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Greek *Salvia sclarea* L. Essential Oils: Effect of Hydrodistillation Time, Comparison of the Aroma Chemicals Using Hydrodistillation and HS-SPME Techniques

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Abstract: Since the essential oil of *Salvia sclarea* is used as a flavouring agent, the effect of different extraction techniques (hydrodistillation & HS-SPME) and duration of hydrodistillation (2, 3 and 4 h) with respect to yield, composition and identification rate of extracted essential oils from Greek cultivated *S. sclarea* aerial blooming parts were investigated. Linalool and linalyl acetate levels seemed to decrease with increasing duration of hydrodistillation, while diterpenes increased dramatically, while the head space analysis showed significantly lower levels of linalool in comparison to its ester. Thus, linalool (5.1-35.8%), linalyl acetate (11.3-37.6%) and sclareol (0.0-41.8%), concerning the oils obtained by hydrodistillation, were the most important metabolites. Solid-phase microextraction yielded mainly oxygenated monoterpenes, especially linalyl acetate (59.3%), followed by *cis*-linalool oxide (8.6%) and linalool (7.8%).

Keywords: *Salvia sclarea*; Solid-phase microextraction; Linalool; Linalyl acetate; Hydrodistillation; Sclareol.

1. Plant Source

Salvia sclarea L. (clary sage) is a biennial or perennial shrub up to 100 cm high belonging to the Labiatae family [1]. It is native to southern Europe and C. Asia [2] and cultivated worldwide.

Aerial parts of cultivated *S. sclarea* were collected during the flowering stage in June 2014 from J. & A.N. Diomedes Botanic Garden, University of Athens, County Attiki. A voucher specimen (No. OT-102) has been deposited in the Herbarium of the University of Athens (ATHU).

2. Previous Studies

In Greece *S. sclarea* is known by the common name “agiannitis” [3] and locally is referred to be used as a diuretic, for coughs, colds, blood cleaning, on wounds and sore eyes [4]. In traditional medicine the herb is used for stomachic and digestive disorders, and in kidney diseases [5]. A strong cytotoxic activity of *S. sclarea* oil equivalent to that of doxorubicin, has been reported [6]. The essential oils or extracts of *S. sclarea* aerial parts have been evaluated for antiinflammatory, analgesic

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[7], antifungal [8], antioxidant, antibacterial [9,10] and opioid receptor binding activity [11]. The essential oil composition of *S. sclarea* has been extensively studied [5,8,12-16 and references therein]. Aim of the present study was the evaluation of the effect of different hydrodistillation times (2, 3, and 4 h) on the yield and composition of Greek cultivated *S. sclarea* essential oils and to compare the results of the conventional hydrodistillation method with those of a HS-SPME method.

3. Present Study

The essential oils were isolated from the fresh flowering stems and leaves by hydrodistillation (HD) for 2, 3 and 4 h using a modified Clevenger-type apparatus with a water-cooled receiver, according to European Pharmacopoeia method [14]. Plant material prior to hydrodistillation was cut in small pieces. The oils were obtained using *n*-pentane as a collecting solvent and subsequently, they were dried over anhydrous sodium sulfate and stored under N_2 atmosphere in amber vials at 4 °C until they were analyzed.

HS-SPME Analysis: The collected fresh flowering stems and leaves were cut roughly with scissors (~2 cm long) and subjected to head space solid phase microextraction (HS-SPME). The SPME sampler coated with carboxen/polymethylsiloxane (Supelco, CAR/PDMS, 75 μm) was used for the extraction of the volatiles. The one centimeter long coated fiber was conditioned in a GC injection port at 280 °C for 2 h, prior to use. The sample (1 g) was placed in a 12 mL vial which was sealed with parafilm and placed in an oven at 45 °C and equilibrated for 15 min. Subsequently, the SPME fiber was pushed through the plastic film for exposure to the headspace of the sample for 15 min and then retracted. After extraction, the SPME sampler was immediately inserted into the GC injector and the fiber thermally desorbed. Each sampling was performed in duplicate. No reconditioning was needed for the fiber before next sampling.

GC-MS: Analysis of the essential oils was performed using a Hewlett Packard (Hewlett Packard GmbH, Waldbronn, Germany) model 5973-6890 GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (220 °C). The transfer line temperature was 250 °C. Helium was used as carrier gas (1 mL/min) and the capillary column used was HP 5MS (30 m × 0.25 mm; film thickness 0.25 μm; Agilent, Palo Alto, CA, USA). The temperature program was the same with that used for the GC analysis; split ratio 1:10. The injected volume was 1 μL of diluted essential oil in *n*-pentane (10% v/v). Total scan time 83.33 min. Acquisition mass range 40-400 amu. The identification of the compounds was based on comparison of their retention indices (RI), their retention times (RT) and mass spectra with those obtained from authentic samples (purchased from Sigma-Aldrich Co., Buchs SG, Switzerland), and/or the NIST/NBS, Wiley libraries (available through Hewlett Packard) and the literature [17].

The essential oils isolated from fresh flowering stems and leaves of cultivated *S. sclarea* obtained at different hydrodistillation times ranged from a yield of 0.26-0.48% (v/w), showing both qualitative and quantitative differences. The relative amounts of the volatile compounds identified in the essential oils are given in Table 1. Forty five components were identified, representing 87.8-99.8% of the oils under study.

The major difference between commercial oils of *S. sclarea* is in the linalool/linalyl acetate contents [18]. In the studied Greek oils the oxygenated monoterpenes constituted the main fraction of the oils obtained in 2 h and 3 h (88.8% and 59.1%, respectively), with linalool acetate (37.6, 21.9%) and linalool (35.8, 19.7%) being the dominant components, followed by *α*-terpineol (11.0, 6.8%). Linalool and linalyl acetate are useful monoterpenes for the perfumery industry known to possess several biological activities such as anti-inflammatory activity [19]. The essential oil obtained in 4 h was characterized by the high amount of diterpenes (52.3%), with sclareol being the dominant component (41.8%). Diterpenes were also present in the oil obtained in 3 h (17.3%), with sclareol being the main compound (13.2%), whereas they were not detected in the oil obtained in 2 h. Sclareol is a natural fragrance compound used widely in the cosmetic and food industries, reported to exhibit anti-inflammatory, bactericide, fungicide and allelopathic acivity [20,21]. This essential oil sample

was the only one in which alkanes (3.9%) have been detected, probably owing to the longer exposure to high temperature during the hydrodistillation. According to the European Pharmacopeia the amount of linalool and its acetylated derivative, typical volatile constituents in clary sage oil, ranges between 6.5-24.0% and 56.0-78.0% respectively, while the amount of sclareol, an oxygen containing diterpene, varies from 0.4 to 2.6% [14]. The essential oil of clary sage has been extensively studied and the afformentioned oxygenated monoterpenes, linalool and linalyl acetate, are the most characteristic and dominant volatiles of *S. sclarea*. Therefore, the results of the analysis of the essential oil obtained in 3 h, are generally in accordance with the previously reported chemical analyses of *S. sclarea* [8,15,22-30]. Remarkable were, though, the higher levels of sclareol in this sample (13.2%) compared to published reports (0.0-5.55%). Although environmental parameters, namely geoclimatic location and growing conditions, can affect the chemical composition of an essential oil, clary sage oil appears to have a quite stable pattern, with certain metabolites being dominant, regardless the origin of the plant material.

Processing fresh clary sage material, the excess moisture causes the major constituent, linalyl acetate, to hydrolyse to linalool and this can be seen in the present analyses (Table 1) [18]. More specifically, monoterpenes extracted by hydrodistillation decrease in concentration as time of extraction increases. This trend of shifting to higher molar mass compounds with prolonged HD time is evident, indicating that the same technique can give substantially different composition just by applying it for a longer duration.

HS-SPME analysis led to the identification of 22 volatile components. Oxygen containing monoterpenes also characterized the HS-SPME sample (82.0%), with linalool acetate dominating (59.3%). Linalool was present in 7.8%, while *cis*-linalool oxide, existing only in traces in sample oil of 3 h, was present in considerable amount (8.6%). In this sample monoterpenes hydrocarbons were present in higher amounts than in the oil samples obtained by HD. Sesquiterpenes were found in lower amount (2.6%), whereas no diterpenes were detected.

Multiple patterns in the concentration of grouped compounds between duration of HD and HS-SPME have been noticed (Table 1). Technically, regarding the different used isolation methods of *S. sclarea* volatiles, the hydrodistilled oils showed higher number of compounds compared to HS-SPME trapped volatiles. Monoterpenes may be susceptible to chemical changes under hydrodistillation. Fragile and thermosensitive constituents may decompose resulting into artifacts due to heating. During hydrodistillation, the most volatile compounds and water-soluble components are lost in the gaseous phase and in the hydrolate phase, respectively. HS-SPME on the other hand is a simple, fast, solvent-free technique for the analysis of volatile metabolites, allowing using a small quantity of plant material, giving a chromatographic fingerprint of the analyzed herb's headspace.

In order to obtain a more complete characterization of plant volatiles, HD and HS-SPME extraction techniques should be used supplementary. Overall if the primary goal is a *S. sclarea* essential oil rich in sclareol, the optimum hydrodistillation time is 4 h. *Salvia sclarea* is often used as an ornamental plant in gardens that is pruned after flowering. According to the results of the present study this plant material, instead of being discarded it could be exploited for the isolation of sclareol, which is industrially used as a natural fragrance compound.

Table 1. Percentage composition of the essential oils obtained by HD and SPME from fresh flowering stems and leaves of *Salvia sclarea*.

RI	Compound*	2 h HD	3 h HD	4 h HD	HS-SPME
990	Myrcene	3.0	1.6	tr	5.8
1017	α-Terpinene	-	tr	-	tr
1024	*p*-Cymene	-	tr	-	3.5
1029	Limonene	tr	0.5	tr	2.4
1031	1,8-Cineole	tr	tr	tr	-
1037	(*Z*)-β-Ocimene	tr	1.1	tr	1.3
1050	(*E*)-β-Ocimene	2.8	1.7	tr	2.2
1067	*cis*-Linalool oxide	-	tr	-	8.6

1084	*trans*-Linalool oxide	-	tr	-	0.7
1086	Terpinolene	tr	0.6	tr	-
1096	Linalool	35.8	19.7	5.1	7.8
1188	α-Terpineol	11.0	6.8	2.7	tr
1229	Nerol	tr	1.5	tr	-
1252	Geraniol	tr	2.3	tr	-
1257	Linalool acetate	37.6	21.9	11.3	59.3
1349	α-Terpinyl acetate	tr	0.1	tr	0.9
1361	Neryl acetate	1.8	2.3	1.0	1.8
1376	α-Copaene	tr	0.6	0.7	0.6
1381	Geranyl acetate	2.6	4.4	1.8	2.9
1387	β-Bourbonene	tr	tr	tr	tr
1388	β-Cubebene	tr	0.2	tr	tr
1390	*β*-Elemene	tr	0.1	tr	tr
1419	(E)-Caryophyllene	tr	0.7	1.2	2.0
1433	β-Gurjenene	tr	tr	tr	tr
1485	Germacrene D	5.2	4.4	4.2	tr
1500	Bicyclogermacrene	tr	0.7	1.3	-
1505	(*E*,*E*)-α-Farnesene	-	0.4	tr	-
1513	γ-Cadinene	-	tr	tr	tr
1523	δ-Cadinene	tr	0.2	tr	-
1565	1,5-Epoxysalvial-(14)-ene	tr	tr	tr	-
1578	Spathulenol	tr	0.4	1.6	-
1583	Caryophyllene oxide	tr	0.1	0.7	-
1653	α-Eudesmol	tr	0.7	tr	-
1826	8,13-Epoxy-15,16-dinorlabd-12-ene	-	1.4	5.2	-
1913	(5*E*,9*E*)-Farnesyl acetone	-	0.5	tr	-
1987	Manool oxide	-	0.6	1.7	-
2010	13-*epi*-Manool oxide	-	0.3	1.1	-
2027	(*E*,*E*)-Geranyl linalool	-	0.1	tr	-
2057	Manool	-	1.5	2.5	-
2140	Isoabienol	-	0.3	tr	-
2222	Sclareol	-	13.2	41.8	-
2700	Heptacosane	-	-	1.3	-
2900	Nonacosane	-	-	1.2	-
3000	Triacontane	-	-	0.6	-
3100	Untriacontane	-	-	0.8	-
	Grouped compounds				
	Monoterpene hydrocarbons	5.8	5.5	tr	15.2
	Oxygen containing monoterpenes	88.8	59.1	21.9	82.0
	Sesquiterpene hydrocarbons	5.2	7.3	7.4	2.6
	Oxygen containing sesquiterpenes	tr	1.7	2.3	-

Diterpenes	-	17.3	52.3	-
Alcanes	-	-	3.9	-
Total identified (%)	99.8	90.9	87.8	99.8

*Constituents listed in order of elution from a HP-5 MS column, RI: retention indices on HP-5 MS column relative to C_9-C_{23} *n*-alkanes, tr: trace (<0.05%), - not detected.

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