Evaluation of supercritical plant extracts on volatile and non volatile biologically active lipophil components

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Key words: supercritical fluid extraction, monoterpenes, sesquiterpenes, phtalides, Asteraceae, Lamiaceae, Apiaceae, sesquiterpene-γ-lactones, parthenolide, cnicin, phytosterols, β-sytosterol, triterpenes, β-amyrin.

Summary: Authors dealt more than ten years with the analysis of supercritical extracts. For extraction (SFE) carbon dioxide was used as supercritical solvent. Fractionation of extracts was carried out by releasing the separations pressure at two stages. The extracts were collected as separate samples successively in time.
The traditional extractions were carried out with steam distillation or by using n-hexane and ethanol in Soxhlet apparatus. For the analysis of volatile compounds GC, GC-MS; of non volatile compounds TLC-densitometry and spectroscopic methods were used.
The following general characteristics were established comparing the composition of steam distillated oils with that of volatile SFE fractions. The SFE fractions were richer in monoterpene-esters and poorer in alcohols than the essential oils prepared by traditional way (clary sage, lavandel). Regarding the distribution of the monoterpene and sesquiterpene compounds, the SFE fractions contained sesquiterpene hydrocarbon in higher percentage than the distillated oils (e.g. β-caryophyllene in *Salvia fruticosa*, β-caryophyllene, γ-muurolene, γ-cadinene in *Ocimum basilicum*). Further the proportion of sesquiterpenes increased in SFE fractions collected successively in time.Significant difference was remarkable in respect of the optical rotationability of lovage oil and SFE fraction which was probably caused by the different ratio between the two ligustilid enantiomers. It was verified in some cases that a part of mono- and sesquiterpenes were present originally in a bounded form (glycosides) in plants. Therefore they appeared in essential oil fractions only after previous acidic treatment (*Thymus, Origanum* species). During the supercritical extraction the azulenogene sesquiterpene lactones did not transform to azulenes (in chamomile, yarrow), but the non volatile SFE fractions of some *Asteraceae* plant contained sesquiterpene-—lacton of unchanged structure in high quantity (e.g. cnicin in blessed thistle, parthenolide in feverfew). Authors obtained also SFE fractions which were rich in triterpenoids and phytosterols (marigold, common dandelion).

Introduction

The supercritical fluid extraction is a special separation technique to obtain extracts and fractions of natural origin for therapeutical or cosmetic use (*Chouchi* et al. 1995; *Dugo* et al. 1995; *Bicchi* et al. 1999).

Authors carry out the analysis of supercritical extracts of plant materials containing biological active volatile and non volatile terpenoids for more than ten years, further were studied the advantages and disadvantages of the supercritical extraction method in comparison of the traditional extraction methods.

The aim of this paper is to give a summary of the work on obtaining SFE fractions rich in volatile compounds (mono-, sesquiterpenes and phenyl-propane (aromatic) derivatives as well as non volatile sesquiterpene-γ-lactones, triterpenes and sterols.

Material and methods

Plant material

Some plant species of *Lamiaceae, Asteraceae* and *Apiaceae* families, – rich in volatile terpenoids or aromatic

compounds as well as sesquiterpene-γ-lactones, sterols and triterpenes in free form – were used in these studies.

Lamiaceae family:

Lavandula officinalis L. (lavandin or lavander)
* *Salvia sclarea* L. (clary sage)
* *Salvia officinalis* L. (sage)
Salvia triloba (syn. *S. fruticosa* L.)
Rosmarinus officinalis L. (rosemary)

Origanum vulgare L. (oregano marjoram)
* *Satureja hortensis* L. (savory)
* *Thymus vulgaris* L. (thyme)
Thymus serpyllum L. (wild thyme)
* *Ocimum basilicum* L. (basil)

Asteraceae family

Chrysanthemum parthenium L. (Beruh) (feverfew)
Matricaria recutita L. (chamomile)
Cnicus benedictus L. (blassed thistle)
Taraxacum officinale L (common dandelion)
Calendula officinalis L. (marigold)

Apiaceae family

** *Anthriscus cerefolium* L. Hoff (chervil)
Levisticum officinale L. (lovage)

A part of plant samples was cultivated* or collected** in Hungary; the other part of them originated from Herbaria, but Turkish oregano and Greek feverfew were studied too.

For extraction the grinded herb of plants; the root of lovage and common dandelion; the flowers of chamomile and marigold were used.

Methods for extraction

Supercritical fluid extraction

The extraction apparatus consisted of the extractor, two separators, storage vessel and gas meters. The raw material after grinding was charged into the extractor. $C0_2$ from the storage vessel was fed through the heat exchanger by means of the high-pressure pump. The compressed $C0_2$ solved the volatile and non-volatile lipophilic substances. Two separators in series were applied to obtain the extract where the press is decreased in two stages.

The pasty SFE product could be collected in the first, the volatile oil containing product in the second separator. A fractionated extraction was carried out by a stepwise increase of the extraction pressure, meanwhile the products were collected at each step in the first or second separator. The accumulated product samples were collected and weighed. The effect of the extraction parameters (pressure, temperature) on the yield were determined by 3^2 full factorial designed experiments. The solvent leaving the separators after condensation flow back into the solvent storage vessel. The total volume of $C0_2$ consumed was measured by the volumetric gas meter (*Oszagyán* et al. 1996; *Rónyai* et al. 1998).

Water steam distillation

The traditional water steam distillation in neutral medium was carried out according to the description of the Hungarian Pharmacopoea Edition 7, in the special apparatus for the extraction of essential oils. (*Ph.Hg. VII.* 1986) For the acidic distillation acidified water (pH – 2 by hydrochloric acid) was used. In certain cases the combination of SFE and steam distillation (steam distillation of the SFE fractions) was used.

Extraction with organic solvents

n-Hexane or petroleum ether and ethanol (96%) were used as organic solvents in Soxhlet apparatus.

The samples for studying triterpenes and sterolos the unsaponifiable matters were prepared. The samples were saponified by treatment with ethanolic KOH and the reaction mixture was extracted with chloroform. The chloroformic extracts (*Ph.Hg.VII.* 1986) were used for quantitative determinations.

Methods for analysis

GC for evaluation the composition of essential oils

For the separation, identification and evaluation of volatile compounds gas chromatographic method was used. The GC analysis was carried out on packed (a) and cappilaric (b) columns, respectively with nitrogen as carrier gas and flam ionisation detector; temperatures of detector and injector were 230 °C and 200 °C, respectively.

A 3 m × 2,3 mm glass spiral column packed with 3% 0V-17 and 30 m × 0,32 mm, silica fuse columns coated with DB-1701 of 25 μm, or specific chiralic column coated with Rt-β DEXm and Rt-β DEXsm of 25 μm thickness were used. The columns were programmed as follows 60°–230 °C at 8 °C/min (a) and 60°–230 °C, 8^0C min. 230 °C isotherm 3 min (b).

GC/MS investigation was performed using a Hewlett-Packard 5890A instrument coupled to a VG-TRIO-2 quadrupole mass spectrometer with a direct capillary interface. DB-1701 fused silica column (30 m × 0,32 mm, 0,25 μm film thickness) was used. The oven tempwerature program was 70 °C (0.5 min), 70°–120 °C (15 °C/min), 120 °C–240 °C 4 °C/min), 240 °C (2 min). Injector temperature was 210 °C.

The oil components were identified by comparing their retention times with those of authentic standards, essential oils of known composition and peak enrichment. The confirmation of identity was done by comparison of their mass spectra with those reported in the literature (*Stenhagen* et al. 1974) and reference compounds.

TLC-densitometry for the analysis of the non volatile sesquiterpene-γ-actones

Silicagel GF 254 (Merck) was used as sorbent with the following solvent systems: benzene-n-hexane-acetonitrile

30:20:21 (v:v) (parthenolide), carbon tetrachloride-acetonitrile 40:15 (v:v) (cnicin). Detection: vanillin-sulphuric acid reagent (see essential oil). The plates were scanned to the direction of elution by Shimadzu-CS-930 densitometer fitted at 520 nm and 600 nm respectively. Parthenolide was used as standard).

IR spectroscopy was used to screen the SFE products for the presence of sesquiterpene-γ-lactones. *Beckman* (Acculab 8) infrared spectrophotometer was used in liquid cell technique and the IR spectra of samples were recorded in 1650–1850 cm^{-1} wavenumber region. Absorption at the region of 1745–1785 cm^{-1} were studied.

TLC-densitometry for analysis of triterpenes and sterols

Kieselgel 60 F_{254} was used as adsorbent with solvent system : n-hexane-ethylacetate (6:2). The plates were treated by sulphuric solution of cerium sulphate then scanned to the direction of elution by Shimadzu-CS-930 densitometer fitted at 600 nm. β-amyrin and β-sytosterine compounds were used as standards.

Results and discussion

Supercritical extraction of volatile compounds

Ratio of monoterpene ester and alcohols

Comparing the essential oil composition of *Lavandula* and *Salvia sclarea* SFE-fractions with that obtained by steam distillation authors established that the linalyl acetate content of SFE fractions were in general higher than that of traditional essential oils, and the *Lavandula* SFE fractions - independent from the plant source (English or French Lavandula) – were in general more valuable, respectively to the higher acetate content than the distilled one. The statement could be explained by the reduced possibility of the hydrolytical processes (*Rónyai* et al. 1999a; *Lemberkovics* et al. 1998b).

The composition of feverfew oils obtained by steam distillation and SFE the yield of camphor and chrysanthenyl acetate were always higher in SFE products (*Table 1*) (*Kéry* et al. 1998).

Table 1 Composition of feverfew essential oil obtained by steam distillation and SFE

Code	Compounds	Percentage occurrence %				
		Essential oil	SFE fractions			
			1.	2.	3.	4.
1	α-pinene	1,1	1,3	trace	–	1,1
2	camphene	4,4	3,0	5,4	4,1	3,2
3	β-pinene	0,4	0,2	0,1	0,4	trace
4	limonene	0,3	trace	0,1	0,2	trace
5	eucalyptol	1,9	1,5	2,1	1,6	1,7
6	γ-terpinene	0,1	0,1	trace	trace	trace
7	linalool	0,2	trace	0,1	0,2	0,1
8	α-thujone	1,2	trace	0,3	0,2	0,1
9	camphor	53,9	65,9	70,9	61,6	67,1
10	borneol	0,2	0,1	trace	trace	0,2
11	chrysanthenyl-acetate	26,9	29,7	29,1	27,6	29,8

The main component of the steam distillated feverfew oils of Greece origin was chrysanthenyl-acetate but the caphor monoterpene ketone was the predominant constituent in the SFE fractions. It is interesting that the composition of essential oil obtained by steam distillation of SFE fraction was in good agreement with the conventional *Chysanthemum* oil. We have to remark that chrysanthenyl alcohol was detectable only in the oils gained by acidic distillation in the samples (*Lemberkovics* et al. 1998b).

Change of ratio of mono- and sesquiterpenes during the fluid extraction successively in time

The investigations were carried out on extraction of *Salvia officinalis, S. fruticosa* and *Ocimum basilicum*.

Authors established that in the SFE fractions of basil the percentage occurrence of the linalool main component as well as the monoterpene compounds (e.g. citronellol, borneol) was lower, but the ratio of sesquiterpenes was higher than that in traditional basil oil (e.g. β-caryophyllene 0.41 → 0.86%, γ-muurolene 2.56 → 4.85% and γ-cadinene 1.88 → 3,34%) (*Rónyai* et al. 1999b).

In the case of sage the change of essential oil composition was studied during the fluid extraction. The results showed that in general the first fractions were richer in monoterpenes (α-, β-pinenes, camphene, limonene, eucalyptol, p-cymene, α-, β-thujone, borneol, camphene, bornyl-acetate) than the latter ones. In the second and third fractions the rate of sesquiterpenes was mostly higher than they were in the first fractions (β-caryophyllene 3 → 4.7%, α-humulene 5.0 → 7.06 %) (a/ *Lemberkovics* et al. 1998).

Distribution of monoterpene hydrocarbons and oxygenated monoterpenes during the supercritical extraction

Comparing the composition of traditional rosemery oil with that of SFE fractions it was established that the main components: α-pinene, eucalyptol, camphor, verbenon were the same in both samples.

Table 2 Comparing of composition of essential oil and SFE fractions of rosemary

Components	Percentage occurrence %					
	Essential oil	SFE fractions				
		6/1	6/2	6/3	6/4	6/5
α-pinene	15,8	15,1	14,9	15,1	4,51	1,30
camphene	3,73	3,15	2,91	2,68	086	0,24
p-cymene	1,33	1,55	1,53	1,33	0,69	0,12
limonene	4,28	5,90	5,32	4,95	2,33	0,55
eucalyptol	16,8	17,4	16,7	18,4	16,3	16,9
(1,8-cineole)	1,88	1,66	1,12	1,61	1,74	2,12
linalool						
camphor	11,4	14,3	13,4	14,1	16,8	19,7
borneol	7,66	7,75	8,20	7,59	10,1	10,9
terpinene-4-ol	0,90	1,05	1,33	1,20	trace	trace
α-terpineol	2,20	2,04	2,34	2,33	2,83	2,65
verbenon	11,0	13,8	13,6	15,3	20,0	23,9
neryl-acetate	1,67	1,22	0,94	0,69	0,86	0,80
caryophyllene	1,84	2,72	2,66	2,67	2,45	1,37

Regarding the percentage occurrence of the components it was provable that the SFE fractions were poorer in hydrocarbons than the essential oil obtained by steam distillation. The first SFE fractions contained the monoterpene hydrocarbons and the oxygenated monoterpenes were present mainly in the latter fractions (*Table 2*) (*Oszagyán*, 1998).

Supercritical extraction of volatile aromatic (phenylpropane derivative) compounds

For studying this question two species from the *Apiaceae* family: *Anthriscus cerefolium* and *Levisticum officinale* were choosed. Fresh chervil was collected by us in Budapest (Népliget).

Eleven major components of the chervil oil and volatile concentrate (SFE) were identified. The compositions of SFE volatile concentrates and distilled oil were significantly different. *Table 3* shows that the methyl chavicol content of distilled oil was much higher and the compound of 1-allyl-2,4-dimethoxy-benzene was considerably lower than that of SFE volatile concentrates.

The oil and water obtained by SFE were almost completely immiscible; however, the volatile components were partly soluble in water (liquid-liquid equilibrium). The chemical composition of the oil recovered from aqueous phase by petroleum ether extraction was significantly different from the composition of the SFE volatile concentrate (*Simándi* et al. 1996).

Comparing the composition of lovage oil with that of lovage SFE fractiones authors established that in all cases the main component was the phtalide-derivative Z-ligustilid (component 13). Its highest concentration (79,3%) was in SFE-3, 77,7% was in oil obtained by steam distillation, 78,0% in hexane and 67,3% ethanole extract (*Table 4*). There was a significant, remarkable difference in the optical rotation value of essential oil (α: +1.4°) and SFE fractions (SFE-2, α: +27.8) which is probably caused by the different ratio of ligustilid enantiomers (Z/E 28.0 →14.3).

Table 3 Comparative percentage composition of chervil oil produced by SFE and steam distillation

Components	Steam distillation	SFE	
		Oil	Aqueous phase*
α-pinene	trace	trace	0,7
camphene	trace	trace	0,3
β-pinene	trace	trace	0,7
myrcene	trace	trace	0,2
limonene	0,2	1,3	19,7
p-cymene	trace	trace	5,2
1,8-cineol	trace	trace	1,0
undecane	trace	trace	1,0
methyl-cavicol	80,0	21,1	28,0
(E)-anethole	0,2	t	1,4
1-allyl-2,4-dimethoxy-benzene	16,0	57,4	1,3

* Oil recovered from aqueous phase by petroleum ether extraction

SFE extraction of phenoloid compounds (aromatic monoterpene derivatives) from Thymus and Origanum species

Authors established that the phenolic components were obtained only partly in SFE fractions relatived to the essential oil obtained by steam distillation. The yield of phenoloid compounds was better, if the SFE fraction was water steam distillated. A fraction rich in phenoloid components was obtained, if the steam distillation was carried out from acidic medium (*Lemberkovics* et.al. 1998b; *Oszagyán* et al. 1996b; *Oszagyán* et al. 2000).

On the bases of our above mentioned results for the characterization of solubility and extractability (in fluid

Table 4 Percentage occurrence of volatile oil components of essential oil and SFE fractions of lovage

Components	F	Percentage occurrence %								
		Essential oil	SFE 1.	SFE 2.	SFE 3/1	SFE 3/2	SFE 3/3	SFE 3/4	Hexanic extract	Alcoholic extract
1	0,37	0,05	0,18	0,08	0,14	–	0,07	–	–	7,3
2	0,38	0,04	0,25	0,15	0,23	0,08	–	–	–	–
3	0,40	0,16	0,54	0,36	0,47	0,27	0,47	0,65	–	–
4	0,45	0,03	0,17	0,16	0,65	0,12	–	–	–	–
5	0,45	0,11	0,66	0,38	0,74	0,26	0,23	0,14	–	–
6	0,54	0,23	0,47	0,34	0,64	0,24	0,16	0,12	trace	–
7	0,61	0,17	0,74	0,55	0,93	0,36	0,26	0,19	trace	–
8	0,68	0,91	0,27	0,29	0,20	0,22	0,41	0,83	–	–
9	0,85	0,22	0,14	0,17	0,18	0,12	0,20	0,11	–	–
10	0,87	1,00	1,28	1,29	1,32	1,33	1,00	1,03	1,30	trace
11	0,89	5,80	3,30	3,31	3,23	3,23	3,33	3,67	3,40	4,60
12	0,93	0,48	0,52	0,51	0,39	0,51	0,80	0,90	0,70	trace
13	1,00	77,7	75,4	79,1	71,6	79,3	77,8	78,4	78,0	67,3
14	1,01	3,15	3,86	2,69	2,69	2,9	2,58	3,07	2,80	2,20
15	1,02	1,60	3,52	3,00	2,60	3,37	2,85	3,42	3,00	1,40
16	1,16	2,78	2,98	5,54	4,80	6,57	4,87	6,47	6,60	2,50

Legend:
F= retention factor relative to Z-ligustilide
11 n-buthylidene-phtalide
12 buthyl-phtalide
13 Z-ligustilide
15 3-buthyl-4,5-dihidrophtalide
16 E-ligustilide

CO_2) of the individual volatile components a factor (measuring number: 100*log P/M) was introduced which contained the hydrophobity (influenced by polarity) and molecular mass of the components *(Table 5)*. The linearic connection between this factor and extractability is demonstrated in *Figure 1 (Oszagyán* et al. 2000).

Table 5 Factor(100* log P/M) of some volatile components for characterization on their fluid extractability

Components	logP	100* logP/M
geranyl-acetate	2,68	1,36
α-terpineol	2,22	1,44
linalyl-acetate	2,87	1,46
linalool	2,27	1,47
terpinene-4-ol	2,28	1,48
verbenon	2,27	1,51
camphor	2,71	1,78
α-pinene	3,25	2,38
caryophyllene	4,99	2,44
cineol	3,77	2,44
camphene	3,40	2,49
carvacrol	3,34	2,22
thymol	3,34	2,22
γ-terpinene	3,09	2,27
p-cymene	3,72	2,77

Legend: **logP**: hydrophobity; **M**: molecular mass

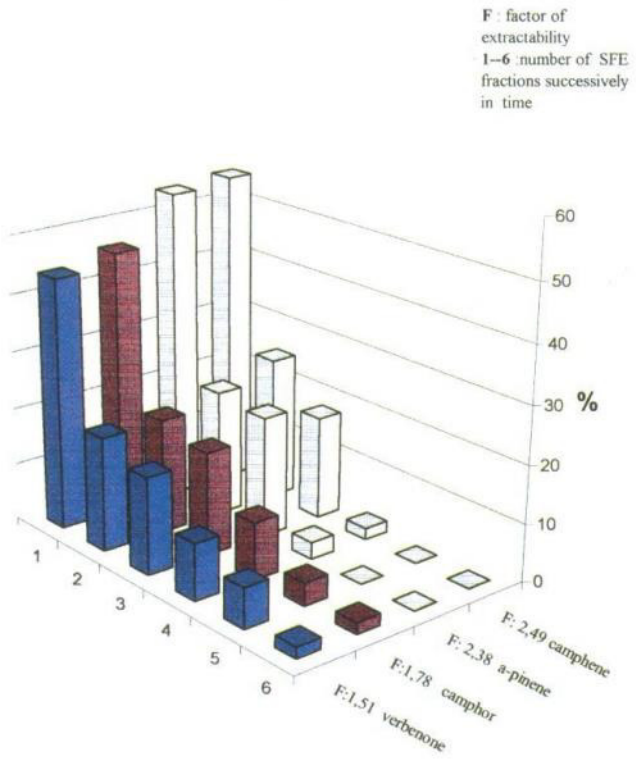

Figure 1 Yield (%) of main components of rosemary during SFE

Supercritical extraction of non volatile terpenoids

Extraction of sesquiterpene—lactones from Asteraceae families

SFE of Chrysanthenum parthenium and Cnicus benedictus

The yield of sesquiterpene—lactones in feverfew is 0.73 g/100 g calculated in parthenolide. The recovery of parthenolide was almost complete by SFE.

The extraction of cnicin from *Cnicus benedictus* was unsuccessful by using factor levels (pressure: 100, 250, 400 bar, temperature 40, 50, 60 °C) of feverfew extraction. Cnicin was absent in the SFE products. When 4% methanol was added to the CO_2 flow to the column, it eluted the lactone fraction containing 70% cnicin (*Kéry* et al. 1998; *Kéry* et al. 1999).

At the same time it was established that the azulenogene guajanolide sesquiterpene-γ-lactones (e.g. matricin) were not present in SFE fractions obtained from chamomile and wormwood respectively. The possibility of hydrolysis and isomerization processes were excluded during the supercritical extraction, therefore the volatile fractions did not contain chamazulene (*Lemberkovics* et al. 1998b).

Supercritical extraction of sterols and triterpenes from Taraxacum officinale, Levisticum officinale and Calendula officinalis

In all cases authors established that the *phytosterol* content (calculated in β-*sytosterol*) and the free *triterpene* content of SFE fractions were higher than the extracts obtained by traditional extraction methods using organic solvents. For example the faradiol and faradiol-monoester triterpene content was 0.06 g and 0.10 g, respectively in alcoholic extract and 5.0 g and 12.0 g, respectively in SFE extract of marigold (*Rónyai* et al. 1998).

Authors established also that the supercritical extraction is more selective for β-*amyrin*, than the traditional extracts obtained by organic solvents. Above mentioned facts were demonstrated by the data on common dandelion: 24,5 g/100 g SFE extract, 13,0 g/100 g n-hexane extract and 1,3 g/100 g ethanole extract (*Kristó* et al. 2000).

Conclusion

The monoterpene-ester content of SFE fractions in general is higher and the alcohol content is lower than those in essential oils obtained by steam distillation. If the steam distillation is carried out for a longer time (~ 5–6 hours) or from acidic medium, the ratio of alcoholic components increases or new alcoholic components appear in oil. This fact proves that a part of volatile terpenes is in bounded form in the plant. Studying the change of composition of SFE fractions succesively in time we could establish that the monoterpene hydrocarbones are present in the first fractions, the oxygenated monoterpenes as well as the sesquiterpenes and volatile phenolic compounds are concentrated in the

latter fractions. During the supercritical extraction the azulenogene sesquiterpene lactones don't transform to azulenes, but the non volatile SFE fraction of some *Asteraceae* plant contain sesquiterpene-γ-lactone of unchanged structure as well as free triterpene and sterol components in high quantities. Summerizing we can establish that the composition of volatile part of SFE fractions is closer to the original aroma – composition of plant then that of essential oil obtained by steam distillation. Further the method is suitable to obtain extracts which are rich in biologically active compounds and could provide good basis for the production of medicaments.

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