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Dalmatian Sage. Part 1. Differing Oil Yields and Compositions from Flowering and Non-flowering Accessions

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The components in essential oils of Dalmatian sage, *Salvia officinalis* L., were identified by ^{1}H- and ^{13}C-NMR spectroscopy and by GC–MS. The diterpene manool was identified for the first time in steam-distilled oil from Dalmatian sage. Several sesquiterpenes were also identified for the first time in Dalmatian sage oils. A rapid GC method was developed and used to analyse 13 oils from a preliminary study of Dalmatian sage accessions. Oil yields from pilot-scale distillations in summer were significantly higher from non-flowering accessions than from flowering accessions. Oils from flowering and non-flowering accessions had different compositions, with significantly higher levels of thujones, β-caryophyllene and viridiflorol in oils from flowering accessions.

KEY WORDS: *Salvia officinalis* L.; Lamiaceae; essential oil; flowering vs. non-flowering; manool; sesquiterpenes; NMR

INTRODUCTION

We are investigating several native and introduced plants that could form the basis of an essential oil and extract industry in New Zealand.[1] One of the introduced species of interest is Dalmatian sage, *Salvia officinalis* L. (family Lamiaceae), which we are investigating for production of both a culinary herb and essential oil.[2] There are many cultivated varieties of *S. officinalis*, some of which have been selected to be non- (or rarely-) flowering.[3] Both flowering and non-flowering types grow well under irrigation in the Mediterranean-like environment of Central Otago, New Zealand.

There does not appear to be a published standard for the composition of Dalmatian sage oil, probably because of the great variability of these oils. This is highlighted in Tucker and Maciarello's review of analyses of *S. officinalis* oils from the Dalmatian coast of the former Yugoslavia and Albania, the traditional source.[4] These oils showed a wide range of levels of the eight main components: camphene, β-pinene, 1,8-cineole, α-thujone, β-thujone, camphor, α-humulene and borneol. Svoboda and Deans reported large differences in the chemical composition in oils from plants from different parts of Europe.[5] A few other sesquiterpenes have been identified, with β-caryophyllene and viridiflorol reaching > 5% in some oils.[4] Further minor components in sage oils have been listed by Heath.[6] This paper describes the identification of further components in Dalmatian sage oils, a rapid gas chromatographic (GC) method to analyse these oils, and oil yields and compositions from flowering and non-flowering accessions.

EXPERIMENTAL

Plant Material

Dalmatian sage accessions were obtained between 1988 and 1992, and established at Redbank Research Station, Clyde, Central Otago (latitude 45°14′S, longitude 169°20′E). Flowering

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Table 1. Dalmatian sage sources, harvest dates and oil yields

Source[a]	Type[b]	Harvest	Charge (kg)	Oil % Fresh[c]	Oil % DM	Oil codes[d]
Holland (CSS)	F	5 Jan 94	80.5	0.13	0.42	1
Budapest, Hungary (BG)	F	8 Jan 94	17.95	0.12	0.56	2
Auckland, New Zealand (CSS)	F	8 Jan 94	16	0.18	0.81	3
Ontario, Canada (CSS)	F	8 Jan 94	26	0.19	0.77	4
New Zealand (DSIR, Sage B)	NF	8 Jan 94	5.5	0.22	0.84	5
Copenhagen, Denmark (BG)	F	8 Jan 94	22.5	0.12	0.51	6
Wunstorf, Germany (CSS)	F	8 Jan 94	11.35	0.12	0.42	7
Dijon, France (BG)	F	8 Jan 94	15.35	0.15	0.53	8
Budakalasz, Hungary (BG)	F	8 Jan 94	9.9	0.11	0.43	9
Firenze, Italy (BG)	F	8 Jan 94	7.3	0.11	0.46	10
Christchurch, New Zealand (CHP)	NF	5 Feb 94	36.35	0.35	0.9	11
New Zealand (DSIR, Sage D)	NF	5 Feb 94	45.7	0.14	0.47	12
New Zealand (DSIR, Lincoln Grey)	NF	6 Feb 94	23.3	0.2	0.58	13

[a]BG = Botanical garden; CSS = Commercial seed supplier; DSIR = Department of Scientific and Industrial Research, Canterbury (now Crop & Food Research): CHP = Commercial herb producer.
[b]F = flowering, NF = non-flowering.
[c]From 30 minutes steam distillation, except 45 minutes from RB94/1.
[d]Used in Figure 3.

types were grown from small quantities of seed obtained from botanical gardens and commercial seed supply companies from around the world. Non-flowering types were grown from cuttings obtained within New Zealand (see Table 1).

All accessions were established in rows 1 m apart with 30 cm spacings between plants within each row. Irrigation was applied regularly during the growing season. Fertilizer was applied annually, according to soil test results to ensure that soil fertility was not limiting crop production. Plants were trimmed to give foliage for distillations once each year during flowering, and had formed hedgerows by summer 1993/94.

In summer 1993/94, mature plants were trimmed to 20–30 cm above ground level when the flowering accessions were just past full flowering (which occurred at the same time for all accessions). The trimmed foliage was weighed and kept at 1°C until distillation was carried out (within 48 h). Three sub-samples of foliage from each accession were dried to constant weight at 80°C to determine moisture content. Herbarium voucher specimens were deposited in the Redbank Research Station Collection (voucher numbers listed in Supplementary Material).

Extraction of Essential Oil

The pilot-scale system described in our previous work with thyme was used for steam distillation of the essential oil.[7] Sage samples were distilled as harvested, i.e. as a mixture of leaf and stem, plus floral parts for flowering accessions. The samples, which varied in weight (see Table 1), were placed directly in the distillation vessel with no pre-treatment and distilled for 30 min. A preliminary trial of a 2 h distillation with this system showed that 99% of essential oil was recovered in the first 30 min. After each distillation the oil was separated and its volume measured before drying over anhydrous sodium sulphate. Sub-samples of oils were stored below 0°C for GC analysis.

Component Isolation

A Dalmatian sage oil from a flowering accession (Accession SO 005) was subjected to silica gel column chromatography (50 g Davisil, 60 Å, 35–70 μm). The column was developed with hexane (200 ml), hexane:ethyl acetate (100 ml each 19:1, 9:1, 4:1, 1:1) and ethyl acetate (100 ml), collecting 10 ml fractions. These were all analysed by GC (see below) and those with similar compositions were combined. ^{1}H- (200 MHz) and ^{13}C- (50 MHz) NMR spectroscopy (Varian Gemini) was used to examine $CDCl_3$ solutions (^{1}H spectra were referenced to $CHCl_3$ at 7.27 ppm and ^{13}C spectra to $CHCl_3$ at 77.08 ppm).

Fractions 8–12, eluted with hexane, contained mostly monoterpene hydrocarbons. Characterization of these fractions by NMR spectroscopy proved too difficult due to the presence of a complex mixture of compounds with similar relative abundances.

Fractions 11 and 12 contained mostly β-caryophyllene, which was identified by ^{1}H- and ^{13}C-NMR spectroscopy.[8,9]

Fractions 13–21 contained mostly α-humulene; ^{1}H-NMR olefinic signals: 5.60 (1H, dt, J = 16,7 Hz), 5.16 (1H, d, J = 16 Hz), 4.96 (1H, br t, J = 7 Hz), 4.88 (1H, br t, J = 7 Hz); ^{13}C-NMR: 141.05, 139.21, 133.17, 127.78, 125.94, 125.03, 42.06, 40.48, 40.04, 39.81, 37.41, 27.17, 23.42, 17.98, 15.13.

Fractions 38–40, eluted with 4:1 hexane:ethyl acetate, contained a mixture of 1,8-cineole, α-thujone, β-thujone, camphor, bornyl acetate and caryophyllene oxide. Due to the complex nature of these fractions, full ^{1}H-NMR analysis was not feasible. However, diagnostic double doublet signals (J = 5,4 Hz) from one of the cyclopropane protons of α- and β-thujone were observed at 0.13 and –0.03 ppm respectively. ^{13}C-NMR data were obtained for α-thujone, the major component of the mixture: 47.38, 39.71, 32.92, 28.89, 25.53, 19.99, 19.72, 18.72, 18.19 ppm (the signal corresponding to the carbonyl carbon of the molecule was not observed).

Fraction 44, eluted with 4:1 hexane:ethyl acetate, contained mostly manool; ^{1}H-NMR olefinic signals: 5.91 (1H, dd, J = 17,11 Hz), 5.21 (1H, dd, J = 17,1) 5.05 (1H, dd, J = 11,1), 4.82 (1H, br d, J = 2), 4.52 (1H, br s); ^{13}C-NMR: 148.77, 145.37, 111.60, 106.52, 73.65, 57.41, 55.66, 42.28, 41.52, 39.80, 39.16, 38.43, 33.69, 32.18 27.73, 24.51, 21.79, 19.47, 17.79, 14.51.[10]

Fractions 46 and 47, also eluted with 4:1 hexane:ethyl acetate, contained mostly viridiflorol. The ^{1}H-NMR spectrum contained a characteristic triplet at 0.12 ppm (J = 9 Hz) due to one of the cyclopropane ring protons; ^{13}C-NMR: 73.63, 58.25, 39.77, 38.50, 37.82, 32.15, 29.14, 28.72, 28.61, 25.82, 22.36, 18.86, 18.44, 16.36, 16.16.

Fractions 49–53, eluted with 1:1 hexane:ethyl acetate, contained mostly borneol; ^{13}C- NMR: 76.44, ~49.7, ~48.0, 45.14, 39.05, 28.31, 25.96, 20.22, 18.71, 13.35.

Some other components were identified using GC co-injection of reference samples, as summarized in Table 2.

GC–MS Analysis

A Dalmatian sage oil from a non-flowering accession was subjected to GC–MS analysis on a Hewlett Packard 5892 GC coupled to a Finnigan-Mat 4500 MS. The column was a 50 m × 0.32 mm (i.d.) Chrompack Sil-5 CB (0.40 μm film), with He carrier gas (1.0 ml/min). The temperature programme was: 65°C for 10 min; then 2°C/min to 280°C; then 280°C for 15 min. Mass spectra were obtained in electron impact mode; 70 eV ionization voltage; scanning range: 35–350 amu; rate, 1 scan/sec. Identifications were by comparison with a library (of *ca.* 10000 spectra) built up on this system from analyses of pure compounds and compounds identified with certainty (using other spectroscopic methods) in mixtures. Apart from the components listed in Table 2, the following minor components were identified (in order of increasing retention time): (*Z*)-salvene, tricyclene, α-thujene, oct-1-en-3-ol, sabinene, *p*-cymene, γ-terpinene, linalol, pinocamphone, (*Z*)-hex-3-enyl 2-methylbutyrate, α-cubebene, α-ylangene, β-bourbonene, β-selinene, α-muurolene, (*E,E*)-α-farnesene, *trans*-calamenene, α-calacorene, α-cadinene and β-calacorene.

GC Analyses

Dalmatian sage oils were analysed as 1% solutions in hexane, using a Perkin Elmer Autosystem gas chromatograph under the control of Perkin Elmer Omega (Version 5.2) software. The column used was a 10 m × 0.25 mm i.d., 0.25 μm film, J & W DB-1, with H_2 carrier gas (linear velocity 55 cm/s). Injections (0.5 μl) were made into a split (100:1) injector set at 260°C. The flame ionization detector was set at 350°C. A temperature programme of 50–250°C at 30°C/min was used to analyse all the oils and fractions.

DB-1 Kovats retention indices (RIs) of major peaks were measured by co-injection of an oil with *n*-alkanes (C8, 9, 10, 12, 14, 16, 18, 20, 22 and 24) on this temperature programme (Table 2). RIs were also measured on a 30 m × 0.25 mm i.d., 0.25 μm film, J & W DB-Wax column (Table 2), with H_2 carrier gas (linear velocity 55 cm/s), and a temperature programme of 50–250°C at 5°C/min.

GC peaks of area greater than 1% in at least one of the 13 oils were labelled peak 1–peak 27 (Table 2). Peaks due to 1,8-cineole, β-caryophyllene and manool were used as references to correct retention time fluctuations between runs. ASCII files of the levels of these 27 peaks in each oil were used for statistical analyses, with SAS Institute software. The unscaled levels of these 27 peaks in each oil were initially analysed using principle components analysis (PCA) to help

Table 2. Dalmatian sage component identifications

Peak	DB-1 RI	DB-Wax RI	Compound	Identification method	Silica eluent
1	933	1003	α-Pinene	MS, DB-1,[a] DB-Wax[b]	Hexane
2	945	1039	Camphene	MS, DB-1, DB-Wax	Hexane
3	969	1079	β-Pinene	MS, DB-1, DB-Wax	Hexane
4	982	1146	Myrcene	MS, DB-1, DB-Wax	Hexane
5	1019	1209	1,8-Cineole+ Limonene	MS	4:1 H:E[c]
6	1025	~1200	(*Z*)-Ocimene	MS	Hexane
7	1085	1416	α-Thujone	NMR, MS	4:1 H:E
8	1095	1435	β-Thujone	NMR, MS	4:1 H:E
9	1117	1507	Camphor	MS, DB-1, DB-Wax	4:1 H:E
10	1146	1702	Borneol	NMR, MS, DB-Wax	1:1 H:E
11	1269	1573	Bornyl acetate	MS	4:1 H:E
12	1375	ND[d]	α-Copaene	MS	–
13	?	ND[d]	α-Gurjunene	MS	–
14	1416	1587	β-Caryophyllene	NMR, MS	Hexane
15	1420	ND	β-Cubebene	MS	–
16	1433	ND	Aromadendrene	MS	–
17	1450	1659	α-Humulene	NMR, MS, DB-Wax	Hexane
18	1470	ND	α-Amorphene	MS	–
19	?	ND	Germacrene D	MS	–
20	1492	ND	Bicyclogermacrene	MS	–
21	1507	ND	Unknown	–	–
22	1515	ND	δ–Cadinene	MS	–
23	1555	ND	Palustrol+Spathulenol	MS	–
24	1568	ND	Caryophyllene oxide	MS, DB-1	Hexane
25	1580	2081	Viridiflorol	NMR, MS	4:1 H:E
26	1592	ND	α-Humulene oxide	MS	–
27	2042	2663	Manool	NMR, MS, DB-1, DB-Wax	4:1 H:E

[a]Coinjection on DB-1.
[b]Coinjection on DB-Wax.
[c]Hexane:ethyl acetate
[d]Not determined; could not reliably correlate between DB-1 and DB-Wax traces.

with recognition of composition patterns. The significances of differences between flowering and non-flowering types were tested by analysis of variance (GLM procedure).

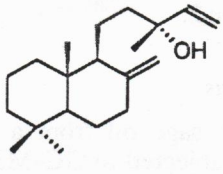
Supplementary Material Available

Full GC analyses of 13 oils (two pages) are available from the authors.

RESULTS AND DISCUSSION

The composition of Dalmatian sage oils has been studied by many researchers, with early work reviewed by Heath.[6] The most useful recent studies are those by Tucker and Maciarello,[4] and by Formacek and Kubeczka,[11] which agree on the major monoterpene and sesquiterpene components. We confirmed the identifications of some of these major components by fractionating an oil from a flowering accession on silica gel and checking NMR spectra (Table 2 and Figure 1). Formacek and Kubeczka give ^{13}C-NMR spectra of essential oil components data for C_6D_6 solutions,[11] whereas the more usual (and cheaper) solvent is $CDCl_3$. Therefore we have reported our ^{13}C-NMR data for purified components in $CDCl_3$. ^{1}H-NMR data are given for selected components with signals at unusual shifts, since we find that high-field (200 MHz or greater) ^{1}H-NMR spectra of oils can be used to indicate the presence of high levels (>10%) of such components.

We also isolated the diterpene alcohol manool (**1**), by comparison of GC retention times and



1 Manool

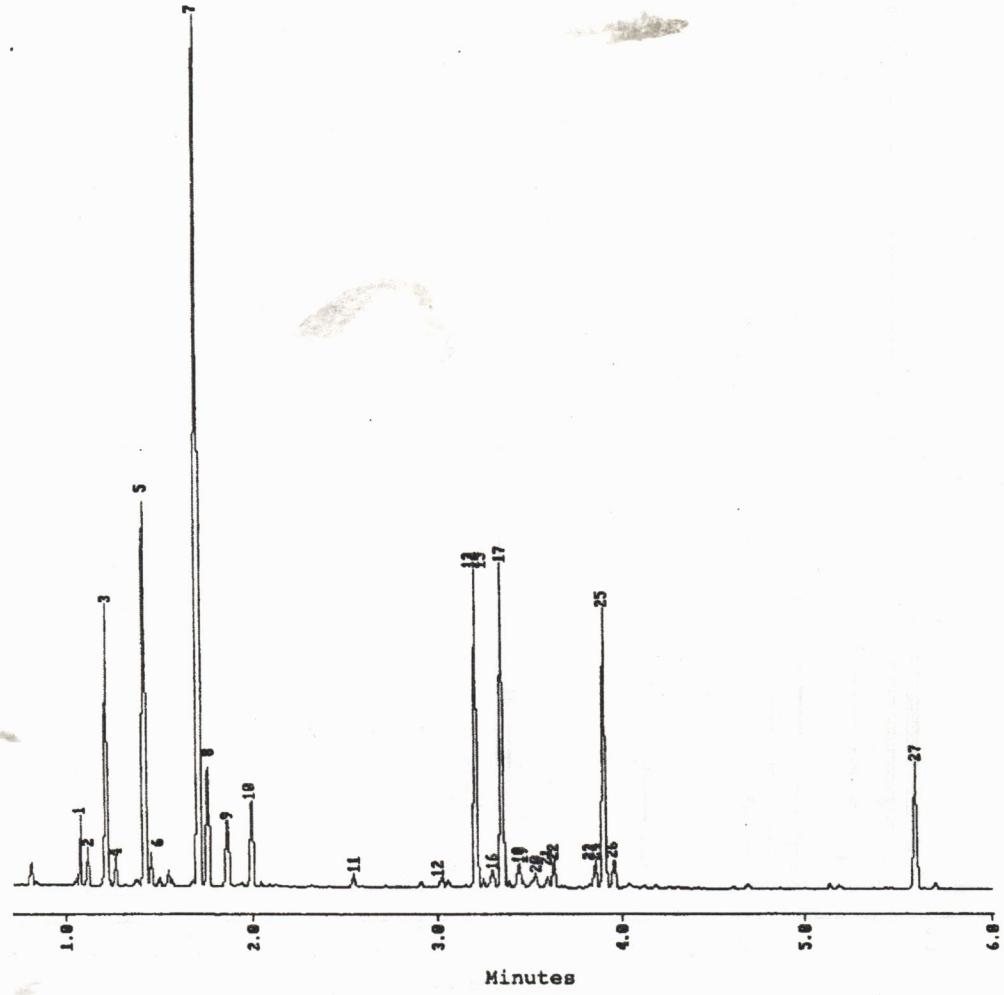


Fig. 1. GC trace of Dalmatian sage oil produced from a flowering accession (oil 4 in Figure 3); see Table 2 for peak identifications

NMR spectra with an authentic sample of manool (from *Halocarpus biformis* (Hook.) Quinn, previously *Dacrydium biforme*[12]). Manool has only been reported once from *S. officinalis*, in an ethanol extract rather than a steam-distilled oil.[13] Manool has been found in a steam-distilled oil from another *Salvia* species, *S. stenophylla* Burch.[14] We detected manool in the oils from all our 13 Dalmatian sage accessions, at levels ranging from 0.1 to 4.8%. We presume that this component has been overlooked, or could not be identified, by other researchers (for example, Rhyu's "peak 97"[15] could well be manool).

We found that our oils from non-flowering accessions of Dalmatian sage had much more complex sesquiterpene compositions than did the oils from flowering accessions (cf. Figures 1 and 2). Therefore we undertook a detailed GC–MS analysis of a non-flowering oil, which led to the identification of several sesquiterpenes that do not seem to have been reported before in Dalmatian sage oils (see Table 2 and Experimental). The GC analyses of oils from different accessions are discussed below.

We have 22 Dalmatian sage accessions growing in Central Otago, New Zealand. Thirteen of these accessions (nine flowering and four non-flowering, see Table 1) are well enough established to give sufficient material for pilot-scale still distillations. We were attempting to predict the oil yields and compositions that would be obtained from commercial production, so leaves

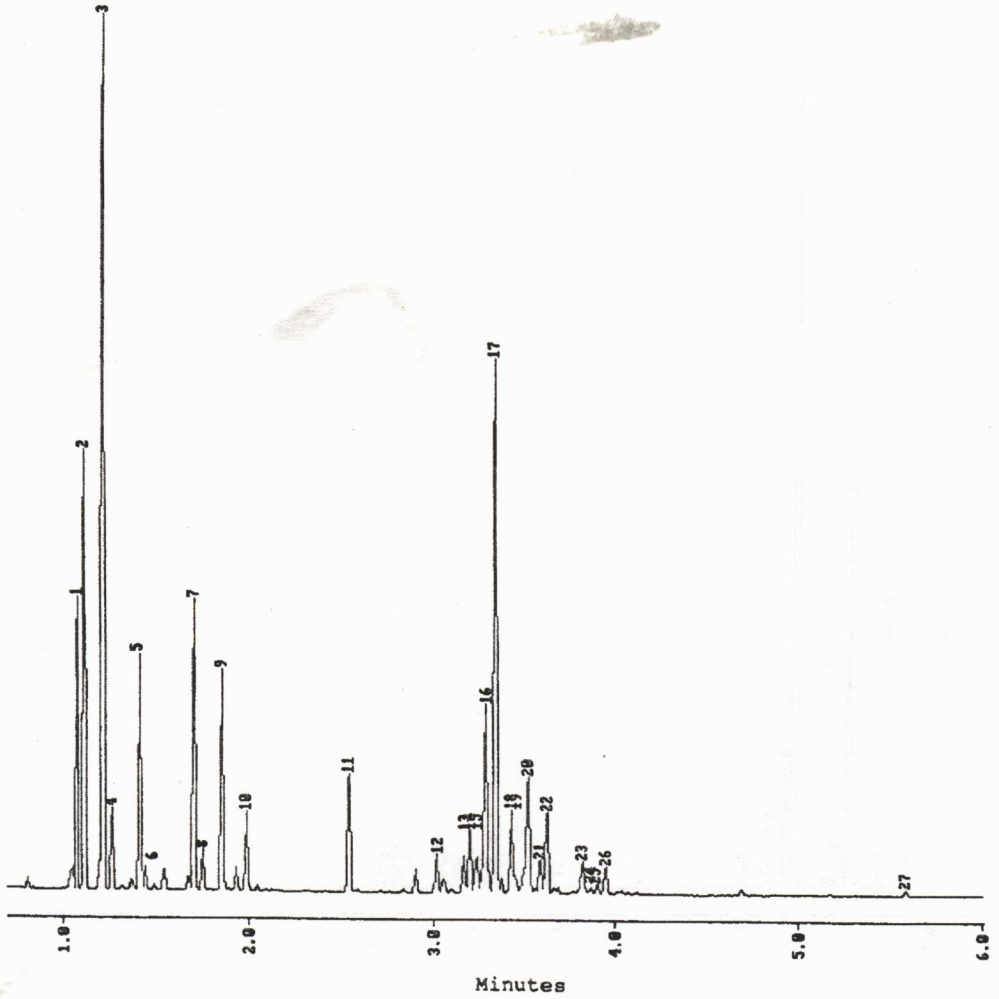


Figure 2. GC trace of Dalmatian sage oil produced from a non-flowering accession (oil 13 in Figure 3); see Table 2 for peak identifications

and flowers were not separated from the stems before steam distillation. All the accessions were harvested in the Southern summer, just past full bloom of the flowering accessions. Oil yields varied from 0.11 to 0.35% of fresh weight (1.1–3.5 ml/kg, see Table 1), or 0.42–0.90% of dry weight. This is similar to the range of yields reported for sage cultivars grown in North America.[4] The flowering accessions gave significantly ($P < 0.05$) lower oil yields (mean 0.14% of fresh weight, SD 0.03) than did the non-flowering accessions (mean 0.23%, SD 0.08). These different yields can be explained by reference to Máthé *et al.*, who studied the monthly variation in oil contents of leaf, stem and generative organs in flowering types of *S. officinalis*.[16] Oil content of leaf was highest late in flowering, but the proportion of leaf per plant was low at this time. Máthé *et al.* found that highest overall yields were obtained from plants in the vegetative stage, i.e. non-flowering.

We developed a fast GC method to analyse the oils from our 13 different Dalmatian sage accessions, and to analyse the many oils from our continuing studies. The 27 major components, that came above 1% in at least one of our oils (Table 2), were separated by this method in just over six minutes (Figures 1 and 2). The levels of these major components in oils from flowering and non-flowering types are summarized in

Table 3. Significant differences in levels of oil components, flowering vs. non-flowering

Components	Mean level[a]		
	Flowering (n = 9)	Non-flowering (n = 4)	Significance[b]
α-Pinene	1.90	4.30	*
Camphene	1.65	6.56	**
β-Pinene	7.48	14.99	*
Myrcene	0.74	1.21	*
1,8-Cineole + Limonene	9.73	4.36	**
(Z)-Ocimene	1.09	0.79	NS
α-Thujone	17.10	6.11	*
β-Thujone	3.99	0.91	**
Camphor	1.92	4.78	**
Borneol	2.59	2.20	NS
Bornyl Acetate	0.66	2.89	**
α-Copaene	0.12	1.13	**
α-Gurjunene	0.09	1.05	**
Caryophyllene	12.27	2.13	**
β-Cubebene	0.30	1.21	**
Aromadendrene	0.41	5.74	**
α-Humulene	14.98	17.27	NS
α-Amorphene	0.82	2.23	**
Germacrene D	0.19	0.97	**
Bicyclogermacrene	0.39	4.15	**
Unknown	0.43	0.99	**
δ-Cadinene	0.82	2.29	**
Palustrol + Spathulenol	0.05	0.30	*
Caryophyllene oxide	0.97	0.26	**
Viridiflorol	9.58	0.69	**
α-Humulene oxide	1.07	1.15	NS
Manool	2.50	0.55	*

[a]Percentage of total volatiles by GC.
[b]NS, $P > 0.05$; *, $0.05 > P > 0.01$; **, $0.01 > P$.

Table 3. The full GC data set (levels of 27 components in 13 oils) was subjected to principal components analysis (PCA) to help to distinguish compositional patterns.[17] The 1st and 2nd principal components (PCs) accounted for 80% of the total variance of the data set. The main contributor to the 1st PC was α-thujone (eigenvector +0.62), followed by β-caryophyllene (eigenvector +0.44) and viridiflorol (eigenvector +0.32). The main contributor to the 2nd PC was α-humulene (eigenvector +0.70). Figure 3 shows the 13 oils plotted in terms of these two PCs, with the flowering and non-flowering types separated on the first PC. Further statistical analyses showed that the flowering and non-flowering types differed significantly in the levels of most of the oil components (Table 3). As expected from the PCA, the flowering oils had higher levels of α-thujone, β- caryophyllene and viridiflorol (cf. Figures 1 and 2). The two oils from flowering accessions which came closest to the oils from non-flowering accessions in the PCA plot (2 and 9 in Figure 3) had low levels of α-thujone, but higher levels of β-thujone. Total thujone levels in the oils from flowering accessions showed a range of 10–31%, compared to a range of 6–8% for the oils from non-flowering accessions.

This distinction between the oil compositions of flowering and non-flowering types of Dalmatian sage does not seem to have been made before. Preliminary results from our analyses of oils from flowering and non-flowering types in winter show the same distinct compositions, so the difference in the summer oils was not due to the oil content of the inflorescences on the flowering accessions.

None of the summer oils described above reached the total thujone level of 50% required for high quality Dalmatian sage oils.[6] However, preliminary results show that winter oils have higher thujone levels. We are continuing our

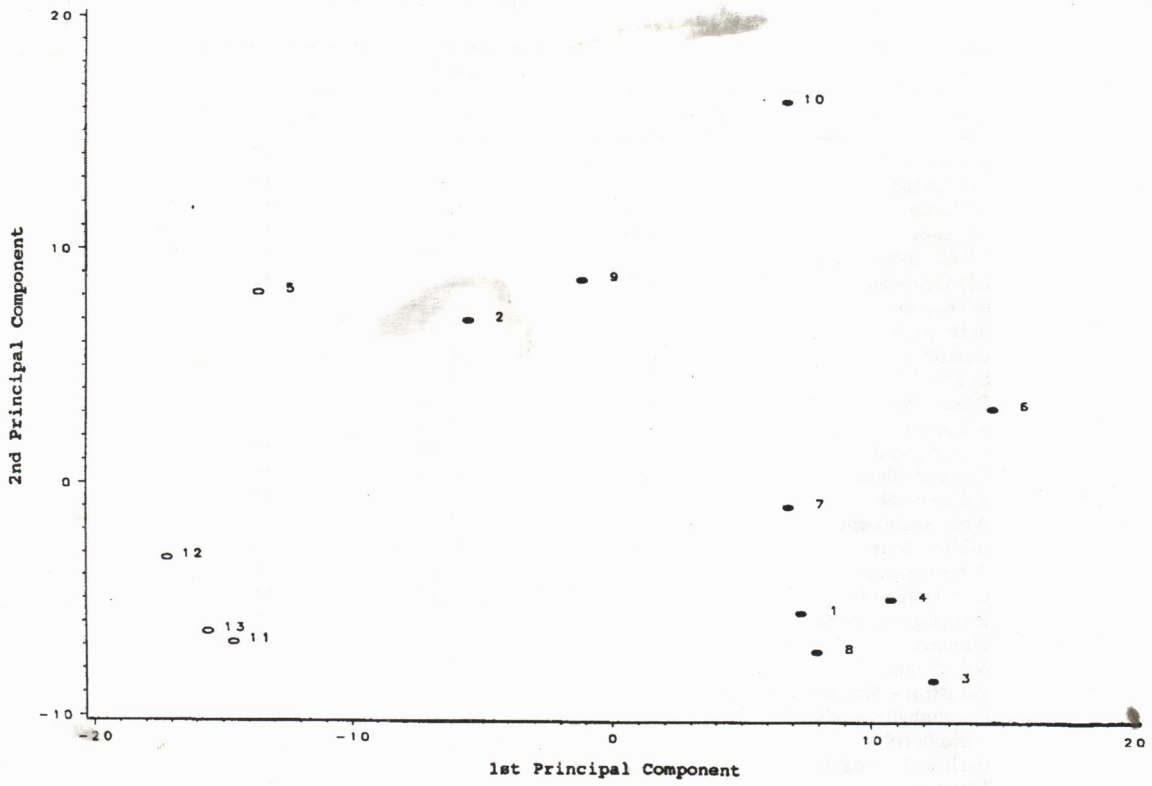


Fig. 3. Oil compositions of Dalmatian sage oils from flowering (●) and non-flowering (○) accessions in terms of the 1st and 2nd principal components

studies on the factors affecting Dalmatian sage oil yields and compositions by comparing oils from flowering and non-flowering types in wholly vegetative states.

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