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Variability of thujone content in essential oil due to plant development and organs from *Artemisia absinthium* L. and *Salvia officinalis* L.

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Summary

The study compared changes in essential oil content and its thujone ratio in two popular herbs (*Artemisia absinthium* L. and *Salvia officinalis* L.), pertaining to plant development and plant organs. Both species were harvested in 2018 at the vegetative, floral budding, flowering and after flowering phases; flowers and leaves were sampled separately. The essential content is always higher in the flowers than in the leaves at the same phenophase in both species we examined. Decreased essential oil content in both organs during the developmental phases was also common to both species. In *S. officinalis*, both leaf and flower oils showed quantitatively different compositional profiles. During plant development, the main component α-thujone decreased significantly in leaf oils, while both thujone isomers demonstrated statistically stable values in flower oils. In *A. absinthium*, leaf and flower oils exhibited similar thujone ratios. During plant development, neither of the thujone isomers changed significantly in leaf oils but the ratio of α-thujone increased in the flowers. It was established, that only the distribution and dynamics of total volatiles showed common features in the two species, while the variability of thujone ratios represents differences specific to each target species. For the praxis, in *S. officinalis* timing of harvest seems to be more important while in *A. absinthium* the ratio of organs may play a more significant role in reaching lower thujone levels of the drug.

Keywords: absinthe, medicinal plants, ontogenesis, flowering, chemotype, chemical variability

Introduction

The monoterpene ketones, α- and β-thujones, are natural substances found in plants, commonly used for flavoring of foods and beverages (Lachenmeier and Uebelacker, 2010). The most well-known product containing thujone is certainly absinthe, produced from wormwood (*Artemisia absinthium* L.). For bitter spirit drinks, such as absinthe, a maximum limit of 35 mg/kg thujone was indicated by the EU Council Directive 88/388/EC. Based on a typical recipe, 2.5 kg of *A. absinthium* yields 100 litres of absinthe (Max, 1990). This is equivalent to 4.4 mg *A. absinthium* oil or 2 mg to 4 mg thujone per drink, which would be far below the level at which acute toxicity effects were observed. Subsequently Lachenmeier and Uebelacker (2010) provided evidence that the current EU limits ensure sufficient protection for consumers. According to the recent regulation of the European Parliament and Council (2008), thujone in chemically pure form is not allowed to be added to foods, but it may be introduced indirectly into foods by using plants containing thujone. The EMA (European Medicines Agency) suggests in its wormwood monograph a daily limit of 3.0 mg thujone/day/person for thujone in *Absinthii herba* for a maximum duration of use of 2 weeks (EMA/HMPC, 2008a). In the case of *Salvia officinalis*, plant material with low thujone content should be preferred and a daily limit set at 5.0 mg thujone/day/person for a maximum duration of use of 2 weeks (EMA/HMPC, 2008b).

Although both α- and β-thujones are also found naturally in considerable concentrations in other essential oils (EO) such as tansy (*Tanacetum vulgare*), white-cedar (*Thuja occidentalis*), and other *Artemisia* and *Achillea* species (Pelkonen et al., 2013), the above mentioned *A. absinthium* and *S. officinalis* species are the most characteristic ones as well as being found in numerous preparations frequently used by consumers.

The mean concentration of α- and β-thujones in *A. absinthium* oils are 5.8% and 12.5% respectively, as calculated from a review of 24 references (Lachenmeier and Nathan-Maister, 2007). In the case of *S. officinalis,* the ratio of α-thujone varied from 1.2 to 45.8% and that of β-thujone between 1.0% and 40.1%) as summarized in Kintzios (2003).

Just as chemosyndromes of the plants may vary according to plant parts and ontogenetic phases (Németh-Zámbori, 2015), the thujone content may vary on a large scale, too. In *A. absinthium*, Ariño et al. (1999) reported qualitatively similar but quantitatively different EO profiles from leaves and flowers. Judzentiene and Budiene (2010) determined higher ratios of thujone in flowers (5.3-10.4% of the oil) than in leaves (0.0-8.9% of the oil). On the other hand, in *S. officinalis* samples collected from a wild region in China, the percentage of β-thujone was higher in leaves (14.86%) than in the flower oil (6.05%) while the ratio of α-thujone was three times higher in the flowers than in the leaf oil (Li et al., 2015). In the case of tansy (*Tanacetum vulgaris*), most leaf oils of twenty samples showed lower concentrations of thujone than those detectable in the inflorescence oils (Judzentiene and Mockute, 2005).

During the plant development of *A. absinthium*, the ratio of the main compound β-thujone reached its highest ratio (51.99%) in the flower oil at floral budding; however, in the leaf oil the highest ratio (63.41%) was measured at the vegetative stage (Nguyen et al., 2018). Ben Farhat et al. (2016) reported that in *S. officinalis*, the proportion of thujone varied depending on the ontogenetic phase and showed a peak at the fruiting stage. In tansy (*Tanacetum vulgaris*), the maximum proportion of α-thujone (44%) was detected in two stages: in the leaf rosette phase and at the beginning of fruit setting (Goudarzi et al., 2015).

While thujone is receiving more and more attention from researchers and producers, reliable data on its variability are both scarce and contradictory. Therefore, the aim of our study was to detect the influencing factors on the variation of thujone-containing EO depending on plant organ and harvesting time (plant development). We asked whether the rows of chemosyndromes could be generalized or whether they are specific for the species, and our study is a comparision of two important thujone-containing species (*A. absinthium* and *S. officinalis*).

Materials and Methods

Plant material and growth conditions

The plant material used in this study consisted of wormwood (*Artemisia absinthium* L.) and sage (*Salvia officinalis* L.). The ac-

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cession of *A. absinthium* originated from Gatersleben Genebank (Germany) with the denomination "Belgien", where thujone chemotype individuals were identified during our previous work (NGUYEN et al., 2017). For *S. officinalis*, we investigated an accession of unknown origin (sage016) maintained in the gene bank of the Department of Medicinal and Aromatic Plants, Szent István University.
The plants were grown in open field plots at the Experimental Station of Szent István University, in Budapest. The soil is sandy-loam, pH 7.8, humus content 1.2%. The plants were cultivated without additional fertilization; mechanical weed control and, in dry periods, irrigation was performed. The experiment was carried out in 2018 with four harvesting times from a 3-year-old cultivated population, based on the phenological stages of each species (Tab. 1).

Chemical analyses

Essential oil extraction

In both species, samples were collected in four phenological stages from perennial, cultivated plantations. Each time, 10 randomly chosen individual plants were harvested and the plant material was dried at room temperature (20-25 °C) in shade for two weeks. After drying, the harvested individual samples were mixed creating one bulk sample representative for the population, for each harvest time. Then, leaves and flowers were separated from stems and each of them were used in three replicates for EO distillation and GC-MS analysis.
50 g dried materials from each sample was hydro-distilled in a Clevenger-type apparatus using 500 ml of water for 2.5 hours according to the method recommended by the VII. Hungarian Pharmacopoeia. The oils were collected, and traces of water removed with anhydrous sodium sulphate. Then, the extracts were separated with a syringe filter and stored in an airtight vial in a refrigerator at 4 °C prior to analysis.

Gas chromatography-Mass spectrometry analysis

The GC-MS analyses were carried out using an Agilent Technologies 6890N instrument equipped with HP-5MS capillary column (5% phenyl, 95% dimethyl polysiloxane, length: 30 m, film thickness: 0.25 nm, I.D. 0.25 mm) and an Agilent Technologies MS 5975 inert mass selective detector. The temperature program was the following: initial temperature 60 °C, then by a rate of 3 °C/min up to 240 °C; the final temperature was kept for 5 min. Carrier gas was helium (1 ml min^{-1}), injector and detector temperatures were 250 °C. Split ratio: 30:1. 10 µl of EO has been diluted by n-hexane to 1 ml and from this the injected quantity was 0.2 µl. Ionization energy was 70 eV. The MS was recorded in full scan mode that revealed the total ion current (TIC) chromatograms (mass range m/z 50-550 µma). Components were identified by comparison of their linear retention indices, which were calculated using the generalized equation of VAN DEN DOOL and DEC. KRATZ (1963) with literature data (Tab. 3; 4); and by matching their recorded mass spectra with those in mass spectra library references (NIST MS Search 2.0 library, Wiley 275).

Statistical analysis

SPSS version 23 was used to analyse the data. The Two-way ANOVA test was conducted on both species to compare the EO content of leaves and flowers harvested at different phenological stages (vegetative, floral budding, flowering and after flowering). For evaluating changes of the concentrations of thujone isomers among the phenophases, one-way ANOVA was used for both the leaf and flower samples. Homogeneity of variances was checked with Levene's test and the normality of variances was checked using the Kolmogorov-Smirnov method.

Results and discussion

Variability of essential oil content

According to the two-way ANOVA test of between-subjects effects, significant differences between both species could be detected in both flower and leaf samples harvested at different phenological stages. Significant differences were detected also between leaf and flower samples collected at the same phenological phases, with a single exception (in *S. officinalis*, after flowering stage). The results are summarized in Tab. 2.
The highest EO content of *S. officinalis* was obtained from flowers in floral budding phenophase (2.82 ml/100 g) and the lowest one was also detected in flowers at after flowering stage (0.73 ml/100 g). Flowers always contained higher levels of volatiles compared with the leaves at the same developmental stage. In both organs, the ac-

Tab. 2: Changes in essential oil content of the leaves and flowers of experimental species during different growth stages

Species	Phenological stage	Essential oil content (ml/100 g) Leaves	Flowers
S. officinalis	Vegetative	2.08 c	-
	Floral budding	1.30 Ab	2.82 Bc
	Flowering	1.15 Abc	1.30 Bb
	After flowering	0.91 Aa	0.73 Aa
A. absinthium	Vegetative	1.72 c	-
	Floral budding	0.90 Ab	1.86 Bb
	Flowering	0.61 Aa	1.69 Bb
	After flowering	-	0.26 a

Lower case letters in columns of each species represent significant differences between phenological phases of the same organ and capital letters in rows represent significant differences between leaves and flowers at the same phenological phase, according to the Games-Howell test at p=0,05.

Tab. 1: Harvesting times and developmental phases of the experimental species

Species	Harvesting time	BBCH Code (HESS et al., 1997)	Description	Denomination of phase
A. absinthium	08.06.2018	40	Harvestable vegetative plant parts or vegetative propagated organs begin to develop	Vegetative
S. officinalis	25.04.2018			
A. absinthium	12.07.2018	55	First individual flowers visible (still closed)	Floral budding
S. officinalis	09.05.2018			
A. absinthium	08.08.2018	65	Full flowering: 50% of flowers open, first petals may have fallen	Flowering
S. officinalis	12.06.2018			
A. absinthium	10.09.2018	67	Flowering finishing: majority of petals fallen or dry	After flowering
S. officinalis	12.07.2018			

cumulation level of volatiles decreased continually during the plant development. Hossein Mirjalili et al. (2006) also reported that EO content of Iranian *S. officinalis* reached the highest level at floral budding (0.9%), then decreased until fruiting. In the case of *A. absinthium*, the results are similar. Highest oil content was found in flowers at the floral budding stage (1.86 ml/100 g) and the lowest values were obtained from flowers after flowering (0.26 ml/100 g). The EO content of the flowers was significantly higher than that of the leaves and its tendency to decrease during the plant development was also detected. In the case of *A. absinthium* the present results corroborate previous data on the tendency to reduce the concentration of volatile compounds during the vegetation period (Nguyen et al., 2018). In several other species a similar phenomenon has been established: the accumulation of volatile compounds reaches a peak at the beginning of flowering and decreases sharply after flowering and further on, during seed ripening (Mohammadi et al., 2015; Németh, 2005). This distribution and the dynamics of oil accumulation may be in connection with the ecological role of the volatile molecules both as defense molecules and/or attractants (Guitton et al., 2010; Németh, 2001).

Variability of thujones in essential oil
***Salvia officinalis* L.**

The qualitative and quantitative results on the composition of *S. officinalis* EO distilled from flowers and leaves are listed in Tab. 3. In total, ten constituents which were higher than 1% of the GC area were identified, representing 91,5-94.1% of the total area.

Leaf oils and flower oils showed different profiles. α-Thujone (16.7-24.7%) is the major component of leaves while it is found only in lower concentrations in the flower oils where viridiflorol was detected as the main component (20.9-28.6%). β-Thujone was present both in leaves and flowers and varied from 2.9% (in flowers) to 8.6% (in leaves). Both isomers of thujone always showed higher ratios in leaves than did in flowers at the same time. Similar to this finding, Perry et al. (1999) also found lower thujone levels in flowers (16%) compared with leaves (31%). Further, a study by Santos-Gomes and Fernandes-Ferreira (2001) indicated that α-thujone content in the oil of stems, leaves and flowers of *S. officinalis* in Portugal represented about 55, 30, and 18% of the total oil, respectively.

During the sampled phenophases, in the leaf samples the highest level of α-thujone accumulation was registered at the first two developmental phases (23.15-24.70%), and it decreased significantly during the flowering period. In flowers, the same dynamics of α-thujone ratio were detected with a peak at the floral budding stage (14.2%), although the differences were not significant. The ratio of β-thujone in the leaves fluctuated, with the highest values after flowering and the lowest ones in floral budding. In flower oils the concentration of β-thujone was statistically stable. Considering the sum of both isomers together, a significant decrease could be ascertained in the case of the leaf oils, with a maximum at floral budding (29.1%) and a minimum after flowering (25.3%), while in the flowers the variation was not significant.

Decreasing ratios of α-thujone in the EO of *S. officinalis* during plant development were reported by Santos-Gomes and Fernandes-Ferreira (2001). Similarly, Ben Farhat et al. (2016) detected a decreasing tendency of β-thujone accumulation during consecutive phenophases. However, contrary to our findings as well as the above mentioned studies, the fruiting stage was determined to be peak accumulation stage for α-thujone in Spain (Ben Farhat et al., 2016) and for both thujone isomers in Iran (Mirjalili et al., 2006). Nevertheless, we have to add that neither of these studies dealt with samples from individual plant organs, but rather investigated the whole shoots.

In parallel with the decrease of thujone content, the other skeleton type compounds – except bornane class – also tended to decrease during plant development, while the ratios of sesquiterpenes (mostly that of viridiflorol and biformene) were increasing. This indicates gene expression or enzyme activity changes at the starting phases of terpene biosynthesis (Tab. 3).

Tab. 3: EO composition[1] (GC area %) of *S. officinalis* obtained from leaves and flowers at different phenological stages

Compounds	RT	LRI[2]	LRI[3]	Leaf					Flower	
				V	FB	F	AF	FB	F	AF
				40	55	65	67	55	65	67
β-Pinene	6.45	981	979[4]	2.1	1.8	0.7	5.7	9.9	5.1	4.7
1,8-Cineol	8.07	1034	1035[4]	8.1	7.9	6.3	7.7	11.3	10.4	3.9
α-Thujone	10.68	1105	1105	23.2[b]	24.7[b]	20.5[ab]	16.7[a]	14.2[a]	12.4[a]	9.8[a]
β-Thujone	11.11	1113	1112	5.7[ab]	4.4[a]	5.1[a]	8.6[b]	3.0[a]	3.4[a]	2.9[a]
Camphor	12.19	1144	1146	11.8	8.8	8.6	5.8	3.9	2.5	3.1
Borneol	13.09	1162	1169[4]	1.8	4.0	3.0	5.9	4.7	7.1	6.0
β-Caryophyllene	23.31	1420	1420	10.1	9.9	10.2	6.4	8.3	5.9	8.2
α-Humulene	24.67	1454	1457[4]	11.3	8.8	9.4	4.5	6.4	4.8	5.8
Viridiflorol	30.39	1598	1495[4]	14.3	18.3	19.6	21.6	20.9	24.6	28.6
Biformene	45.59	2022	2026[5]	4.9	3.5	8.4	8.8	10.7	18.1	20.0
Total monoterpenes (%)				52.59	51.6	44.2	50.3	47.0	40.8	30.5
Total sesquiterpenes (%)				40.61	40.6	47.6	41.2	46.4	53.4	61.0
Total identified percentage				93.20	92.2	91.7	91.5	93.4	94.1	91.5

V: vegetative; FB: floral budding; F: flowering; AF: after flowering

Letters in rows represent significant differences between phenological phases of the same organ

[1] Components reaching 1% of GC area are listed

[2] Linear retention indices calculated relative to the elution ranking of n-alkanes (C_9-C_{20}) on HP-5MS column

[3] Linear retention indices according to Adams (2007)

[4] Linear retention indices from literature Ben Farhat et al. (2016) on HP-5MS column

[5] Linear retention indices from literature Petrović et al. (2006) on HP-5MS column

***Artemisia absinthium* L.**

The total area of the identified components in *A. absinthium* EO varied between 86.7% and 97.6%. During evaluation of the components which are higher than 1% of GC area, 12 compounds were identified and listed in Tab. 4.

Both leaves and flowers accumulated β-thujone as the main volatile component and α-thujone was the second largest compound. It was observed that the 'α' isomer always showed higher ratios in leaves than in flowers at the same sampling time. In case of the 'β' isomer higher ratios in the flowers were registered only until flowering.

During plant development, in the leaf samples the ratio of both isomeric forms of thujone reached the highest percentages at the floral budding stage: 25.4% of α-thujone and 66.8% of β-thujone were measured. However, the fluctuation that we noted does not reflect significant differences. In the flowers, the maximum concentration of α-thujone appeared at the flowering stage (24.5%), where this peak value is significant, while β-thujone reached the highest ratios at the after flowering stage, with no statistical difference compared to the former stages (60.9%).

The sum of both thujone isomers in leaves did not show significant differences among the developmental phases we studied. However, in the case of flowers a significant change was registered with increasing percentages in the later phenophases from 68.3% (at floral budding) to 81.3% (after flowering).

CARNAT et al. (1992) reported that the content of both thujone isomers decreased during the harvesting periods from July to November; however, these data refer to the whole aerial parts of *A. absinthium*. No reference was found in this study, either for the plant organs separately or for exact information regarding the phenophases. Our previous work showed that the ratio of the main compound β-thujone reached the highest ratio in the flowers at the floral budding stage and decreased significantly after that; while in the leaves, the highest value of this compound was measured at the vegetative stage and fluctuated after that (NGUYEN et al., 2018). The changes of α-thujone are less characteristic and do not follow the pattern of the other thujone isomer.

During the different developmental stages and for both organs, *A. absinthium* oils were dominated by the monoterpene fraction (73.7-94.4%) while sesquiterpenes were present in relatively lower percentages (3.2-15.6%) both in leaf and flower oils. The highest concentration of monoterpene compounds was detected in the leaf oils at the floral budding stage (94.4%) and in flower oils at the after flowering stage (77.2%), which were in harmony with the accumulation dynamics of β-thujone. In parallel with this, the ratio of sesquiterpenes increased following the after flowering phase and reached 15.6% as highest ratio (in leaves). As changing tendencies were different for each skeleton class of monoterpenes and sesquiterpenes, they might be resulted by changes at the level of terpene synthases.

Conclusion

The accumulation of volatile compounds as EO showed similar organic distribution and accumulation dynamics in both species of our study. The EO content of the flowers was always superior compared with the leaves at the same phenological phase both in *A. absinthium* and *S. officinalis*. The highest level of EO content was found in leaves at the vegetative stage (2.08 ml/100 g in *S. officinalis* samples and 1.72 ml/100 g in *A. absinthium* samples); and in flowers at the floral budding stage (2.82 ml/100 g in *S. officinalis* samples and 1.86 ml/100 g in *A. absinthium* samples). After the peaks, the concentrations decreased during the development of plants.

Between the two species, the distribution of the thujone isomers is a characteristic difference. Our results confirmed former descriptions (NGUYEN et al., 2017): the accumulation level of β-thujone was higher than that of α-thujone in each sample of *A. absinthium*. On the other hand, *S. officinalis* oils contained higher α-thujone percen-

Tab. 4: EO composition[1] (GC area %) of *A. absinthium* obtained from leaves and flowers at different phenological stages

Compounds	RT	LRI [2]	LRI[3]	Leaf			Flower		
				V	FB	F	FB	F	AF
				40	55	65	55	65	67
Sabinene	6.36	976	969	1.5	0.3	0.2	1.7	1.4	-
1,8-Cineol	8.07	1034	1030[4]	0.9	0.5	0.2	1.2	0.7	0.2
α-Thujone	10.68	1105	1105[4]	23.0[a]	25.4[a]	23.0[a]	15.9[a]	24.5[b]	20.3[b]
β-Thujone	11.11	1113	1112	61.7[a]	66.8[a]	49.5[a]	52.4[a]	51.5[a]	60.9[a]
Lavandulol	13.18	1166	1165	1.1	1.4	0.8	2.6	0.8	0.3
β-Caryophyllene	23.31	1420	1417	1.1	0.7	1.9	1.4	1.0	1.2
β-Selinene	25.98	1486	1489	1.8	1.5	3.2	3.1	1.4	2.9
Neryl-isobutanoate	26.26	1492	1490	-	-	1.4	1.4	-	1.3
Neryl-isovalerate	29.54	1583	1582	-	-	1.6	-	-	3.3
Geranyl 2-methyl butanoate	29.80	1591	30.25	-	-	3.4	-	-	4.7
Selin-11-en-4-α-ol	32.56	1661	1658	-	0.9	2.8	-	-	1.1
Chamazulene	35.10	1733	1730	2.5	0.1	0.1	7.0	0.9	0.9
Total monoterpenes (%)				88.3	94.4	73.7	73.7	78.8	81.7
Total sesquiterpenes (%)				5.6	3.2	15.6	13.0	14.3	13.4
Total identified percentage				93.8	97.6	89.3	86.7	93.1	95.1

V: vegetative; FB: floral budding; F: flowering; AF: after flowering

Letters in rows represent significant differences between phenological phases of the same organ

[1] Components reaching 1% of GC area are listed

[2] Linear retention indices calculated relative to the elution ranking of n-alkanes (C_9-C_{20}) on HP-5MS column

[3] Linear retention indices according to ADAMS (2007)

[4] Linear retention indices from SHAROPOV et al. (2012) on HP-5MS column

tages compared with the other isomer, which coincides with previous data (Kintzios, 2003; Ben Farhat et al., 2016). At the same time, in leaves of both species and in the flowers of *A. absinthium* the above mentioned characteristic thujone isomer is the major component of the EO; however, in *S. officinalis* the flowers accumulated viridiflorol in the highest concentrations.

The ratios of both thujone isomers in leaves show higher percentages than in flowers at the same harvesting time, which is a common feature for both *A. absinthium* and *S. officinalis* in the study. Differences between the species were nevertheless registered according to the dynamics and variations of the percentages during the study phases. In *S. officinalis*, the leaf oil presented significant changes concerning both α- and β-thujones as well as their sum, while these ratios were statistically stable in the flower oil.

To the contrary, *A. absinthium* leaf oil proved to be more stable, while the ratio of both thujones and their sum increased in the flower oil during plant development.

These findings may show that the accumulation tendency of total volatiles is a more common feature of these species and – according to the references – of many other EO bearing ones. However, the proportional changes of thujone isomers in the EO exhibit characteristic differences between *S. officinalis* and *A. absinthium*, which might represent the different ecological roles of these monoterpenes for the study species. For the praxis, in *S. officinalis* timing of harvest seems to be more important while in *A. absinthium* the ratio of organs may play a more significant role in reaching lower thujone levels of the drug. According to this, a later harvest of *S. officinalis*, preferably in flowering stage would be advantageous and *A. absinthium* herb should contain more leaves than flowers.

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