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The Essential Oil Composition of *Helichrysum italicum* (Roth) G. Don: Influence of Steam, Hydro and Microwave-Assisted Distillation

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Abstract: *Helichrysum italicum* (Roth) G. Don (Asteraceae), also known as immortelle, usually grows in the Mediterranean area. The composition of the essential oil (EO) of immortelle is a mixture of various aromatic substances, mainly monoterpenes and sesquiterpenes. Distillation is the most widely used method for extraction of EO immortelle, although the yield is very low (<1%). In this work, we aim to investigate how the use of different distillation methods affects the yield and chemical composition of immortelle EO. For this purpose, we applied two conventional methods: steam distillation (SD) and hydrodistillation (HD), and a modern (environmentally friendly) technique—microwave-assisted distillation (MAD). Wild immortelles from four different locations in Croatia were collected and carefully prepared for extraction. Each sample was then analyzed by gas chromatography–mass spectrometry (GC-MS). GraphPad Prisma statistical software was used to study the statistics between different groups of connections and analyze the data on the number of connections. The results show that HD gives a significantly higher yield (0.31 ± 0.09%) compared to MAD (0.15 ± 0.03%) and SD (0.12 ± 0.04%). On the other hand, the highest number of chemical compounds was identified with MAD (95.75 ± 15.31%), and most of them are subordinate compounds with complex structures. SD isolated EOs are rich in derived acyclic compounds with the highest percentage of ketones. The results show that the application of different distillation methods significantly affects the composition of the obtained immortelle EO, considering the yield of EO, the number of isolated, derived and non-derived compounds, chemotypes and compounds with simple (acyclic) and complex structures.

Keywords: *Helichrysum italicum*; essential oil; immortelle; extraction; chemical composition; terpenes; chemotypes

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1. Introduction

Helichrysum italicum (Roth) G. Don is known as immortelle or curry plant and belongs to the family Asteraceae. It usually grows on dry, sandy and rocky soils in the Mediterranean region at a wide range of altitudes: up to 2200 m above sea level [1,2]. In the Croatian flora, it is present along the coastal belt and on the islands in the form of two subspecies: *microphyllum* and *italicum* [3]. *H. italicum* is a 30–70 cm tall shrub with flowers grouped in yellow heads [4]. On the branched stems, the 1–3 cm long leaves are alternate and twisted at the edges. The flowers and leaves are traditionally used for therapeutic purposes to relieve respiratory, digestive, and skin problems [5].

The traditional methods for extracting essential oils (EO) of immortelle are steam distillation (SD) and hydrodistillation (HD). HD is the oldest and simplest method for extracting EO. In general, extraction is based on the evaporation of volatile organic compounds (VOCs) from the plant material immersed in an aqueous medium. The main advantage of HD is its ability to isolate joints at temperatures up to 100 °C [6]. The disadvantage is that the compounds are hydrolyzed. This is due to the influence of water and increased temperature [7]. Compared to HD, SD does not require the plant material to be directly immersed in a water medium. SD begins by heating the water in the steam generator. As the steam passes through the plant material, the structure of the plant tissue is broken and the glands burst, resulting in the release of compounds [8]. To increase the yield of EOs and thus prevent the loss of VOCs, innovative techniques such as supercritical CO_2 extraction [9] and microwave assisted distillation (MAD) are used [10,11]. Another advantage of innovative techniques in the extraction of EOs is that they can be performed in a much shorter time compared to conventional methods. These extraction methods and techniques are applied to immortelle, in order to obtain the highest quality essential oil for further use [12].

Essential oils are complex mixtures of volatile organic compounds synthesized by the plant as secondary metabolites to: (I) for defense against herbivores, insects and microorganisms; (II) for communication with plants of the same species; and (III) in response to various environmental stimuli [13]. Since the EO of *H. italicum* is widely used in pharmaceutical, cosmetic, and food industries, some studies have already been conducted on the chemical composition of EO [14–16]. The use of the EO of *H. italicum* generally depends on the chemotype. Three groups of chemotypes are rich in EO: (I) nerol and its esters are the most commonly described in the literature; (II) α- and β-selinene; and (III) γ-curcumene [17,18].

This work focuses on the EO chemical composition of wild immortelle in Croatia (four different sites on the Adriatic Sea). For this purpose, three different distillation methods are applied: steam, hydro and microwave assisted distillation. The chemical composition of each sample was determined in the same way using gas chromatography–mass spectrometry (GC-MS). Based on the results obtained, the yield of EO was determined for each method. Groups of compounds (by functional groups) and chemotypes were also defined. It was determined which extraction method isolated the most organic compounds and which contained the most derivative, i.e., non-derivative compounds.

2. Materials and Methods

2.1. Plant Materials

The collection of above-ground parts of wild immortelle (Figure 1A) was carried out in July 2018 at four different sites in the coastal area of Dalmatia (Croatia). The selected sites (see Table 1) were far from major settlements, industry and main roads. At least 20 individual plants were collected at each site. Plants that were damaged or dirty were not harvested. The collection was done according to botanical [19] and legal regulations (permit for collecting non-timber forest products 112/2018). The identification of the plant material was performed by Professor Goran T. Anačkov, and the specimens were confirmed and deposited in the Herbarium of the Department of Biology and Ecology (BUNS Herbarium) of the Faculty of Natural Sciences of the University of Novi Sad. Drying of the plant material was carried out for 15 days at room temperature in the dark. After drying, the plant material was cut into 1–3 cm pieces, homogenized (Figure 1B) and stored in the dark until extraction.

2.2. Steam Distillation (SD)

SD was performed with water-soaked plant material to allow more efficient penetration of steam through the plant material (76 ± 37 g). The steam was generated with a steam generator connected to the distillation apparatus by a pipe system. The temperature of the steam was determined by the boiling point of water at atmospheric pressure. After 2.5 h

of collecting the distillate, EO was collected in a small amount of *n*-pentane to verify the separation of EO from water. The EOs obtained were dried with anhydrous sodium sulfate and then stored at 4 °C in the dark.

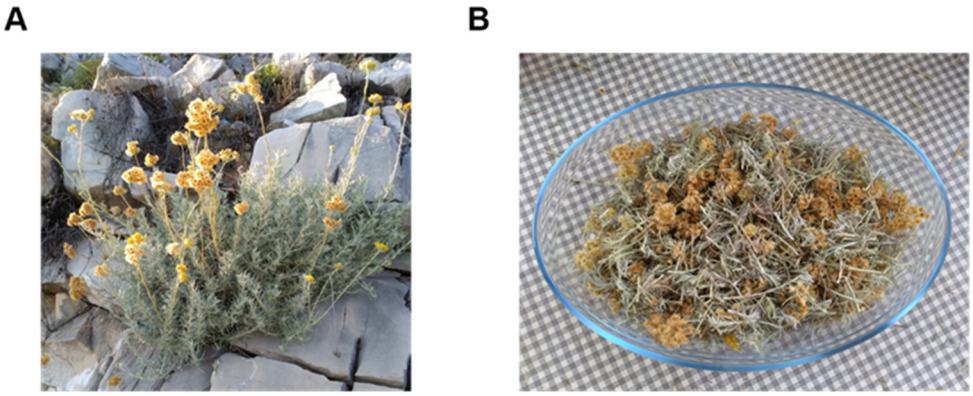

Figure 1. Acquisition and preparation of plant material for distillation purposes; (**A**)—wild-growing immortelle (*H. italicum* (Roth) G. Don) in its natural environment, (**B**)—dried and cut material after homogenization.

Table 1. Location and yield of essential oils *H. italicum* (Roth) G. Don.

No.	Name of Location	Yield (%) SD	HD	MAD	Latitude	Longitude	Approx. Elevation
1	Plano (PL)	0.08	0.19	0.18	43°33′42.08″ N	6°16′55.80″ E	243 m
2	Kaštela (KA)	0.09	0.31	0.10	43°34′48.63″ N	16°19′41.63″ E	418 m
3	Marina (MA)	0.16	0.31	0.17	43°30′31.58″ N	16°7′52.31″ E	13 m
4	Kornati (KO)	0.16	0.42	0.16	43°49′32.24″ N	15°16′18.37″ E	48 m

2.3. Hydrodistillation (HD)

HD was performed in a Clevenger apparatus. Dried plant material (112 ± 35 g) was immersed in water in a distillation flask. The distillation lasted for 2.5 h. The essential oils were collected in *n*-pentane, which was also dried with anhydrous sodium sulfate and stored in the dark at 4 °C until use.

2.4. Microwave-Assisted Distillation (MAD)

MAD was performed in a microwave extraction system (Milestone "ETHOS X") in a laboratory oven (1900 W maximum). Dried plant material (140 ± 15 g) was soaked in water before being placed in a distillation flask. Distillation lasted 35 min at atmospheric pressure and the maximum power was 500 W (98 °C) at a wave frequency of 2.45 GHz. The system for cooling the steam was located above the oven. The distillate was collected with an *n*-pentane trap in a side tube, dried over anhydrous sodium sulphate, and stored at 4 °C until analysis.

2.5. GC–MS Analysis

Analysis of the obtained EO samples was performed using a gas chromatograph (GC) model 7890A (Agilent Technologies, Santa Clara, CA, USA) in conjunction with a selective mass detector (MS) 5975C (Agilent Technologies). The injection temperature was set at 250 °C, and the injection volume was 1 µL. A nonpolar HP-5MS column (30 m × 0.25 mm × 0.25 µm) was used for chromatographic separation of the compounds, with the stationary phase consisting of 5% phenylmethylpolysiloxane. The carrier gas was high purity helium with a flow rate of 1 mL min-1. The oven temperature was programmed as follows: Hold at 70 °C for 2 min, heat to 200 °C at 3 °C min^{-1}, and hold for 18 min. The split ratio was 1:50, the ionization energy was 70 eV, the ion source temperature was

230 °C, the MS quad temperature was 150 °C, and the transfer line was 280 °C. The mass scan interval was 30–350 mass units. The retention indices were determined relative to the retention time of the *n*-alkanes (C_9–C_{25}). Compounds were identified based on the retention indices and comparison of the spectra with spectra from the Wiley 9 and NIST 14 databases and with previously published work. The amount of each compound was calculated by integrating the area under the peak [9].

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 9). The data distribution was examined with the Shapiro–Wilk test, while the two-way test ANOVA with Tukey post hoc test was used to examine the statistical significance between the different groups of preparations. For data analysis of the yield of EO, the one-way ANOVA with Tukey post hoc test was used. Kruskal–Wallis and Dunn tests were used to analyze the data for the number of compounds identified. Results are presented as mean ± standard deviation. Only results with a calculated probability p of less than 0.05 are considered statistically significant. Symbols: * for $p < 0.05$, ** for $p < 0.001$, *** for $p < 0.0001$, and **** for $p < 0.00001$.

3. Results

Isolation of EOs

In this work, we compared how the use of different distillation methods affects the yield and composition of immortelle EO. Wild immortelle was used for SD, HD and MAD. The yield of essential oils of immortelle obtained by each distillation method was calculated from the mass of plant material used during the distillation process and the mass of EO in pure and concentrated states. HD gave a significantly higher yield (0.31 ± 0.09%) of EO compared to SD (0.12 ± 0.04%) and tended to give a higher amount of oil compared to MAD (0.15 ± 0.03%). Considering the duration of the distillation method used, both HD and SD had the same distillation time (2.5 h), but HD isolated twice the amount of EO compared to SD. MAD, on the other hand, had a much shorter distillation time (35 min), but produced a similar amount of EO as SD. The yields of EO obtained by all three methods are consistent with those previously reported for immortelle, where yields of less than 1% were also reported [6,18].

After isolation of the essential oils, GC-MS analysis was performed. A total of 120 compounds were identified in 12 samples of immortelle EOs. The MAD method yielded the EO with the highest number of identified compounds (73–106), which was significantly higher compared to the samples obtained with SD (55–79), but close to HD, which yielded EO with (90–96). SD isolated samples of immortelle EO in which the lowest number of compounds was identified. One possible reason is that SD is limited compared to the other two distillation methods because steam cannot penetrate dry cell membranes [20], which limits the isolation of volatile compounds to the plant surface. Table 2 shows the chemical composition of EOs of immortelle for each sample. In samples from different sites obtained by the same distillation method, the same compounds are present in different proportions. The differences are due to genetic, environmental, and climatic factors that affect plant growth and biosynthesis of the compounds [21,22].

A total of 37 common compounds were identified in all samples, regardless of the distillation method and plant material used, and are highlighted in Table 2. Figure 2A shows the statistics of the compounds identified in at least one sample with a proportion greater than 5%. The most important compound in the essential oils of immortelle was neryl-acetate, which was the most frequently isolated in the isolates from SD (about 10%), but did not show statistically significant differences from the other distillation methods. The high content of neryl-acetate was also reported by other authors for Helichrysum italicum Roth. G. Don ssp. Italicum [18,23] and related species [24]. Other compounds with high abundance were α-pinene, Italidione I, II and III, γ-curcumene, ar-curcumene, α- and β-selinene, and rosifoliol. Italidione I was significantly increased in SD compared to the

other methods used, and Italidione II was significantly increased in SD compared to MAD. MAD isolated significantly more α-pinene compared to SD and tended to isolate more than HD. In the samples obtained with HD, γ-curcumen was not detected, while SD and MAD isolated a relatively high amount of γ-curcumen. The absence of γ-curcumene in the samples obtained by HD can be explained by its photosensitivity [25], which is enhanced in the aqueous medium by the effect of high temperature. In addition, we tested whether the chemotype of EO changed. SD and HD produced EOs with a nerol-rich chemotype, whereas MAD isolated an EO with a mixed chemotype that had similar amounts of nerol, curcumene, and selinene compounds in the isolates (Figure 2B). Since the starting material was the same for all three methods, these changes could only have been caused by the distillation methods themselves.

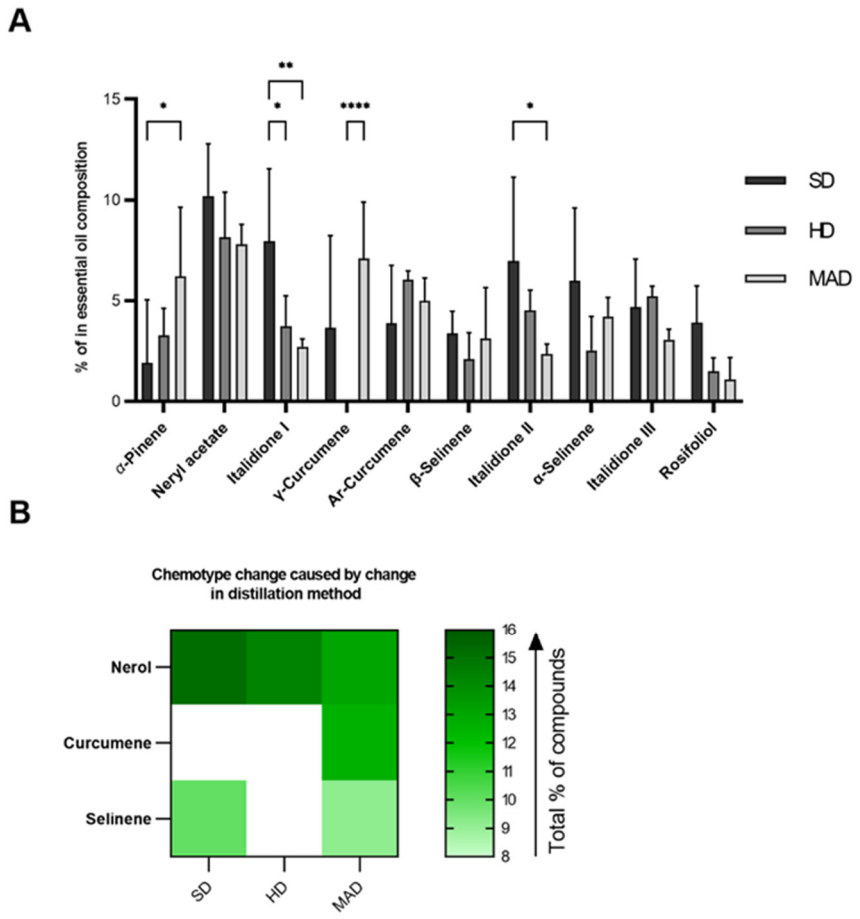

Figure 2. Application of three different distillation methods (SD—steam distillation, HD—hydrodistillation and MAD—microwave-assisted distillation) produces EOs with different compositions and chemotype. (**A**) Compounds identified in at least one sample in a proportion greater than 5%, (**B**) Heat map of total amount of nerol, curcumene and selinene compounds identified in EOs for each distillation method used. * $p < 0.05$, ** for $p < 0.001$, *** for $p < 0.0001$, and **** for $p < 0.00001$.

Table 2. Chemical composition of essential oils of *H. italicum* (Roth) G. Don. The proportion of the compounds is expressed in %.

RT	Compound	RI-E	RI-L	PL-SD	KA-SD	MA-SD	KO-SD	PL-HD	KA-HD	MA-HD	KO-HD	PL-MAD	KA-MAD	MA-MAD	KO-MAD	ID
3184	**4-Methyl-3-hexanone**	<900	-	0.15	0.25	0.16	0.50	0.09	0.04	0.09	0.28	0.17	0.08	0.07	0.19	MS
3362	(2E)-Hexenal	<900	855 *	0.03	0.06	nd	nd	0.07	0.01	0.09	0.05	0.06	0.05	0.05	0.05	MS
3581	Hexan-1-ol	<900	870 *	nd	nd	nd	nd	nd	nd	0.01	0.01	0.02	nd	0.01	0.01	MS
4138	Heptanal	902	901	0.01	nd	nd	nd	0.02	nd	0.02	0.04	0.02	nd	0.01	0.02	RI, MS
4931	***α*-Pinene**	943	942	0.09	6.60	0.56	0.36	3.07	5.06	3.15	1.75	5.50	11.24	4.19	3.81	RI, MS
5196	*α*-Fenchene	954	952 *	nd	0.03	0.11	nd	0.06	0.14	0.34	0.45	0.31	0.35	0.31	0.45	RI, MS
5242	Camphene	956	954	nd	0.13	0.02	nd	0.12	0.14	0.23	0.28	0.37	0.32	0.24	0.26	RI, MS
5362	*2,4*-Thujadiene	961	956	nd	nd	nd	nd	nd	nd	0.01	0.02	0.03	nd	0.01	0.02	RI, MS
5929	*β*-Pinene	982	979	nd	0.07	nd	nd	0.08	0.08	0.10	0.39	0.19	0.12	0.09	0.34	RI, MS
6104	6-Methyl-5-hepten-2-one	988	987	0.07	0.08	0.04	nd	0.06	0.03	0.05	0.07	0.08	nd	0.03	0.06	RI, MS
6234	*β*-Myrcene	992	992	nd	nd	nd	nd	0.04	0.02	0.04	0.08	0.05	0.03	0.04	0.08	RI, MS
6295	(E)-dehydroxy-Linalool Oxide	994	993 *	0.08	0.07	0.07	0.13	0.12	0.04	0.06	0.15	0.08	nd	0.04	0.07	RI, MS
6539	**Isobutyl 2-methylbutanoate**	1003	1004	0.03	0.05	0.10	0.13	0.06	0.03	0.11	0.15	0.12	0.06	0.12	0.12	RI, MS
6666	α-Phellandrene	1007	1007	nd	nd	nd	nd	nd	nd	nd	nd	0.03	nd	0.02	0.02	RI, MS
6724	(Z)-dehydroxy-Linalool Oxide	1010	1008 *	0.03	nd	0.02	nd	0.02	nd	0.01	0.04	0.02	nd	nd	0.01	RI, MS
7017	α-Terpinene	1020	1017	nd	nd	0.02	nd	nd	nd	nd	nd	0.15	0.08	0.09	0.08	RI, MS
7285	***o*-Cymene**	1030	1032 *	0.01	0.17	0.06	0.40	0.20	0.34	0.66	1.68	0.23	0.16	0.29	1.12	RI, MS
7433	**Limonene**	1035	1032	0.08	2.53	0.96	0.72	0.82	2.06	2.76	2.49	2.38	3.31	2.72	2.85	RI, MS
7486	1,8-Cineole	1036	1033	0.99	0.81	1.29	0.60	0.12	0.44	0.45	nd	0.61	0.68	0.31	0.16	RI, MS
7760	(Z)-β-Ocimene	1045	1037 *	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	0.01	0.02	RI, MS
7904	Benzeneacetaldehyde	1050	1049	nd	nd	nd	nd	nd	nd	0.01	0.10	0.02	nd	nd	0.02	RI, MS
7988	(E)-β-Ocimene	1052	1050 *	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	nd	0.03	RI, MS
8078	**Isobutyl angelate**	1055	1051 *	0.17	0.35	0.75	0.87	0.14	0.22	0.70	0.67	0.35	0.31	0.76	0.53	RI, MS
8366	γ-Terpinene	1063	1064	nd	0.13	0.14	nd	nd	nd	nd	nd	0.38	0.21	0.24	0.28	RI, MS
RT	Compound	RI-E	RI-L	PL-SD	KA-SD	MA-SD	KO-SD	PL-HD	KA-HD	MA-HD	KO-HD	PL-MAD	KA-MAD	MA-MAD	KO-MAD	ID
8836	cis-Linalool oxide (furanoid)	1076	1072 *	0.12	nd	nd	nd	0.11	0.07	0.07	0.13	0.05	nd	0.02	0.05	RI, MS
9392	α-Terpinolene	1090	1086 *	0.08	0.40	0.17	nd	nd	nd	nd	nd	0.48	0.40	0.31	0.23	RI, MS
9400	*trans*-Linalool oxide (furanoid)	1091	1086 *	nd	nd	nd	nd	0.09	0.05	0.08	0.07	nd	nd	nd	nd	RI, MS
9484	**2-Nonanone**	1093	1093 *	0.06	0.12	0.28	0.44	0.17	0.08	0.25	0.32	0.14	0.07	0.11	0.19	RI, MS
9712	**2,4-Dimethyl-heptane-3,5-dione**	1098	-nd	0.22	0.23	0.05	0.25	0.69	0.19	0.25	0.77	0.23	0.07	0.10	0.31	MS
9769	α-Pinene oxide	1100	1095	nd	nd	nd	nd	nd	0.24	0.19	nd	nd	nd	nd	nd	RI, MS
9870	**Linalool**	1103	1104	2.81	2.09	2.31	2.68	0.86	1.00	1.77	1.38	0.94	0.86	1.26	1.46	RI, MS
9927	2-Methylbutyl 2-methylbutyrate	1104	1107	0.06	0.17	0.22	0.32	0.17	0.14	nd	nd	0.23	0.18	0.27	nd	RI, MS
10,395	**Fenchol**	1118	1115 *	0.39	0.87	0.43	0.57	0.44	0.35	0.38	0.43	0.46	0.29	0.23	0.31	RI, MS
10,801	α-Campholenal	1130	1126 *	0.05	nd	nd	nd	0.34	0.06	0.07	0.23	0.07	nd	nd	0.03	RI, MS
11,070	cis-Limonene oxide	1137	1134 *	nd	nd	nd	nd	0.03	0.03	0.08	0.09	nd	nd	nd	nd	RI, MS
11,235	trans-Limonene oxide	1142	1138 *	nd	nd	nd	nd	0.03	0.04	0.07	0.13	nd	nd	nd	nd	RI, MS

Table 2. *Cont.*

11,346	**trans-Pinocarveol**	1144	1140	0.70	0.58	0.12	0.53	1.27	0.28	0.17	0.07	0.58	0.14	0.10	0.39	RI, MS
11,568	trans-Verbenol	1150	1144	0.06	nd	nd	nd	0.27	0.11	0.07	0.07	0.04	nd	0.02	0.06	RI, MS
11,700	Camphene hydrate	1154	1149	0.15	0.32	0.05	0.22	nd	nd	nd	nd	0.20	0.11	nd	0.09	RI, MS
11,859	**2-Methylbutyl angelate**	1158	-	0.77	1.57	2.06	2.38	0.91	1.16	2.34	2.08	1.15	1.39	2.27	1.57	MS
12,217	Pinocarvone	1166	1161 *	0.20	0.07	nd	0.29	0.36	0.76	0.06	0.05	0.14	nd	0.03	0.12	RI, MS
12,427	**endo-Borneol**	1171	1168	0.63	1.44	0.40	0.67	0.94	0.76	0.76	0.76	0.93	0.57	0.49	0.57	RI, MS
12,864	**Terpinen-4-ol**	1181	1178	0.53	0.60	0.57	1.93	0.25	0.30	0.50	1.15	0.53	0.25	0.41	1.00	RI, MS
13,272	**4,6-Dimethyloctane-3,5-dione**	1190	-	3.01	3.99	1.58	3.88	1.99	2.00	2.39	2.46	1.58	1.20	1.22	1.50	MS
13,485	**α-Terpineol**	1195	1195	2.16	3.42	1.16	2.25	1.92	1.80	1.71	1.84	1.57	1.09	1.04	1.22	RI, MS
13,676	Myrtenol	1199	1195 *	0.17	0.11	nd	nd	0.16	0.09	0.14	0.17	0.13	nd	0.06	0.09	RI, MS
13,923	Decanal	1206	1206	0.33	0.18	0.09	nd	nd	nd	nd	nd	nd	nd	nd	nd	RI, MS
14,612	trans-Carveol	1224	1223	0.25	0.16	nd	nd	0.33	0.11	0.21	0.37	0.16	nd	nd	0.15	RI, MS

RT	Compound	RI-E	RI-L	PL-SD	KA-SD	MA-SD	KO-SD	PL-HD	KA-HD	MA-HD	KO-HD	PL-MAD	KA-MAD	MA-MAD	KO-MAD	ID
15,002	**Nerol**	1234	1229	2.84	2.28	2.20	2.70	1.71	1.14	2.33	2.12	2.57	1.00	1.78	2.43	RI, MS
15,253	Hexyl 2-methylbutanoate	1240	1236 *	nd	nd	nd	nd	nd	nd	0.08	0.12	0.06	nd	0.04	0.10	RI, MS
15,427	(Z)-Neral	1245	1242	0.22	nd	nd	0.42	0.25	0.22	0.32	0.33	0.06	nd	0.04	0.18	RI, MS
15,578	D-Carvone	1248	1242 *	nd	nd	nd	nd	0.06	0.03	0.06	0.08	0.02	nd	nd	0.03	RI, MS
15,920	3-Methylpentyl angelate	1257	1252 *	nd	0.07	0.07	nd	0.08	0.08	0.18	0.15	0.05	nd	0.07	0.08	RI, MS
16,642	(E)-Neral	1273	1267 *	0.12	nd	nd	0.20	0.12	0.09	0.13	0.14	0.03	nd	0.03	0.08	RI, MS
17,050	Neryl formate	1282	1285 *	0.08	nd	nd	0.20	0.23	0.06	0.11	0.30	nd	nd	nd	nd	RI, MS
17,285	**Hexyl angelate**	1287	-	0.10	0.34	0.42	0.41	0.37	0.48	0.85	0.74	0.45	0.31	0.43	0.79	MS
17,603	2-Undecanone	1294	1293 *	0.05	nd	0.09	0.17	0.12	0.06	0.18	0.29	0.09	0.05	0.07	0.18	RI, MS
17,858	trans-Pinocarvyl acetate	1299	1300	nd	nd	nd	nd	0.06	nd	0.02	0.06	0.02	nd	nd	0.01	RI, MS
18,316	4-Hydroxy-3-methylacetophenone	1312	1323	0.14	nd	nd	0.17	0.17	0.07	0.11	0.26	0.05	nd	0.03	0.09	RI, MS
18,568	(E,E)-2,4-Decadienal	1318	1318	0.03	nd	nd	nd	nd	nd	nd	nd	0.03	nd	0.01	nd	RI, MS
19,921	α-Terpinyl acetate	1352	1349 *	nd	nd	nd	nd	nd	0.03	0.06	0.10	0.03	nd	0.03	0.03	RI, MS
20,736	**Neryl acetate**	1371	1365	6.38	11.05	12.26	11.06	7.58	8.68	10.84	5.45	8.02	8.26	8.54	6.32	RI, MS
20,807	Ylangene	1373	1374	nd	0.39	0.25	nd	nd	0.15	nd	nd	nd	0.18	0.50	nd	RI, MS
21,064	**α-Copaene**	1378	1376 *	0.02	4.26	2.62	0.64	1.54	1.99	2.48	1.96	2.52	4.31	3.55	3.52	RI, MS
21,414	trans-β-Damascenone	1386	1386 *	0.08	nd	nd	nd	0.07	0.07	0.11	0.04	0.04	nd	0.01	0.04	RI, MS
21,590	Sativen	1390	1391 *	nd	0.12	nd	nd	nd	0.07	nd	nd	nd	0.07	0.11	0.10	RI, MS
21,858	β-Longipinene	1396	1400 *	nd	nd	nd	nd	nd	nd	nd	nd	0.04	nd	0.06	0.08	RI, MS
22,034	**Isoitalicene**	1400	1397 *	0.05	0.12	0.12	0.92	0.24	0.10	0.14	0.17	0.19	0.15	0.18	0.31	RI, MS
22,214	Italicene	1405	1409	0.10	2.68	2.51	nd	3.38	1.84	2.48	2.62	3.08	3.54	3.53	4.17	RI, MS
22,384	α-Gurgujene	1409	1408	nd	nd	0.10	nd	nd	nd	nd	nd	0.06	0.07	0.24	0.06	RI, MS
22,519	α-Cedrene	1413	1411 *	nd	nd	0.07	nd	0.11	0.04	0.06	0.09	0.09	nd	0.10	0.10	RI, MS
22,663	**cis-α-Bergamotene**	1417	1415	0.08	0.79	0.69	0.31	0.73	0.43	0.61	0.81	1.26	0.94	0.76	1.29	RI, MS
22,837	**Caryophyllene**	1421	1420	0.09	1.85	3.15	0.12	0.70	0.27	1.11	0.78	2.70	3.13	4.28	2.76	RI, MS
23,462	**trans-α-Bergamotene**	1437	1436	0.15	0.74	0.67	0.22	0.79	0.40	0.61	0.80	1.29	0.90	0.78	1.25	RI, MS

Table 2. *Cont.*

RT	Compound	RI-E	RI-L	PL-SD	KA-SD	MA-SD	KO-SD	PL-HD	KA-HD	MA-HD	KO-HD	PL-MAD	KA-MAD	MA-MAD	KO-MAD	ID
23,564	Aromandendrene	1440	1439	0.02	nd	0.18	nd	nd	nd	nd	nd	nd	nd	0.46	nd	RI, MS
23,812	**Italidione I**	1446	-	6.91	5.78	5.73	13.30	3.88	5.67	2.03	3.34	3.02	2.55	2.27	3.02	MS
24,287	**Neryl propanoate**	1458	1452*	3.10	2.18	2.13	1.59	4.80	3.83	2.32	3.54	3.23	1.96	2.00	3.09	RI, MS
24,320	Alloaromadendrene	1459	1459	1.63	0.39	0.82	nd	nd	nd	nd	nd	nd	0.44	0.79	nd	RI, MS
24,367	cis-β-Farnesene	1460	1457	0.23	0.27	0.40	nd	nd	nd	nd	nd	0.82	0.22	0.30	0.96	RI, MS
24,616	α-Acoradiene	1466	1466 *	nd	0.20	0.52	nd	0.60	0.49	0.48	0.97	0.64	0.37	0.51	0.80	RI, MS
24,744	**β-Acoradiene**	1469	1470 *	0.87	0.15	0.46	0.65	0.55	1.14	0.42	0.72	0.56	0.34	0.48	1.14	RI, MS
25,066	γ-Selinene	1477	1470	0.31	1.46	1.00	nd	1.32	1.24	1.22	1.31	1.80	2.34	1.57	1.75	RI, MS
25,219	γ-Curcumene	1480	1480	0.19	4.78	9.66	nd	nd	nd	nd	nd	6.74	10.06	8.17	3.43	RI, MS
25,333	α-Amorphene	1483	1484	nd	nd	nd	nd	0.69	0.40	0.72	0.17	nd	nd	nd	nd	RI, MS
25,521	Ar-Curcumene	1487	1484	nd	4.29	4.29	6.92	5.81	5.85	6.70	5.80	4.04	4.18	5.36	6.44	RI, MS
25,565	**β-Selinene**	1488	1489	3.78	4.46	3.40	1.85	1.97	3.09	3.03	0.26	2.77	6.78	1.85	1.05	RI, MS
25,793	**Italidione II**	1493	-	12.07	3.17	3.96	8.61	4.69	4.68	3.16	5.55	2.23	1.75	2.91	2.51	MS
25,899	α-Selinene	1496	1494	11.23	3.58	3.45	5.60	3.58	3.51	2.98	nd	4.39	5.47	3.47	3.40	RI, MS
26,118	α-Muurolene	1501	1502	nd	1.40	1.59	nd	3.73	1.50	1.39	1.56	1.06	1.14	1.75	1.34	RI, MS
26,344	β-Cadinene	1507	-	nd	0.33	0.81	nd	nd	nd	nd	nd	0.92	0.26	0.96	0.37	MS
26,458	β-Bisabolene	1510	1511	nd	nd	nd	nd	0.27	0.25	0.23	0.47	nd	0.15	nd	0.41	RI, MS
26,551	β-Curcumene	1513	1515 *	nd	0.22	0.47	nd	nd	nd	nd	nd	0.55	0.39	0.50	0.36	RI, MS
26,673	γ-Cadinene	1516	1513 *	0.44	0.55	0.67	0.27	0.85	0.69	0.80	0.48	0.63	0.61	0.86	0.78	MS
26,816	7-epi-α-Selinene	1520	1522	nd	nd	nd	nd	nd	nd	nd	nd	0.16	nd	0.08	0.12	RI, MS
26,998	δ-Cadinene	1525	1523 *	0.33	1.62	1.92	0.40	nd	nd	nd	nd	1.31	2.07	2.24	1.60	RI, MS
27,036	cis-Calamene	1526	1529	nd	nd	nd	nd	0.58	0.61	0.86	0.82	nd	nd	nd	nd	RI, MS
27,336	Cadina-1,4-diene	1534	1534 *	nd	nd	nd	nd	nd	nd	nd	nd	0.29	0.49	0.36	nd	RI, MS
27,418	Italicene ether	1536	1537 *	0.92	0.48	0.63	0.52	0.99	0.54	0.50	0.74	0.43	nd	0.21	0.70	RI, MS
27,535	α-Cadinene	1539	1538 *	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.32	0.52	nd	RI, MS
27,590	2-Phenylethyl tiglate	1541	-	0.45	0.40	0.66	0.59	0.61	0.88	0.45	0.98	0.45	nd	nd	0.96	MS
27,742	α-Calacorene	1545	1542	nd	0.27	0.36	nd	0.29	0.21	0.43	0.38	0.45	0.25	0.56	0.49	RI, MS
RT	Compound	RI-E	RI-L	PL-SD	KA-SD	MA-SD	KO-SD	PL-HD	KA-HD	MA-HD	KO-HD	PL-MAD	KA-MAD	MA-MAD	KO-MAD	ID
28,615	(E)-Nerolidol	1567	1563 *	0.18	nd	0.21	nd	0.29	0.27	0.34	0.25	0.22	0.16	0.45	0.37	RI, MS
28,805	**Caryophyllene alcohol**	1572	1569 *	0.57	0.11	0.36	0.26	0.34	0.32	0.48	0.33	0.29	0.11	0.35	0.36	RI, MS
29,244	**Italidione III**	1583	-	6.15	3.19	2.14	7.21	5.60	5.47	4.48	5.30	2.95	2.83	2.63	3.83	MS
29,905	**Guaiol**	1599	1599	0.92	0.20	0.59	0.28	0.55	0.30	0.53	0.55	0.57	0.19	0.55	0.38	RI, MS
30,328	Rosifoliol	1611	1600 *	5.52	1.91	5.39	2.79	1.79	0.74	1.18	2.25	0.95	nd	2.61	0.71	RI, MS
30,937	1-epi-Cubenol	1628	1628 *	0.60	0.52	0.45	0.72	0.25	0.83	0.37	0.49	0.15	nd	0.14	nd	RI, MS

Table 2. *Cont.*

31,172	**γ-Eudesmol**	1634	1635	1.80	0.28	0.42	0.49	0.71	0.17	0.55	0.22	0.47	0.19	0.18	0.44	RI, MS
31,524	**τ-Cadinol**	1644	1644	1.08	0.36	0.68	0.40	0.59	0.97	0.82	0.64	0.44	0.28	0.52	0.42	RI, MS
31,718	α-Muurolol	1649	1646	nd	0.09	0.16	nd	nd	0.24	0.23	0.13	0.10	0.06	0.23	0.14	RI, MS
31,884	**β-Eudesmol**	1654	1650 *	3.01	0.32	0.51	1.21	1.03	0.67	0.56	0.86	0.68	0.21	0.43	0.53	RI, MS
32,046	**Neointermedeol**	1658	1660 *	3.78	1.13	1.72	2.59	2.64	2.31	1.38	1.29	1.45	0.91	0.89	0.81	RI, MS
32,464	Bulnesol	1669	1666	nd	nd	nd	nd	nd	nd	nd	nd	0.19	nd	0.21	nd	RI, MS
32,562	**β-Bisabolol**	1672	1672 *	0.84	0.23	0.47	0.39	1.07	0.53	0.94	0.86	0.56	0.15	0.52	0.71	RI, MS
32,782	Cadalene	1678	1676 *	nd	nd	nd	nd	0.62	0.39	0.38	0.33	0.13	nd	0.15	0.26	RI, MS
33,015	epi-α-Bisabolol	1684	1684 *	nd	nd	nd	nd	0.36	0.35	0.08	0.46	0.14	nd	0.04	0.17	RI, MS
33,094	α-Bisabolol	1686	1685	0.67	0.06	0.27	nd	0.75	0.12	0.51	0.55	0.25	0.14	0.35	0.39	RI, MS
34,119	Pentadecanal	1714	1714 *	0.52	nd	0.17	nd	0.34	0.28	0.26	0.25	0.18	nd	nd	0.17	RI, MS
34,697	Neryl hexanoate	1731	1730 **	0.17	0.24	0.49	nd	0.96	0.57	0.40	0.55	0.97	0.81	0.41	0.78	RI, MS
35,669	Xanthorrhizol	1759	1753 *	0.20	nd	0.08	nd	0.30	0.16	0.17	0.09	0.16	nd	0.08	0.18	RI, MS
			TOTAL	93.51	96.96	96.23	94.30	88.13	83.84	84.70	81.17	90.89	94.71	91.68	89.31	

RT–average retention time; RI-E–experimental retention indices; RI-L–retention indices according literature (NIST); SD–steam distillation, HD–hydrodistillation, MAD–microwave-assisted distillation; Plano (PL), Kaštela (KA), Marina (MA); *—retention indices according [26]; **—retention indices according [27]; ID–manner of identification of compounds (according retention indices (RI) and/or mass spectrum (MS)).

4. Discussion

The differences obtained prompted us to examine what were the main changes in the composition of EO when different distillation methods were used. EO are complex mixtures of volatile compounds consisting mainly of two groups, terpenes and phenolic compounds [8]. All the essential oils we obtained were very rich in terpenes, which accounted for more than 60% of all EO compositions. The differences in the proportion of terpene compounds are shown in Figure 3A. Derived monoterpenes and non-derived sesquiterpenes accounted for the majority of EO compositions, 52.4 ± 11.2% at SD, 46.5 ± 5.2% at HD, and 60.30 ± 3.4% at MAD. Sesquiterpenes are the most represented in all three methods, except for two samples obtained by SD (PL-SD and KO-SD). Statistically, MAD isolated more sesquiterpenes than HD and tended to isolate more than SD, while SD isolated significantly more other (non-terpene) compounds than MAD. When sesquiterpenes were further partitioned, the difference in sesquiterpene content came from subordinate sesquiterpenes. The high content of sesquiterpenes in the immortelle EO can be explained by the growing season and the time of harvest of the plant material. In the early stages of plant development, monoterpenes are the predominant group of compounds, while sesquiterpenes are present during and after flowering [28]. Compared to monoterpenes, sesquiterpenes have a higher boiling point, so they are less sensitive to temperature changes and their content is usually higher in summer [21]. Immortelle EOs obtained with MAD contained a significantly higher proportion of subordinate sesquiterpenes compared to other samples. When EOs obtained by different distillation methods were compared, no significant differences were found in the proportion of subordinate monoterpenes, monoterpene derivatives, and sesquiterpene derivatives.

We also compared the presence of heteroatoms (mainly oxygen) in the compounds and defined these compounds as derived in the text. The results show a significant difference in the derivation of compounds, with MAD isolating almost twice as many underived compounds compared to SD and HD, while SD and HD produced more derived compounds than MAD (Figure 3B). Comparing the specific functional groups of the derived compounds, alcoholic compounds were present in a significantly higher proportion in the samples obtained from SD than in the samples obtained from MAD. The percentage of ketone compounds showed a significant difference between all distillation methods used, with SD isolating the most ketones, followed by HD. MAD isolated the least amount of ketones. Regarding the content of esters and other compounds (ethers, aldehydes, phenols, furans, and epoxides), no significant differences were observed in the samples studied. There are two possible explanations for this phenomenon: heat penetration into the material is more efficient at MAD than at HD, while it is least efficient at SD [20,29,30]. If the derived compounds occur closer to the surface of the plant material, then the derived compounds are more abundant in SD than in MAD. The problem with this explanation is that MAD did not have the highest yield or the most identified compounds in the EOs. In addition, the compounds isolated from MAD are not the sum of the compounds isolated from SD and HD and the compounds isolated from the deeper parts of the plant, but MAD isolated other compounds that led to the chemotype change. Another possibility is that the oxygenated compounds were formed during the distillation process. At SD and especially at HD, EO is exposed to water and heat for a long time (2.5 h), while at MAD, the interaction of water and EO is relatively less and shorter (no additional water, everything comes from the material itself and the distillation takes much less time (35 min)).

According to the compound structure, the identified compounds were classified into simple (acyclic) compounds and compounds with complex structures (containing at least one ring in the structure). The results are shown in Figure 3C. Immortelle EO samples obtained from SD contain a significant proportion of acyclic compounds compared to MAD, while MAD isolates a significantly higher proportion of complex compounds compared to the other two methods. In addition, compounds with complex structures were divided into monocyclic, bicyclic, and tricyclic compounds. MAD isolated statistically significant differences only for bicyclic compounds compared to HD, but on average it isolated more monocyclic, bicyclic,

and tricyclic compounds compared to the other two methods used. It has been previously reported that cyclic monoterpenes can be converted to acyclic monoterpenes under the influence of heat [31]. This is the explanation for the formation of oxygenated compounds, which are more abundant in SD and HD compared to MAD. In SD and HD, the plant materials are heated in an aqueous environment, which increases the probability of addition and decyclization reactions between isolated compounds. Under the distillation conditions of MAD, heat and water could not react with volatile compounds, which reduced the structural complexity of the compounds and the amount of derived compounds.

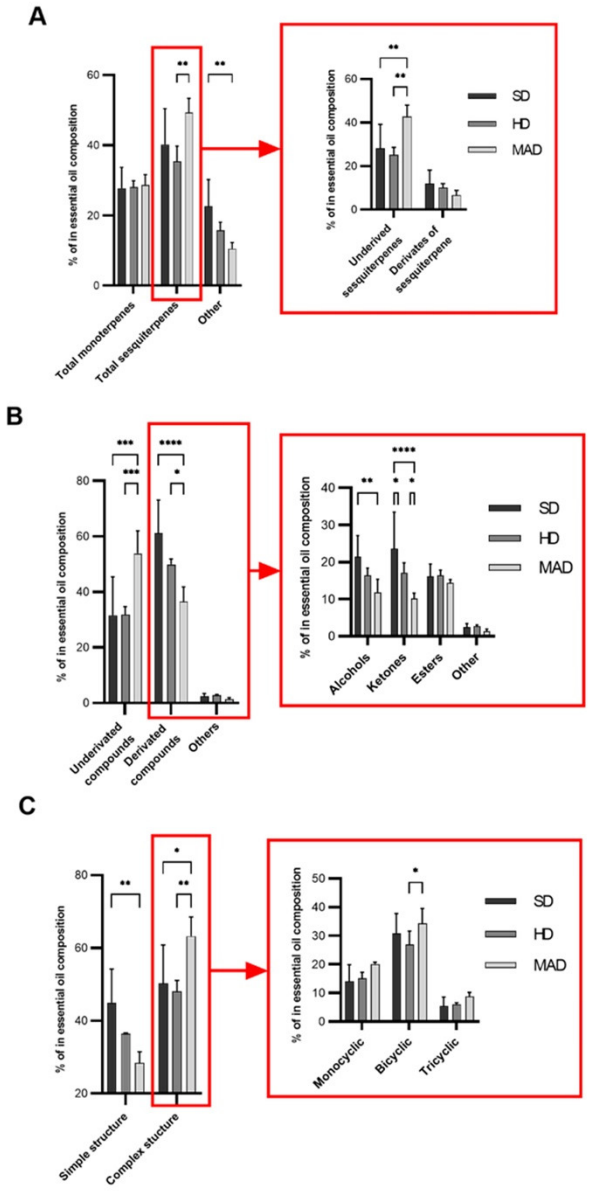

Figure 3. Application of three different distillation methods (SD–steam distillation, HD–hydrodistillation and MAD–microwave-assisted distillation) induces changes in isolated terpene composition, functional groups and compound structural complexity. (**A**) Total amount of monoterpenes, derived monoterpenes, sesquiterpenes, derived sesquiterpenes and other compounds identified in EOs. (**B**) Total amount of underived and derived compounds identified in EOs with further subdivision of derived compounds onto alcohols, ketones, esters and others. (**C**) Total amount of compounds with simple (acyclic) and complex (at least one ring in the structure) compounds identified in EOs. Complex compounds were further divided into monocyclic, bicyclic and tricyclic compounds. * for $p < 0.05$, ** for $p < 0.001$, *** for $p < 0.0001$, and **** for $p < 0.00001$.

In summary, SD isolated EOs rich in derived acyclic compounds with the highest amount of ketones. MAD isolated EOs rich in derived compounds with complex structures. HD had intermediate results in terms of functional groups and structural complexity of the isolated compounds. This can probably be caused by the transformation of the compounds.

This study has shown that the application of different distillation methods significantly affects the composition of the obtained Immortelle EO, considering the yield of EO, a range of isolated, derived and non-derived compounds, chemotypes and compounds with simple (acyclic) and complex structures. The differences in yield and composition obtained by different extraction methods play an important role in the choice of extraction method in practice.

5. Conclusions

Three different types of distillation methods were used: SD, HD, and MAD, to investigate how the use of different distillation methods affects the yield and chemical composition of Immortelle EO. HD gives a significantly higher yield ($0.31 \pm 0.09\%$) of EO compared to SD ($0.12 \pm 0.04\%$) and MAD ($0.15 \pm 0.03\%$). A total of 120 compounds were identified in 12 samples of immortelle EOs, most of which were terpenes, accounting for more than 60% of all EO compositions.

Using the same plant starting material, we found differences in the chemotype of EO. SD and HD produced EOs of the nerol-rich chemotype, whereas MAD isolated EO of the mixed chemotype, which had similar amounts of nerol, curcumene, and selinene compounds in the isolates. In addition, the traditionally used SD produced EOs rich in oxygenated acyclic compounds, which are highly sought after in the perfume industry. HD produced more EO than the other methods with a similar composition to SD. MAD produced EOs rich in non-derived compounds with complex structure and had the highest compound diversity of all three methods.

Considering the common standard quality markers EO of immortelle (neryl-acetate and α-pinene), all three distillation methods yielded EO. Considering the further use and desired chemical composition EO, it is necessary to consider the appropriate distillation methods.

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