). In the CO_2 extractor, the parameters 100 bar, 40 °C and 30 min. were considered optimal.

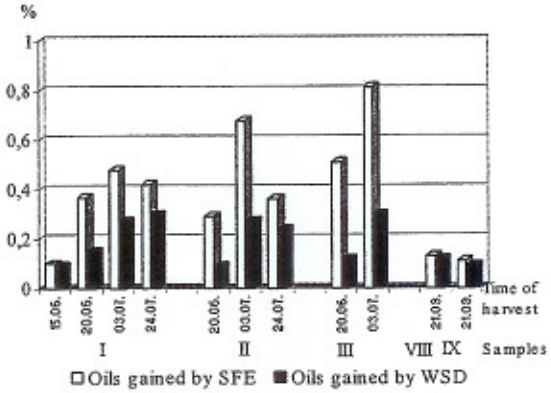


Fig. 4. Yields of essential oils obtained by different extraction methods

The variation in the oil composition of three populations (of various origins) was followed during the vegetation period. No changes in the proportions of the four main components were found in one population (Fig. 5), but the other two exhibited opposite changes (Figs. 6 and 7). Further studies are necessary to explain these differences.

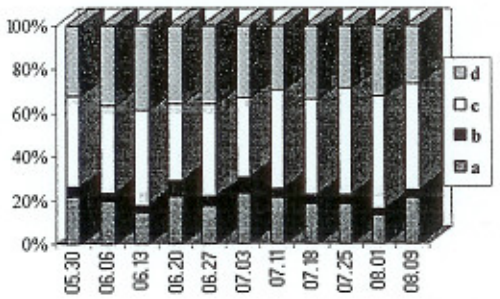


Fig. 5. Proportions of the main components in the volatile oil of *Hyssopus officinalis* L. (sample II)

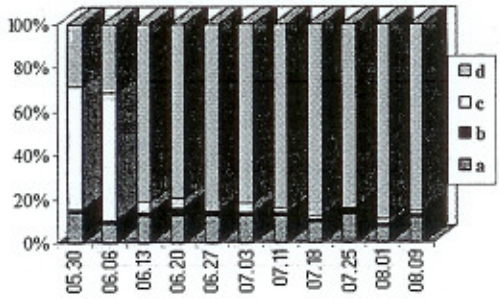


Fig. 6. Proportions of the main components in the volatile oil of *Hyssopus officinalis* L. (sample I)

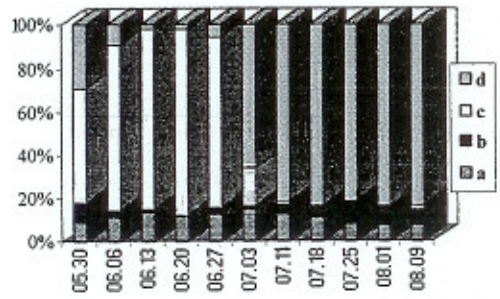
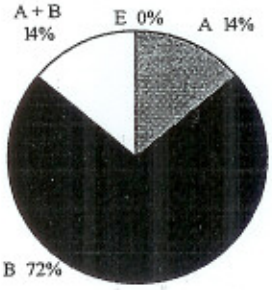
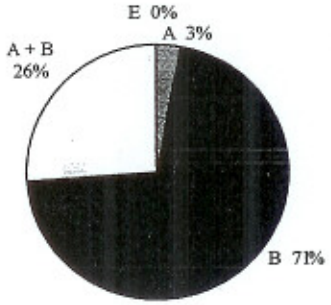


Fig. 7. Proportions of the main components in the volatile oil of *Hyssopus officinalis* L. (sample III)

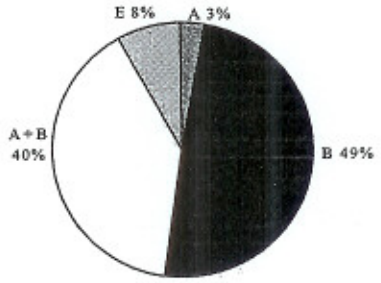
Our observations indicated that selection on the basis of the flower colour does not guarantee the homogeneity of the oil composition. It was therefore worth differentiating between populations as concerns the main components of the oils, e.g. pinocamphone, isopinocamphone, pinocamphone + isopinocamphone, limonene and oils with miscellaneous composition (Fig. 8).



Distribution of the individuals in strain marked 7.



Distribution of the individuals in strain marked 9.



Distribution of the individuals in strain marked 10.

A: isopinocamphone is the main component of the oil (more than 50% of the oil)
B: pinocamphone is the main component of the oil (more than 50% of the oil)
A+B: pinocamphone and isophinocamphone are together the main components (20-50%)
E: another compound is the main component of the oil (more than 30% of the oil)

Fig. 8. Distribution of the main components in the volatile oil of the offspring of *Hyssopus officinalis* L.

Chemical heterogeneity in oil composition was observed among the offspring of a plant with a particular chemical composition. When only the main four components (a: β-pinene, b: limonene, c: pinocamphone, d: isopinocamphone) were considered, clear and mixed lines alike could be found among the offspring independently of the original composition.

The above observations justify the necessity and usefulness of comparative chemical studies in hyssop selection, and these are currently ongoing.

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Hyssopus officinalis L. produkcióbiológiai és kémiai változékonyságának tanulmányozása*

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Summary

Varga, E., Hajdú, Zs., Veres, K:, Máthé, I., Németh, É., Pluhár, Zs. and Bernáth, J.: ***Investigation of production biological and chemical variation of Hyssopus officinalis L.***

Hyssopus officinalis L. (Lamiaceae family) has been cultivated in Central Europe for a long time. This essential oil containing species serves not only as spice but in many countries including Hungary, it is used as a folk medicine against certain respiratory diseases. Despite this fact, little is known about the variation of its productivity under Central European climatic conditions. The cultivated populations of hyssop can be characterised by a significant heterogenity. In the course of its breeding the uniformity of flower colour (e.g. blue form), and increase in the oil content are the main achievable purposes. The purpose of this work was to investigate both the variability of strains of different origin and the time-dependent variations of its production parameters. The optimum of phytomass was obtained at the beginning of July. The essential oil content as well as compounds of the non volatile fractions were also investigated. The non volatile fractions for rosmarinic, caffeic acids were analysed mainly by TLC and densitometry. Both compounds were present in all samples and they are suitable for the characterisation of the plant.

The essential oils were gained with Water Steam Distillation (WSD) and Supercritical Fluid Extraction (SFE) with CO_2. The oils were analysed by GC, GC-MS techniques. In the essential oil composition of the populations studied significant heterogenity could be observed. In the case of applying SFE extraction the oil composition is more uniform, similarly to the obtained by WSD adding hexane. The heterogenity can be experienced in the offsprings too. If only the main four components (β-pinene, limonene, pinocamphone, isopinocamphone) are regarded, among the offsprings clear and mixed lines alike can be found.

Results of these experiments justify the necessity and usefulness of selection which is going on.

Összefoglalás

A Hyssopus officinalis (Lamiaceae) közismert gyógy- és fűszernövény, melyre nagyfokú morfológiai és kémiai heterogenitás jellemző. Virágzó hajtását és illóolaját használják. Több országban termesztik, köztük Magyarországon is.

Jelen közleményben a szerzők beszámolnak a növény egyedfejlődése során mért fitomassza adatokról, egyes, a növény jellemzésére alkalmas nem illó komponensek – rozmaringsav és kávésav – mennyiségi változásainak nyomon követéséről. Tanulmányozták az illóolaj-összetételt a származási hely és gyűjtési idő függvényében, valamint az egyedszelekcióval nyert utódokban. Eredményeik szerint a csupán külsőleges bélyegekre történő szelekció nem biztosít homogén illóolaj-összetételt. Ehhez szükség van a párhuzamosan végzett kémiai szelekcióra is.

Módszerként denzitometriát, vízgőzdesztillációt, szuperkritikus CO_2-extrakciót, valamint GC és GC-MS eljárást alkalmaztak. Kidolgozták a vizsgálatokhoz szükséges metodikát.

Eredményeink a hazai termesztésben és a további kutatásokban egyaránt hasznosíthatók.

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Bevezetés

A gyógynövényexport és a hazai gyógynövény-felhasználás területén napjainkban növekvő hangsúlyt kapnak a minőségi és a gazdaságossági szempontok. Morfológiai és kémiai vonatkozásban változékony fajok esetén – e csoportba tartozik a *Hyssopus officinalis* is – a minőség biztosítása számos problémát vet fel.

A növény gyógyászati és egyéb irányú (élelmiszeripari, kozmetikai) felhasználása megkívánja azon tényezők minél teljesebb ismeretét, melyek lehetővé teszik elegendő mennyiségű, a nemzetközi és hazai elvárásoknak megfelelő minőségű, magas hatóanyag tartalmú növényi anyag biztosítását.

A Lamiaceae családba tartozó *Hyssopus officinalis* – izsóp – közismert évelő félcserje. A növény a Kaszpi-tengertől a Mediterráneumig honos, Közép-Európában régóta termesztik. A termesztett növény a *Hyssopus officinalis subsp. officinalis* formaköréhez tartozik, melyre a kopasz hajtás és a kék, rózsaszín ill. fehér virágszín a jellemző. Számos kémiai változatát is leírták [1].

Már az ókorban ismert gyógynövény. Virágzó hajtását görcsoldó hatása miatt alkalmazzák. Köhögéscsillapító és légcsőhurutot enyhítő teakeverékek alkotórésze. Főzete és kivonata gombaölő és izzadásgátló hatású. Hazai gyógytermékek alkotórésze [2]. Felhasználása fűszernövényként is gyakori, illóolaját a konzerv-, likőr- és a kozmetikaiparban használják [1–3].

Az izsóp virágzó hajtásainak illóolaj tartalma 0,3–1%, β-pinén, pinokámfon és izopinokámfon főbb komponensekkel. Az illóolajban változékony összetétellel előforduló számos egyéb vegyületet is leírtak. A föld feletti rész az illóolajon kívül tartalmaz flavonoidokat, cseranyagot, keserűanyagokat és gyantát [4–12].

Az izsóp a VII. Magyar Gyógyszerkönyvben nem hivatalos. A drogot képező virágzó hajtás (*Hyssopi herba*) és a belőle nyert illóolaj (*Aetheroleum hyssopi*) minősítése szabványelőirat szerint történik.

A hajtásdrog szerepel a magisztrális gyógyszerrendeléshez felhasználható gyógyszeranyagok sorában, az úgynevezett „pozitív listán".

Az izsóp morfológiai és kémiai polimorfizmusának megfelelően a termesztett populációkat nagyfokú heterogenitás jellemzi. A korszerű fajtanemesítésben a virágszín egyöntetűségének biztosításán túl az illóolaj tartalom növelése a cél. Az illóolaj összetételére és az egyéb tartalomanyagokra vonatkozóan napjainkban is hiányosak az ismeretek.

Hazai vonatkozásban nem ismert a növény fejlődési dinamikája, ismeretlenek a fitomassza adatok, nincs elég adat a kémiai összetétel változékonyságára. Az is tisztázásra vár, hogy fajtanemesítésnél elegendő-e az egységes fenotípus biztosítása. Kérdés, hogy a homogén virágszínnel együtt jár-e a kémiai összetétel állandósága és ily módon kiküszöbölhetők-e az illóolaj minőségi összetételében megnyilvánuló különbségek.

Jelen közleményünkben beszámolunk a növény egyedfejlődése során mért fitomassza adatokról, egyes, a növény jellemzésére alkalmas nem illó komponensek – így a rozmaringsav és kávésav – mennyiségi változásainak nyomon követéséről. Tanulmányoztuk az illóolaj-összetételt a származási hely és gyűjtési idő függvényében, valamint az egyedszelekcióval nyert utódok illóolaj-összetételét vizsgáltuk, figyelembe véve a Magyar Szabvány és a Nemzetközi Minőségi Tanúsítvány (ISO) vonatkozó előírásait. Kidolgoztuk a vizsgálatokhoz szükséges metodikát.

Kísérleti rész

Anyagok

Kísérleteink vizsgálati anyagát külföldi botanikus kertekből nemzetközi magcsere útján beszerzett és Magyarországon felnevelt *Hyssopus officinalis ssp. officinalis* képezte. A növényi anyagot az MTA Ökológiai és Botanikai Kutatóintézete (Vácrátót), valamint a Kertészeti és Élelmiszeripari Egyetem Gyógynövénytermesztési Tanszéke (Budapest) biztosította.

Különböző korú illetve újravetett állományokból származó szárított, valamint nyers növényi anyaggal dolgoztunk.

I. táblázat

Vácrátóti izsóp populációk eredete és vetési ideje

Származási hely (jelölés)
St. Gallen (I*)
Zürich (II*)
Frankfurt (III*)
Latvia (IV*)
Halle (V*)
Montreal (VI*)
Antwerpen (VII**)
Salaspils (VIII**)
Wroclaw (IX**)

*vetési idő 1991. 04. 10.

** vetési idő 1993. 11. 11.

Módszerek

A fitomasszára vonatkozó méréseket 1991-ben vetett illetve 1993-ban újravetett állományokkal végeztük. A sorozatméréseket két éven át (1994–95) folytattuk májustól októberig. Meghatározott időpontokban 5–5 hajtást gyűjtöttük és mértük a hajtások hosszát. Ezt követően a begyűjtött anyagot 35–40 °C-on légszáraz állapotig szárítottuk. Vizsgáltuk a különböző állományok közötti különbségeket, meghatároztuk a levél és generatív szervek, szárhozam, valamint az összhajtás tömegét.

A nem illó komponensek ellenőrzését TLC-denzitometriás módszerrel végeztük [13, 14]. A kivonat készítéséhez minden esetben 0,5–0,5 g légszáraz, frissen őrölt (IV) hajtásdrogot használtunk. A kivonás megkezdése előtt az egyes mintákat 5 ml petroléterrel rázatva előtisztítottuk, ezután 5 ml 70%-os MeOH-lal, kémcsőben 10 percen át tartó ultrahangos keveréssel (SONOMATIC® 375, 40 kHz) szobahőmérsékleten kivontuk. A kivonatot szűrőpapíron szűrtük.

Állófázis: TLC-Szilikagél 60 (Merck).

Mintafelvitel: 20 és 40 µl (25-A-RN pipetta).

Összehasonlító anyagok: 1, 2, 5, 7, 10 µl 0,05%-os rozmaringsav oldat, illetve 0,01%-os kávésav oldat.

Mozgófázis: toluol–etilacetát–hangyasav (5 + 4 + 1)

Kamra: Camag, telítés 30 perc.

Fronttávolság: 9,5 cm.

A denzitometriás mérések előtt a kromatogramokat 10 percig sötét helyen tartottuk. A mérés a kifejlesztés irányában történt.

Mérés: SHIMADZU CS-9301 PC denzitométerrel 325 nm-en, fluoreszcenciás üzemmódban az I-es szűrő alkalmazásával.

Az illóolaj kinyerése nyers, illetve száraz állapotban lévő hajtásokból történt vízgőzdesztillációval és szuperkritikus szén-dioxid extrakcióval.

A vízgőzdesztillációt a VII. Magyar Gyógyszerkönyv szerint végeztük, valamint hexán zárófázis (0,4 ml, HPLC tisztaság) alkalmazásával, mindkét esetben 30 g drogból kiindulva 500 ml vízzel 2 órán keresztül. A zárófázis nélkül nyert, illetve a hexánba desztillált illóolajat analizáltuk.

A szuperkritikus szén-dioxid extrakció esetén az extraktumot homogenizált, V. szita finomságúra őrölt hajtásdrogból 40 °C, 100 bar nyomáson, 30 perc extrakciós idő mellett nyertük.

A laboratóriumi szuperkritikus szén-dioxid extraktor típusa: Isco SFX 2–10 (max. extrakciós nyomás 510 bar, max. hőmérséklet 110 °C), melyhez Model 260 típusú pumpaegység (űrtartalma: 266 ml), egy vezérlőegység és egy analitikai tisztaságú szén-dioxidot tartalmazó palack kapcsolódik. Az extrakció konstans nyomás és változó, de átlagosan 0,6 ml/min. mintánkénti áramlási sebesség mellett folyt. A kivonatot mintánként 4 ml hexánban fogtuk fel.

Az illóolajjal végzett analitikai vizsgálatokat gázkromatográfiás módszerrel (GC) HP 8590 SERIES II (FID detektor) készülék segítségével végeztük az alábbi paraméterek mellett: injekciós blokk hőmérséklete: 250 °C; kolonna: HP-5 30 m x 0,35 mm x 0,25 m; hőmérsékleti program: 60 °C/2 min – 5 °C/min – 220 °C/2 min; vivőgáz analitikai tisztaságú nitrogén.

A tömegspektroszkópiával kombinált gázkromatográfiás mérés (GC-MS) FINNIGAN GCQ tömegspektrométer segítségével történt, melynek körülményei a fentiekkel mindenben megegyeztek, a vivőgáz kivételével, amely ebben az esetben analitikai tisztaságú hélium volt.

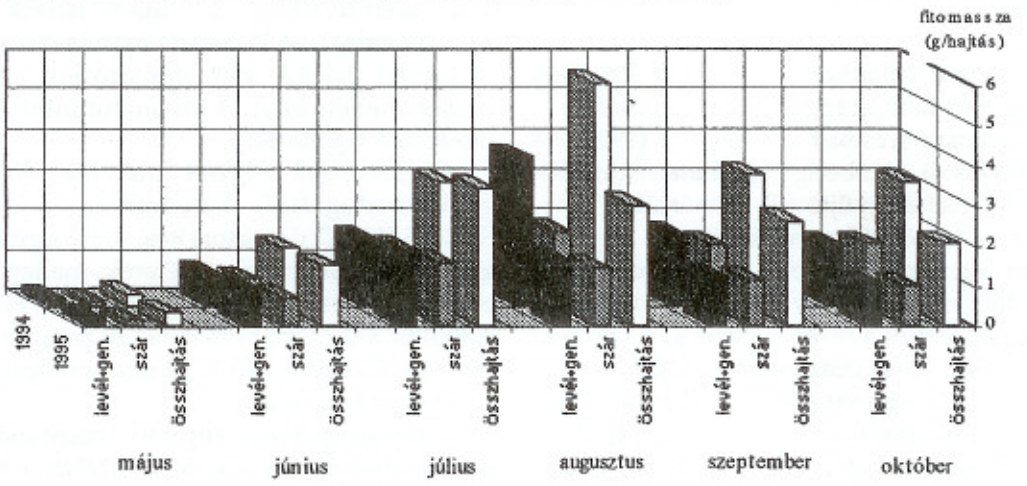
Egyedszelekciós vizsgálatokhoz az egyes populációkból növekedési erély, kék virágszín és illóolajhozam alapján kijelölt anyatöveket és többszörösen szelektált és továbbszaporított utódaikat, összesen 18 törzs 25–25 egyedét tanulmányoztuk.

Eredmények és értékelésük

A növény fejlődési dinamikáját két éven át követve részletekbe menő adatokat szolgáltatunk a hajtáshosszra és a fitomasszára vonatkozóan *(II. táblázat, 1. ábra)*.

A különböző állományok között lényeges eltéréseket nem tapasztaltunk, a növények hazai fejlődése a mag eredetétől függetlenül egyenletes volt. A gyűjtési időpontok megválasztásánál figyelembe vettük a növények fenológiai állapotát. A korai és a teljes virágzás alatt (július második fele és augusztus első fele között) mértük a maximum értékeket. Az összhajtás és a szervek tömege folyamatosan csökkent a terméképződés során. Ez összefüggésbe hozható a hajtások öregedésével, lepusztulásával. A tendencia mindkét évben megegyezett, azzal a különbséggel, hogy a mérés első évében (1994) a hozam magasabb volt. Ennek magyarázata feltehetően környezeti tényezőkre (hőmérséklet, fény, csapadék stb.) vezethető vissza. Maximális nyersanyaghozam a korai és a teljes virágzás időszakában végzett betakarítással biztosítható, mely hazai körülmények között július közepétől augusztus közepéig tart.

A növény analitikai vizsgálatát az irodalomból már ismert tartalomanyagokra alapoztuk. A ve-



1. ábra. Izsóp fitomassza változása a gyűjtési idő függvényében

II. táblázat

Hajtáshossz és fitomassza változások a gyűjtési idő függvényében

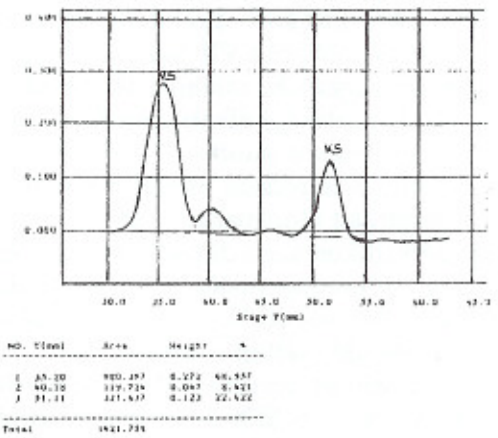
Gyűjtési időpontok (hónap)	hajtáshossz (cm)		fitomassza (g/hajtás)					
			Levél +generatív sz.		szár		összhajtás	
	1994	1995	1994	1995	1994	1995	1994	1995
05. (hajtás)	12,6	19,5	0,2	0,2	0,1	0,12	0,30	0,32
06. (bimbós)	18,8	24,2	0,7	0,88	0,58	0,69	1,28	1,48
07. (korai virágzás)	49,5	60,7	1,66	1,77	1,42	1,56	3,08	3,42
08. (teljes virágzás)	53,7	60,2	3,68	1,60	1,84	1,42	5,52	2,98
09. (korai termés képz.)	53,9	60,5	1,74	1,23	1,50	1,11	3,24	2,59
10. (termés képződés)	53,2	59,9	1,50	1,05	1,54	0,98	3,06	2,03

gyületek egyik csoportját az izsóp nem illó komponensei, másik csoportját az illóolajban lévő összetevők képezték.

A nem illó komponensek közül azok jöhettek számításba, amelyek a drog minősítésekor sorozatvizsgálatokhoz felhasználhatók. Erre alkalmas vegyületnek bizonyult a kávésav és a rozmaringsav. A két vegyület változó mennyiséggel valamennyi vizsgált mintában megtalálható, és az egyéb nem illó anyagok mellett a kivonatok koncentrálása nélkül vizsgálható. A rozmaringsav mennyisége (max. 0,24%) többszöröse a kávésavénak (max. 0,01%) *(2. ábra)*.

A rozmaringsav és a kávésav előfordulása a növényben nemcsak analitikai szempontból érdemel figyelmet. A vegyületekkel végzett farmakológiai vizsgálatok eredményei alapján a növény eddigi gyógyászati hasznosítása mellett további terápiás lehetőségek is felvetődnek.

Az izsóp gyógyászati és egyéb irányú felhasználása elsősorban a növény illóolajtartalmával függ össze. Az illóolaj összetételének tanulmányozása munkánk során is fokozott hangsúlyt kapott. A vonatkozó irodalom és saját tapasztalataink szerint az illóolaj nagy számú komponenst tartalmaz, de a legtöbb esetben kiemelhető közülük néhány számottevő mennyiségben előforduló vegyület [15]. Mintáink döntő többségében fő komponens a



2. ábra. Antwerpeni (VII.) minta kivonatának denzitogramja (1995. júliusi gyűjtés) (RS: rozmaringsav; KS: kávésav)

β-pinén (**a**), pinokámfon (**c**) és izopinokámfon (**d**) volt. Kiemelt vegyületnek tekintettük még a limonént (**b**), amely változó mennyiséggel csaknem mindegyik illóolajban előfordult *(3. ábra)*.

A Magyar Szabvány az izsóp és illóolaja fő komponenseiként a β-pinént, pinokámfont és izopinokámfont jelöli meg. Az ISO előiratának megfelelő izsópolaj pinokámfon és izopinokámfon tartalma együttesen 50%. Az illóolajösszetétel vizsgálatánál ezeket a minősítési szempontokat tekintettük irányadónak, a kiemelt négy vegyület összmennyiségét 100%-nak véve, egymáshoz viszonyított arányukat határoztuk meg.

A különböző eredetű magból Magyarországon felnevelt növényeknek csak egy része felelt meg az illóolajra vonatkozó hazai és nemzetközi elvárásoknak. Az általunk követett vegyületek egymáshoz viszonyított aránya is igen változatos volt, függetlenül a növény származási helyétől. Figyelemre méltó, hogy a vizsgált illóolaj minták egyike fő komponensként tartalmazta a limonént.

Az azonos eredetű állományok illóolaj összetételének különbsége és a növény gyűjtésének időpontja között nem találtunk összefüggést. A legnagyobb eltéréseket a virágzást megelőző és a virágzást követő időszakban észleltük, amikor az illóolajtartalom a növényben 0,1% alatt volt. Az eltérő eredetű, azonos időben gyűjtött, alacsony illóolaj tartalmú mintákat ugyanez a változékonyság jellemzi *(4. ábra)*.

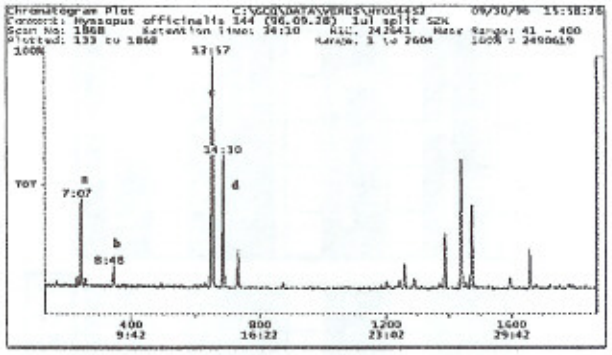
III. táblázat

Vízgőzdesztillációhoz és szuperkritikus extrakcióhoz felhasznált izsóp minták

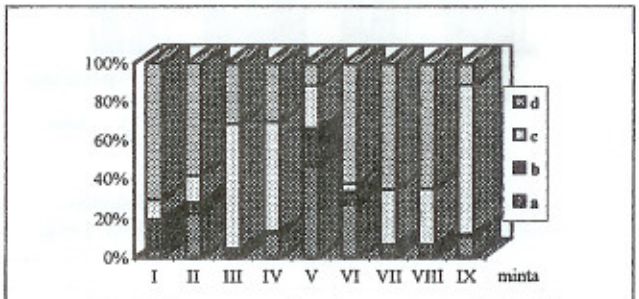
Minta jele*	Fenofázis	Gyűjtés ideje
I	hajtás	06. 15.
I	bimbós	06. 21.
II	bimbós	06. 21.
II	korai virágzás	07. 03.
III	bimbós	06. 21.
III	korai virágzás	07. 03.
III	teljes virágzás	07. 27.
VIII	termés képződés	09. 21.
IX	termés képződés	09. 21

*minták jelölésének magyarázata az I. táblázatban

Ugyanazon drogból vett minták *(III. táblázat)* különböző módszerrel – vízgőzdesztillációval, szuperkritikus szén-dioxid extrakcióval – nyert illóolajának összehasonlító minőségi analízise fontos felismerést eredményezett. Összefüggést találtunk a növény illóolajtartalma, a kinyerésre alkalmazott módszer használhatósága, valamint az illóolaj összetétele között. Magas illóolajtartalom esetén (07. 27.) az alkalmazott kinyerési módszertől független az illóolajösszetétel *(5. ábra)*.



3. ábra. Hyssopus officinalis L. illóolajának (hallei minta) totál ion kromatogarmja



4. ábra. Fő komponensek aránya az izsóp illóolajában (különböző eredetű októberi minták)

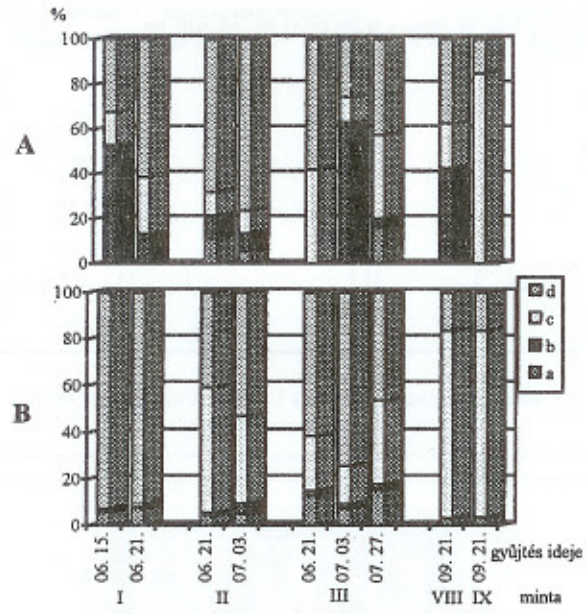
Alacsony illóolajtartalmú mintáknál azonban csak a hexán segédfázis alkalmazásával végzett vízgőzdesztilláció, valamint a szuperkritikus szén-dioxid extrakció biztosított azonos összetételt és szolgáltatott értékelhető adatokat *(6. ábra)*.

Egyedszelekciós vizsgálataink azt bizonyították, hogy a külsőleges bélyegekre, valamint az illóolaj hozamra történő szelekció nem biztosít homogén illóolajösszetételt. Az egységes kék virágszínre és illóolajtartalomra történő szelekció esetén is megjelentek főkomponensként pinokámfont, izopinokámfont, pinokámfont és izopinokámfont, valamint egyéb anyagokat (pl. limonént) tartalmazó populációk. Az utódokban is tapasztalt kémiai heterogenitás *(7. ábra)* arra hívja fel a figyelmet, hogy a szelekció során a párhuzamos kémiai kontroll elengedhetetlenül szükséges.

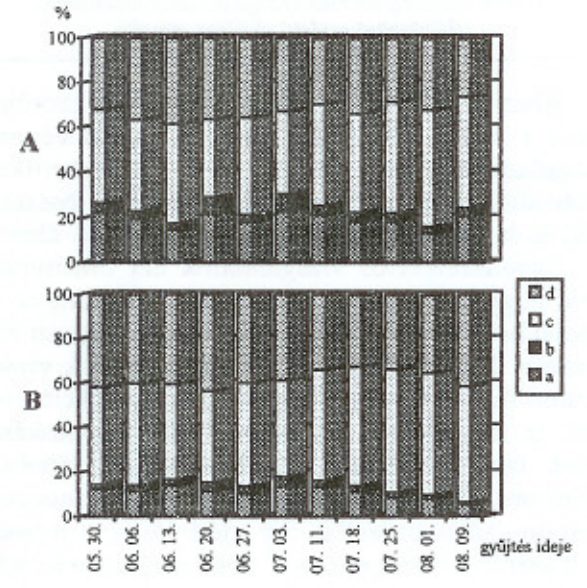
Jelen eredményeink felhasználásával tovább folytatjuk egyedszelekciós vizsgálatainkat.

Köszönetnyilvánítás

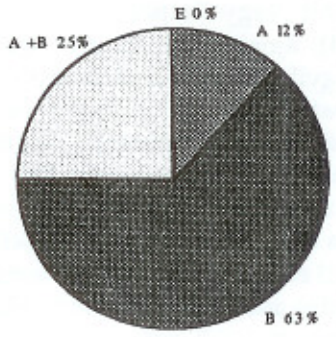
A cikkben szereplő kísérleti munka a T 026098 számú tematikus OTKA pályázat támogatásával készült.



5. ábra Fő komponensek aránya az izsóp illóolajában (A: vízgőzdesztilláció B: szuperkritikus extrakció, 1994)



6. ábra. Frankfurti (III.) mintából nyert illóolaj összetételének követése a vegetációs periódus folyamán (A: vízgőzdesztilláció hexán zárófázissal, B: szuperkritikus extrakció, 1995)



7. ábra. Egyedek megoszlása a 10/6/15-ös törzs utódai között Fő komponensek aránya az utódokban

A: izopinokámfon a fő komponens 50% az illóolajban
B: pinokámfon a fő komponens 50% az illóolajban
A+B: mindkét fenti komponens 20–50%-ban van jelen az illóolajban
E: egyéb komponens jelenik meg számottevő mennyiségben (30% az illóolajban)

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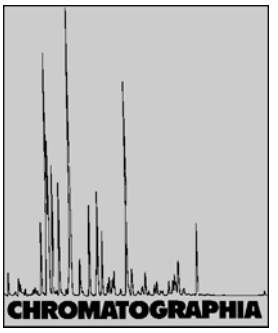
Investigation of the Composition and Stability of the Essential Oils of *Origanum vulgare* ssp. *vulgare* L. and *O. vulgare* ssp. *hirtum* (Link) Ietswaart*



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Key Words

Gas chromatography
GC-MS
Essential oil
Origanum

Summary

The essential oils of *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart and *Origanum vulgare* L ssp. *vulgare* (Fam. Lamiaceae), cultivated in Hungary, have been studied by GC and GC-MS and the qualitative and quantitative chemical composition of the essential oils in the two species have been compared. *O. vulgare* ssp. *hirtum* oil was found to contain carvacrol (76.4%), γ-terpinene (6.6%), thymol (0.23%), and *p*-cymene (4.7%) as the main constituents whereas the major compounds in *O. vulgare* ssp. *vulgare* oil were *p*-cymene (22.3%), caryophyllene oxide (10.2%), sabinene (7.9%), γ-terpinene (5.1%), thymol (0.34%), and spathulenol (4.8%). The stability of content and composition of the oils during the flowering period (economically beneficial period) were observed. The effect of long-term storage on the composition of the oil was also investigated for both the crude and distilled oil of *Origanum vulgare* ssp. *vulgare*.

Introduction

Origanum is used throughout the world as a very popular spice, under the vernacular name oregano. Although the name 'oregano' is given to many species of a variety of genera, most oregano spice products originate from species of the genus *Origanum* (Family Lamiaceae). This species is of great economic importance, not only as a spice but also in many other ways, because its essential oils have antimicrobial, cytotoxic, and antioxidant activity [1–4]. These essential oil-containing species are also used in folk medicine in many countries.

The genus *Origanum* is very diverse both morphologically and chemically and many transitional forms occur worldwide. Until 1980 there was no satisfactory classification of the genus, but since then the Ietswaart system has become widely accepted [5]. This postulates that *Origanum vulgare* L. has six subspecies with differences in the indumentum, the number of sessile glands on the leaves, bracts, and calyces, and the size and colour of the bracts and flowers. The most widely used and consumed subspecies *Origanum vulgare* ssp. *vulgare* L., is native throughout Europe, Iran, India, and China and *Origanum vulgare* ssp. *hirtum* (Link) Ietswaart is indigenous to Albania, Greece, and Turkey [5], but alien in Central European Hungary. Ecologically these species prefer warm, sunny habitats and loose, often rocky, calcareous soils, low in moisture content.

Our work has focused on qualitative and quantitative investigation of the essential oils of the *O. vulgare* ssp. *vulgare* and *O. vulgare* ssp. *hirtum* populations cultivated in Hungary under Central European (continental) climatic conditions. It is a part of a series of wide-ranging investigations into *O. vulgare* ssp. *vulgare* and *O. vulgare* ssp. *hirtum* conducted in Hungary for several years [6]. The main question to be answered here was how well the quality of the crops of the two subspecies, growing under relatively strange environmental conditions in Hungary could be characterized by GC and GC-MS analysis of their essential oils.

Experimental

The plant material was gathered from perennial populations of *Origanum vulgare* ssp. *vulgare* L. and *Origanum vulgare* ssp. *hirtum* (Link) Ietswaart cultivated in the Experimental Station of the 'Szent István' University, Budapest. The essential oil content was investigated throughout the generative phases of the vegetative period

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Table I. Composition of the essential oils of the subspecies examined (oils were distilled from flowering plants).

RI*	RI**	Compound	*Origanum vulgare* ssp. *hirtum* (%)	*Origanum vulgare* ssp. *vulgare* (%)
931	926–930	α-Thujene	0.86	0.40
939	935–938	α-Pinene	0.72	0.31
953	950–951	Camphene	0.27	0.04
976	973–974	Sabinene	–	7.93
980	978–980	β-Pinene	0.69	0.45
991	987–989	β-Myrcene	1.73	0.61
1018	1015–1018	α-Terpinene	1.24	0.63
1026	1024–1025	*p*-Cymene	4.67	22.31
1031	1030–1033	Limonene	0.39	0.62
1040	1037–1038	*cis*-Ocimene	–	3.76
1050	1047–1049	*trans*-Ocimene	–	3.56
1062	1058–1060	γ-Terpinene	6.63	5.11
1098	1097–1102	Linalool	–	0.28
1160	1161–1162	Pinocamphone	–	0.18
1167	1168–1169	Borneol	0.57	–
1173	1174–1175	Isopinocamphone	–	1.32
1177	1178–1180	Terpin-4-ol	0.49	0.44
1189	1187–1190	α-Terpineol	–	0.83
	1285–1289	Unknown	–	0.13
1290	1291–1293	Thymol	0.30	0.34
1298	1297–1302	Carvacrol	76.44	–
1384	1380–1384	β-Bourbonene	–	1.44
1418	1417–1420	β-Caryophyllene	1.60	4.09
1480	1477–1480	Germacrene-D	–	4.18
1576	1576	Spathulenol	–	4.86
1581	1579–1582	Caryophyllene oxide	–	10.26

* Kovats retention indices according to published data [8]. ** Kovats retention indices measured in this work.

of both species from May to August in 2000. The flowering shoots were detached and the dried in air (approx. 2 weeks at room temperature); the plant material (30 g) was then ground and steam distilled for 2 h in accordance with Ph. Hg. VII [7]. The composition of the oil obtained was studied by GC and GC-MS.

GC was performed with an HP 5890 Series II gas chromatograph (FID) equipped with a 30 m × 0.35 mm × 0.25 μm HP-5 fused silica capillary column. The oven temperature was programmed at 3° min^{-1} from 60 to 210 °C and then at 5° min^{-1} to 250 °C which was held for 2 min. The detector and injector temperatures were 250 °C, the carrier gas nitrogen, and split sample injection was used.

GC-MS was performed with a Finnigan GCQ ion-trap bench-top mass spectrometer. All conditions were as for GC except the carrier gas was He at a linear velocity at 31.9 cm s^{-1} and compounds were separated on a 30 m × 0.25 m × 0.25 μm DB-5 MS column. Positive-ion electron impact ionization was used; the mass range was 40–400 amu. The constituents were identified by comparing their Kovats indices with those of authentic reference compounds (Table I), by comparison of their mass spectra with published MS data [8], and by computer library search (database supplied with the instrument). The equivalence was always >85%. The reproducibility of GC measurements is illustrated in Table II, in which the Kovats indices of some oregano oil components are listed. Kovats indices were calculated by use of the equation published by Kovats [9]. Coefficients of variation were never outside the range CV% = 0.04–0.24. The identities of compounds in samples (listed in Table II) were also confirmed by observing the increase in the sizes of particular peaks in the chromatograms on addition of authentic compounds to the samples.

For stability studies the air-dried plant material was stored at room temperature; the oil distilled from it was stored at −18 °C; each was stored for one year. For reference, analytical data obtained from freshly distilled oil from the same oregano population are given in Table III. The oil was obtained from an air-dried sample taken from the same population but distilled within two weeks of collection of the plant.

Solvents and other chemicals used were analytical grade.

Results and Discussion

Our studies revealed significant differences between the essential oil content of the two subspecies. The oil content of *O. vulgare* ssp. *vulgare* was much lower (max. 0.2%) than that of *O. vulgare* ssp. *hirtum* (max. 4.3%). Similar results were obtained from *Origanum* populations growing in their natural Mediterranean habitats in Greece [10].

The essential oil content of *Origanum vulgare* ssp. *hirtum* in the pre-flowering vegetative and flowering stages was high (2.4–4.3%); during seed ripening it decreased significantly (to 1.1%). No developmental stage-dependent variation was observed for *O. vulgare* ssp. *vulgare* from the late vegetative to the post-flowering periods. It was, therefore, established that during the flowering, economically important, period the oil content of the two subspecies was markedly quantitatively different. During this period the oil content of both subspecies remained quite stable but at a level an order of magnitude lower in *O. vulgare* ssp. *vulgare* than in *O. vulgare* ssp. *hirtum*. Consequently, the samples gathered from the same popula-

Table II. Examples of the reproducibility of measured Kovats retention indices (RI) in GC.

Compounds	Individual tests			Distilled oils		Mean (n = 5)	± s
	1st injection	2nd injection	3rd injection	Sample 1	Sample 2		
β-Pinene	980	978	978	980	980	979	1.1
Limonene	1031	1030	1030	1033	1033	1031	1.5
Linalool	1097	1098	1097	1102	1102	1099	2.6
Borneol	1168	1168	1168	1169	1169	1168	0.5
Carvacrol	1299	1297	1302	1299	1299	1299	1.4
β-Caryophyllene	1419	1417	1418	1420	1420	1419	1.3

tions of each species at slightly different times of the flowering period could statistically be regarded as duplicates. The composition of the essential oils was determined by GC and GC-MS.

Table I shows the Kovats indices and the ratios (percentages of total volatile fractions) of the components. Kovats indices were calculated mainly on the basis of GC-MS analysis [9] and were compared with published data [8]. Identification of the compounds was confirmed by chromatography of authentic samples of thymol, β-pinene, limonene, linalool, and β-caryophyllene, and comparison of mass spectra with those in databases.

Table I includes only those components present at a level of 0.01%, or more, of the total oil content. In *Origanum vulgare* ssp. *vulgare* oil 23 of 26 components from the two subspecies could be identified, whereas in *O. vulgare* ssp *hirtum* oil only 14 were identified. Thirteen components were found in one of the two subspecies only.

The main components of the oils were different for the two subspecies. *O. vulgare* ssp. *hirtum* oil contained carvacrol (76.4%), γ-terpinene (6.6%), and *p*-cymene (4.7%) as main constituents whereas the major compounds in *O. vulgare* ssp. *vulgare* oil were *p*-cymene (22.3%), caryophyllene oxide (10.2%), sabinene (7.9%), γ-terpinene (5.1%), and spathulenol (4.8%); no carvacrol was found. Differences between the minor components from the two subspecies were also found (Table I); this means that they differ not only in their oil content but in their chemical character also, although the main constituents of both subspecies are aromatic compounds – carvacrol and *p*-cymene, respectively.

Variations in the composition of the oils are apparent from the average values and their variances. For the main components the variance was less than 10%, which is indicative of rather stable composition. Because the samples were harvested at different times, this stability also reflects the stability of the composition of the oil throughout the flowering period. We were interested in the effect of the type of storage on the composition of the oil of *O. vulgare* ssp. *vulgare*. The plant and its essential oil were stored for one year and thereafter another oil sample was distilled from the plant and the fresh and stored oils were compared. Table III shows there were relatively few differences between the sesquiterpene fractions of the freshly distilled and the stored oils, although the monoterpene content has changed markedly (Table III and Figure 1). These changes mean that the characteristic components of the distilled oil, e.g. *p*-cymene, α-terpinene and others, decreased in concentration (to less than 0.01%) or disappeared from the oil. This implies that distillation of the plant close to the time of utilization is preferable if essential oils of natural composition are required.

To summarize the results of our experiments, we conclude that the two subspecies can be well-characterized by their essential oil content. The oil content of *O. vulgare* ssp. *hirtum* is significantly higher than that of *O. vulgare* ssp. *vulgare*. The oil composition reflects the genetically fixed chemical character of the plants. For plants grown in Hungary and crops harvested during the generative stage of development, only one oil analysis of each subspecies seems to be sufficient to make a rather good estimate of the expected oil content ($CV\%$ = 0.11 or 0.25 respectively) and composition. Because the character of the oils of the two subspecies was different throughout the flowering period a single measurement should be sufficient to distinguish between the subspecies. Comparison with the differences observed in Greece, where both subspecies are native, showed that chemical differences between the two subspecies have not changed under Hungarian environmental conditions.

Gas chromatograms obtained on DB-5 or HP-5 columns (Agilent Technologies), GC-MS data (Finnigan GCQ), and Kovats indices also proved very suitable for determination of the composition of *Origanum* oils. In routine measurements retention times for the main components can also furnish comparable and reproducible results. These results will be used in our continuing selection work on oregano.

Table III. Comparison of samples of *Origanum vulgare* ssp. *vulgare* oil.

RI*	RI**	Compound	A (%)	B (%)	C (%)
931	926– 930	α-Thujene	0.28	0.82	–
939	935– 938	α-Pinene	0.28	1.77	–
953	950– 951	Camphene	0.09	0.30	–
976	973– 974	Sabinene	4.33	5.14	0.69
980	978– 98	β-Pinene	0.60	1.03	1.90
991	987– 989	β-Myrcene	0.94	0.49	0.29
1018	1015–1018	α-Terpinene	0.90	0.78	–
1026	1024–1025	*p*-Cymene	12.41	25.33	6.62
1031	1030–1033	Limonene	0.55	0.59	0.45
1040	1037–1038	*cis*-Ocimene	6.27	0.71	0.24
1050	1047–1049	*trans*-Ocimene	6.88	1.51	0.10
1062	1058–1060	γ-Terpinene	10.18	4.53	–
1098	1097–1102	Linalool	0.36	0.60	0.80
1160	1161–1162	Pinocamphone	0.22	–	2.19
1167	1168–1169	Borneol	–	0.28	0.37
1173	1174–1175	Isopinocamphone	0.86	–	4.23
1177	1178–1180	Terpin-4-ol	0.15	0.81	1.24
1189	1187–1190	α-Terpineol	0.63	0.68	1.18
1242	1238–1241	Carvacrol methyl ether	–	0.32	0.41
	1285–1289	Unknown	1.59	–	–
1290	1291–1293	Thymol	0.34	0.90	2.14
1298	1297–1302	Carvacrol	–	0.12	0.13
1384	1380–1384	β-Bourbonene	1.60	1.75	3.07
1418	1417–1420	β-Caryophyllene	5.28	2.53	5.62
1480	1477–1480	Germacrene-D	7.79	2.16	1.23
1576	1576	Spathulenol	2.69	5.98	7.06
1581	1579–1582	Caryophyllene oxide	5.53	12.83	14.30

* Kovats retention indices according to published data [8]. ** Kovats retention indices measured in this work. A: Essential oil distilled from air-dried plant. B: Essential oil distilled from dried plants stored for one year. C: Essential oil stored for one year at –18 °C.

Acknowledgements

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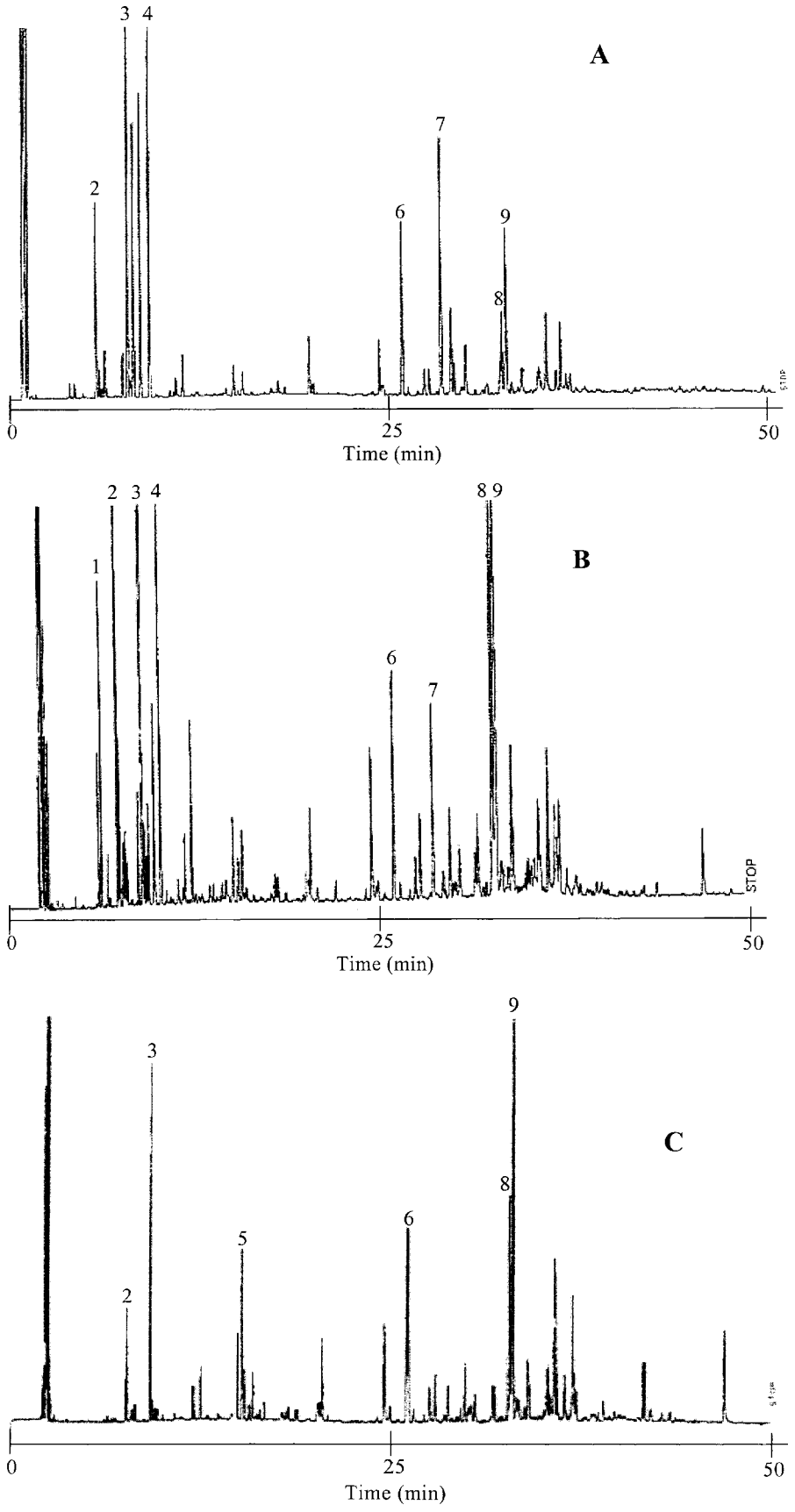

Figure 1. Gas chromatogram obtained from *Origanum vulgare* ssp. *vulgare* oil: **A**. *Origanum vulgare* ssp. *vulgare* oil distilled from air dried plant; **B**. *Origanum vulgare* ssp. *vulgare* oil of distilled from dried plants stored for one year. **C**. *Origanum vulgare* ssp. *vulgare* oil stored for one year at –18 °C. Peaks: **1** = *a*-pinene, **2** = sabinene, **3** = *p*-cymene, **4** = γ-terpinene, **5** = isopinocamphone, **6** = β-caryophyllene, **7** = germacrene-D, **8** = spathulenol, **9** = caryophyllene oxide.

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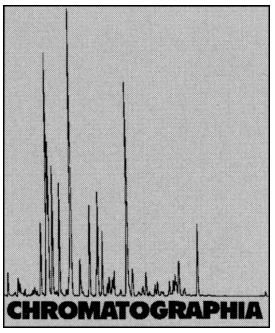
Gas Chromatographic Method for Routine Determination of Oleanolic and Ursolic Acids in Medicinal Plants



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Key Words

Gas chromatography
Solid-phase extraction
Derivatization
Oleanolic and ursolic acid
Medicinal plants

Summary

A rapid and simple gas chromatographic method has been established for routine analysis of free oleanolic and ursolic acids in dried samples of medicinal herbs. Soxhlet extraction of triterpenes was followed by solid-phase extraction (SPE). Amounts of the compounds were measured by gas chromatography after silylation of the purified samples. Experiments were performed to establish the optimum conditions (e.g. solvent, and mode and duration of extraction) for calibration curve linearity, sensitivity, reproducibility, and recovery. The conditions used for derivatization and gas chromatographic analysis resulted in an improvement on literature data. The method devised enables accurate routine measurement of many samples in quite a short time (e.g. for chemotaxonomical screening, or quality control of herbal drugs). The practical application of the method was illustrated on five *Lamiaceae* species.

Introduction

Oleanolic acid (OA) and ursolic acid (UA) (Figure 1) occur widely in the plant kingdom in the form of free acids or aglycones of triterpenoid saponins. These compounds have numerous similarities in chemical and pharmacological character. Their main medicinal properties are reported to include hepatoprotective, anti-inflammatory, antitumour, adaptogenic, and antihyperlipidaemic effects [1].

Surprisingly, the literature contains relatively little information on the distribution of these triterpenic acids (TA) in the plant kingdom, despite their wide-ranging and pharmacological importance. The lack of a simple and accurate routine method seems an obvious reason. It seems inconsistent that publications dealing with quantification of OA and UA cover a wide spectrum of analytical methods [2–7]. Because the subjects of the investigations were generally a few species only, earlier authors have not concentrated on introduction of simple and rapid methods [8, 9].

Many publications report the isolation of OA and/or UA from plant material [10–12]. Such data cannot characterize exactly the amounts of active components in the plants because of losses that might occur during long isolation procedures.

Gas chromatography (GC) was the first method enabling parallel determination of OA and UA in samples derivatized by methylation, acetylation [13], or silylation [4, 13–15]. There are many examples of similar investigations of plants containing either OA or UA alone [8, 16–20].

In comparison with the most recent methods [6, 7, 21, 22], the lower cost of instrumentation and its general use in routine laboratory work are the main advantages of GC. On attempting to repeat the only English-language report of the successful partition of OA and UA [5], we achieved only partial separation. Accordingly, we chose GC because of its precision, high speed, and ability to achieve baseline separation of the two TA. When many samples are to be analysed these are essential requirements.

Methods described in articles dealing with a similar problem could be adopted only with limitations, because of complicated extraction and purification procedures [4, 13, 14] or the different samples studied (e.g. olive oil) [15]. For this reason, special efforts were made to devise a procedure which ensures simple and quantitative extraction.

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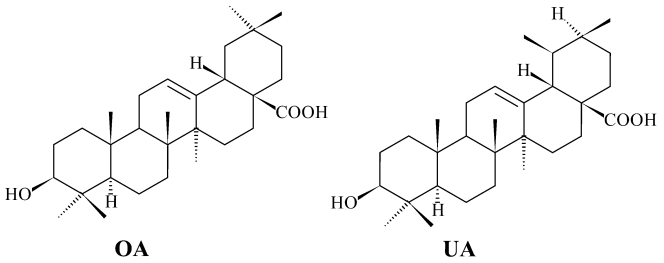

Figure 1. The chemical structures of oleanolic acid (OA) and ursolic acid (UA).

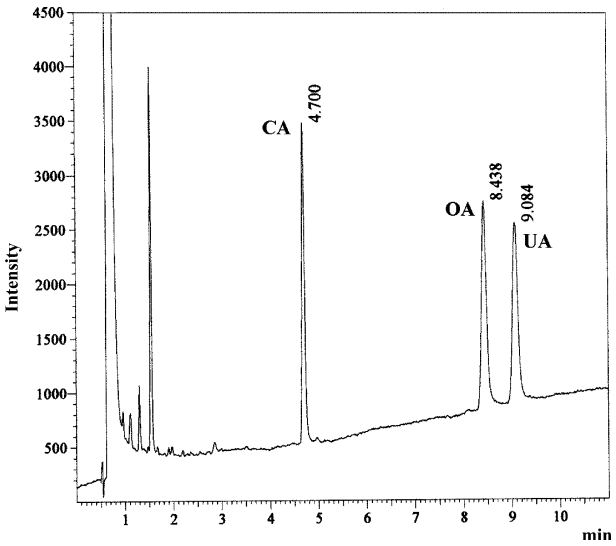

Figure 2. Gas chromatogram obtained from a mixture of authentic oleanolic acid (OA), ursolic acid (UA), and cholesteryl acetate (CA).

Experimental

The plant material (herb) was gathered in July 1999 from the experimental field of the Vácrátót Institute, dried at 30 °C, and powdered. All the organic solvents used were of analytical grade (Merck, Germany). During the optimization procedure authentic OA and UA (Roth, Germany) were used. The internal standard was cholesteryl acetate (CA; Sigma, USA). Two stock solutions of different composition were used: solution A contained 1.086 mg OA and 1.003 mg UA, and solution B contained 4.289 mg CA; both were dissolved in 10 mL methanol. SPE was performed with Chromabond cartridges containing 500 mg adsorbent (Macherey–Nagel, Germany) and a Chromabond vacuum manifold (Macherey–Nagel). For silylation the prepurified samples (0.4–0.8 mg) dried at 80 °C were dissolved in 200 μL dry pyridine (Fluka, Germany) in 1.5-mL vessels (La Pha Pack, Germany) and derivatized by adding 400 μL *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA/1% TMCS) (Supelco, USA). Aluminium-backed silica gel plates (Merck, Germany) with chloroform–methanol, 19:1, as mobile phase were used for TLC monitoring. The spots of OA/UA and CA were visualized by spraying the plates with anisaldehyde–sulfuric acid reagent and subsequent heating (105 °C, 5 min). The two TA (OA/UA) were detected together.

Gas chromatography was performed with a Shimadzu (Japan) GC-2010 instrument equipped with flame ionization detection (FID) and fitted with a 12 m × 0.15 mm × 0.25 μm BP1 column. Nitrogen was used as carrier gas at a linear velocity of 40 cm s^{-1}, head pressure 210 kPa. The volume injected was always 1.0 μL, the split ratio was 1:50, and the instrument was used in flow-control mode. The injector, detector, and column temperatures were 300 °C. These conditions, established as the first step of the optimization procedure, were used during subsequent experiments for quantification of the OA and UA content of samples.

To check the need for heating to achieve derivatization, solutions A (400 μL) and B (100 μL) were placed in vessels, solvent was removed by evaporation, and the samples were heated at room temperature for 30 min (S1) or at 80 °C for 30, 60, 120, 180, or 300 min (S2–S6, respectively).

When the optimum silylation conditions had been determined calibration plots were drawn. Five solutions were prepared, T_1 (20 μL solution A + 100 μL solution B); T_2 (100 μL solution A + 100 μL solution B); T_3 (400 μL solution A + 100 μL solution B); T_4 (750 μL solution A + 100 μL solution B); and T_5 (1000 μL solution A + 100 μL solution B), and used to determine the linear correlation ranges and the smallest detectable amount.

To study conditions for SPE, solution A (400 μL) and solution B (100 μL) were placed in measuring flasks (5 mL), the methanol was removed by evaporation at 60 °C in an oven, and the residues were re-dissolved in 5 mL 20% aqueous methanol. These solutions were transferred to Chromabond C18, Florisil, and SB cartridges previously conditioned with 5 mL methanol and 5 mL water. Each column was then washed with 5 mL water. To determine the appropriate composition for elution 5-mL portions of 40, 50, 60, 70, 80, 90, and 100% aqueous methanol were passed successively through the cartridges and the TA and CA content of the eluates was checked by TLC as described above.

Recovery from SPE was studied by applying the established clean-up procedure to three solutions prepared in the same way as described above. The amounts of OA and UA were determined by GC analysis of the dried and silylated samples.

The next stage of the work was optimization of the extraction procedure. *Melissa officinalis* L., *Salvia officinalis* L., and *Hyssopus officinalis* L. were used as model plants during these experiments. Two parallel extractions were performed for each sample.

To choose the optimum extraction solvent, benzene, ethyl acetate, acetone, pure methanol, and 70% aqueous methanol were compared. Powdered plant material (0.5 g) was extracted for 60 min with the organic solvent (45 mL) in a Promax 2020 shaker (Heidolph, Germany). The fractions were then combined and diluted to 50 mL. Portions (1 mL) of

these samples were then dried and the residues were re-dissolved in 5 mL 20% aqueous methanol. The amounts of OA and UA extracted were determined by using the optimized GC and SPE procedures described above.

The best solvent was then used to select the most effective method of extraction by comparison of results obtained use of a shaker, an ultrasonic bath, and a Soxhlet apparatus. Because shaking had already been evaluated (see above) this was not repeated – the earlier results were accepted. To test the effect of ultrasound, drug powder (0.5 g) in organic solvent (45 mL) was placed in an ultrasonic bath (Tesla, Czechoslovakia) for 60 min. The final volume was again diluted to 50 mL. Finally, plant material (1.0 g) was placed in a Soxhlet apparatus, extracted with 90 mL solvent for 1 h, and the solutions obtained were diluted to 100 mL.

To determine the optimum duration of extraction the powdered samples were extracted in a Soxhlet apparatus for 2, 3, 4, 5, 6, 7, or 8 h. (The 1-h treatment was not repeated – the results obtained earlier were accepted.)

To evaluate the repeatability of instrumental measurement of each compound gas chromatographic analysis of plant extract and a test mixture (T_3) were repeated five times. When reproducibility was studied, the overall method was considered. Samples (12×0.8 g) of powdered *Salvia officinalis* were subjected to the overall process, and the results were compared statistically.

In addition to the model plants mentioned above, the species *Marrubium vulgare* L. and *Satureja montana* L. (also belonging in the *Lamiaceae* family) were also examined by this procedure to demonstrate its practical application.

Results and Discussion

The chemicals used for silylation (pyridine and BSTFA/1% TMCS) and the internal standard (CA) were identical with those described elsewhere [14]. The pyridine is not merely a simple solvent, because it can facilitate the derivatization, similarly to TMCS. The application of CA is advantageous because it has not yet been demonstrated to occur in plants.

During selection of the GC conditions, the results of earlier reports [4, 15] were also taken into consideration. In our work, however, isothermal conditions were satisfactory, rather than a temperature programme. A gas chromatogram obtained from a mixture of authentic compounds can be seen in Figure 2.

Study of the effect of heating for different times showed that the silylation procedure needs 5 days at room temperature (sample S1), accepted by other authors [14, 15]. There are also many examples in the literature of heating of the reaction mixture [4, 17]. The results of our experiments indicated that heating at 80 °C for 2 h was sufficient (sample S4) – further increasing the reaction time did not effect the measured data (samples S5 and S6). In contrast with our expectations, the derivatized samples remained stable for as long as 1 week.

The calibration data and the limits of detection are listed in Table I. The

Table I. Performance data for the gas chromatographic method for oleanolic and ursolic acids.

	Oleanolic acid	Ursolic acid
Slope and intercept of calibration plot ($Y = CX + D$)	$C = 0.733$; $D = 0.009$	$C = 0.727$; $D = 0.011$
Number of data points	5	5
R^2 value	0.9999	0.9995
Limit of detection (ng)	2	2
SPE recovery (%)	98.90	99.50
Repeatability (C.V., %)		
Test mixture	3.49	1.55
Plant sample	4.86	1.03
Reproducibility (C.V., %)	5.52	6.48

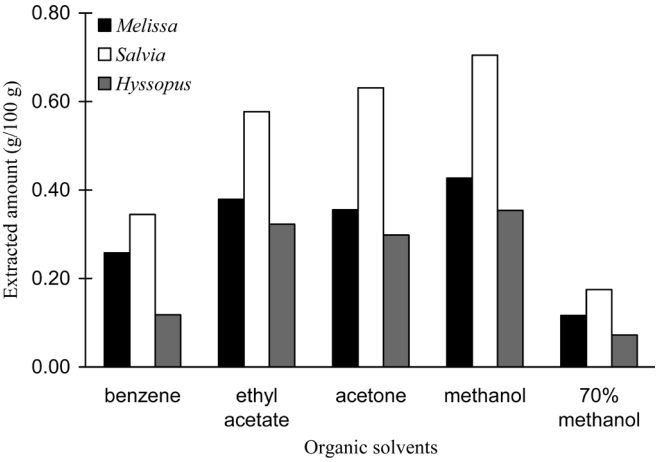

Figure 3. Amounts of oleanolic and ursolic acids extracted by different organic solvents.

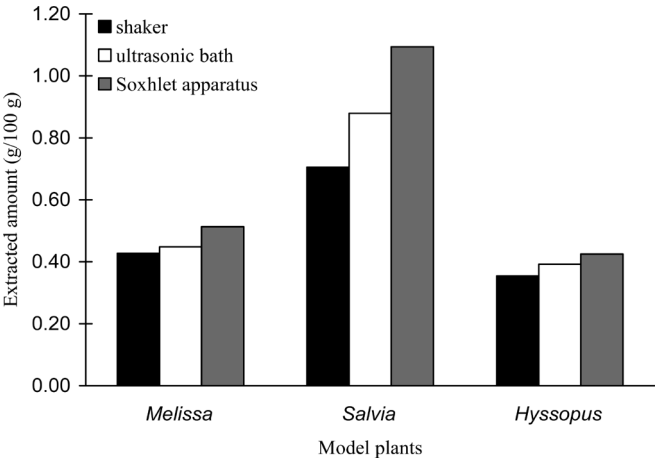

Figure 4. Amounts of oleanolic and ursolic acids extracted by use of different extraction techniques.

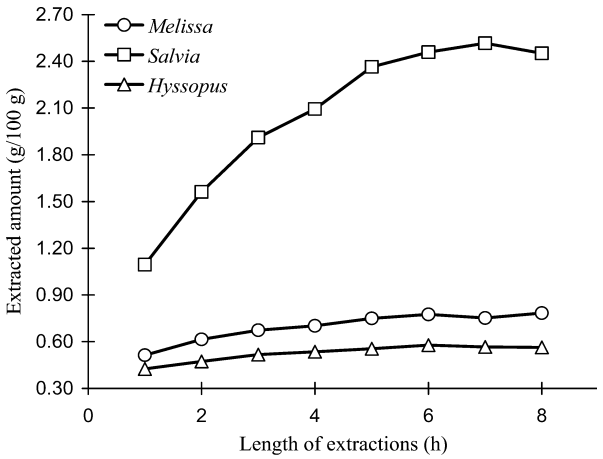

Figure 5. Amounts of oleanolic and ursolic acids extracted by use of the Soxhlet procedure for different periods of time.

Table II. Oleanolic acid (OA) and ursolic acid (UA) content of different *Lamiaceae* species, determined by use of the gas chromatographic method.

No.	*Lamiaceae* species	OA content (% dry weight)	UA content (% dry weight)
1	*Hyssopus officinalis* L.	0.143	0.407
2	*Marrubium vulgare* L.	0.016	0.032
3	*Melissa officinalis* L.	0.170	0.612
4	*Salvia officinalis* L.	0.653	1.861
5	*Satureja montana* L.	0.138	0.507

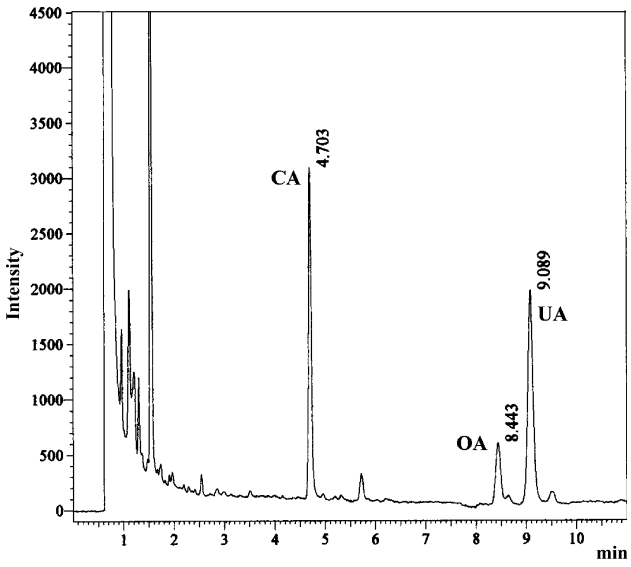

Figure 6. Gas chromatogram obtained from an extract of *Salvia officinalis* L. herb. OA = oleanolic acid, UA = ursolic acid, CA = cholesteryl acetate.

relationships between the amounts present and the peak areas measured were found to be linear in the ranges 0.0–181.0 ng for OA and 0.0–167.2 ng for UA. This was confirmed by the R^2 values. The smallest detectable quantity of the compounds was 2 ng.

Only the Chromabond SB cartridges, which contain silica modified with quaternary ammonium groups, were found suitable for SPE of OA and UA; those used for determination TA in olive oil [15] were packed with a bonded aminopropyl phase. Both adsorbents have anion exchanger character but that of Chromabond SB is much stronger. The Chromabond C18 and Florisil columns did not retain the compounds. When eluents containing increasing concentrations of methanol were tested, elution of OA and UA began at 50% aqueous methanol, so even 40% aqueous methanol can be used to wash the samples on the cartridges.

The data in Table I clearly show that the SPE recovery is > 98% for both OA and UA, a level similar to that reported in the literature [15].

Figure 3 shows that the best extraction was achieved with methanol. The results obtained for 70% aqueous methanol show that increasing the polarity further reduced the amounts of TA extracted. (For simplicity, Figures 3–5 depict the combined amounts of OA and UA.)

The different efficacies of the various extraction techniques are illustrated in Figure 4. The benefits of Soxhlet extraction are obvious. Shaking, which had been used to select the optimum extraction solvent, was the least successful technique. Soxhlet extraction for 6 h proved optimum. Figure 5 shows that extraction for longer does not enhance the yield of TA. In earlier works on the determination of free OA and UA the extraction was more complicated because of the lack of SPE [4, 13]. Hydrolysis of the glycosides also made the process more complex [8, 14, 16, 17].

Table I shows that the repeatability is better for UA than for OA in both studies. The reproducibility was satisfactory – the values for OA were between 0.673 and 0.820, and those for UA were between 1.729 and 2.122. Because biological samples were involved, the coefficients of variation were also acceptable (OA 5.52% and UA 6.48%).

The results obtained from GC determination of OA and UA in five *Lamiaceae* species are presented in Table II. Of these species, *Salvia officinalis* contained the largest amounts of both the acids studied; amounts of OA and UA were lowest in *Marrubium vulgare*. A gas chromatogram obtained from an extract of sage is depicted in Figure 6.

Acknowledgement

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Study of the oleanolic and ursolic acid contents of some species of the Lamiaceae

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Abstract

The oleanolic and ursolic acid contents from 88 taxa of Lamiaceae (19 genera, 66 species, 8 subspecies, 9 varieties and 5 hybrids) were investigated using gas chromatography. Both triterpenoid acids were present in all of the investigated taxa, but the plants belonging in the subfamily Nepetoideae produced significantly higher amounts than those in the subfamily Lamioideae. The oleanolic acid content ranged from traces to 1.840% dry weight, and that of ursolic acid from traces to 4.019% dry weight.

Keywords: Lamiaceae; Subfamily Lamioideae; Subfamily Nepetoideae; Triterpenoids; Oleanolic acid; Ursolic acid; Chemotaxonomy; Gas chromatography

1. Introduction

Oleanolic acid (OA) and ursolic acid (UA) are common constituents of plants. These two triterpenes may occur as aglycones of saponins and as free acids. Reports on their wide-ranging occurrence in one of the most important groups of medicinal plants, the family Lamiaceae, usually also describe the isolation of free OA and UA besides other compounds (Mendes et al., 1989; Bruno and Ciriminna, 1993; Kuo et al., 2000; Ryu et al., 2000; Tezuka et al., 2000; Tan et al., 2002). Both OA and UA have many important pharmacological effects, which are rather similar because of the closeness of their chemical structures. The literature furnishes numerous data on their anti-inflammatory (Safayhi and Sailer, 1997; Baricevic et al., 2001), hepatoprotective (Liu et al., 1995), antitumour (Ovesna et al., 2004), anti-HIV (Kashiwada et al., 1998), antimicrobial (Mallavadhani et al., 2004.), antifungal (Rocha et al., 2004), gastroprotective (Rodriguez et al., 2003), hypoglycemic (Perez et al., 1998) and antihyperlipidemic (Ma, 1982) properties. They are relatively non-toxic and have been used in cosmetics and health products (Liu et al., 1995). Unfortunately, insufficient information is available concerning the distributions of OA and UA in the Lamiaceae, because the published quantitative investigations generally extended to only a few species, and mainly qualitative data are to be found on the

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presence of these compounds. Erdtman (1945) divided the family Lamiaceae into two subfamilies on the basis of the pollen morphology, the Lamioideae and the Nepetoideae. The Lamioideae were subdivided into several separate subfamilies by subsequent authors, e.g. into Ajugoideae, Chloanthoideae, Lamioideae, Pogostemonoideae, Scutellarioideae, Teucrioideae and Viticoideae by Cantino et al. (1992). The chemistry of the Nepetoideae and Lamioideae sensu Erdtman (1945) is very different, such as the contents of volatile oils, rosmarinic and caffeic acids, phenylethanoid glycosides and betaines (Cantino and Sanders, 1986; Máthé et al., 1993; Blunden et al., 1996; Máthé, 1997; Janicsák et al., 1999; Pedersen, 2000; Grayer et al., 2003; Hohmann et al., 2003).

Previous TLC densitometric results revealed that there are also significant quantitative differences in the amounts of OA/UA between the subfamilies Lamioideae and Nepetoideae sensu Erdtman. Therefore we decided to determine the presence and quantities of OA and UA in representative species of both subfamilies.

The closeness of the chemical structures of these two triterpenoid acids makes their TLC separation very difficult, and we therefore applied a recently developed GC method (Janicsák et al., 2003) for their quantitative estimation. We report here on the contents of OA and UA in 88 taxa of Lamiaceae.

2. Materials and methods

2.1. Plant material

Herbs of the species of Lamiaceae studied (in the case of the 4 species of *Dracocephalum*, only leaves) were collected in July, 1999 from the experimental field of the Institute of Ecology and Botany, Vácrátót, Hungary. The experimental field is approximately 30 km north of Budapest. Its climate is continental, with a mean annual temperature of 10.3 °C, and a mean annual precipitation of 525 mm. The plants were mainly grown from seeds obtained via a botanical garden seed exchange programme. They were identified by the botanist of our research group. Voucher samples of each species have been deposited in the herbarium of the Institute. Samples were dried at 40 °C.

2.2. Extraction and analysis

Powdered samples (0.8 g) were extracted with methanol (90 ml) for 6 h in a Soxhlet apparatus. Finally, the solutions obtained were diluted to 100 ml (stock solutions).

For the clean-up procedure (SPE), 4 ml of water was added to 1 ml of each stock solution to make 5 ml samples of each of the 20% aqueous methanolic mixtures. These solutions were transferred to Chromabond SB cartridges. The exact parameters of the SPE have been described by Janicsák et al. (2003).

For silylation, the SPE-cleaned extracts dried at 80 °C were dissolved in 200 μl of dry pyridine (Fluka, Germany) in vessels with a volume of 1.5 ml (La Pha Pack, Germany) and derivatized by adding 400 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (Supelco, USA). To achieve silylation, the samples were heated at 80 °C for 2 h.

Gas chromatography was performed with an HP 5890 Series II instrument (Hewlett Packard/now Agilent Technologies/, USA) equipped with an FID detector. The other measurement parameters were as follows: column: HP 5 (30 m × 0.32 mm × 0.25 μm); split ratio: 1:10; carrier gas: nitrogen; injector, detector and column temperature: 300 °C; head pressure: 8 psi; injected volume: 1.0 μl; analysis time per sample: 22 min.

3. Results and discussion

Fourteen representative species of the Lamioideae sensu Cantino et al. (1992) and 57 species of the Nepetoideae were studied, but no representatives of Cantino's other six subfamilies which are part of the Lamioideae sensu Erdtman (1945). The investigated taxa, together with their OA and UA contents, are listed in Table 1. As expected on the basis of our earlier results (Máthé, 1997), the OA and UA contents were significantly lower in the species belonging to the Lamioideae than in the Nepetoideae. In comparison with previous data on the rosmarinic and caffeic acid contents of species of Lamiaceae (Janicsák et al., 1999), smaller differences in OA and UA contents were observed among different subspecies and genera, e.g. *Salvia nemorosa* (OA: 0.265–0.441%; UA: 0.925–1.306%) and *Dracocephalum* species (OA: 0.051–0.269%; UA: 0.260–0.817%).

Table 1
Oleanolic and ursolic acid contents of Lamiaceae taxa (% dry weight)

Lamiaceae taxa	Voucher	OA	UA
Subfamily Lamioideae			
Ballota nigra L.	9446	0.013	0.039
Leonurus cardiaca L.	9779	*	0.016
Marrubium alysson L.	8201	0.009	0.014
M. incanum Desr.	8676	*	0.008
M. peregrinum L.	8570	0.036	0.040
M. supinum L.	L1336	*	*
M. thessalum Boiss. Et Heldr.	8202	0.009	0.017
M. vulgare L.	9960	*	0.030
Phlomis tuberosa L.	T/020	*	0.027
Physostegia virginiana (L.) Benth.	ÜL418	*	0.009
Sideritis syriaca L.	8253	0.059	0.068
Stachys grandiflora (Stev.) Benth.	L918	*	*
S. sylvatica L.	T046	0.031	0.036
S. officinalis (L.) Trevis.	9446	0.013	0.014
Subfamily Nepetoideae			
Calamintha nepeta (L.) Savi	8254	0.075	0.263
Dracocephalum bipinnatum Rupr.[a]	DP99	0.166	0.494
D. diversifolium Rupr.[a]	DD99	0.254	0.817
D. moldavica L.[a]	DM99	0.051	0.260
D. peregrinum L.	7053	0.269	0.722
D. rupestre Hance[a]	DRP99	0.127	0.502
D. ruyschiana L.	6535	0.246	0.674
Elsholtzia strauntonii Benth.	L676	0.118	0.398
Hyssopus officinalis L.	8697	0.139	0.314
H. officinalis L. l. *ruber* (Mill.) Gams	9747	0.150	0.350
H. officinalis L. l. *cyaneus* Alef.	8272	0.154	0.378
H. officinalis L. l. *albus* Alef.	8273	0.202	0.547
H. officinalis L. ssp. *montanus* (Jordan et Fourr.) Briq.	1347	0.352	0.719
Lavandula angustifolia Mill.	L775	0.479	1.397
Lavandula angustifolia Mill. 'Munstead'	L1030	0.334	0.911
L. angustifolia Mill. ssp. *pyrenaica* (DC.) Guinea	L979	0.409	1.279
Melissa officinalis L.	L570	0.140	0.581
Melissa officinalis L. *cultivar*	9181	0.142	0.531
M. officinalis L. cv. Lemon Balm.	9285	0.114	0.411
M. officinalis L. ssp. *altissima* (Sibth. & Sm.) Arc.	L981	0.063	0.218
Monarda citriodora Cerv.	L561	0.120	0.175
M. didyma L.	8225	0.069	0.187
M. fistulosa L.	8227	0.038	0.071
Nepeta cataria L.	9062	0.213	0.516
N. grandiflora M.B.	9065	0.529	0.935
N. mussinii Spreng.	6547	0.051	0.104
N. pannonica L.	L828	0.080	0.182
Nepeta × *faassenii* Berg.	9163	1.416	2.923
Origanum heracleoticum L.	L588	0.060	0.129
Origanum kopetdaghense A. Boriss.	L592	0.145	0.482
O. tyttanthum Gautsch.	L587	0.059	0.147
Salvia aethiopis L.	10627	0.017	0.054
S. amplexicaulis Lam.	8070	0.328	1.055
S. argentea L.	8305	0.116	0.218
S. austriaca Jacq.	8164	0.025	0.049
S. candidissima Vahl	1262	0.041	0.064
S. deserta Schang.	ÜL29	0.333	0.637
S. dumetorum Andrz	8001	0.129	0.450
S. dumetorum Andrz fl. *Rosea*	8582	0.065	0.154
S. fugax Pobed.	8003	0.192	0.434
S. glutinosa L.	T044	0.056	0.120

Table 1 (*continued*)

Lamiaceae taxa	Voucher	OA	UA
S. hians Royle	1221	0.220	0.597
S. jurisicii Kos.	8948	0.627	0.117
S. kuznetzovii Sosn.	ÜL309	0.111	0.216
S. lavandulifolia Vahl.	6302	1.840	4.019
S. nemorosa L.	8146	0.441	0.925
S. nemorosa L. l. *albiflora* Schur.	T040	0.292	0.950
S. nemorosa L. l. *coerulea* Priszter	T042	0.150	0.507
S. nemorosa L. ssp. *illuminata* (Klokov) Soó	ÜL307	0.328	1.261
S. nemorosa L. ssp. *moldavica* (Klokov) Soó	8037	0.265	0.943
S. nemorosa L. ssp. *tesquicola* (Klokov et Pobed.) Soó	7006	0.415	1.306
S. nubicola Wall.	T059	0.128	0.387
S. nutans L.	10142	0.148	0.364
S. officinalis L.	5676	1.559	3.825
S. pratensis L.	8156	0.120	0.256
S. pratensis L. ssp. *bertolonii* (Vis.) Soó	6899	0.339	0.533
S. przewalskii Maxim.	L747	0.140	0.391
S. sclarea L.	10622	0.045	0.145
S. × *simonkaiana* Borb.	6617	0.201	0.567
S. staminea Benth.	L1518	0.165	0.066
S. × *sylvestris* L.	6999	0.186	0.558
S. tomentosa Mill.	8007	0.362	1.047
S. transsylvanica (Schur) Schur	7008	0.320	0.758
S. verbenaca L.	L1716	0.393	0.313
S. verticillata L.	8182	0.579	0.594
S. verticillata L. ssp. *amasiaca* (Freyn et Bornm.) Bornm.	ÜL175	0.392	0.998
S. virgata Jacq.	6319	0.293	0.354
S. viscosa Jacq.	1077	0.101	0.137
Satureja coerulea Janka	6558	0.176	0.644
S. montana L.	6562	0.131	0.490
Thymus serpyllum L.	L1646b	0.368	1.398
T. vulgaris L.	L1645a	0.265	0.643
T. × *citriodorus* (Pers.) Schreb.	T061	0.174	0.517
T. × *citriodorus* (Pers.) Schreb. 'Variegata'	T062	0.214	0.526

*In trace amounts.

[a] Only the leaves were investigated.

The OA contents varied from traces (*Leonurus cardiaca*, *Marrubium incanum*, *Marrubium supinum*, *Marrubium vulgare*, *Phlomis tuberosa*, *Physostegia virginiana* and *Stachys grandiflora*) to 1.840% (*Salvia lavandulifolia*). Other species with high OA levels were *Salvia officinalis* (1.559%) and *Nepeta* × *faassenii* (1.416%). Low OA contents were found in *Marrubium alysson* (0.009%) and *Marrubium thessalum* (0.009%). As indicated above, all the lowest OA-producing taxa belong to the subfamily Lamioideae.

With the exceptions of 3 species of *Salvia* (*Salvia jurisicii*, *Salvia staminea* and *Salvia verbenaca*), the levels of UA were always higher than those of OA in the samples tested. The UA contents ranged between traces and 4.019%. In the Lamioideae, the lowest UA contents were recorded for *M. supinum* and *S. grandiflora* (traces) and the highest for *Sideritis syriaca* (0.068%), whereas in the Nepetoideae, *Salvia austriaca* exhibited the lowest level (0.049%) and *S. lavandulifolia* the highest (4.019%).

High concentrations (over 2.5%) of UA were detected in *Nepeta* × *faassenii* and *S. officinalis*. Relatively high concentrations of UA were also found in the subfamily Nepetoideae in *Lavandula angustifolia* (1.397%), *L. angustifolia* ssp. *pyrenaica* (1.279%), *Salvia amplexicaulis* (1.055%) *S. nemorosa* ssp. *illuminata* (1.261%), *S. nemorosa* ssp. *tesquicola* (1.306%), *S. tomentosa* (1.047%) and *Thymus serpyllum* (1.398%), whereas low levels were observed in *Monarda fistulosa* (0.071%), *Salvia aethiopis* (0.054%), *Salvia candidissima* (0.064%) and *S. staminea* (0.066%).

The two triterpenoid acids were present in all of the taxa investigated. However, there are significant quantitative differences between the two subfamilies, similarly as for other chemotaxonomic markers, such as rosmarinic and caffeic acids, betaines, volatile oil components and phenylethanoid glycosides (Máthé et al., 1993; Blunden et al., 1996;

Máthé, 1997; Janicsák et al., 1999; Hohmann et al., 2003). The subfamily Lamioideae appears to be poorer in both OA and UA than the subfamily Nepetoideae. The mean values for the Lamioideae were OA: 0.012% and UA: 0.023%, while those for the Nepetoideae were OA: 0.263% and UA: 0.638%.

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Chemical Composition and Antimicrobial Activities of Essential Oils of Four Lines of *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart Grown in Hungary

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The essential oils of four lines of *Origanum vulgare* L. subsp. *hirtum* (Link) Ietswaart cultivated in Hungary were analysed by GC and GC-MS methods. These oils were found to contain carvacrol, γ-terpinene and *p*-cymene as main constituents. The antimicrobial activities of the various oils and their authentic individual components were tested on Gram-positive and Gram-negative bacterial strains, two *Saccharomyces cerevisiae* strains and two *Candida albicans* strains. No difference in sensitivity was found between *Escherichia coli, Staphylococcus epidermidis* and the yeast strains tested, but there were marked differences in sensitivity between the proton pump-deficient mutant of *E. coli* and its wild type as regards the growth inhibition and MIC values.

Keywords: *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart, essential oil, GC-MS, antibacterial activity.

Origanum is used throughout the world as a very popular spice, under the vernacular name oregano. Oregano, one of the most important Mediterranean spices, exhibits rather high diversity from both taxonomical and chemical aspects [1]. Though the name 'oregano' is devoted to many species of various genera, most oregano spice products originate from *Origanum* species (Family Lamiaceae). It is of great economic importance, not only as a spice, but also since its essential oil exerts antimicrobial, cytotoxic and antioxidant activities [2-4].

Until 1980, there was no satisfactory classification of the *Origanum* genus, but since then the Ietswaart's system has become widely accepted [5]. This postulates that *O. vulgare* L. has six subspecies with differences in the indumentum, the number of sessile glands on the leaves, bracts and calyces, and the size and color of the bracts and flowers. *O. vulgare* subsp. *hirtum* (Link) Ietswaart is native to Albania, Greece and Turkey [6], but alien to Hungary. Ecologically, this species prefers warm, sunny habitats, and loose, often rocky, calcareous soils, low in moisture content.

Our present study focused on the quantity and nature of the essential oils in *O. vulgare* subsp. *hirtum* populations cultivated in Hungary, i.e. under continental climatic conditions. It forms part of a series of wide-ranging investigations into *O. vulgare* subsp. *hirtum* that have been conducted in Hungary for several years [1]. The antibacterial activity of essential oils and their main constituents was recognized long ago [4,7]. In the present work, the antibacterial properties of the essential oils from the aerial parts of four lines of *O. vulgare* subsp. *hirtum*

Table 1: Comparison of the *Origanum vulgare* subsp. *hirtum* oils (2004).

Compounds[a]	RI[b]	% in samples				Identification
		A	B	C	D	
α-Thujene	930	1.7	1.8	1.6	1.5	c
α-Pinene	938	0.9	0.8	0.8	0.7	c, d
Camphene	951	0.3	0.3	0.3	0.2	c
Sabinene	974	0.5	0.4	0.1	0.3	c, d
β-Pinene	980	2.1	2.1	2.1	1.4	c, d
α-Phellacrene	1006	0.1	0.2	0.2	0.1	c
α-Terpinene	1018	0.9	1.0	2.1	0.4	c, d
p-Cymene	1025	9.5	6.3	6.1	4.2	c, d
Limonene	1030	0.4	0.4	0.4	0.3	c, d
γ-Terpinene	1060	7.2	5.5	16.0	1.3	c, d
cis-Sabinene hycrate	1071	0.5	0.5	0.4	0.2	c
Terpinolene	1087	0.2	0.3	0.2	0.2	c
Borneol	1168	0.3	0.5	0.5	0.2	c, d
Terpinen-4-ol	1178	0.3	0.4	0.2	0.3	c
Carvacrol methyl ether	1242	-	-	-	0.7	c
Thymol	1291	0.3	0.3	3.1	0.3	c, d
Carvacrol	1297	70.8	73.7	61.7	84.2	c, d
β-Caryophyllene	1420	1.4	2.6	3.3	1.5	c, d
Germacrene-D	1480	0.3	-	-	-	c
Total		97.7	97.1	99.1	98.0	

[a]Compounds listed in sequence of elution from a CB-5 MS column.
[b]Kovats retention indices calculated against C_9 to C_{24} *n*-alkanes on a CB-5 MS column
[c]Comparison of mass spectra with MS libraries and retention indices.
[d]Comparison with authentic compound.

were evaluated. These lines were obtained from seeds of a previously selected strain so they should be regarded as offspring of the same population. Our comparative study shows that, though the oil content may vary significantly, the oil composition and the antimicrobial activities of the lines remained similar, and proved to be rather stable.

The oil contents of the *O. vulgare* subsp. *hirtum* lines were high (3.8-7.0%, v/w). Similar results were earlier published on *Origanum* populations growing in their natural Mediterranean environment [8]. The compositions of the essential oils were determined by GC and GC-MS techniques. Table 1 presents the results of the qualitative and quantitative analyses. The nineteen identified constituents accounted for 97.1-99.1% of the oils. These oils were found to contain carvacrol (61.7-84.2%), γ-terpinene (1.3-16.0%) and *p*-cymene (4.2-9.5%) as main constituents. The essential oil of sample **C** also contained an appreciable quantity of thymol (3.1%). The compositional characteristics indicated that all these lines belong to the "carvacrol group" described by Pasquier [9]. The concentrations of the minor components in the four samples varied, but the differences were not significant.

All of the *O. vulgare* subsp. *hirtum* oils exerted broad antimicrobial effects on the tested bacterial and fungal strains. The agar diffusion method furnished semiquantitative data on the bacteriostatic and fungistatic effects of the oils (Table 2). The zones of inhibition revealed that the oils had similar activities against bacteria and yeasts. The most sensitive strain among the tested bacteria was the proton pump-deficient *Escherichia coli* AG100A. The MIC values varied from 0.04 μL/mL to 0.24 μL/mL for the bacterial strains, and from 0.08 μL/mL to 0.20 μL/mL for the fungal strains tested. The MIC values demonstrated a difference in sensitivity between the two *E. coli* AG strains. The proton pump-containing *E. coli* AG100 displayed MIC values that were 2-3 times as high as those for the proton pump-deficient mutant *E. coli* AG100A. These results suggest that the antimicrobial effects of the tested oils are probably based on proton pump-related mechanisms. The different compositions of the four samples of *O. vulgare* subsp. *hirtum* did not appear to have any appreciable influence on the antimicrobial effects tested. The antimicrobial activity of the oil is clearly due to the high activity of carvacrol, as described earlier [7].

Eleven different components of those commonly found in *O. vulgare* subs. *hirtum* essential oils were investigated with regard to their antimicrobial activity (Table 3). The most pronounced effects were shown by thymol and carvacrol (MIC values: 0.31-2.50 μL/mL), but the sensitivities of the bacteria and fungi differed. In the case of carvacrol, *E. coli* AG100 and AG100A (a proton pump-deficient mutant of *E. coli* AG100) did not differ in susceptibility, whereas when they were treated with the other compounds, *E. coli* AG 100A was the more likely to be inhibited. This suggests that carvacrol has a different effect on this bacterial strain: binding to the proton pump may not occur. Limonene and α-terpinene are efficient antimicrobial agents.

The sensitivities of the bacteria and fungi to the oil components were similar, though the effects on the *Saccharomyces* strains were more explicit than those on the *Candida* strains. The mitochondrium-containing *Saccharomyces* strain (*S. cerevisiae* 0425 52C, Grand) was not more susceptible than its mitochondrium-less mutant (*S. cerevisiae* 0425 δ/1).

Experimental

Plant material: The plant material was gathered from the perennial populations of *Origanum vulgare* subsp. *hirtum* cultivated at the Experimental Station of the Faculty of Horticultural Science, Corvinus University of Budapest, Hungary. In open field

Table 2: Bacteriostatic and fungistatic activities of various plant oils.

Samples	Concentration (v/v%)	*E. coli* F'lac	*E. coli* AG100	*E. coli* AG100A	*S. epidermidis*	*S. cerevisiae* δ/1	*S. cerevisiae* 52C	*C. albicans* 10231	*C. albicans* 14053
A	5	36	28	44	25	40	35	38	35
B	5	30	27	46	23	42	38	35	32
C	5	35	25	45	17	40	43	30	36
D	5	45	31	58	27	42	45	40	43
Penicillin	1 mg/mL	24	24	24	23	-	-	-	-
Gentamycin	1 mg/mL	32	32	32	33	-	-	-	-
Fluconazole	2 mg/mL	-	-	-	-	19	27	31	40
DMSO	100	0	0	0	0	0	0	0	0

Zones of growth inhibition (in mm) of microorganisms on nutrient agar plates

Table 3: MIC determination of selected plant oils and individual oil components on various bacterial and fungal strains.

Samples	*E. coli* F'lac	*E. coli* AG100	*E. coli* AG100A	*S. epidermidis*	*S. cerevisiae* δ/1	*S. cerevisiae* 52C	*C. albicans* 10231	*C. albicans* 14053
A[a]	0.20	0.20	0.12	< 0.04	0.12	0.12	0.16	0.20
B[a]	0.16	0.20	0.08	0.08	0.12	0.12	0.16	0.16
C[a]	0.16	0.24	0.08	< 0.04	0.12	0.12	0.16	0.20
D[a]	0.16	0.20	0.08	0.08	0.08	0.12	0.16	0.16
α-Pinene[a]	0.62	0.62	0.31	1.25	0.16	0.16	1.25	0.63
Sabinene[a]	2.50	> 5	1.25	2.50	0.31	0.16	2.50	2.50
β-Pinene[a]	1.25	3.75	0.62	1.25	0.31	0.08	0.62	0.62
α-Terpinene[a]	2.50	3.75	1.25	1.25	0.62	1.25	> 5	5.00
p-Cymene[a]	20.0	7.50	5.00	2.50	0.62	1.25	2.50	2.50
Limonene[a]	0.62	0.62	0.31	1.25	0.16	0.16	1.25	0.62
γ-Terpinene[a]	10.0	> 20	7.50	5.00	1.25	5.00	15.0	8.75
Borneol[a]	5.00	2.50	1.25	25.0	2.50	5.00	2.50	5.00
Thymol[a]	1.25	2.50	0.33	0.31	0.16	0.08	0.08	0.16
Carvacrol[a]	0.16	0.16	0.16	2.50	0.31	0.31	1.25	1.25
β-Caryophyllene[a]	> 20	20.0	1.25	20.0	> 20	> 20	> 20	> 20
Penicillin[b]	8.0	8.0	1.6	4.0	-	-	-	-
Gentamycin[b]	1.2	4.0	2.0	0.8	-	-	-	-
Fluconazole[b]	-	-	-	-	56.0	64.0	16.0	0.8
DMSO	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20

The tabulated numbers indicate MICs as the volumes of oils and components resulting in complete inhibition
[a] μL/mL
[b] μg/mL

experiments, several individuals from a non-homogeneous line were investigated. The aerial parts of the plants were collected during the flowering stage in July 2004. Voucher specimens have been deposited in the Genbank reserved living material, Department of Medicinal and Aromatic Plants, Corvinus University of Budapest, under the numbers OH-1/1 (**A**); OH-1/2 (**B**); OH-1/8 (**C**) and OH-9/11 (**D**).

Isolation of the essential oil: The flowering shoots were cut off and steam-distilled for 2 h, according to the method in the Pharmacopoea Hungarica VII [10]. The essential oils obtained were dried over anhydrous sodium sulfate, filtered and stored at –18ºC until tested and analyzed. The yields were calculated based on the dry weight of the plant materials.

Gas chromatography: The GC analysis was carried out with an HP 5890 Series II gas chromatograph (FID), using a 30 m x 0.35 mm x 0.25 μm HP-5 fused silica capillary column. The temperature program was from 60°C to 210°C at 3°C min^{-1}, and from 210°C to 250°C (2 min hold) at 5°C min^{-1}. The detector and injector temperature was 250°C and the carrier gas was N_2, with split sample introduction.

Gas chromatography-mass spectrometry: GC-MS analysis was performed with a FINNIGAN GCQ ion trap bench-top mass spectrometer. All conditions were as above except that the carrier gas was He at a linear velocity of 31.9 cm sec^{-1} and two capillary columns were used with different stationery phases (DB-5MS and SolGel-WAX; 30 m x 0.25 mm x 0.25 μm). The positive ion electron ionization mode was used, with a mass range of 40-400 amu. Identification of the compounds was based on comparisons with published MS data [11] and a computer library search (the database was delivered together with the instrument), and also by comparison of their Kovats

'indices with those of authentic compounds and with literature values. The identification was confirmed with the aid of authentic samples. Kovats indices were calculated mainly from the GC-MS analysis results [12].

Bacteriostatic and fungistatic activities: Bacterial and fungal strains: *Escherichia coli* K12 LE140, *Staphylococcus epidermidis* (clinical isolate), *Escherichia coli* AG100, *E. coli* AG100A, *Saccharomyces cerevisiae* 0425 δ/1, *S. cerevisiae* 0425 52C, *Candida albicans* ATCC 10231, and *C. albicans* ATCC 14053.

The bacteriostatic and fungistatic activities were determined using an agar-well diffusion method. The essential oils were dissolved in DMSO at concentrations of 2-10 v/v%, depending on the quantity of the oil. Different nutrient agars were used: MTY (minimal-trypton-yeast) for the *E. coli* F'lac and *S. epidermidis* strains, LB (Luria-Bertani) for the *E. coli* AG 100 and AG 100A strains, and 2xYPD (yeast extract-peptone-dextrose) for the yeast strains. The culture broth was inoculated with the bacterial and yeast strains and incubated overnight either at 37°C (bacteria) or at room temperature for 48 h (fungi). Each preculture was diluted 10-100-fold in physiological saline solution (0.9% w/v) before plating on the nutrient agar. Wells 10 mm in diameter were punched into the agar and filled with 50 μL of either the oil solutions or DMSO solvent blank. After incubation, the antimicrobial activity was evaluated by measuring the diameter of the inhibition-zone observed (Table 2).

The MIC (minimal inhibitory concentration) values were determined with the broth dilution assay. The overnight cultures of bacteria and the 24 h cultured yeast strains were diluted 100-fold in physiological saline solution and 50 μL inocula was added to the appropriate nutrient broth. The solutions of the essential oils (dissolved in DMSO at 1%, v/v) were added to the cultures in increasing concentration, and the samples were then incubated for 24 h at 37°C for the bacteria, and for 48 h at room temperature for the yeasts. The MIC was defined as the lowest concentration of the test sample that resulted in complete inhibition of visible growth in the broth (Table 3). DMSO was used as a negative control, while penicillin, gentamycin and fluconasol were used as a positive control.

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