





Article

Essential Oils from Azorean *Cryptomeria japonica* Female Cones at Different Developmental Stages: Variations in the Yields and Chemical Compositions

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Abstract: *Cryptomeria japonica* (Thunb. ex L.f.) D. Don (Cupressaceae) is widely cultivated in the Azores archipelago (Portugal) for landscaping and timber production, generating a huge amount of biomass residues. Among these, female cones (FC) emerge as a source of particularly valuable essential oils (EOs), namely, as promising broad-spectrum antimicrobial agents. However, phytochemical research on *C. japonica* FC EOs remains scarce. This study evaluated the EO yields and chemical compositions of immature and mature FC (IFC and MFC) from the same batch of Azorean *C. japonica*. IFC and MFC EOs, obtained via hydrodistillation, presented different yields (0.72% vs. 1.12% *w/w*, dry weight), and their composition, determined via gas chromatography/mass spectroscopy, revealed that the monoterpenes class was dominant (51.84% vs. 69.13%), followed by the sesquiterpenes (36.44% vs. 26.28%) and diterpenes (10.96% vs. 3.03%) classes. However, the correspondent oxygen-containing terpenes classes were 1.5–2.0 times higher in IFC. Thus, the maturation process revealed an increase in EO yield and α -pinene content (19.5% vs. 41.3%) but a decrease in other important bioactive terpenes/terpenoids (terpinen-4-ol, bornyl acetate, elemol, γ -eudesmol, phyllocladene, and nezukol) content. In conclusion, IFC and MFC EOs, due to their unique composition, may have differential commercial applications and, consequently, contribute to increasing the commercial potential of *C. japonica*'s EO industry.

Keywords: Azores; *Cryptomeria japonica*; immature and mature female cones; forestry waste biomass; essential oil; GC/MS analysis; α -pinene; diterpene hydrocarbons; oxygen-containing terpenes; sustainable circular bioeconomy



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1. Introduction

Forestry and timber production biomass residues (e.g., bark, leaves, strobili, and roots) can constitute a valuable natural source of bioactive compounds such as essential oils (EOs) with potential applications in different fields (e.g., food, cosmetic, pharmaceutical, medical, and agriculture). In fact, the EOs market has been increasing substantially in the last few decades, which can be attributed to the generally recognized as safe (GRAS) status for the majority of EOs, among other key factors such as, for example, the great interest of the scientific community in the discovery of novel drugs or botanical pesticides from natural resources [1]. However, it should be highlighted that the chemical composition/biological activities, as well as the quantity of an EO and, consequently, its specific commercial

applications and price, reflect the influence of both exogenous and endogenous factors, namely, environmental factors (abiotic or biotic), genetic variability, and variation among different plant parts and the different developmental stages [2].

Strobili (cones), a type of the aforementioned residues, are exclusively born by coniferous trees and shrubs [3]. Conifers represent a large group of gymnosperms with significant ecological and economic roles in many forest ecosystems and forest-dependent industries. Among others, conifers contribute substantially to global carbon sequestration, soil and water conservation, and forest biodiversity [4]. Coniferous forests are also the most important sources of biogenic volatile terpene compounds emissions [5].

Terpenes are simple hydrocarbons composed of multiple isoprene units $(C_5H_8)_n$. Meanwhile terpenoids are terpene derivatives containing different functional groups, such as alcohols, aldehydes, or ketones. Terpene compounds are biosynthesized in superior plants by the well-known malonic acid pathway and, possibly, by the non-mevalonate pathway consuming triose phosphate. Along the biosynthetic pathways, the terpene synthase (TPS) is the critical enzyme that catalyzes the formation of hemi-, mono-, sesqui-, or diterpene specialized metabolites. Moreover, vast variations of terpenoids arise from the different TPS types present in the plants [6]. Conifer plants produce a complex mixture of mono-, sesqui-, and diterpene compounds, which are found in volatile emissions and oleoresin secretions. These compounds are involved in the physical and chemical defense responses against pathogen and herbivore attacks and can help to protect against abiotic stress, thus contributing to the evolutionary diversification and colonization success of conifers [7,8].

Cryptomeria japonica (Thunb. ex L.f.) D. Don (Cupressaceae) is a coniferous evergreen tree, which has been used for various purposes, for example, as an ornamental plant, a windbreak, and a source of timber. It is a particularly popular and commercially valuable tree in Japan and Azores archipelago forests [9]. In the latter archipelago, *C. japonica*, known in Portuguese language as “criptoméria”, was introduced from Japan in the mid-19th century.

The Azores archipelago, an autonomous region of Portugal composed of nine volcanic islands, is located in the northern Mid-Atlantic Ridge, with a distance of more than 1000 km to the American and the European coast [10,11]. In the Azores, there are two major forest types: native and exotic forests. Native forests, which occur mostly above 500 m elevation, support numerous endemic tree species, such as *Laurus azorica* (Seub.) Franco (Lauraceae), *Ilex azorica* Gand. (Aquifoliace), *Erica azorica* Hochst. ex Seub. (Ericaceae), and *Juniperus brevifolia* (Hochst. ex Seub.) Antoine (Cupressaceae), as well as endemic shrub species such as *Vaccinium cylindraceum* Sm. (Ericaceae) and *Myrsine retusa* Aiton (Myrsinaceae). On the other hand, exotic forests occur principally at mid and lower elevations and are mostly represented by plantations of *C. japonica* and *Eucalyptus globulus* Labill. (Myrtaceae) used for forestry, as well as by patches of the invasive *Pittosporum undulatum* Vent. (Pittosporaceae) and *Acacia melanoxylon* R. Br. (Fabaceae) species [12]. Currently, *C. japonica* occupies 60% of the total wood-producing forest area in the Azores [10].

Being a monoecious species, both *C. japonica* reproductive organs, i.e., female and male cones, occur on the same tree. Male cones are ovoid or ellipsoid and arise in clusters near the apex of young shoots, while female cones (FC) are globular and formed at the apices of branchlets. Pollination occurs from February to April, and seeds mature in October. Concerning the FC, the young or immature ones (IFC) emerge from a rosette of leaves and are nearly 5 mm in diameter (Figure 1A). At the time of pollination, these possess a flat top, becoming almost globular within one month. In contrast, mature FC (MFC) have a tapering apex and a 1–2 cm diameter (Figure 1B). These contain 20–30 spirally arranged megasporophylls (scales), which confer on them a spiny appearance [13].

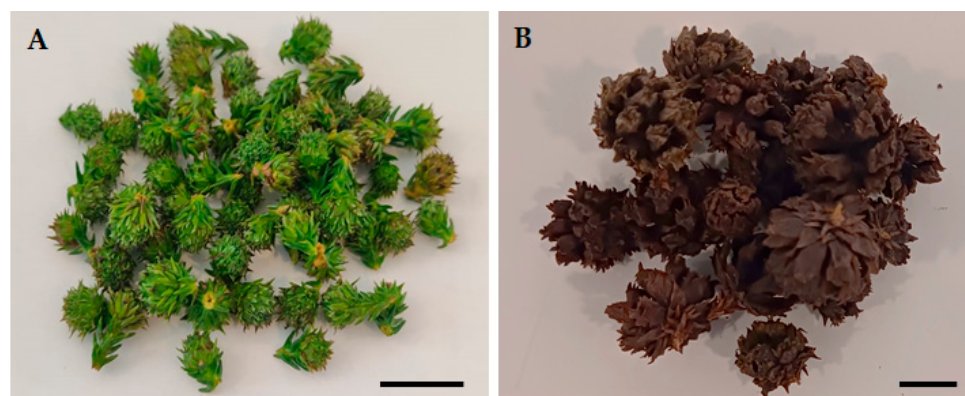


Figure 1. Fresh female cones (FC) of Azorean *Cryptomeria japonica* used in this study: (A) immature FC; (B) mature FC. Bar = 1 cm.

The Azorean *C. japonica* foliage is the primary biomass residue from the timber industry and forest operations, representing, currently, the main source for local EO production as well, although it can be obtained from other plant parts [8]. According to Mizushima and Kuriyama [14], the *C. japonica* EO has been used in medicine and art, as well as perfumery and aromatherapy. Moreover, although the biological properties of EOs of *C. japonica* from different origins may vary, they all possess some degree of pesticidal activity with potential applications in the agrochemical (as repellent, larvicidal, insecticidal, acaricidal, or molluscicide) industry. In addition, all *C. japonica* EO chemotypes exhibited antimicrobial properties, hence their great potential as food preservatives and/or antibiotic natural alternatives and applications in the cosmeceutical/pharmaceutical industry as active ingredients [15]. Nevertheless, *C. japonica* biomass residues remain a relatively untapped source of diverse specialized metabolites with valuable multi-bioactivities, as recently compiled in our critical reviews on the phytochemistry and biological properties of the EOs [16] and organic extracts [9] from this plant.

Previous research by the authors on Azorean *C. japonica* EO, intended to improve the commercial value of this eco-friendly natural product, revealed that their composition and bioactivity are influenced by the different distillation methods used [10,17]. In addition, in our earlier study [8] on the biological properties of EOs extracted via hydrodistillation (HD) from different parts of Azorean *C. japonica*, we observed that FC EO was the most promising source of multi-bioactivities. Thus, in this study, we focus our attention on this Azorean *C. japonica* anatomical part. However, it is important to note that the existing literature lacks comprehensive information on the chemical composition of EOs from FCs at various maturity stages and their associated biological activities. Thus, the present comparative research was conducted to determine the EO yields and chemical compositions of IFC and MFC from the same batch of Azorean *C. japonica* to evaluate the influence of FC maturity stage on the aforementioned EO parameters and, consequently, to add more value to the *C. japonica*'s EO industry.

2. Materials and Methods

2.1. Plant Material

The foliage of *C. japonica* was collected during the pollination stage in early March 2023 (winter season) from healthy plants belonging to a tree population located on Lomba da Maia (latitude, 37° 48'32.7"N; longitude, 25° 20'06.5"W; altitude, 440 m) in the northeast region of São Miguel Island (Azores archipelago, Portugal). The plant material was taken to a laboratory at the University of the Azores, where the cones attached to the foliage sample were immediately removed. Male cones were sparse and thus discarded, whereas FC were divided into IFC and MFC (Figure 1) and stored at −20 °C until further use. A portion of both the fresh IFC and MFC was dried at room temperature (20 °C) until a constant weight for moisture content determination. A voucher specimen was prepared (voucher number:

AZB 4581) and deposited in the Herbarium AZB–Ruy Telles Palhinha at the University of the Azores.

2.2. Essential Oil (EO) Extraction via Hydrodistillation (HD) Process

To obtain the EOs, IFC and MFC samples were subjected to HD using a Clevenger-type apparatus. The sample-to-water ratio was 1.5:10 g/mL, and the distillation time was approximately 3 h, starting from the first distillate droplet. The isolated EOs were dehydrated with anhydrous Na₂SO₄, filtered, weighed on an analytical scale, and stored in sealed amber vials at 4 °C until further chemical analysis. Each HD was performed in triplicate.

The EO yield was calculated on a biomass dry weight (d.w.) basis, as shown in Equation (1), where EO_m is the weight of EO; and sample_m is the fresh weight of sample. The EO density (ρ) was calculated through the Equation (2), where EO_v represents the volume of EO.

$$\%EO \text{ yield} = \frac{EO_m}{Sample_m - \%Moisture} \times 100 \quad (1)$$

$$Density, \rho \text{ (g/cm}^3\text{)} = \frac{EO_m}{EO_v} \quad (2)$$

2.3. Essential Oil (EO) Composition Analysis

The EO chemical profiles of IFC and MFC samples were determined via gas chromatography/mass spectroscopy (GC/MS) following our previously published method [1]. The analyses were conducted using a Shimadzu GCMS–QP2010 Ultra gas chromatograph–mass spectrometer (Shimadzu Corp., Tokyo, Japan). The EO samples were dissolved in methylene chloride (0.1 g/mL), and an aliquot of 0.1 μL was injected on a ZB–5MSPlus (5% phenyl; 95% methyl siloxane) capillary column (60 m length × 0.25 mm i.d., 0.25 μm film thickness) from Phenomenex Inc. (Torrance, CA, USA). Helium was used as the carrier gas with a flow rate of 36.3 cm/s. The split ratio and injector temperature were 24.4:1 and 260 °C, respectively. The detector temperature was also 260 °C, and the oven's temperature was set at 2 °C/min from 50 °C to 260 °C, being then held at 260 °C for 5 min. The transfer line and ion source temperatures were 300 °C and 260 °C, respectively. Mass spectra were recorded at 70 eV, where the mass scan range was 40–400 amu, with a scan time of 0.3 s. The identity of the EO components (EOCs) was established as described in Arruda et al. [1] by comparing their retention indices (RI), relative to *n*-alkane standard indices, and their GC/MS spectra using two MS databases: (1) a lab-made library with commercially available standards and components of reference Eos; and (2) other libraries, namely, FFNSC4.0, NIST11, and Wiley10. For quantification, EOCs' raw percentage was calculated by integrating total ion current (TIC) chromatogram peaks without correction factors as mean values of three injections from each sample.

2.4. Statistical Analysis

Statistical comparisons were made with a two-tailed unpaired Student's *t* test, assuming equal variance. A Shapiro–Wilk test was used to assess normality. A *p*-value of 0.05 was considered statistically significant. All determinations were performed in triplicate, and the data were expressed as mean ± standard deviation (SD). All analyses were conducted using SPSS version 27.0 software (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Essential Oil (EO) Yield, Density, and Colour

Table 1 illustrates that HD of IFC and MFC yielded, respectively, 0.72% and 1.12% (*w/w*, d.w.) of EOs with the same color and density, which are lower than that of water. The yield from MFC aligns with previous research findings (0.67–1.66%, *w/w*, d.w.) [8,18]. The MFC EO yield was significantly higher than that of IFC EO (*p* < 0.01). This trend is in

good agreement with results found by Salehi Shanjani et al. [19] in the yields of FC EOs from *Juniperus excelsa* M. Bieb (Cupressaceae) at different maturity stages.

Table 1. Yield, density, and colour of the essential oil isolated via hydrodistillation from Azorean *Cryptomeria japonica* immature and mature female cones (IFC and MFC).

Sample	Moisture Content (%)	Essential Oil		
		Yield (% w/w, d.w.)	Density (g/cm ³)	Color
IFC	68.00 ± 0.24 ^a	0.72 ± 0.00 ^b	0.91 ± 0.01 ^a	yellowish
MFC	5.80 ± 0.09 ^b	1.12 ± 0.04 ^a	0.91 ± 0.01 ^a	yellowish

Values are mean ± SD ($n = 3$). Within each column, values followed by different superscript letters indicate statistically significant differences at $p < 0.05$. d.w.—dry weight.

The results revealed that the EO yield in *C. japonica* FC increased by 1.5 times with maturation process, perhaps due to the changes that the FC undergo to enable seed dispersion. It is known that EO yield can be influenced by diverse factors, including the plants' overall growth and tissue development, associated with the distinct biological methods of genotype and respective phenotype expression. For example, phytohormones, like brassinosteroids (mainly found in pollen grains and immature seeds), are of extreme importance to various vegetal metabolic pathways of chemical production, along with general biomass increase, with the case of EOs being possibly influenced by these compounds' effects on plants' tissues, including an increase in their yield [20].

Moreover, some EOCs present high toxicity, being therefore biosynthesized and stored in specialized structures (such as ducts and cavities) [8,21]. The development of these secretory structures (both in number and size) represents a phenotypical part of the referred controlled genetic expression, which can contribute to the increase in EO yield, as Godger et al. [22] observed in their study of leaf EO from *Eucalyptus polybractea* R.T. Baker. In the present study's case, since the overall environmental and laboratory methodology conditions are controlled, with both IFC and MFC originating from the same tree population and being processed via the same methodology, it can be interpreted that the previously mentioned results, in terms of EO yield in FC, are attributed to different growth regulators and other primary compounds that possess specific functions in the maturation process of the FC, as well as to morphological characteristics derived from these phenomena.

3.2. Essential Oil (EO) Composition Analysis

Figure 2A,B show the total ion current (TIC) chromatogram determined via GC/MS of the IFC and MFC EO samples, respectively, and their chemical compositions are presented in Table 2. It should be noted that Table 2 shows the EOCs grouped according to their chemical class, namely, monoterpene hydrocarbons (MH), oxygen-containing monoterpenes (OCM), sesquiterpene hydrocarbons (SH), oxygen-containing sesquiterpenes (OCS), diterpene hydrocarbons (DH), and oxygen-containing diterpenes (OCD).

Table 2. Composition of the essential oil isolated via hydrodistillation from Azorean *Cryptomeria japonica* immature and mature female cones (IFC and MFC).

N.	Component	RI	Relative %	
			IFC	MFC
1	Cyclofenchene	876	-	t
2	Bornylene	902	-	t
3	Tricyclene	918	0.17 ± 0.00	0.20 ± 0.00
4	α-Thujene	921	0.45 ± 0.01	0.99 ± 0.02
5	α-Pinene	929	19.53 ± 0.45	41.32 ± 1.31
6	α-Fenchene	943	t	0.82 ± 0.03
7	Camphene	944	1.04 ± 0.03	0.85 ± 0.02

Table 2. Cont.

N.	Component	RI	Relative %	
			IFC	MFC
8	Thuja-2,4(10)-diene	948	-	t
9	Sabinene	967	3.86 ± 0.04	5.38 ± 0.51
10	β-Pinene	972	1.10 ± 0.13	1.55 ± 0.09
11	Myrcene	984	2.63 ± 0.28	2.28 ± 0.28
12	α-Phellandrene	1002	1.17 ± 0.02	0.10 ± 0.00
13	δ-3-Carene	1004	0.31 ± 0.04	0.70 ± 0.10
14	α-Terpinene	1012	1.83 ± 0.16	0.67 ± 0.14
15	p-Cymene	1019	0.13 ± 0.02	1.06 ± 0.15
16	Limonene	1024	1.54 ± 0.17	1.11 ± 0.03
17	β-Phellandrene	1026	0.58 ± 0.03	0.79 ± 0.11
18	γ-Terpinene	1052	2.69 ± 0.25	1.02 ± 0.08
19	Terpinolene	1080	0.91 ± 0.03	0.87 ± 0.01
20	p-Cymenene	1085	-	0.10 ± 0.01
21	<i>trans</i> -Sabinene hydrate	1064	-	0.11 ± 0.01
22	Fenchone	1083	-	0.05 ± 0.00
23	Linalool	1094	0.07 ± 0.00	t
24	<i>cis</i> -Sabinene hydrate	1095	-	t
25	endo-Fenchol	1114	t	0.05 ± 0.01
26	<i>cis</i> -p-Menth-2-en-1-ol	1119	0.24 ± 0.03	0.09 ± 0.00
27	<i>trans</i> -Pinocarveol	1135	-	0.05 ± 0.00
28	<i>trans</i> -p-Menth-2-en-1-ol	1137	0.17 ± 0.01	0.08 ± 0.00
29	Camphor	1141	0.08 ± 0.01	0.37 ± 0.05
30	Camphene hydrate	1149	0.11 ± 0.01	0.06 ± 0.00
31	Pinocamphone	1155	-	0.05 ± 0.00
32	Borneol	1166	0.07 ± 0.00	0.19 ± 0.02
33	Isopinocamphone	1170	-	0.20 ± 0.03
34	Terpinen-4-ol	1175	11.21 ± 1.08	5.97 ± 0.51
35	p-Cymen-8-ol	1181	-	0.06 ± 0.00
36	α-Terpineol	1189	1.05 ± 0.03	0.97 ± 0.04
37	<i>cis</i> -Piperitol	1191	0.06 ± 0.00	t
38	Verbenone	1201	-	t
39	<i>trans</i> -Piperitol	1203	0.09 ± 0.00	0.06 ± 0.00
40	Linalyl acetate	1244	t	0.10 ± 0.00
41	Bornyl acetate	1278	1.55 ± 0.09	0.43 ± 0.02
42	Isobornyl acetate	1280	0.06 ± 0.00	-
43	α-Terpinyl acetate	1339	0.10 ± 0.00	0.29 ± 0.01
44	β-Cubebene	1381	-	t
45	β-Elemene	1382	0.06 ± 0.00	0.41 ± 0.03
46	β-Ylangene	1411	-	0.06 ± 0.00
47	β-Cariophyllene	1412	t	0.07 ± 0.00
48	β-Copaene	1422	-	0.24 ± 0.02
49	<i>trans</i> -β-Farnesene	1445	-	t
50	α-Humulene	1448	-	t
51	Selina-4,11-diene	1465	-	0.06 ± 0.00
52	<i>trans</i> -Cadina-1(6),4-diene	1467	-	0.05 ± 0.00
53	Germacrene D	1473	0.09 ± 0.00	0.18 ± 0.01
54	Valencene	1480	-	0.08 ± 0.00
55	β-Selinene	1481	-	0.36 ± 0.03
56	<i>trans</i> -Muurolo-4(14),5-diene	1484	-	t
57	α-Selinene	1488	-	0.40 ± 0.03
58	α-Muurolole	1490	0.06 ± 0.00	0.05 ± 0.00
59	β-Bisabolene	1499	-	t
60	γ-Cadinene	1505	t	0.11 ± 0.01
61	δ-Cadinene	1510	0.56 ± 0.03	0.31 ± 0.02
62	<i>trans</i> -Cadina-1,4-diene	1524	t	-
63	Germacrene B	1550	t	t

Table 2. Cont.

N.	Component	RI	Relative %	
			IFC	MFC
64	β -Dihydroagarofuran	1497	0.05 \pm 0.00	t
65	Cubebol	1507	-	t
66	Elemol	1540	11.01 \pm 0.05	7.00 \pm 0.02
67	(E)-Nerolidol	1552	0.06 \pm 0.00	0.07 \pm 0.00
68	Germacren D-4-ol	1568	0.25 \pm 0.01	0.17 \pm 0.01
69	β -Oplophenone	1594	t	t
70	5-epi-7-epi- α -Eudesmol	1597	0.42 \pm 0.03	0.05 \pm 0.00
71	1,10-di-Epicubenol	1610	-	0.05 \pm 0.00
72	10-epi- γ -Eudesmol	1613	1.07 \pm 0.01	0.12 \pm 0.00
73	1-Epicubenol	1619	t	-
74	Agarospirol	1621	-	0.89 \pm 0.02
75	γ-Eudesmol	1623	11.41 \pm 0.21	2.82 \pm 0.03
76	Hinesol	1631	0.07 \pm 0.00	0.27 \pm 0.01
77	τ -Cadinol	1633	0.28 \pm 0.00	0.12 \pm 0.00
78	epi- α -Cadinol	1635	0.31 \pm 0.02	0.12 \pm 0.01
79	δ -Cadinol	1637	0.09 \pm 0.00	0.05 \pm 0.00
80	β-Eudesmol + α-Eudesmol	1646	10.32 \pm 1.00	11.81 \pm 0.29
81	7-epi- α -Eudesmol	1652	0.10 \pm 0.00	0.09 \pm 0.00
82	Bulnesol	1656	0.12 \pm 0.00	-
83	α -Eudesmol acetate	1773	-	t
84	Isopimara-9(11),15-diene	1892	0.08 \pm 0.00	t
85	Sclarene	1918	0.33 \pm 0.02	0.05 \pm 0.00
86	Rosa-5,15-diene	1920	0.98 \pm 0.05	0.24 \pm 0.03
87	Kryptomenen	1923	0.24 \pm 0.01	0.06 \pm 0.00
88	Pimaradiene	1934	0.35 \pm 0.01	0.07 \pm 0.00
89	Sandaracopimara-8(14),15-diene	1953	0.23 \pm 0.00	0.08 \pm 0.00
90	Phyllocladene	2009	6.54 \pm 0.09	1.32 \pm 0.01
91	Kaur-16-ene	2032	-	0.07 \pm 0.00
92	Abietatriene	2040	0.16 \pm 0.01	0.09 \pm 0.00
93	Nezukol	2119	1.77 \pm 0.03	0.45 \pm 0.02
94	Sandaracopimarinal	2169	0.13 \pm 0.01	0.18 \pm 0.01
95	Phyllocladanol	2198	t	0.16 \pm 0.01
96	6,7-Dehydroferruginol	2301	t	0.12 \pm 0.00
97	<i>trans</i> -Ferruginol	2304	0.09 \pm 0.00	0.12 \pm 0.00
Identified components (%)			99.24 \pm 0.89	98.44 \pm 1.29
Unidentified components (%)			0.76 \pm 0.00	1.56 \pm 0.01
Grouped components (%)				
1–20	Monoterpene hydrocarbons		36.93 \pm 1.27	59.87 \pm 0.04
21–43	Oxygen-containing monoterpenes		14.91 \pm 0.94	9.26 \pm 0.63
44–63	Sesquiterpene hydrocarbons		0.84 \pm 0.04	2.57 \pm 0.13
64–83	Oxygen-containing sesquiterpenes		35.60 \pm 1.33	23.71 \pm 0.61
84–92	Diterpene hydrocarbons		8.91 \pm 0.05	2.00 \pm 0.03
93–97	Oxygen-containing diterpenes		2.05 \pm 0.04	1.03 \pm 0.04

Values are mean \pm SD ($n = 3$). RI—retention indices on a ZB-5MSPlus capillary column. t—traces (< 0.05%). Components higher than 5.00% are highlighted in boldface.

As shown in Table 2, a total of 97 EOCs were identified in the Azorean *C. japonica* FC EOs. Among these, 68 and 93 EOCs were present in the IFC and MFC EO samples, representing 99.24% and 98.44% of the total composition, respectively. A total of 64 EOCs (nos. 3–7, 9–19, 23, 25, 26, 28–30, 32, 34, 36, 37, 39–41, 43, 45, 47, 53, 58, 60, 61, 63, 64, 66–70, 72, 75–81, 84–90, and 92–97) were present in both EO samples.

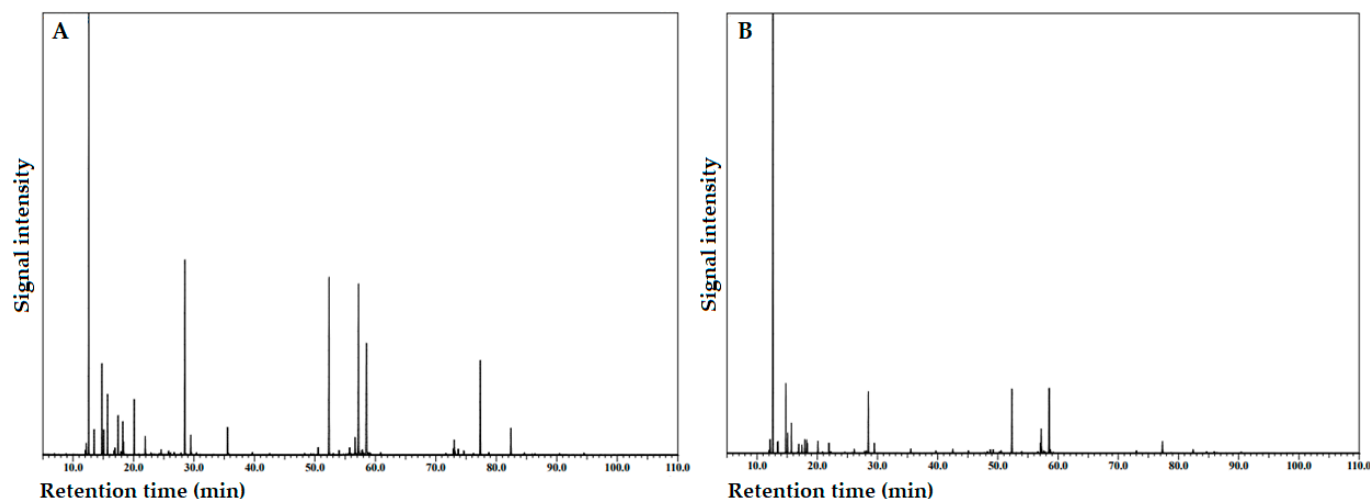


Figure 2. Total ion current (TIC) chromatogram on a ZB-5MSPlus capillary column of the essential oil isolated from Azorean *Cryptomeria japonica* female cones (FC): (A) immature FC; (B) mature FC.

Seven major EOCs ($\geq 5\%$) were identified in the FC EOs: α -pinene, sabinene, terpinen-4-ol, elemol, γ -eudesmol, $\alpha+\beta$ -eudesmol, and phyllocladene. In the IFC EO sample, their content decreased as follows: α -pinene (19.53%) \gg γ -eudesmol (11.41%) \approx terpinen-4-ol (11.21%) \approx elemol (11.01%) \approx $\alpha+\beta$ -eudesmol (10.32%) $>$ phyllocladene (6.54%) $>$ sabinene (3.86%); while in the MFC EO sample, they decreased in the following order: α -pinene (41.33%) \gg $\alpha+\beta$ -eudesmol (11.81%) $>$ elemol (7.00%) \approx terpinen-4-ol (5.97%) \approx sabinene (5.38%) $>$ γ -eudesmol (2.82%) $>$ phyllocladene (1.32%). This last EO composition was in accordance with our previous research [8]. Although the main component in both EO samples was α -pinene, this MH was significantly higher in MFC EO. Contrariwise, the maturation process revealed a remarkable decrease in terpinen-4-ol, elemol, γ -eudesmol, and phyllocladene content. In parallel with our study, α -pinene is also the major constituent of IFC and MFC EOs from *Juniperus communis* L., both of which possess high commercial value [23].

Another seven important EOCs ($\geq 1.5\%$) identified in Azorean *C. japonica* FC EOs were also found in both the IFC EO and MFC EO samples, namely, β -pinene (1.10% vs. 1.55%), myrcene (2.63% vs. 2.25%), α -terpinene (1.83% vs. 0.67%), limonene (1.54% vs. 1.11%), γ -terpinene (2.69% vs. 1.02%), bornyl acetate (1.55% vs. 0.43%), and nezukol (1.77% vs. 0.45%). Again, the maturation process revealed a decrease in the EOCs content, except for the MH β -pinene.

Regarding terpene classes, in the IFC EO sample, their content decreased as follows: MH (36.30%) \approx OCS (35.62%) $>$ OCM (14.91%) $>$ DH (8.91%) $>$ OCD (2.05%) $>$ SH (0.84%); while in MFC EO sample, they decreased in the following order: MH (59.88%) \gg OCS (23.75%) $>$ OCM (9.27%) $>$ SH (2.58%) \approx DH (2.00%) $>$ OCD (1.03%). Although the main terpene classes in both EO samples were MH and OCS, the ratio between MH and OCS is 2.5 times higher in MFC EO. Furthermore, although OCM was the third representative terpene class in both EO samples, this class was followed by DH in IFC EO, while SH was the fourth representative class in MFC EO. Finally, concerning the ratio between total terpenes and total oxygen-containing terpenes (terpenoids), its value is 2.1 times higher in IFC EO. Thus, IFC EO is richer in DH and terpenoids than MFC EO.

Overall, the presence and/or relative contents of several EOCs of the EO samples under study varied significantly with the maturity stage. Our results are in good agreement with those from similar studies of other Cupressaceae species, namely, *Juniperus oxycedrus* L. [15], *J. excelsa* [19], and *Cupressus arizonica* Greene [24], which reported that the maturation process of their FC led to notable qualitative and quantitative variations in diverse EOCs. In addition, Salehi Shanjani et al. [19] reported that biosynthesis/catabolism appear to be a key factor involved in the accumulation of α -pinene (and possibly other terpenes), as

the cones mature similarly in the case of EO yield variation with the influence of various primary metabolic terpenes and other compounds on genetic expression and derived phenotype characteristics.

It is known that biosynthesis of EOCs is commonly restricted to only a subset of organs, tissues, or developmental stages and may be tightly regulated by internal or external stimuli [6,7,25]. Indeed, we may observe that MFC EO presents an additional 25 compounds compared to IFC EO, which might be an indication that some terpene biosynthetic pathways or enzymes (such as TPSs) involved in precursor pathways may not yet be fully activated in IFC.

Numerous studies indicate these variations in EO's chemical composition as a result of other factors besides each plant's endogenous features, including environmental conditions (e.g., climatic variations) and biotic interactions, typically occurring as a potential defense mechanism response against a wide range of herbivores and pathogens [7]. This factor may represent a possible explanation for the increase in EOCs with recognized antibacterial, antifungal, and/or insecticidal activity, like α -pinene and terpinen-4-ol [10,26,27].

In the present study, both IFC and MFC samples were collected from the same batch of Azorean *C. japonica* during the same seasonal period, thus, any variations in their EO chemical compositions appear to be solely attributable to differences in maturity stages, possibly related to the catabolic and biosynthetic pathways of EOCs.

4. Conclusions

The interest of the scientific community and EO markets in *C. japonica* biomass residues (wastes/byproducts) as a source of EO is rapidly increasing. In our continuous search for *C. japonica* residues valorization and incentives for the local *C. japonica*'s EO industry, we recently reported that Azorean *C. japonica* strobili (cones), which can be obtained from forestry waste biomass in an economic and sustainable way, can be used to produce EO with potential application as an antimicrobial agent against some bacteria and fungi [8]. Thus, determining the optimal maturity stage of this plant organ for maximizing the yields of desirable EOCs is a necessity. Indeed, to the best of our knowledge, this is the first study that evaluated the influence of the developmental stage of *C. japonica* female cones on their EO yields and chemical compositions.

The comparison of EOs from immature vs. mature female cones from Azorean *C. japonica* has demonstrated significant changes in their yield and chemical profile. In fact, in the cones' maturation process, the EO yield, as well as mono- and sesquiterpene hydrocarbons content, increased by 1.5, 1.6, and 3.1 times, respectively, while the oxygen-containing monoterpenes, oxygen-containing sesquiterpenes, diterpene hydrocarbons, and oxygen-containing diterpenes content decreased by 1.6, 1.5, 4.4, and 2.0 times, respectively. Thus, to obtain EO with a higher yield and α -pinene content, mature cones are recommended; however, for obtaining an EO with a specific quality, such as remarkable amounts of oxygen-containing terpenes and diterpene hydrocarbons, immature cones must be used.

It should also be highlighted that the major EOCs ($\geq 5\%$) identified in the Azorean *C. japonica* female cones EOs were α -pinene, sabinene, terpinen-4-ol, elemol, γ -eudesmol, $\alpha+\beta$ -eudesmol, and phyllocladene. All these EOCs have already demonstrated valuable biological properties [10].

This research shows that the maturity stage of *C. japonica*'s female cones plays a critical role in the potential industrial use of their EOs due to their distinct chemical profiles which, in turn, are expected to affect their biological activities. Ongoing studies will involve the bioactivity screening of these *C. japonica* female cones' EO samples to evaluate their potential as alternative and sustainable raw materials for medical, food, and/or agrochemical applications.

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Abbreviations

DH, diterpene hydrocarbons; d.w., dry weight; EO, essential oil; EOC, essential oil component; FC, female cones; GC/MS, gas chromatography/mass spectroscopy; GRAS, generally recognized as safe; HD, hydrodistillation; IFC, immature female cones; MFC, mature female cones; MH, monoterpene hydrocarbons; OCD, oxygen-containing diterpenes; OCM, oxygen-containing monoterpenes; OCS, oxygen-containing sesquiterpenes; RI, retention indices; SH, sesquiterpene hydrocarbons; TIC, total ion current; TPS, terpene synthase.

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