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Diversity of Volatile Compounds in the *Inula candida* / *I. verbascifolia* Group (Asteraceae-Inuleae) and Its Impact on Species Delimitation

Aikaterini Koutsaviti [1], Maria Eleftheria Zografaki [1], Nikolaos M. Fyllas [2], Olga Tzakou [1,*] and Theophanis Constantinidis [3,*]

[1] Section of Pharmacognosy and Chemistry of Natural Products, Department of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece
[2] Biodiversity Conservation Laboratory, Department of Environment, University of the Aegean, 81100 Mytilene, Greece
[3] Section of Ecology & Systematics, Department of Biology, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15784 Athens, Greece
* Correspondence: tzakou@pharm.uoa.gr (O.T.); constgr@biol.uoa.gr (T.C.)

Abstract: The members of the *Inula candida* / *I. verbascifolia* group are perennial and chasmophytic plants attributed to four species and eleven entities at subspecific or varietal level. They are mostly confined to Greece. Volatile compounds of above-ground flowering parts of twenty-three populations covering ten taxonomic entities were obtained after hydrodistillation and analyzed by gas chromatography and mass spectrometry (GC-MS). In most cases, the total percentage of identified constituents reached 89% or above. Seventy-two components were identified. Oxygenated sesquiterpenes prevailed in the *I. candida* subgroup (47.3–71.5%), with *epi*-α-cadinol present in all members (13.4–42.7%) but rarely found in the *I. verbascifolia* subgroup. Considerable amounts of the hydrocarbon aldehydes decanal, undecanal and particularly tridecanal (0.5–35.0%, rarely absent) were predominately found in the *I. verbascifolia* members but were mostly absent in the *I. candida* subgroup. Isoalantolactone (12.4–49.5%), identified only in *I. subfloccosa*, and γ-(*Z*)-curcumen-12-ol, found only in *I. candida* subsp. *limonella* (22.6–42.1%), may serve as chemotaxonomic markers. Two different chemotypes can be distinguished within *I. verbascifolia* subsp. *aschersoniana*: a *trans*-muurola-4(14),5-diene-, γ-cadinene-rich chemotype from north-eastern Greece and a 1-*epi*-cubenol-, tridecanal-rich chemotype from central Greece. Different statistical algorithms were used to interpret the chemical diversity and identify the most appropriate number of clusters for the taxa. Cluster analyses indicated that the optimum number of clusters that best explain the metabolomic variability of the taxa is two. The degree of membership for each population based on the fuzzy k-means algorithm supported the *I. verbascifolia* subsp. *aschersoniana* samples within the *I. candida* subgroup, whereas *I. subfloccosa* may belong to any of the two clusters formed, although it also appears to have some unique characteristics.

Keywords: *Inula* taxa; Greece; essential oils; chemical diversity; statistical evaluation; chemotaxonomy

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1. Introduction

Inula L., together with *Blumea* DC., form the largest genera within Asteraceae tribe Inuleae, each comprising about 100 species [1,2]. In its traditional circumscription, *Inula* is not monophyletic; several related genera interfere with *Inula* in molecular phylogenetic trees, thus making *Inula* paraphyletic [3]. To resolve this situation, a rearrangement of the genus *Pentanema* was recently proposed [4], to comprise several species previously ascribed to *Inula*.

Within traditional *Inula*, an assemblage of clump-forming, woody-based species characterized by the presence of a whitish-tomentose indumentum covering leaves and stems and a predominantly chasmophytic habitat constitute the *Inula candida* / *I. verbascifolia*

group, distributed in southern Italy, parts of the Balkan Peninsula, Anatolia and Lebanon [5]. In most floristic works, this group comprises four or five independent but very variable species [6,7]. *I. verbascifolia* (Willd.) Hausskn. has the broadest distribution, with population records ranging from south-east Italy to parts of the Balkan Peninsula, western Anatolia and Lebanon [5]. Five subspecific entities are recognized within this species [6]: *I. verbascifolia* subsp. *aschersoniana* (Janka) Tutin, *I. verbascifolia* subsp. *heterolepis* (Boiss.) Tutin, *I. verbascifolia* subsp. *methanaea* (Hausskn.) Tutin, *I. verbascifolia* subsp. *parnassica* (Boiss. & Heldr.) Tutin and *I. verbascifolia* subsp. *verbascifolia*. Some authors, however, prefer to consider these taxa as independent species [7,8]. The second species, *I. candida* (L.) Cass., is equally variable but with a narrower distribution [5]. It includes four infraspecific entities mostly accepted at subspecies (one at variety) level: *I. candida* subsp. *candida*, *I. candida* subsp. *decalvans* (Halácsy) Tutin, *I. candida* subsp. *limonella* (Heldr.) Rech. f. and *I. candida* var. *rotundifolia* Halácsy [6,8,9]. The whole last subgroup is endemic to Greece [5,6]. Similar to *I. verbascifolia*, the infraspecific taxa of *I. candida* have occasionally been erected to species level [8]. The third species of the group, *I. subfloccosa* Rech. f., is a very localised plant known from the southern parts of Evvia Island (Greece) [6]. It resembles *I. verbascifolia* but has floccose and glandular leaves [6]. The remaining two members of the group are *I. anatolica* Boiss. and *I. fragilis* Boiss., both endemic to Anatolia (Turkey) [5,7,8]. Older literature [10] included the last two species to the *I. candida* / *I. verbascifolia* group, but recent approaches exclude *I. anatolica* from the group [8] because of its smaller size, less tomentose leaves and lanceolate outer phyllaries.

When the taxonomic diversity of the group is linked with distribution ranges, it becomes obvious that Greece hosts the highest number of taxa (10) followed by Turkey (3 or 4). The Greek populations are present in almost all the phytogeographical regions of the country and cover a broad elevation range, from sea level to ca. 1900 m a.s.l. or higher. Still, for reasons difficult to justify, the group is absent from the islands of the central Aegean, thus forming the ''Kykladenfenster'' [11]. Some rare taxa are very localised, confined to a small portion of an island or a couple of mountains only (e.g., *I. subfloccosa* and *I. candida* subsp. *decalvans*, respectively).

Recent authors have stressed the need for a thorough modern revision of the group [12]. The morphological diversity in the group is not yet fully understood and sometimes *I. candida* is difficult to distinguish from *I. verbascifolia*. *I. subfloccosa* has been considered of uncertain status, perhaps related to the subgroup of *I. verbascifolia* [12]. A recent molecular approach transferred two members of the latter subgroup to *Pentanema*, as *Pentanema verbascifolium* (Willd.) D. Gut. Larr et al. and *P. aschersonianum* (Janka) D. Gut. Larr. et al. [4]. However, incongruencies were observed between the ITS nrDNA and the concatenated plastid markers phylogenies. The plastid tree, in particular, allocated the obviously related *I. verbascifolia* and *I. aschersoniana* (*I. verbascifolia* subsp. *aschersoniana*) into different clades.

Inula is a genus containing bioactive compounds [13]. *Inula helenium* L., commonly known as elecampane, has been used in traditional medicine for centuries. Several *Inula* constituents exhibit anti-tumor/cytotoxic activity, antibacterial/antifungal actions, and have antidiabetic properties, among others [14]. Proper identification of the species is essential in reviewing biological activities and the knowledge of taxonomic relatives may be beneficial in the pharmacological evaluation of various *Inula* members.

This contribution is an attempt to use metabolomics as an aid to assess species delimitation and evaluate taxonomic relationships within the *I. candica* / *I. verbascifolia* group. Volatile compounds have been used successfully in elucidating relationships in various Asteraceae genera, as, for example, *Achillea* [15], *Aldama* [16], *Anthemis* [17], *Helichrysum* [18], *Senecio* [19], and *Solidago* [20]. They may offer useful chemotaxonomic markers and insights into common biosynthetic ways, presumably a consequence of genetic proximity. To this target, the goals of this study can be summarized as follows: (a) to collect and analyze volatiles in most members of the *I. candica* / *I. verbascifolia* group and, if possible, to investigate more than one population per taxonomic entity; (b) to identify and evaluate the chemical diversity present in volatile compounds obtained under the same experimental

conditions; and (c) to use metabolomics as variables in cluster analyses in order to assess species identities and discuss taxonomic implications.

In this work we consider all members of the group as belonging to *Inula*. For most taxa, no nomenclatural combinations under *Pentanema* exist.

2. Materials and Methods

2.1. Plant Material

The plants of the *I. candica* / *I. verbascifolia* group are perennial herbs, with several stems emerging from a suffruticose base. Most populations flower from middle spring to middle summer or later, depending on the altitude and the exposure. Our investigation covered 10 taxa of the group and 23 populations, from various localities. The accepted taxonomic classification, scientific names and detailed information for each studied population are presented in Table 1. The collection altimeter readings of our samples ranged from 20 to 1750 m a.s.l. Voucher material from each population was identified according to relevant literature [6,7,21], dried in a plant press and deposited in the ATHU Herbarium of National and Kapodistrian University of Athens. All the material used for the analytical procedures was collected when in flower. Two to three healthy stems with leaves and inflorescences were selected per plant and cut off from their base (aerial stems). Approximately 5–8 plants were sampled per population. The collected stems were combined to create a pooled sample and left to dry in a shady, well-ventilated place for several days.

Table 1. The populations of the *Inula candida/I. verbascifolia* group investigated, with localities, altimeter, and coordinates. The code number of the last column is also used as an identifier in the chemical and cluster analyses.

Species	Subspecies/Variety	Locality	Altimeter (m)	Coordinates (WGS 84)	Code Number
Inula candida					
	subp. *candida*	Kithira Island, Chora village	270	36°14′ N, 22°59′ E	I1
	subsp. *decalvans*	Kriti Island, Mt. Dikti, northern parts	1380	35°07′ N, 25°29′ E	I5
	subsp. *limonella*	Evvia Island, Mt. Dirfis, near the shelter	1110	38°36′ N, 23°51′ E	I2
	subsp. *limonella*	Peloponnisos, Mt. Parnonas, upper parts	1750	37°16′ N, 22°36′ E	I3
	subsp. *limonella*	Peloponnisos, Mt. Koulochera, upper parts	1060	36°49′ N, 22°59′ E	I4
	subsp. *limonella*	Sterea Ellas, Mt. Kitheronas, near the summit	1310	38°10′ N, 23°14′ E	I19
	var. *rotundifolia*	Peloponnisos, Mt. Krithina, near the top	580	36°28′ N, 23°08′ E	I6
Inula verbascifolia					
	subsp. *aschersoniana*	Thessalia, Mt. Ossa, near the shelter	1480	39°48′ N, 22°41′ E	I7
	subsp. *aschersoniana*	Macedonia, NE of Kavala	710	41° 01′ N, 24°27′ E	I22
	subsp. *aschersoniana*	Macedonia, N of Krioneri village	460	41° 02′ N, 24°22′ E	I23
	subsp. *heterolepis*	Samos Island, Mt. Kerkis, foothills	450	37°43′ N, 26°35′ E	I8
	subsp. *heterolepis*	Rodos Island, NNW of Laerma village	260	36°11′ N, 27°57′ E	I9
	subsp. *heterolepis*	Kalimnos Island, between Masouri and Arginondas settlements	50	37°01′ N, 26°56′ E	I10
	subsp. *methanea*	Sterea Ellas, Mt. Imittos, Kesariani suburb	630	37°58′ N, 23°49′ E	I11
	subsp. *methanea*	Sterea Ellas, Mt. Pateras, upper parts	980	38°06′ N, 23°27′ E	I12
	subsp. *methanea*	Sterea Ellas, E of Erithres village	430	38°13′ N, 23°23′ E	I20
	subsp. *parnassica*	Sterea Ellas, near Proussos village	770	38°45′ N, 21°39′ E	I13
	subsp. *parnassica*	Sterea Ellas, Mt. Parnassos, between Arachova and Livadi	1130	38°29′ N, 22°33′ E	I14
	subsp. *parnassica*	Sterea Ellas, E of Delfi archaeological site	540	38°28′ N, 22°30′ E	I21
	subsp. *verbascifolia*	Kefallinia Island, Livadi village	20	38°15′ N, 20°25′ E	I15
	subsp. *verbascifolia*	Kefallinia Island, Angonas village	200	38°18′ N, 20°29′ E	I16
Inula subfloccosa		Evvia Island, W of Mt. Ochi	390	38°02′ N, 24°25′ E	I17
Inula subfloccosa		Evvia Island, between Agios Dimitrios and Kalliani villages	280	38°07′ N, 24°26′ E	I18

2.2. Collection of Volatile Compounds

Dried aerial parts from each population were cut into small pieces and subjected to hydrodistillation for 3 h using a modified Clevenger-type apparatus with a water-cooled

receiver to reduce overheating artifacts. The isolated essential oils were taken up in pentane, dried over anhydrous sodium sulfate and stored at 4 °C until analyzed.

2.3. Chemical Analysis of Essential Oils

Gas chromatography (GC) analysis for the quantitative determination of components was carried out on an SRI 8610C gas chromatograph equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm; film thickness 0.25 μm), a split/splitless injector and a FID detector. For the identification of components, GC-mass spectrometry (GC-MS) analysis was carried out using a Hewlett-Packard 6890 gas chromatograph equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm; film thickness 0.25 μm), a split/splitless injector and a Hewlett- Packard 5973 MS detector operating in electron ionization mode at 70 eV. In both cases, the GC analysis parameters were the following: injection was performed at 200 °C in a split ratio 1:10, while detection was performed at 250 °C; the carrier gas was He at a flow rate of 2 mL/min; the oven temperature was 60 °C at the time of the injection, raised to 300 °C at a rate of 3 °C /min and subsequently held at 300 °C for 10 min.

The identification of the chemical constituents was based on comparison of their relative retention times and mass spectra with those obtained from authentic standards (Sigma Chemical Co., St. Louis, MO, USA, PhytoLab GmbH and Co., Vestenbergsgreuth, Germany) and/or reported in the NIST/NBS and Wiley libraries and the literature [22]. The relative amounts of volatile compounds of the essential oils were calculated with the peak area normalization method.

2.4. Statistical Analyses

The concentration of each chemical component in samples from the 23 studied populations was organized in a taxon × chemical component table. This table was analyzed using different clustering algorithms to identify the optimum and most meaningful number of clusters that could be delimited. We initially used the Ward's hierarchical agglomerative [23] and the "k-means" [24] clustering methods. In addition, because of the high dimensionality of our taxon × chemical component matrix, we applied the k-means clustering algorithm on the coordinates of the first axes of a principal components analysis that accounted for 70.0% of the total volatile compounds variance ("kmeans-PCA"). The same method was applied on the pre-transformed taxon × chemical component matrix using the Hellinger transformation ("kmeans-PCA (Hellinger)"), in order to deal with the fact that many chemical components were not systematically identified across all samples, or their concentrations were very low (traces) and were thus included in the matrix with a value of zero. Finally, we used the "fuzzy k-means" clustering algorithm [25], a soft clustering method, where each object (population, in our case) could belong to multiple clusters with a different likelihood.

For each of the above algorithms, we evaluated the goodness of the clustering structure using three internal validation metrics. Internal clustering validation metrics quantify the effectiveness of a clustering algorithm by evaluating the ratio of "cluster separation" to "cluster compactness" [26]. The silhouette index (SI) is based on the pairwise differences of between- and within-cluster distance, with higher values used to identify the optimum number of clusters. The Dunn index (DI) estimates the ratio of the minimum pairwise distance between objects in different clusters to the maximum distance among clusters. Higher DI indicate a better performance of the clustering algorithm [27]. The Calinski–Harabasz index (CHI) is based on the average between and within cluster sum of squares, with higher values suggesting a better classification.

Instead of following the above procedure in a purely arithmetic way (i.e., sequentially increasing the number of clusters, estimating the validation metrics and then selecting the optimum number of clusters), we applied all the clustering algorithms for only two, three and ten clusters representing the minimum (meaningful) number of clusters, the number of taxonomically defined species, and the number of taxonomic entities that have

been identified in our dataset. By comparatively evaluating the internal validation metrics, we then identified the optimum number of clusters derived from the different clustering algorithms and contrasted the outputs with the "fuzzy k-means" clustering method. All data analyses and visualizations have been performed with the R statistical language [28] and the factoextra [29], cluster [30], fclust [31] and clusterCrit [32] packages.

3. Results

3.1. Chemistry and Main Constituents

All plant samples were effectively analyzed, and seventy-two chemical components were identified. The total percentage of identified constituents reached 89% or above (94.3 ± 7.0%), with the exception of I9 sample, i.e., *Inula verbascifolia* subsp. *heterolepis* from Rodos Island, where essential oil analysis of 67.0% was achieved (Table 2).

The *Inula candida* subgroup is represented by four subspecific entities and seven samples in our study. Sesquiterpenes dominate the essential oil of this group (I1-I6 and I19), especially oxygenated derivatives (47.3–71.5%). The main metabolite present in all samples was epi-α-cadinol, varying between 13.4% and 42.7% (24.2 ± 10.2%). The *I. candida* subsp. *limonella* essential oils were, however, richer in γ-(*Z*)-curcumen-12-ol (36.3 ± 9.2%), an oxygenated sesquiterpene not found in the other *I. candida* samples. Sesquiterpene hydrocarbons were detected in lesser amounts, with (*E*)-caryophyllene (4.4 ± 4.6%) reaching highest percentage in *I. candida* subsp. *candida* essential oil (I1, 11.5%). Additionally, aromatic compounds were detected (5.8–14.3%), with (3*Z*)-hexenyl benzoate (7.3 ± 2.1%) basically constituting the major component. Monoterpenes (4.1–14.2%) were identified in considerably lower amounts with linalool (7.2 ± 3.3%) being the most abundant metabolite of this chemical class. An unusual 12.0% presence of dictamnol and a 20.1% of abienol was found in var. *rotundifolia* from Mt. Krithina (I6). The presence of the labdane diterpene abienol is of interest, as this class of compounds is not common in Asteraceae. This diterpene appears in the oils of all taxa of the *I. candida* subgroup (5.0 ± 9.8%), except that of *I. candida* subsp. *decalvans*; in the *I. verbascifolia* subgroup it is present in only two samples (1.3% for I8 and 4.7% for I9), while in the essential oils of *I. subfloccosa* it is present in low amounts (0.6 ± 0.8%). In the literature, (12*Z*)-abienol is referred to as a component of *I. crithmoides* L. (synonym of *Limbarda critmoides* (L.) Dumort.) essential oils derived from aerial parts [33–35].

The *Inula verbascifolia* subgroup is represented by five subspecies and fourteen samples in our analyses; sampling covers a wider geographic range, compared to that of *I. candida*. Its chemical profile was more heterogeneous, showing qualitative and quantitative differences but also variations related to the main compounds. The essential oils of *I. verbascifolia* subsp. *aschersoniana* (I22, I23 and I7) were all rich in sesquiterpenes; however, the samples collected from the Kavala area (I22 and I23) exhibited a particularly similar chemical profile, not only with respect to the main compounds but also their quantities, compared to I7 from Mt. Ossa. The Kavala samples were characterized by sesquiterpene hydrocarbons and their oxygenated analogues (51.9–75.3% and 22.1–33.9%, respectively), while no aromatic components were observed. Major components in these samples were *trans*-muurola-4(14),5-diene (19.6 ± 3.7%), γ-cadinene (19.7 ± 9.6%), δ-cadinene (16.8 ± 5.4%), and the oxygenated 1-*epi*-cubenol (13.6 ± 3.3%), i.e., sesquiterpenes that were either detected in notably lower amounts or absent in I7 and the rest of the investigated *Inula* essential oils. Sample I7 from Mt. Ossa, on the other hand, was significantly richer in oxygenated sesquiterpenes, which almost dominated its chemical composition (47.8%), with *epi*-α-cadinol (30.5%) as the major component. Additionally, a considerable percentage in aldehydes (20.9%) was also observed in I7, along with aromatic compounds (13.6%), constituents that were detected only in traces or not at all in the Kavala material (I22 and I23).

Table 2. Chemistry of the essential oils obtained from the aerial parts of the *Inula candida* and *I. verbascifolia* members, together with the related *I. subfloccosa.* Numbers refer to percentages (%), estimated in relation to the total oil yield.

Components [a]/Populations	**RR [b]**	**I1**	**I5**	**I2**	**I3**	**I4**	**I19**	**I6**	**I7**	**I22**	**I23**	**I8**	**I9**	**I10**	**I11**	**I12**	**I20**	**I13**	**I14**	**I21**	**I15**	**I16**	**I17**	**I18**
Hexanal	801	tr	tr	tr	tr	tr	tr	tr	tr	-	-	tr	tr	2.6	1.3	-	tr	tr	tr	tr	tr	-	tr	tr
(2E)-Hexenal	855	tr	tr	1.0	0.7	1.0	1.3	tr	1.1	tr	-	tr	tr	4.1	tr	tr	tr	tr	4.3	tr	tr	tr	tr	tr
Hexanol	870	tr	tr	0.4	tr	tr	0.8	tr	tr	tr	-	tr	tr	tr	0.9	tr	tr	tr	3.6	tr	tr	-	tr	tr
α-Pinene	939	tr	tr	tr	-	tr	tr	tr	-	2.2	3.8	1.5	2.7	11.2	10.0	12.0	tr	-	tr	tr	tr	tr	tr	-
1,8-Cineole	1031	tr	-	tr	-	-	-	-	-	-	-	tr	tr	5.1	6.1	9.2	2.3	tr	-	-	tr	-	tr	-
γ-Terpinene	1059	-	tr	-	tr	tr	tr	tr	-	-	-	tr	tr	tr	0.6	2.5	tr	-	tr	tr	tr	-	tr	-
Linalool	1096	2.5	12.5	5.5	5.3	6.3	8.6	9.8	2.6	tr	-	1.1	tr	4.3	1.3	2.5	tr	2.4	13.3	5.2	5.3	tr	5.3	2.9
Nonanal	1100	1.9	1.5	0.7	1.1	0.5	0.6	tr	1.4	tr	tr	0.9	tr	2.0	1.9	3.0	tr	1.2	7.0	4.1	2.8	3.6	tr	tr
cis-Chrysanthenol	1164	-	-	-	-	-	-	-	-	-	-	tr	tr	2.0	19.9	4.5	tr	-	-	4.8	-	-	-	-
Terpinen-4-ol	1177	tr	tr	-	-	tr	tr	tr	-	-	-	tr	tr	tr	1.5	2.3	tr	-	tr	tr	tr	-	tr	-
α-Terpineol	1188	1.6	1.7	0.8	tr	tr	0.7	0.9	tr	-	-	tr	tr	tr	5.8	2.0	1.3	3.0	tr	6.1	tr	tr	tr	tr
Decanal	1201	tr	tr	0.3	1.0	0.6	tr	tr	2.2	-	-	1.2	tr	2.4	4.5	14.1	4.4	9.1	6.9	3.0	2.8	5.3	tr	tr
Carvacrol	1299	tr	tr	tr	tr	tr	-	tr	tr	-	-	tr	tr	tr	tr	1.2	tr	tr	-	8.0	tr	tr	tr	-
Undecanal	1306	tr	tr	-	tr	tr	tr	tr	3.4	tr	tr	3.4	tr	3.9	tr	tr	tr	0.7	7.2	2.8	11.1	15.3	-	-
Isobutyl benzoate	1329	tr	tr	-	tr	tr	tr	tr	0.6	-	-	tr	tr	tr	0.8	1.0	tr	3.2	3.5	2.6	tr	tr	tr	-
β-Bourbonene	1388	-	-	-	-	tr	tr	-	-	-	-	-	-	-	tr	1.4	1.3	-	2.6	tr	-	-	-	-
(*Z*)-Jasmone	1392	-	tr	tr	tr	tr	tr	1.8	-	-	-	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	-	3.1	1.3
Dodecanal	1408	-	tr	-	-	-	tr	-	1.9	-	-	3.4	3.1	4.1	tr	tr	tr	-	6.1	3.0	5.5	7.6	-	-
(*E*)-Caryophyllene	1419	11.5	4.8	0.4	tr	1.7	2.2	9.9	tr	tr	-	1.3	4.4	1.3	3.2	4.6	3.0	3.4	3.7	8.7	tr	tr	12.0	1.9
Dictamnol	1429	-	3.5	-	-	tr	-	12.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
allo-Aromadendrene	1460	tr	tr	0.6	tr	0.9	tr	tr	tr	-	-	1.3	tr	tr	tr	1.3	tr	tr	-	tr	tr	-	8.6	tr
Dodecanol	1470	-	-	-	-	tr	-	-	tr	-	-	tr	-	-	-	-	-	tr	tr	tr	tr	3.7	-	-
trans-Cadina-1(6),4-diene	1476	-	tr	tr	tr	tr	tr	-	-	tr	2.7	-	-	-	tr	tr	-	-	-	-	-	-	-	-
ar-Curcumene	1480	-	2.8	0.7	1.0	tr	tr	-	tr	-	-	0.8	tr	tr	-	-	-	1.6	-	-	-	-	tr	tr
γ-Curcumene	1482	-	5.0	1.0	4.1	tr	1.6	-	-	-	-	tr	-	tr	-	tr	-	-	-	-	-	-	tr	tr
Germacrene D	1485	tr	tr	-	-	tr	-	tr	tr	-	-	tr	-	-	0.7	2.4	7.0	-	tr	3.4	-	-	-	tr
β-Selinene	1490	tr	tr	tr	-	tr	tr	-	-	-	-	-	tr	-	1.2	tr	tr	tr	-	tr	-	-	4.7	1.4
trans-Muurola-4(14),5-diene	1493	-	tr	tr	tr	tr	tr	tr	-	22.2	17.0	-	-	-	0.5	tr	-	-	-	tr	-	-	-	-
γ-Amorphene	1495	-	-	-	tr	-	-	-	-	-	-	-	-	-	4.2	-	-	-	-	-	-	-	-	-
Bicyclogermacrene	1500	tr	-	-	-	tr	tr	tr	-	-	-	5.3	tr	1.6	-	4.6	2.5	-	-	tr	3.1	tr	6.0	1.4
Tridecanal	1510	-	-	-	-	-	-	-	10.9	-	-	14.3	15.4	12.4	0.5	1.6	2.5	1.6	25.9	13.8	37.5	35.0	-	-
δ-Amorphene	1512	tr	tr	tr	-	-	-	tr	tr	-	-	-	-	-	4.0	-	-	-	-	-	-	-	tr	tr
γ-Cadinene	1513	1.2	tr	1.0	1.3	1.5	tr	tr	-	26.4	12.9	-	-	-	-	tr	tr	-	-	-	-	-	tr	tr
δ-Cadinene	1523	tr	tr	1.0	3.5	1.4	4.5	tr	1.0	20.6	12.9	1.1	3.9	tr	7.8	2.7	9.4	tr	tr	2.3	tr	-	tr	tr
Lilial	1528	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	5.9	-	-
trans-Calamenene	1529	tr	-	tr	-	tr	-	-	-	6.1	6.4	-	-	-	tr	-	-	-	-	-	-	-	-	-
Liguloxide	1536	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	23.8	-	-	tr	tr	tr	1.3
(3*Z*)-Hexenyl benzoate	1566	9.8	5.8	6.3	7.2	10.6	6.7	4.8	11.2	-	-	8.0	7.4	7.6	1.1	4.0	5.7	8.6	6.5	10.1	tr	-	8.8	1.6
Spathulenol	1578	4.5	tr	-	-	-	tr	-	-	-	-	12.8	2.4	16.8	1.9	7.3	3.4	tr	tr	tr	2.8	tr	20.2	13.8

Table 2. *Cont.*

Components [a]/Populations	RR [b]	I1	I5	I2	I3	I4	I19	I6	I7	I22	I23	I8	I9	I10	I11	I12	I20	I13	I14	I21	I15	I16	I17	I18
Hexyl benzoate	1580	4.5	tr	0.3	tr	2.5	tr	5.6	tr	-	-	-	tr	tr	tr	tr	3.2	tr	tr				tr	-
Caryophyllene oxide	1583	8.8	tr	3.0	1.8	1.9	4.3	8.4	2.9	-	-	1.3	tr	5.3	0.9	2.7	tr	5.2	4.7	8.8	tr	tr	7.6	3.2
Component 1 [c]	1589	-	-	-	-	-	-	-	tr	-	-	-	4.7	-	-	-	-	tr	tr	tr	-	-	tr	-
Component 2 [d]	1611	-	-	-	-	-	-	-	-	-	-	-	28.0	-	-	-	-	-	-	tr	-	-	tr	tr
β-Copaen-4-α-ol	1590	5.0	tr	-	-	-	-	-	-	-	-	-	tr	0.6	tr	1.8	-	-	-	-	tr	-	-	-
1-*epi*-Cubenol	1628	-	-	tr	tr	tr	tr	tr	-	11.2	15.9	tr	-	-	3.7	tr	-	-	-	-	-	-	tr	-
Muurola-4,10(14)-dien-1-β-ol	1631	tr	tr	-	-	-	tr	tr	2.4	-	-	-	-	3.3	tr	1.2	2.7	-	-	tr	-	-	tr	tr
Isospathulenol	1634	tr	-	-	-	-	-	-	-	-	-	4.5	tr	-	-	tr	tr	-	-	-	tr	-	tr	tr
epi-α-Cadinol	1640	24.9	42.7	20.4	17.0	32.4	13.4	18.8	30.5	5.8	9.0	9.6	8.6	5.8	-	2.1	5.2	6.3	tr	tr	2.7	tr	tr	-
Caryophylla-4(12),8(13)-dien-5α-ol	1641	2.6	-	-	-	-	tr	tr	tr	-	-	-	-	tr	tr	tr	tr	tr	-	tr	-	-	tr	tr
Caryophylla-4(12),8(13)-dien-5β-ol	1642	5.3	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	tr	1.9	-	tr	-	-	2.4	tr
α-Muurolol	1646	-	-	-	-	-	-	-	-	-	-	2.0	tr	tr	tr	tr	-	2.8	tr	tr	-	tr	-	-
Cubenol	1647	tr	-	-	-	-	-	-	-	-	-	-	-	-	3.9	-	-	-	-	-	-	-	-	-
β-Eudesmol	1650	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	tr	5.7	-	-	4.0	8.1
Himachalol	1653	-	-	4.3	3.5	-	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Cadinol	1654	8.4	14.2	4.3	3.5	10.8	4.2	6.3	11.2	5.1	9.0	3.1	4.2	2.4	0.8	1.8	5.3	0.6	tr	tr	tr	tr	-	-
Helifolenol A	1675	-	-	0.1	tr	1.6	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Germacra-4(15),5,10(14)-trien-1-α-ol	1686	tr	tr	-	-	-	-	tr	-	-	-	-	-	-	tr	tr	3.1	-	-	tr	-	-	-	-
γ-(*Z*)-Curcumen-12-ol	1729	-	-	39.4	41.0	22.6	42.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Hexyl-(*E*)-cinnamaldehyde	1749	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	9.9	-	-
7,14-Anhydro-amorpha-4,9-diene	1756	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.4
β-(Z)-Curcumen-12-ol	1757	-	tr	tr	tr	tr	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.1	tr
Benzyl benzoate	1760	tr	tr	1.4	tr	tr	tr	tr	1.8	tr	-	0.6	5.8	tr	tr	tr	3.8	7.5	tr	tr	tr	tr	tr	tr
Epoxy-pseudoisoeugenyl isobutyrate	1793	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.5	-	-	-	-	-	-	-
2-Ethyl-hexyl salicylate	1807	-	-	-	tr	tr	tr	tr	tr	-	-	tr	tr	tr	tr	-	-	1.1	-	-	tr	tr	-	-
Hexadecanal	1822	tr	tr	tr	tr	tr	tr	tr	-	-	-	1.3	tr	-	-	-	tr	0.9	-	tr	3.7	tr	tr	tr
Hexahydrofarnesyl acetone	1840	3.9	tr	tr	tr	tr	tr	tr	0.8	-	-	tr	tr	tr	tr	tr	tr	1.7	4.1	3.1	tr	2.6	tr	tr
Benzyl salicylate	1865	tr	tr	0.8	tr	tr	tr	tr	tr	-	-	tr	4.4	tr	tr	tr	tr	1.6	-	tr	tr	tr	tr	tr
Hexadecanol	1875	-	tr	-	tr	-	tr	tr	tr	-	-	-	-	-	-	-	tr	-	tr	tr	tr	3.4	tr	-
Isoalantolactone	1952	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.4	49.5
Heneicosane	2100	tr	tr	tr	tr	tr	tr	-	tr	-	-	tr	tr	tr	-	-	tr	3.4	-	tr	tr	tr	tr	tr
Component 3 [e]	2126	tr	tr	tr	tr	tr	tr	tr	tr	-	-	tr	tr	tr	tr	tr	7.5	-	tr	tr	-	-	tr	tr
Abienol	2149	tr	-	2.8	7.4	2.3	2.5	20.1	-	-	-	1.3	4.7	-	-	-	-	-	-	-	-	-	tr	1.1
Pentacosane	2500	tr	tr	tr	tr	tr	tr	tr	0.8	tr	-	tr	tr	tr	tr	tr	4.6	tr	tr	3.8	tr	tr	tr	tr
Heptacosane	2700	tr	tr	tr	tr	tr	tr	tr	1.4	tr	-	3.5	tr	tr	tr	tr	5.8	tr	tr	tr	7.0	tr	tr	tr

Table 2. *Cont.*

Components [a]/Populations	**RR [b]**	**I1**	**I5**	**I2**	**I3**	**I4**	**I19**	**I6**	**I7**	**I22**	**I23**	**I8**	**I9**	**I10**	**I11**	**I12**	**I20**	**I13**	**I14**	**I21**	**I15**	**I16**	**I17**	**I18**
Nonacosane	2900	tr	tr	tr	tr	tr	tr	tr	3.5	-	-	7.0	tr	tr	tr	tr	6.4	tr	tr	tr	15.3	4.5	tr	tr
TOTAL		**96.4**	**94.5**	**96.5**	**99.4**	**98.6**	**98.0**	**98.4**	**91.6**	**99.6**	**89.6**	**90.6**	**67.0**	**98.8**	**89.0**	**93.8**	**92.4**	**91.6**	**99.4**	**99.3**	**99.6**	**96.8**	**98.2**	**89.9**
HYDROCARBONS																								
Alkanes/Alkenes		tr	tr	tr	tr	tr	tr	tr	5.7	tr	-	10.5	tr	tr	tr	tr	16.8	3.4	tr	3.8	22.3	4.5	tr	tr
Alcohols		tr	tr	0.4	tr	tr	0.8	tr	tr	tr	-	tr	tr	tr	0.9	tr	tr	tr	3.6	tr	tr	7.1	tr	tr
Aldehydes		1.9	1.5	2.0	2.8	2.1	1.9	tr	20.9	tr	tr	24.5	18.5	31.5	8.2	18.7	6.9	13.5	57.4	26.7	63.4	66.8	tr	tr
TERPENES																								
Monoterpenes		4.1	14.2	6.3	5.3	6.3	9.3	10.7	2.6	2.2	3.8	2.6	2.7	22.6	45.2	36.2	3.6	5.4	13.3	24.1	5.3	tr	5.3	2.9
Sesquiterpene hydrocarbons		12.7	12.6	4.7	9.9	5.5	8.3	9.9	1.0	75.3	51.9	9.8	8.3	2.9	21.6	17.0	23.2	5.0	6.3	14.4	3.1	tr	31.3	4.7
Oxygenated sesquiterpenes		63.4	60.4	71.5	66.8	69.3	68.5	47.3	47.8	22.1	33.9	33.3	15.2	34.2	11.2	16.9	19.7	42.3	8.8	17.6	5.5	2.6	52.8	79.6
Diterpenes		tr	-	2.8	7.4	2.3	2.5	20.1	-	-	-	1.3	4.7	-	-	-	-	-	-	-	-	-	tr	1.1
AROMATIC COMPONENTS		14.3	5.8	8.8	7.2	13.1	6.7	10.4	13.6	-	-	8.6	17.6	7.6	1.9	5.0	22.2	22.0	10.0	12.7	tr	15.8	8.8	1.6

[a] Compounds listed in the order of elution on a HP-5MS column under the specified chromatographic conditions. [b] Relative retention indices calculated from the retention times of the compounds in relation to those of a series of n-alkanes (C_8–C_{29}) analyzed under the same chromatographic conditions. [c] m/z (rel. int., %): 220 (0.8), 206 (10), 191 (9), 173 (6), 162 (100), 147 (98), 133 (10), 119 (10), 105 (26), 91 (47), 79 (15), 67 (10), 55 (10), 41 (12). [d] m/z (rel. int., %): 222 (0.7), 206 (27), 191 (12), 173 (7), 162 (100), 147 (87), 133 (10), 120 (78), 105 (52), 91 (48), 79 (28), 67 (15), 55 (15), 41 (22). [e] m/z (rel. int., %): 204 (2), 189 (5), 175 (32), 149 (21), 135 (29), 121 (27), 109 (50), 95 (43), 81 (100), 69 (60), 55 (42), 41 (57).

Inula verbascifolia subsp. *heterolepis* essential oils (I8, I9 and I10) were characterized by the presence of aldehydes (18.5–31.5%), particularly of tridecanal (14.0 ± 1.5%). Sesquiterpenes were also a major chemical class in the essential oils of this subspecies, particularly the oxygenated derivatives (15.2–34.2%) with spathulenol possessing high percentage especially in I8 (12.8%) and I10 (16.8%) from Samos and Kalimnos Islands, respectively. The material from Rodos Island (I9) was also relatively rich in that chemical group but *epi*-α-cadinol was the most important representative (8.6%). The same sample showed a higher percentage of aromatic compounds (17.6%) compared to the rest of subsp. *heterolepis* essential oils (7.6–8.6%), and afforded some chemical compounds that were difficult to match well with those of the libraries or authentic samples. They are presented in Table 2 as Components 1 and 2, along with their mass fragmentation pattern. Worth noting is also the variation in monoterpenes (2.6–22.6%), with I10 from Kalimnos being strikingly richer, particularly in α-pinene (11.2%), a constituent detected in significantly lower percentage in the sample from Samos (1.5%) and Rodos (2.7%).

Inula verbascifolia subsp. *methanea* essential oils (I11, I12 and I20) were also characterized by a high percentage of sesquiterpenes (32.8–42.9%); however, samples I11 and I12 were almost equally rich in monoterpenes (36.2–45.2%), a chemical group found generally only in relatively low amounts in the rest of the investigated samples. The majority of the studied *Inula* essential oils have a low content in monoterpenes, with the exception of *I. crithmoides* (*Limbarda crithmoides*) [33,34,36] and *I. graveolens* (L.) Desf., also known as *Dittrichia graveolens* (L.) Greuter aerial parts oils [37,38], respectively. I20 from Erithres was instead strikingly richer in aromatic compounds (22.2%), particularly in epoxy-pseudoisoeugenyl isobutyrate (9.5%), a compound not encountered in any other sample. Aromatic compounds were detected in much lower amounts in the rest of subsp. *methanea* samples (1.9–5.0%). *I. verbascifolia* subsp. *methanea* from Mt. Parnitha showed a particularly high content in linalool (21.2%), *epi*-α-cadinol (19.5%) and (*Z*)-nuciferol (16.6%), components that were detected in either low amounts or were absent in previously investigated samples [39].

Inula verbascifolia subsp. *parnassica* essential oils showed notable quantitative variations. All samples were characterized by a constant presence of aldehydes (13.5–57.4%); however, I14 was significantly richer in that chemical class, with tridecanal (25.9%) being the main essential oil constituent. I14 from Proussos, the least abundant sample of this group in aldehydes (13.5%), was instead richer in oxygenated sesquiterpenes and specifically in liguloxide (23.8%), a compound only scarcely encountered in all the investigated samples. All three investigated subsp. *parnassica* essential oils showed also notably high levels of aromatic components (10.0–22.0%). Comparing the chemical profile of a subsp. *parnassica* sample collected from Mt. Parnassos [39] to the investigated samples herein, evident is the lack of aldehydes and the high abundance of methyl salicylate (23.4%), a metabolite not identified in the essential oils of the present study.

The essential oils of *Inula verbascifolia* subsp. *verbascifolia* are differentiated from the rest of the *I. verbascifolia* subgroup by a rather high percentage of aldehydes (more than 60%), mainly tridecanal (36.3 ± 1.8%), followed by undecanal (13.2 ± 3.0%). Even though both samples were collected from Kefallinia Island, I15 (collected in May) showed higher percentage in alkanes/alkenes (22.3%), while I16 (collected in August) was richer in aromatic compounds (15.8%). The essential oil of *I. verbascifolia* (subsp. *verbascifolia*) collected from Italy showed a different chemical profile and was characterized by high amounts of acids (hexadecanoic acid 10.4% and (*Z*,*Z*)-9,12-octadecadienoic acid 6.5%), compounds that were not detected in our samples. Aldehydes were not detected in significant amounts in the Italian sample [36].

Overall, the subspecies of *I. verbascifolia* hardly follow any distinctive taxon-specific pattern in their chemical constituents, apart from *I. verbascifolia* subsp. *verbascifolia*, as mentioned above.

Inula subfloccosa's essential oils were characterized by oxygenated sesquiterpenes (52.8–79.6%) and particularly by the sesquiterpene lactone isoalantolactone (31.0 ± 26.2%), an eudesmanolide not encountered in any other member investigated in this study. Isoalan-

tolactone, along with its isomer alantolactone, has been reported in the root essential oil of *I. helenium* [40,41]. Sample I18 dominated by that chemical group (79.6%), also showing the highest isoalantolactone levels compared to I17, which was relatively richer in sesquiterpene hydrocarbons compared to I18 (31.3% and 4.7%, respectively), with (*E*)-caryophyllene (12.0%) as the main representative of that chemical class.

Overall, the essential oils of the analyzed samples were found poor in monoterpenes (traces, rarely up to 45.2%) and diterpenes (rarely up to 20.1%). In opposite, sesquiterpenes and particularly, oxygenated sesquiterpenes (up to 79.6%) prevailed in the group, with the *I. candida* subgroup exhibiting a predominance of *epi*-α-cadinol. Total aldehydes were high in *I. verbascifolia* (up to 66.8%), but low in *I. candida* (up to 2.8%). Alcohols were altogether insignificant. Aromatic constituents ranged between traces and 22.2% with (3*Z*)-hexenyl benzoate as the main representative component in all studied *Inula* essential oils, except the samples I22 and I23 (*I. verbascifolia* subsp. *aschersoniana* from northern Greece).

3.2. Population Clustering Based on Chemical Composition

The evaluation of each clustering algorithm based on the three internal validation metrics with increasing number of clusters (2, 3 and 10) is summarized in Table 3. With the exception of the hierarchical agglomerative method, the comparative evaluation of the internal validation metrics for most algorithms suggested that the optimal number of clusters were two (2).

Table 3. Internal cluster validation metrics for different algorithms and number of clusters. Values in bold indicate the per algorithm optimal number of clusters. SI is the silhouette index, DI is the Dunn index and CHI is the Calinski–Harabasz index.

Number of Clusters	Algorithm	SI	DI	CHI
2	hierarchical agglomerative	0.218	0.410	6.730
3		0.243	0.337	6.000
10		**0.269**	**0.703**	**8.146**
2	k-means	0.154	**0.396**	**4.550**
3		**0.220**	0.396	4.504
10		0.064	0.333	3.160
2	k-means PCA	**0.113**	0.239	**3.906**
3		0.047	0.239	2.531
10		0.042	**0.333**	3.686
2	k-means PCA (Hellinger)	0.146	0.311	**4.211**
3		**0.155**	0.311	4.036
10		0.060	**0.333**	3.168
2	fuzzy k-means	0.178	**0.396**	**4.995**
3		**0.222**	0.396	4.504
10		0.064	0.333	3.160

For a preselected number of two clusters, the main groups identified by all classification methods were the *I. candida* and the *I. verbascifolia* clusters. The hierarchical clustering algorithm (Figure A1a, Appendix A) grouped together all *I. candida* members, while populations of *I. verbascifolia* and *I. subfloccosa* were all classified to the second cluster, with the exception of *I. verbascifolia* subsp. *aschersoniana* (I7) from Mt. Ossa. The k-means method classified together all *I. candida* and *I. subfloccosa* populations, including the three *I. verbascifolia* subsp. *aschersoniana* populations (Figure A1b, Appendix A). The rest of the *I. verbascifolia* populations were grouped together in the second cluster. When the k-means method was implemented on *Inula* populations scores of the first eight PCA axes (accounting for 69.8% of the total variance), the separation between the two key groups were smaller, with *I. verbascifolia* populations grouped within the *I. candida* cluster (Figure A1c, Appendix A). Finally, the k-means clustering of the Hellinger pre-transformed PCA scores (eight axes: 72.7% of the total variance), yielded a clear classification of the *I. candida* and the *I. ver-*

bascifolia subgroups, with the two *I. subfloccosa* and the two northern *I. verbascifolia* subsp. *aschersoniana* populations (I22 and I23) grouped together within the *I. candida* subgroup (Figure A1d, Appendix A). The changeability of the clustering outcome, particularly for the two *I. subfloccosa* populations was highlighted in the fuzzy k-mean algorithm (Figure 1). In this case, the two main (*I. candida* and *I. verbascifolia*) clusters were again identified, with the three *I. verbascifolia* subsp. *aschersoniana* populations grouped within the *I. candida* cluster.

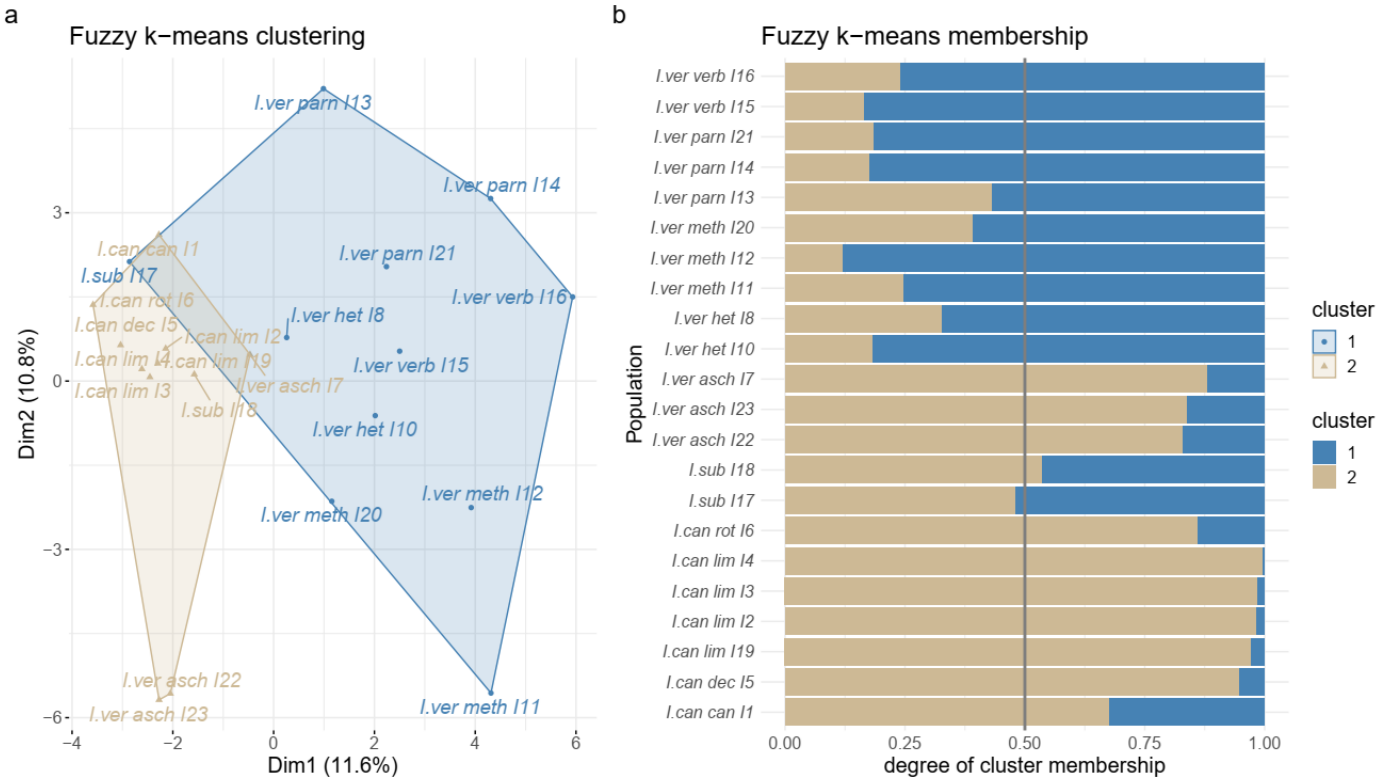

Figure 1. (**a**) Classification of each *Inula* population based on the fuzzy k-means clustering algorithm. Blue color refers to cluster 1 (mainly *I. verbascifolia*) and brown color refers to cluster 2 (mainly *I. candida*). (**b**) Degree of membership of each population based on the fuzzy k-means clustering algorithm. Population I9 (*I. verbascifolia* subsp. *heterolepis*) has the least number of compounds identified and was excluded from several analyses.

Interestingly, this algorithm estimated a borderline cluster membership for both *I. subfloccosa* populations (close to 50% for each subgroup), suggesting that essentially these populations could equally belong to any one of the two clusters formed.

4. Discussion

4.1. Chemical Diversity Overview and Chemotaxonomic Markers

The analyses of the volatile compounds in the essential oils of the *Inula candida* / *I. verbascifolia* group revealed a diverse pattern of metabolomic profiles that may be useful from a chemotaxonomic point of view.

As a rule, sesquiterpenes are the predominant group of components in all populations studied, particularly within taxa of the *I. candida* subgroup (57.2–76.8%) and *I. subfloccosa* (84.3–84.1%), apart from the *I. verbascifolia* subsp. *verbascifolia* volatiles (samples I15, I16). The *I. candida* subgroup compounds have a stable significant percentage of oxygenated sesquiterpenes (47.3–71.5%), in contrast to those of the *I. verbascifolia* subgroup (2.6–47.8%). *Epi*-α-cadinol is a chemotaxonomically important oxygenated sesquiterpene present in considerable amounts in all members of the *I. candida* subgroup (13.4–32.4%) but is rarely found in the *I. verbascifolia* subgroup in amounts higher than 9.5%, with the notable exception of I7 (*I. verbascifolia* subsp. *aschersoniana*). Interestingly, (*Z*)-curcumen-12-ol is a

characteristic sesquiterpene found only in the four *I. candida* subsp. *limonella* populations examined (22.6–42.1%) and could serve as a potential chemotaxonomic marker for this particular subspecies. Furthermore, isoalantolactone characterizes specifically the *I. subfloccosa* samples (12.4–49.5%) and may be another chemical marker that emerges within the group.

In agreement with the literature [42], monoterpenes are generally present at a low percentage, except for the two samples of *I. verbascifolia* subsp. *methanea* (I11, I12), where they dominate (45.2% and 36.2%, respectively), having *cis*-chrysanthenol, α-pinene and 1,8-cineole as main components. Their presence may presumably help identifying this particularly subspecies and differentiate it from its relatives.

The presence of the abienol diterpene is of interest: it appears in all taxa of the *I. candida* subgroup (tr-20.1%), except *I. candida* subsp. *decalvans*, a local subspecies of Crete Island. Within the *I. verbascifolia* subgroup, it is present in only one sample (I8–1.3%), whereas in *I. subfloccosa* it is present in low amounts (tr-1.1%).

The two *I. verbascifolia* subsp. *aschersoniana* samples (I22, I23) from Northern Greece lack aromatic compounds, although these components are present in all *Inula* taxa studied. These two populations are characterized by a high percentages of *trans*-muurola-4 (14),5-diene (17.0–22.2%), not found in any other *Inula* member examined. Therefore, within subsp. *aschersoniana* two different chemotypes can be distinguished: a *trans*-muurola-4(14),5-diene-, γ-cadinene-rich chemotype from north-eastern Greece and a 1-*epi*-cubenol-, tridecanal-rich chemotrype from central Greece.

Finally, the presence of the hydrocarbon aldehydes decanal, undecanal and tridecanal can help differentiate most members of the *I. verbascifolia* subgroup from the *I. candida* subgroup, where they are found at very low percentages or are absent. *I. verbascifolia* subsp. *verbascifolia*, in particular, is quite rich in tridecanal (35.0–37.5%). In *I. subfloccosa*, hydrocarbon aldehydes are practically absent.

4.2. Impact of Chemical Diversity to Species Delimitation

Clustering methods and experimental allocation of the taxa participating in our study into clusters best supported the existence of two sets of members: the *Inula candida* and the *I. verbascifolia* aggregates. The alternatives (three clusters and ten clusters) were also examined using different algorithms but showed no rational groupings in relation to the taxonomic interpretation of the results. As a consequence, we were able to verify only two taxonomic entities at species level, *Inula candida* and *I. verbascifolia*, as accepted by several authors [6,21,43]. We discourage, at the same time, the recognition of subspecific entities as independent species [7,8]. However, within species, chemical variation is pronounced and some distinct metabolomic profiles emerge, as in the cases of *I. candida* subsp. *limonella*, *I. candida* subsp. *decalvans*, *I. verbascifolia* subsp. *methanea* and Greek samples of *I. verbascifolia* subsp. *verbascifolia*. These local assemblages fit the geographically delimited group of populations that coincides with the subspecies concept. Var. *rotundifolia* may also merit subspecific recognition, as its high percentages of dictamnol and abienol indicate. It is a local member of the *I. candida* subgroup, and only one population was available for our analyses. Its deviating metabolomic profile corroborates its morphological differentiation from its allied taxa.

Two particular taxa need further attention. *I. verbascifolia* subsp. *aschersoniana* appears quite heterogenous, with its two northernmost populations (I22, I23) being similar to each other with respect to chemical compounds but differing considerably from the southern population (I7) and all the remaining *I. verbascifolia* samples. Two chemotypes seem to exist within this subspecies. Most clustering analyses attributed them to the *I. candida* subgroup and the fuzzy k-means analysis indicated a high degree of membership to the group that contained several *I. candida* members (Figure 1b). This attribution should be further investigated in future research. Currently, it is not supported by morphology. Interestingly, subsp. *aschersoniana* (as I. *aschersoniana*) and *I. verbascifolia* were members of the same monophyletic clade with a high statistical support in the nuclear ITS phylogeny tree of *Inula* [4] but are placed in different clades in the plastid tree derived from the same samples.

However, no *I. candida* members were involved in this study [4]. A wider sampling and further analysis of subsp. *aschersoniana* populations would presumably shed light to the chemical variation (and affinities) of this subspecies.

Inula subfloccosa could not be unequivocally placed to any particular cluster in our statistical analyses. Fuzzy k-means clustering gave its two samples an almost 50% membership to either *I. candida* or *I. verbascifolia* clusters. *I. subfloccosa* is a local species chemically characterized by copious isoalantolactone amounts. Its "mobile" placement to either *I. candida* or *I. verbascifolia* subgroups, depending on the applied clustering algorithms, could presumably be interpreted as the result of hybridization between members of the two subgroups. However, *I. subfloccosa* has distinct compounds in its essential oil composition and its unique, incense to camphoraceous odor is evident when collected and differrent from all other *Inula* members examined. Furthermore, no respresentatives of the *I. verbascifolia* subgroup are known to grow in its vicinity [12] and recent hybridization events are rather improbable. The elucidation of its taxonomic and phylogenetic relationships may need a detailed morphological evaluation and molecular approaches.

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Appendix A

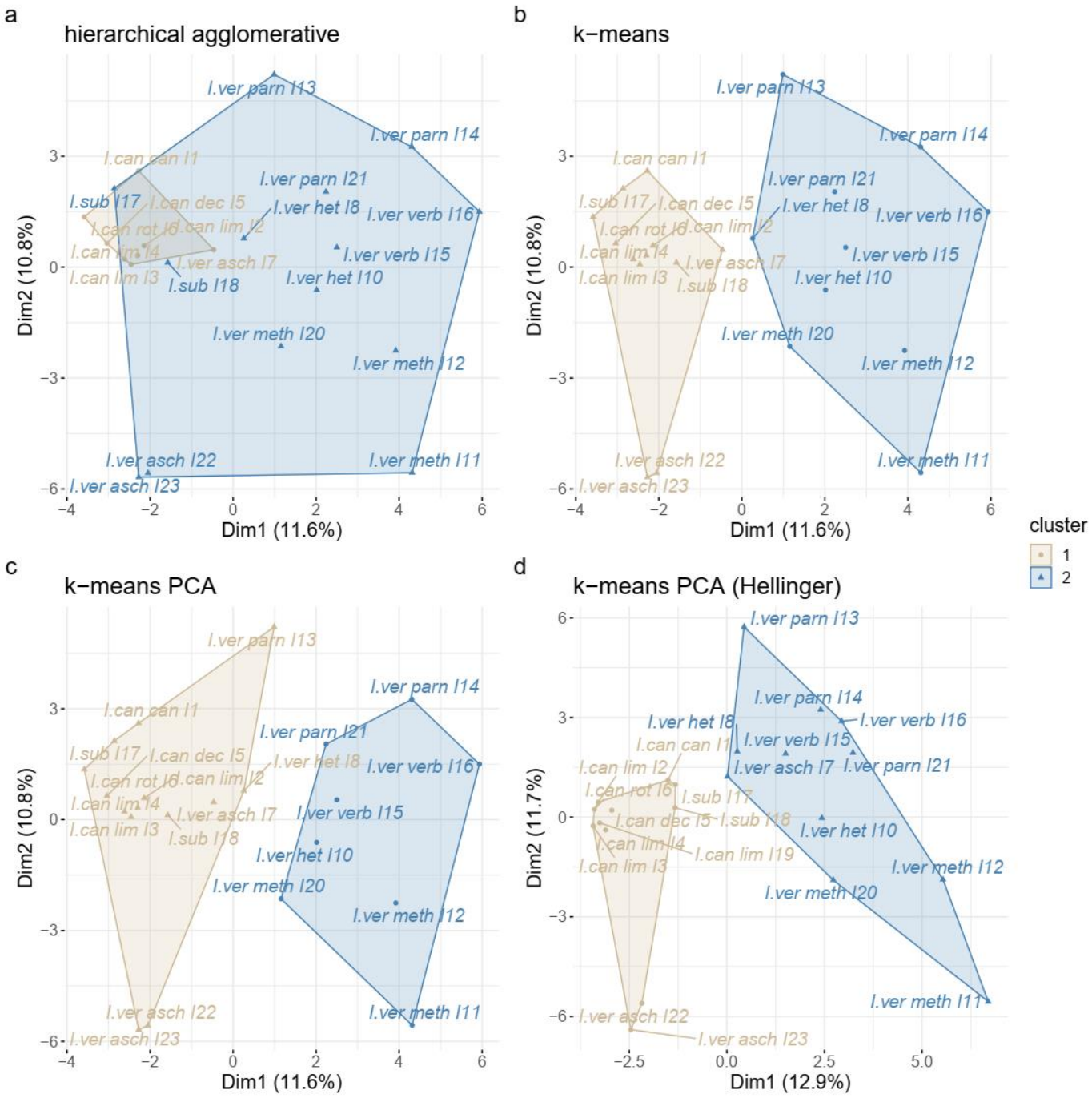

Figure A1. Classification of the *Inula* populations based on (**a**) the hierarchical agglomerative algorithm, (**b**) the k-means algorithm, (**c**) the k-means algorithm on the coordinates of the first eight PCA axes (accounting for 69.8% of the volatile compounds variance) and (**d**) the k-means algorithm on the coordinates of the first eight PCA (72.7%) of the Hellinger pre-transformed volatile compounds matrix.

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