

## Article

# HPTLC Analysis and Chemical Composition of Selected *Melaleuca* Essential Oils

Aimé Vázquez, Nurhayat Tabanca \*  and Paul E. Kendra 

United States Department of Agriculture–Agricultural Research Service (USDA–ARS), Subtropical Horticulture Research Station (SHRS), Miami, FL 33158, USA; aime.vazquez@usda.gov (A.V.); paul.kendra@usda.gov (P.E.K.)  
\* Correspondence: nurhayat.tabanca@usda.gov; Tel.: +1-786-5737077

**Abstract:** Tea tree oil (TTO) is a volatile essential oil obtained by distillation, mainly from the Australian native plant *Melaleuca alternifolia* (Maiden & Betche) Cheel (Myrtaceae). In this study, a comparative analysis of the chemical constituents of seven tea tree oils (*M. alternifolia*) and four other *Melaleuca* spp. oils (*M. cajuputi*, (M<sub>Ca</sub>), two chemotypes of *M. quinquenervia*, (M<sub>N<sub>e</sub></sub> and M<sub>N<sub>i</sub></sub>), and *M. ericifolia* (M<sub>R<sub>o</sub></sub>)) was carried out using gas chromatography–mass spectrometry (GC–MS) and high-performance thin-layer chromatography (HPTLC). Among the seven TTOs, terpinen-4-ol (37.66–44.28%),  $\gamma$ -terpinene (16.42–20.75%),  $\alpha$ -terpinene (3.47–12.62%),  $\alpha$ -terpineol (3.11–4.66%), and terpinolene (2.75–4.19%) were the most abundant compounds. On the other hand, the most abundant compounds of the other *Melaleuca* oils varied, such as 1,8-cineole (64.63%) in M<sub>Ca</sub> oil, (E)-nerolidol (48.40%) and linalool (33.30%) in M<sub>N<sub>e</sub></sub> oil, 1,8-cineole (52.20%) in M<sub>N<sub>i</sub></sub> oil, and linalool (38.19%) and 1,8-cineole (27.57%) in M<sub>R<sub>o</sub></sub> oil. HPTLC fingerprinting of *Melaleuca* oils enabled the discrimination of TTO oils from other *Melaleuca* spp. oils. Variation was observed in the profile of the R<sub>f</sub> values among EOs. The present study shows that HPTLC is one of the best ways to identify and evaluate the quality control in authenticating TTOs, other *Melaleuca* EOs, or EOs from other species within the Myrtaceae.

**Keywords:** tea tree oil; cajeput oil; nerolina oil; niaouli oil; rosalina oil; terpinene-4-ol; GC-MS; TLC



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## 1. Introduction

Plant essential oils (EOs), originally used in the perfume and aromatherapy market, have gained widespread acceptance in other industries. Tea tree oil (TTO), for example, an essential oil isolated from *Melaleuca alternifolia* (Maiden & Betche) Cheel (Myrtaceae) [1], has long been recognized as a safe and effective topical antiseptic in Australia [2–5]. During World War II, it was considered a necessary commodity for first aid kits and was also used as an insect repellent [6]. Current commercial products that contain TTO include assorted ointments, lotions, shampoos, soaps, toothpastes, and mouthwashes [2,5,7]. Additionally, many EOs have since been evaluated for their antimicrobial [8,9], insect attractant [10,11], repellent [12], or insecticidal properties [13–17]. Isman [18] investigated the potential effect of several EOs as attractants, repellents, and toxicants against insects and other organisms.

Since EOs consist of concentrated plant terpenoids, they have provided an ideal substrate for the development of host-based (kairomone) lures for invasive pests in Florida, including the redbay ambrosia beetle *Xyleborus glabratus* Eichhoff, the tea shot hole borer *Euwallacea perbrevis* Schedl [19], and the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), which has been a destructive pest in Europe, the Middle East, Australia, Central and South America, and Hawaii, USA [20–22]. Behavioral studies conducted at the USDA–ARS in Miami, Florida [23,24], have indicated that tea tree oil (TTO) has a strong short-range attractive effect on sterile male medflies in laboratory bioassays [17,25–31]. Therefore, it has good potential as an economical new attractant for male medflies, and the identification of the key components of TTO is important for developing lures for pest management.

In recent experiments [27], we used preparative thin-layer chromatography (prep TLC) to separate TTO into five fractions. Bioassays conducted after these separations revealed that two TTO fractions are responsible for the observed attraction in male medflies. Furthermore, TLC-based bioassays played an important role in the isolation of insect kairomones from complex mixtures such as EOs. Although it is not a new technique, TLC continues to be a valuable tool as a preparative technique for a variety of studies in both chemical and biological fields [25,32–35]. While gas chromatography–mass spectrometry (GC-MS) is exceptional in the identification of unknown chemicals, it works best for trace analysis [36]. In addition, the sample is destroyed in the process of obtaining its fingerprint fragments for proper identification. In cases where a much larger sample is needed or it is required to remain intact for further analysis, TLC proves to be a more suitable method. With the development of high-performance thin-layer chromatography (HPTLC), this separation technique has evolved from a mostly qualitative procedure into a quick and cost-effective quantification method used by the European Pharmacopoeia for the quality control of some EOs [37]. The automated sample application, and the capability of using a universal HPTLC standard mix [38], ensures accurate sample and standard amounts, and provides reproducible quantitative results.

Various *Melaleuca* species, and even other Myrtaceae, are often confused under the common name ‘tea tree’, (e.g., “swamp tea tree”, *M. cajuputi*; “paperbark tea tree”, “broad-leaved tea tree”, or “broad-leaved paper bark”, *M. quinquenervia*; “black tea tree” or “river tea tree”, *M. bracteata*; “lemon scented tea tree”, *Leptospermum petersonii*, etc.). Moreover, kanuka and manuka EOs derived from *Kunzea ericoides* and *Leptospermum scoparium*, respectively, are referred to as New Zealand TTOs [2,3,5,6,39,40].

Considering the diversity of compounds present in *Melaleuca* EOs, and their current and potential applications, including prospective IPM strategies, it is important to study the chemical composition and degree of variability in commercially available *Melaleuca* EOs. In this study, HPTLC methods were developed for the evaluation of seven TTOs and four different *Melaleuca* spp. oils selected mainly from open markets in the United States. Separation patterns of various brands of TTO, as well as other *Melaleuca* oils, were compared to ensure the presence and consistent amount of chemicals attractive for male medflies.

## 2. Results and Discussion

### 2.1. Identification of Components in *Melaleuca* EOs

The identification of the components from the seven *M. alternifolia* EOs (TT<sub>AA</sub>, TT<sub>AS</sub>, TT<sub>EG</sub>, TT<sub>FC</sub>, TT<sub>NG</sub>, TT<sub>PT</sub>, and TT<sub>SAT</sub>) and four other *Melaleuca* EOs (M<sub>CA</sub>, M<sub>Ne</sub>, M<sub>Ni</sub>, and M<sub>Ro</sub>) (Table 1) was achieved on the GC-MS using a non-polar DB-5 column. One hundred thirty-eight compounds were identified in total among the eleven *Melaleuca* EOs, accounting for 99.49–99.97% of the total composition (Table 2).

The TTOs were characterized by a high amount of terpinen-4-ol (37.66–44.28%), followed by  $\gamma$ -terpinene (16.42–20.75%),  $\alpha$ -terpinene (3.47–12.62%),  $\alpha$ -terpineol (3.11–4.66%), terpinolene (2.75–4.19%), *p*-cymene (1.66–11.47%),  $\alpha$ -pinene (1.29–2.66%), aromadendrene (1.20–1.90%), 1,8-cineole (1.17–4.79%), ledene (0.47–1.69%), and limonene (0.30–1.63%).

The GC-MS data indicated that the components of the other *Melaleuca* EOs varied notably from each other. The M<sub>CA</sub> oil contained 1,8-cineole (64.43%) as a principal component, followed by  $\alpha$ -pinene (5.48),  $\alpha$ -terpineol (5.44%),  $\beta$ -caryophyllene (3.82%),  $\gamma$ -terpinene (3.24%),  $\alpha$ -humulene (1.81%),  $\beta$ -selinene (1.28%),  $\alpha$ -selinene (1.12%),  $\beta$ -pinene (1.11%), and terpinolene (1.04%). The most abundant constituents identified in the M<sub>Ne</sub> oil were largely (*E*)-nerolidol (48.40%) and linalool (33.30%), whereas 1,8-cineole (52.20%),  $\alpha$ -pinene (9.75%), (*E*)-nerolidol (6.89%), viridiflorol (6.23%),  $\alpha$ -terpineol (5.08%), limonene (2.83%),  $\beta$ -caryophyllene (2.66%),  $\beta$ -pinene (2.56%), and ledene (1.76%) were identified as the main constituents in the M<sub>Ni</sub> oil. Linalool (38.19%) and 1,8-cineole (27.57%) were the main constituents of the M<sub>Ro</sub> oil.

Table 1. Species, sample codes, and sources of oils used in this study.

Species	Code	Source
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>AA</sub>	Aromappeal (Puritan's Pride, Inc.), Oakdale, NY, USA
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>AS</sub>	Apothecary Shoppe, Portland, OR, USA
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>EG</sub>	Eden's Garden, San Clemente, CA, USA
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>FC</sub>	Floracopeia, Grass Valley, CA, USA
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>NG</sub>	Nature's Gift, Madison, TN, USA
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>PT</sub>	Plant Therapy, Inc, Twin Falls, ID, USA
<i>M. alternifolia</i> (Maiden & Betche) Cheel	TT <sub>SAT</sub>	SAT Group, Kannauj, India
<i>M. cajuputi</i> Powell	M <sub>Ca</sub>	Nature's Gift, Madison, TN, USA
<i>M. quinquenervia</i> (Cav.) S.T. Blake	M <sub>Ne</sub>	Nature's Gift, Madison, TN, USA
<i>M. quinquenervia</i> (Cav.) S.T. Blake	M <sub>Ni</sub>	Nature's Gift, Madison, TN, USA
<i>M. ericifolia</i> Sm.	M <sub>Ro</sub>	Nature's Gift, Madison, TN, USA

Table 2. Comparative percentage composition of the *Melaleuca* EOs.

#	* RI Exp	** RILit	Compounds	TT <sub>AA</sub>	TT <sub>AS</sub>	TT <sub>EG</sub>	TT <sub>FC</sub>	TT <sub>NG</sub>	TT <sub>PT</sub>	TT <sub>SAT</sub>	M <sub>Ca</sub>	M <sub>Ne</sub>	M <sub>Ni</sub>	M <sub>Ro</sub>
1	938	930	$\alpha$ -Thujene RI, MS	0.85 ± 0.10	0.77 ± 0.05	0.76 ± 0.04	0.48 ± 0.02	0.97 ± 0.06	0.79 ± 0.01	0.30 ± 0.10	0.77 ± 0.10	0.27 ± 0.03	1.07 ± 0.28	0.17 ± 0.00
2	946	939	$\alpha$ -Pinene RI, MS, Std	1.89 ± 0.06	2.66 ± 0.03	2.40 ± 0.41	1.67 ± 0.05	2.48 ± 0.16	1.39 ± 0.02	1.29 ± 0.16	5.48 ± 0.11	0.57 ± 0.12	9.75 ± 0.13	5.09 ± 0.03
3	954	954	Camphene RI, MS	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.01 ± 0.00	0.15 ± 0.02	0.00	0.04 ± 0.02	0.28 ± 0.05
4	969	960	Benzaldehyde RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.38 ± 0.04	0.09 ± 0.04	0.00
5	970	975	Sabinene RI, MS, Std	0.72 ± 0.03	0.05 ± 0.00	0.36 ± 0.04	0.45 ± 0.06	0.94 ± 0.03	0.64 ± 0.07	0.08 ± 0.02	0.18 ± 0.04	0.00	0.00	0.00
6	989	979	$\beta$ -pinene RI, MS, Std	0.73 ± 0.01	0.88 ± 0.07	0.89 ± 0.10	0.97 ± 0.16	1.06 ± 0.02	0.80 ± 0.08	0.93 ± 0.01	1.11 ± 0.07	0.94 ± 0.08	2.56 ± 0.33	0.64 ± 0.03
7	998	990	Myrcene RI, MS, Std	0.48 ± 0.06	0.58 ± 0.05	0.63 ± 0.04	0.62 ± 0.12	0.77 ± 0.03	0.41 ± 0.06	0.63 ± 0.02	0.65 ± 0.03	0.60 ± 0.16	0.72 ± 0.20	0.61 ± 0.02
8	1009	991	6-Methyl-5-hepten-2-ol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19 ± 0.03	0.00	0.00
9	1010	1002	$\alpha$ -Phellandrene RI, MS, Std	0.14 ± 0.07	0.54 ± 0.02	0.45 ± 0.07	0.47 ± 0.10	0.53 ± 0.08	0.33 ± 0.10	0.43 ± 0.13	0.50 ± 0.02	0.09 ± 0.01	0.11 ± 0.03	0.03 ± 0.01
10	1012	1003	Pseudolimonene RI, MS	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.00	0.00	0.00	0.00
11	1018	1011	$\delta$ -3-Carene RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20 ± 0.04
12	1021	1014	1,4-Cineole RI, MS, Std	0.00	0.00	0.04 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13	1022	1017	$\alpha$ -Terpinene RI, MS, Std	6.14 ± 0.03	12.62 ± 0.41	9.62 ± 0.14	8.35 ± 0.13	8.89 ± 0.37	7.63 ± 0.19	3.47 ± 0.06	0.22 ± 0.01	0.13 ± 0.03	0.00	0.17 ± 0.04
14	1030	1024	<i>p</i> -Cymene RI, MS, Std	5.18 ± 0.55	1.66 ± 0.15	3.51 ± 0.04	3.59 ± 0.27	3.22 ± 0.28	3.71 ± 0.17	11.47 ± 0.19	0.79 ± 0.11	0.03 ± 0.02	0.07 ± 0.02	1.21 ± 0.06
15	1039	1029	Limonene RI, MS, Std	1.63 ± 0.23	0.30 ± 0.09	1.10 ± 0.07	1.15 ± 0.02	0.87 ± 0.03	1.10 ± 0.05	0.51 ± 0.19	0.10 ± 0.00	0.19 ± 0.03	2.83 ± 0.18	3.03 ± 0.05
16	1039	1029	$\beta$ -Phellandrene RI, MS, Std	0.10 ± 0.01	0.26 ± 0.01	0.86 ± 0.04	0.83 ± 0.06	0.03 ± 0.00	0.44 ± 0.04	0.39 ± 0.01	0.07 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.00
17	1040	1031	1,8-Cineole RI, MS, Std	2.85 ± 0.38	1.17 ± 0.01	3.76 ± 0.12	3.28 ± 0.07	4.79 ± 0.14	3.90 ± 0.08	4.50 ± 0.09	64.63 ± 1.19	1.91 ± 0.16	52.20 ± 0.28	27.57 ± 0.56
18	1049	1037	(Z)- $\beta$ -Ocimene RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.69 ± 0.10	0.00	0.04 ± 0.01
19	1062	1050	(E)- $\beta$ -Ocimene RI, MS, Std	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.00	0.74 ± 0.10	0.03 ± 0.01	0.76 ± 0.08
20	1071	1059	$\gamma$ -Terpinene RI, MS, Std	16.42 ± 0.83	18.91 ± 0.46	19.58 ± 0.10	19.60 ± 0.10	16.58 ± 0.45	20.75 ± 0.20	16.61 ± 0.41	3.24 ± 0.20	0.12 ± 0.05	0.57 ± 0.00	1.84 ± 0.01
21	1081	1070	<i>cis</i> -Sabinene hydrate RI, MS	0.00	0.01 ± 0.00	0.14 ± 0.04	0.20 ± 0.09	0.29 ± 0.07	0.07 ± 0.02	0.09 ± 0.01	0.01 ± 0.01	0.00	0.00	0.00
22	1084	1072	<i>cis</i> -Linalool oxide RI, MS, Std	0.00	0.00	0.30 ± 0.14	0.00	0.00	0.00	0.00	0.00	0.07 ± 0.00	0.00	0.13 ± 0.13
23	1098	1088	Terpinolene RI, MS, Std	2.83 ± 0.05	3.61 ± 0.01	4.19 ± 0.04	3.63 ± 0.21	3.39 ± 0.12	3.16 ± 0.06	2.75 ± 0.04	1.04 ± 0.05	0.26 ± 0.10	0.45 ± 0.07	2.14 ± 0.02
24	1107	1096	Linalool RI, MS, Std	0.07 ± 0.02	0.13 ± 0.01	0.45 ± 0.15	1.12 ± 0.03	1.04 ± 0.04	0.13 ± 0.06	0.22 ± 0.02	0.26 ± 0.04	33.30 ± 0.63	0.47 ± 0.29	38.19 ± 0.06
25	1115	1097	Hotrienol RI, MS	0.00	0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.00	0.12 ± 0.06	0.00	0.15 ± 0.02
26	1123	1121	<i>cis</i> - <i>p</i> -Menth-2-en-1-ol	0.14 ± 0.08	0.21 ± 0.06	0.21 ± 0.05	0.00	0.37 ± 0.08	0.28 ± 0.01	0.36 ± 0.05	0.00	0.00	0.00	0.00
27	1125	1122	<i>trans</i> - <i>p</i> -Menth-2-en-1-ol	0.21 ± 0.01	0.18 ± 0.01	0.17 ± 0.02	0.15 ± 0.02	0.31 ± 0.04	0.22 ± 0.01	0.28 ± 0.04	0.00	0.00	0.00	0.00
28	1168	1166	$\delta$ -Terpinol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12 ± 0.01	0.00	0.11 ± 0.01	0.12 ± 0.01
29	1183	1177	Terpinene-4-ol RI, MS, Std	44.28 ± 0.90	38.62 ± 0.33	37.66 ± 0.24	40.36 ± 0.53	38.42 ± 0.22	40.55 ± 0.67	39.57 ± 1.37	0.71 ± 0.03	0.27 ± 0.00	0.62 ± 0.01	0.79 ± 0.01
30	1188	1182	<i>p</i> -Cymen-8-ol RI, MS	0.14 ± 0.04	0.05 ± 0.02	0.12 ± 0.00	0.17 ± 0.03	0.11 ± 0.01	0.10 ± 0.02	0.19 ± 0.10	0.02 ± 0.01	0.00	0.00	0.10 ± 0.01
31	1198	1188	$\alpha$ -Terpineol RI, MS, Std	3.74 ± 0.05	3.11 ± 0.01	3.35 ± 0.07	3.61 ± 0.09	4.66 ± 0.03	3.40 ± 0.04	4.20 ± 0.09	5.44 ± 0.10	0.46 ± 0.01	5.08 ± 0.06	4.05 ± 0.08
32	1206	1196	<i>cis</i> -Piperitol RI, MS, Std	0.08 ± 0.02	0.07 ± 0.00	0.07 ± 0.00	0.03 ± 0.00	0.08 ± 0.01	0.09 ± 0.01	0.13 ± 0.02	0.00	0.00	0.00	0.00
33	1218	1208	<i>trans</i> -Piperitol RI, MS	0.14 ± 0.01	0.09 ± 0.01	0.10 ± 0.00	0.05 ± 0.00	0.08 ± 0.00	0.15 ± 0.00	0.14 ± 0.06	0.00	0.00	0.00	0.00
34	1226	1216	<i>trans</i> -Carveol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.01
35	1232	1221	<i>cis</i> -Sabinene hydrate acetate RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08 ± 0.00	0.00	0.00
36	1236	1225	Citronellol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.02 ± 0.00
37	1238	1229	Nerol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.00	0.16 ± 0.01
38	1243	1237	Ascaridole RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.05 ± 0.03	0.00	0.00	0.00	0.00
39	1245	1238	Neral	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09 ± 0.01
40	1253	1252	Geraniol RI, MS, Std	0.05 ± 0.05	0.00	0.04 ± 0.03	0.02 ± 0.00	0.03 ± 0.00	0.00	0.02 ± 0.02	0.04 ± 0.02	0.19 ± 0.02	0.02 ± 0.01	0.20 ± 0.00
41	1264	1258	2-Phenyl ethyl acetate RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08 ± 0.02
42	1271	1267	Geraniol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12 ± 0.01
43	1273	1269	<i>trans</i> -Ascaridol glycol RI, MS	0.12 ± 0.02	0.01 ± 0.00	0.04 ± 0.00	0.05 ± 0.02	0.02 ± 0.00	0.04 ± 0.01	0.06 ± 0.02	0.00	0.00	0.00	0.00
44	1291	1288	<i>cis</i> -Ascaridol glycol RI, MS	0.04 ± 0.02	0.01 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.00	0.01 ± 0.00	0.04 ± 0.01	0.00	0.00	0.00	0.00
45	1295	1290	Thymol RI, MS, Std	0.00	0.00	0.00	0.01 ± 0.001	0.00	0.00	0.02 ± 0.01	0.00	0.00	0.00	0.00
46	1306	1299	Carvacrol RI, MS, Std	0.01 ± 0.01	0.00	0.02 ± 0.01	0.02 ± 0.01	0.00	0.00	0.02 ± 0.01	0.00	0.00	0.00	0.00
47	1330	1324	Methyl geranate RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07 ± 0.03
48	1343	1338	$\delta$ -Elemene RI, MS	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.05 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.00	0.00	0.02 ± 0.00
49	1355	1347	$\alpha$ -Terpinyl acetate RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64 ± 0.01	0.00	1.30 ± 0.02	0.00

Table 2. Cont.

#	* RI Exp	** RI Lit	Compounds	TTAA	TTAS	TT <sub>EG</sub>	TT <sub>FC</sub>	TT <sub>NG</sub>	TT <sub>PT</sub>	TT <sub>SAT</sub>	M <sub>Ca</sub>	M <sub>Ne</sub>	M <sub>Ni</sub>	M <sub>Ro</sub>
50	1356	1348	$\alpha$ -Cubebene RI, MS	0.01 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.07 ± 0.00	0.03 ± 0.00	0.06 ± 0.02	0.00	0.00	0.00	0.00
51	1359	1359	Eugenol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.00	0.00
52	1376	1375	$\alpha$ -Ylangene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13 ± 0.02	0.00	0.00	0.00
53	1378	1376	Isoledene RI, MS	0.06 ± 0.01	0.08 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.06 ± 0.00	0.05 ± 0.01	0.06 ± 0.02	0.00	0.00	0.00	0.15 ± 0.02
54	1380	1376	$\alpha$ -Copaene RI, MS, Std	0.10 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	0.07 ± 0.00	0.13 ± 0.00	0.06 ± 0.02	0.13 ± 0.02	0.08 ± 0.01	0.00	0.03 ± 0.00	0.02 ± 0.01
55	1382	1381	Geranyl acetate RI, MS, Std	0	0	0	0	0	0	0	0	0.00	0.00	0.02 ± 0.01
56	1395	1390	$\beta$ -Elemene RI, MS, Std	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.06 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.05 ± 0.03	0.00	0.00	0.32 ± 0.02
57	1403	1402	$\alpha$ -Funebrene RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03 ± 0.01	0.00
58	1404	1403	Methyl eugenol RI, MS, Std	0.00	0.00	0.01 ± 0.00	0.01 ± 0.01	0.00	0.00	0.02 ± 0.00	0.00	0.00	0.00	0.00
59	1409	1408	Isocaryophyllene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.01	0.02 ± 0.01	0.00	0.00
60	1410	1409	$\alpha$ -Gurjunene RI, MS	0.41 ± 0.01	0.58 ± 0.01	0.36 ± 0.01	0.18 ± 0.06	0.45 ± 0.01	0.35 ± 0.01	0.43 ± 0.06	0.06 ± 0.03	0.00	0.07 ± 0.02	0.39 ± 0.01
61	1412	1411	$\alpha$ -Cedrene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06 ± 0.03	0.00	0.00
62	1417	1416	$\beta$ -Maaliene RI, MS	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.00	0.01 ± 0.00	0.00	0.02 ± 0.00	0.00	0.00	0.00	0.03 ± 0.01
63	1421	1419	$\beta$ -Caryophyllene RI, MS, Std	0.37 ± 0.01	0.74 ± 0.02	0.29 ± 0.01	0.21 ± 0.01	0.56 ± 0.02	0.28 ± 0.03	0.40 ± 0.04	3.82 ± 0.12	2.40 ± 0.05	2.66 ± 0.05	0.16 ± 0.01
64	1429	1425	$\gamma$ -Maaliene RI, MS	0.06 ± 0.01	0.08 ± 0.00	0.07 ± 0.00	0.05 ± 0.01	0.06 ± 0.00	0.04 ± 0.00	0.06 ± 0.01	0.01 ± 0.01	0.00	0.01 ± 0.01	0.10 ± 0.01
65	1433	1433	$\beta$ -Gurjunene RI, MS	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.01 ± 0.00	0.00	0.02 ± 0.00	0.05 ± 0.01	0.00	0.00	0.10 ± 0.00
66	1435	1433	$\alpha$ -Maaliene RI, MS, Std	0.07 ± 0.01	0.09 ± 0.00	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.00	0.05 ± 0.00	0.07 ± 0.02	0.05 ± 0.01	0.00	0.00	0.12 ± 0.01
67	1439	1441	Aromadendrene RI, MS, Std	1.51 ± 0.02	1.90 ± 0.02	1.20 ± 0.03	1.25 ± 0.04	1.24 ± 0.01	1.51 ± 0.19	1.27 ± 0.09	0.65 ± 0.04	0.00	0.16 ± 0.08	3.38 ± 0.02
68	1443	1443	Selina-5,11-diene RI, MS	0.16 ± 0.01	0.22 ± 0.01	0.14 ± 0.01	0.08 ± 0.04	0.17 ± 0.00	0.11 ± 0.01	0.15 ± 0.02	0.06 ± 0.01	0.00	0.00	0.26 ± 0.01
69	1448	1451	Amorpha-4,11-diene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07 ± 0.03	0.00	0.00
70	1450	1453	trans-Muurolo-3,5-diene RI, MS	0.11 ± 0.01	0.19 ± 0.01	0.12 ± 0.01	0.03 ± 0.00	0.31 ± 0.01	0.14 ± 0.01	0.18 ± 0.03	0.02 ± 0.01	0.00	0.01 ± 0.00	0.06 ± 0.01
71	1453	1454	$\alpha$ -Humulene RI, MS, Std	0.08 ± 0.00	0.12 ± 0.00	0.07 ± 0.00	0.05 ± 0.00	0.15 ± 0.02	0.07 ± 0.01	0.09 ± 0.01	1.81 ± 0.08	0.40 ± 0.01	0.40 ± 0.03	0.03 ± 0.00
72	1457	1456	(E)- $\beta$ -Farnesene RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.93 ± 0.40	0.12 ± 0.05	0.00
73	1459	1460	Alloaromadendrene RI, MS, Std	0.67 ± 0.01	0.96 ± 0.01	0.53 ± 0.02	0.47 ± 0.02	0.62 ± 0.01	0.54 ± 0.01	0.62 ± 0.05	0.36 ± 0.02	0.00	0.51 ± 0.05	1.41 ± 0.02
74	1464	1466	$\alpha$ -Acoradiene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.00	0.00
75	1467	1470	$\beta$ -Acoradiene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03 ± 0.01	0.00	0.00
76	1471	1475	10-epi- $\beta$ -Acoradiene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02 ± 0.01	0.00	0.00
77	1473	1476	trans-Cadina-1(6),4-diene RI, MS	0.36 ± 0.01	0.55 ± 0.02	0.36 ± 0.01	0.20 ± 0.01	0.45 ± 0.02	0.42 ± 0.02	0.45 ± 0.05	0.00	0.02 ± 0.01	0.00	0.00
78	1474	1477	$\gamma$ -Gurjunene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35 ± 0.01
79	1476	1479	$\gamma$ -Muurolole RI, MS	0.04 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.03 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.05 ± 0.01	0.45 ± 0.03	0.00	0.07 ± 0.01	0.08 ± 0.01
80	1480	1480	ar-Curcumene RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13 ± 0.04	0.00	0.00
81	1481	1482	$\gamma$ -Curcumene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09 ± 0.02	0.00	0.00
82	1483	1484	$\alpha$ -Amorphene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30 ± 0.01	0.00	0.00	0.00
83	1484	1485	Germacrene D RI, MS	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.00	0.18 ± 0.02	0.00
84	1486	1490	$\beta$ -Selinene RI, MS	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.06 ± 0.00	0.05 ± 0.01	0.08 ± 0.02	1.28 ± 0.05	0.00	0.20 ± 0.01	0.23 ± 0.01
85	1488	1490	Alloaromadendr-9-ene RI, MS	0.11 ± 0.01	0.14 ± 0.01	0.07 ± 0.04	0.08 ± 0.01	0.11 ± 0.00	0.07 ± 0.01	0.11 ± 0.02	0.00	0.00	0.18 ± 0.00	0.29 ± 0.01
86	1491	1492	$\delta$ -Selinene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.35 ± 0.01	0.00	0.00	0.00
87	1492	1493	cis- $\beta$ -Guaiene RI, MS	0.19 ± 0.01	0.28 ± 0.01	0.18 ± 0.01	0.11 ± 0.01	0.31 ± 0.01	0.16 ± 0.01	0.21 ± 0.03	0.11 ± 0.01	0.00	0.01 ± 0.01	0.16 ± 0.01
88	1494	1496	Ledene RI, MS	1.14 ± 0.01	1.62 ± 0.02	0.86 ± 0.03	0.47 ± 0.03	1.06 ± 0.01	1.38 ± 0.05	1.69 ± 0.05	0.19 ± 0.03	0.16 ± 0.06	1.76 ± 0.17	0.98 ± 0.02
89	1495	1498	$\alpha$ -Selinene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.12 ± 0.08	0.00	0.00	0.00
90	1496	1499	(Z,E)- $\alpha$ -Farnesene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64 ± 0.14	0.00	0.00
91	1497	1500	Bicyclogermacrene RI, MS	0.91 ± 0.01	0.96 ± 0.06	0.67 ± 0.02	0.31 ± 0.01	0.72 ± 0.02	0.91 ± 0.04	0.60 ± 0.03	0.00	0.00	0.02 ± 0.01	0.62 ± 0.02
92	1498	1500	$\alpha$ -Muurolole RI, MS	0.17 ± 0.01	0.20 ± 0.00	0.14 ± 0.00	0.11 ± 0.01	0.22 ± 0.00	0.16 ± 0.01	0.20 ± 0.02	0.06 ± 0.01	0.00	0.09 ± 0.01	0.03 ± 0.00
93	1501	1501	Epizonarene RI, MS	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.19 ± 0.01	0.00	0.00	0.00
94	1503	1502	trans- $\beta$ -Guaiene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09 ± 0.07	0.00	0.00
95	1505	1505	(Z)- $\alpha$ -Bisabolene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23 ± 0.03	0.00	0.00
96	1506	1505	(E,E)- $\alpha$ -Farnesene RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.67 ± 0.38	0.11 ± 0.04	0.00
97	1508	1512	$\delta$ -Amorphene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19 ± 0.01
98	1512	1513	$\gamma$ -Cadinene RI, MS	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.07 ± 0.01	0.00	0.25 ± 0.02	0.06 ± 0.01
99	1514	1515	(Z)- $\gamma$ -Bisabolene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21 ± 0.05	0.00	0.00
100	1517	1522	7-epi- $\alpha$ -Selinene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02 ± 0.01	0.00	0.00	0.06 ± 0.01
101	1518	1522	trans-Calameene RI, MS	0.07 ± 0.06	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00	0.01 ± 0.00	0.00	0.00	0.00	0.00	0.00
102	1521	1523	$\delta$ -Cadinene RI, MS	1.35 ± 0.01	1.71 ± 0.02	1.22 ± 0.04	1.06 ± 0.04	1.32 ± 0.02	1.78 ± 0.14	1.79 ± 0.09	0.23 ± 0.03	0.13 ± 0.04	0.35 ± 0.01	0.17 ± 0.03
103	1527	1529	Zonarene RI, MS	0.75 ± 0.02	0.58 ± 0.04	0.33 ± 0.02	0.30 ± 0.01	0.37 ± 0.02	0.54 ± 0.04	0.48 ± 0.06	0.04 ± 0.01	0.04 ± 0.01	0.00	0.03 ± 0.02
104	1529	1531	(E)- $\gamma$ -Bisabolene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19 ± 0.05	0.00	0.00
105	1531	1534	trans-Cadina-1,4-diene RI, MS	0.22 ± 0.01	0.28 ± 0.01	0.19 ± 0.01	0.14 ± 0.01	0.29 ± 0.01	0.24 ± 0.01	0.27 ± 0.02	0.00	0.00	0.00	0.00
106	1535	1538	$\alpha$ -Cadinene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06 ± 0.03	0.00	0.00	0.01 ± 0.00
107	1540	1545	$\alpha$ -Calacorene RI, MS	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.00	0.01 ± 0.00	0.04 ± 0.03	0.00	0.00	0.00	0.03 ± 0.03
108	1542	1546	Selina-3,7(11)-diene RI, MS	0.00	0.00	0.01 ± 0.00	0.00	0.00	0.01 ± 0.00	0.00	0.16 ± 0.02	0.00	0.00	0.00
109	1548	1547	(E)- $\alpha$ -Bisabolene RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.41 ± 0.11	0.00	0.00
110	1559	1561	Germacrene B RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11 ± 0.01	0.00	0.00	0.00
111	1560	1563	(E)-Nerolidol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	48.40 ± 1.21	6.89 ± 0.44	0.00
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Table 2. Cont.

#	* RI Exp	** RI Lit	Compounds	TTAA	TTAS	TT EG	TTFC	TTNG	TTPT	TT SAT	M <sub>Ca</sub>	M <sub>Ne</sub>	M <sub>Ni</sub>	M <sub>Ro</sub>
125	1626	1623	10- <i>epi</i> - $\gamma$ -Eudesmol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02 ± 0.01	0.00	0.00	0.00
126	1630	1628	1- <i>epi</i> -Cubanol RI, MS	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.17 ± 0.01	0.07 ± 0.00	0.06 ± 0.01	0.15 ± 0.01	0.00	0.00	0.06 ± 0.03	0.01 ± 0.00
127	1632	1631	Muurola-4,10(14)-dien-1 $\beta$ -ol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03 ± 0.00	0.02 ± 0.00	0.00	0.00
128	1633	1632	$\gamma$ -Eudesmol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15 ± 0.01	0.00	0.00	0.00
129	1639	1640	T-Cadinol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.00	0.00	0.00
130	1643	1646	$\alpha$ -Muurolol RI, MS	0.06 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.07 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.00	0.00	0.03 ± 0.00	0.02 ± 0.00
131	1645	1646	Cubanol RI, MS	0.00	0.01 ± 0.00	0.00	0.02 ± 0.01	0.00	0.00	0.02 ± 0.00	0.00	0.00	0.06 ± 0.01	0.00
132	1649	1650	$\beta$ -Eudesmol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10 ± 0.01	0.00	0.00	0.00
133	1652	1653	$\alpha$ -Eudesmol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18 ± 0.01	0.00	0.01 ± 0.00	0.00
134	1668	1671	Bulnesol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.00	0.00	0.01 ± 0.00	0.00
135	1679	1684	<i>epi</i> - $\alpha$ -Bisabolol RI, MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02 ± 0.00	0.00	0.00
136	1681	1685	$\alpha$ -Bisabolol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03 ± 0.01	0.00	0.00
137	1710	1715	( <i>E,Z</i> )-Farnesol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.01	0.00	0.00
138	1720	1723	( <i>Z,E</i> )-Farnesol RI, MS, Std	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 ± 0.01	0.00	0.00
Total				99.70 ± 0.07	99.85 ± 0.04	99.65 ± 0.00	99.84 ± 0.03	99.87 ± 0.01	99.97 ± 0.02	99.73 ± 0.26	99.49 ± 0.37	99.72 ± 0.11	99.79 ± 0.13	99.53 ± 0.05

\* RI<sub>exp</sub>: Retention indices (RIs) calculated from the current study; \*\* RI<sub>lit</sub>: RI from the literature [41–57]. Identification method: RI: retention index; MS: computer matching of the mass spectra libraries and comparison with the literature data; Std: standards compounds were purchased. TTO and samples and their corresponding major components are represented in blue. Other *Melaleuca* oils and their respective major components are highlighted in gray.

The International Standard, ISO 4730, requires terpinen-4-ol chemotype to be present in commercial TTO production [58]. ISO standards allow terpinen-4-ol between 30 and 48%, along with  $\gamma$ -terpinene (10–28%),  $\alpha$ -terpinene (5–13%), 1,8-cineole (<0.01–15%),  $\alpha$ -terpineol (1.5–8%), *p*-cymene (0.5–8%),  $\alpha$ -pinene (1–6%), and terpinolene (1.5–5%), and containing a mixture of minor terpenoids with sabinene (<0.01–3.5%), aromadendrene (<0.01–3.0%),  $\delta$ -cadinene (<0.01–3.0%), ledene (viridiflorene, <0.01–3.0%), limonene (0.5–1.5%), globulol (<0.01–1.0), and viridiflorol (<0.01–1.0%) [ISO]. Our GC-MS analysis revealed that all the TTO samples fitted into the terpinen-4-ol chemotype (37.7–44.3%) (Table 2). The therapeutic use of TTO is attributed to the concentration of terpinen-4-ol and 1,8-cineole (eucalyptol), yet 1,8-cineole has been reported to cause skin and mucous membranes irritation [59–61]. Therefore, low concentrations of 1,8-cineole are preferred to maximize the therapeutic use of TTO, and it is critical to distinguish between TTOs and other commercially available *Melaleuca* oils.

*M. cajuputi* oil has three chemotypes. Chemotype 1 contains a high concentration (50–70%) of 1,8-cineole, while chemotype 2 contains a lower concentration of 1,8-cineole (31%), and chemotype 3 contains no 1,8-cineole. *M. cajuputi* subsp. *cajuputi* is the main source of cajuput oil, which does contain 1,8-cineole [61]. Our *M. cajuputi* (M<sub>Ca</sub>) oil from Thailand was found to be dominated by a 1,8-cineole-rich chemotype (Table 2).

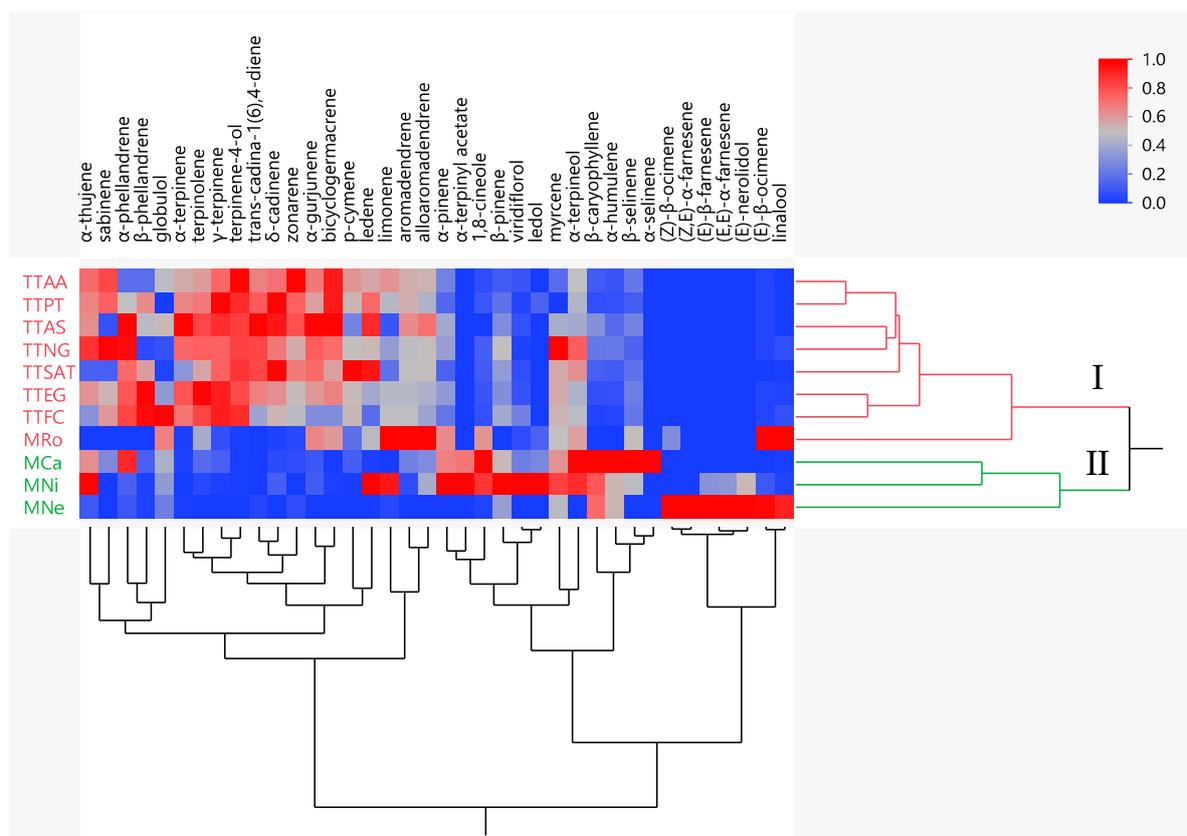
*M. quinquenervia* can be a source of 1,8-cineole-rich essential oil [61]. Four chemotypes were reported for *M. quinquenervia* [62]; the cineole chemotype (1) contains 1,8-cineole (55.0–65.0%),  $\alpha$ -pinene (7.0–12.0%), limonene (6.0–12.0%),  $\alpha$ -terpineol (4.0–10.0%),  $\beta$ -pinene (1.5–4.5%), viridiflorol (1.0–3.5%),  $\beta$ -caryophyllene (0.01–2.0%), and myrcene (0.01–2.0%), and is called niaouli oil; the linalool chemotype (2) contains (*E*)-nerolidol (61.1%), linalool (23.9%), 1,8-cineole (2.6%),  $\alpha$ -pinene (1.9%), terpinene-4-ol (1.8%), viridiflorol (1.6%), and  $\beta$ -caryophyllene (1.1%), and is called nerolina oil; the nerolidol chemotype (3) contains (*E*)-nerolidol (75.7–92.5%),  $\beta$ -caryophyllene (0.5–8.7%), 1,8-cineole (0.01–6.6%), caryophyllene oxide (0.1–6.1%),  $\alpha$ -pinene +  $\alpha$ -thujene (0–4.5%),  $\delta$ -cadinol (0–2.5%), viridiflorol (0.1–1.7%), and  $\alpha$ -terpineol + viridiflorene (ledene) (0–1.5%), and is also called niaouli oil; and the viridiflorol chemotype (4) contains viridiflorol (40.0–45.0%), 1,8-cineole (30.0–35.0%), (*E*)-nerolidol (3.0–6.0%), and ledol (0.01–4.0%), and is called niaouli oil, too. Our *M. quinquenervia* oil (M<sub>Ni</sub>) from Madagascar represents chemotype 1, and the oil (M<sub>Ne</sub>) from Australia represents chemotype 2 (Table 2).

*M. ericifolia* is from native Australian plants and is known as the lavender tea tree or rosalina oil. The major compounds of rosalina oil were identified as linalool (35.0–55.0%), 1,8-cineole (18.0–26.0%), and  $\alpha$ -pinene (5.0–12.0%) [61,62]. Our rosalina oil (M<sub>Ro</sub>) from Australia contained a high abundance of linalool and 1,8-cineole, followed by  $\alpha$ -pinene



means a strong difference. Factor loadings and squared cosine ( $\cos^2$ ) indicate the importance of components representing the individual components for a given principal component (Table 4). The  $\cos^2$  similarity always tends to be 1, showing a high linear correlation relationship of the variables with the components. The highest  $\cos^2$  values were in F1:  $\gamma$ -terpinene (0.902), terpinene-4-ol (0.900), terpinolene (0.886),  $\delta$ -cadinene (0.844), *trans*-cadinene-1(6),4-diene (0.828), bicyclogermacrene (0.798),  $\alpha$ -gurjunene (0.798), zonarene (0.762), and  $\alpha$ -terpinene (0.724). Similarly, the highest  $\cos^2$  value for F2 was  $\alpha$ -terpineol (0.858). When the relationship between the factor loadings and their percentage contributions to the matrix was analyzed, it could be concluded that  $\alpha$ -thujene,  $\alpha$ -pinene,  $\beta$ -pinene, 1,8-cineole, linalool,  $\alpha$ -terpinyl acetate,  $\alpha$ -terpineol,  $\beta$ -caryophyllene,  $\alpha$ -humulene, (*E*)- $\beta$ -farnesene, (*E*)-nerolidol, viridiflorol, and ledol had a negative relationship with the TTOs. Higher percentages of compounds then appear to be of interest for the selection of quality assessment of tea tree *M. alternifolia* EOs.

HCA classified *Melaleuca* EOs in two main groups (Figure 2): group I clustered samples with high contents of terpinene-4-ol,  $\gamma$ -terpinene,  $\alpha$ -terpinene, and terpinolene. Although  $M_{Ro}$  did not belong to *M. alternifolia* (TTOs), it was grouped in cluster I due to its high levels of interfering compounds such as limonene, aromadendrene, and alloaromadendrene. The Euclidean distance between TTAA and TTPT was 1.74, and between TTAA and  $M_{Ro}$  it was 7.51. Group II clustered samples  $M_{Ca}$ ,  $M_{Ni}$ , and  $M_{Ne}$ , which presented high contents of 1,8-cineole, linalool, and (*E*)-nerolidol, with intermediate values of  $\alpha$ -pinene,  $\alpha$ -terpineol, and  $\beta$ -caryophyllene, and lower contents of terpinene-4-ol,  $\gamma$ -terpinene, terpinolene, and  $\alpha$ -terpinene, indicating that the TTOs contained significantly higher concentrations of terpinene-4-ol when compared to the other *Melaleuca* EOs.



**Figure 2.** Two-way dendrogram of the hierarchical cluster analysis (HCA) performed on the chemical composition of the seven tea tree (*M. alternifolia*) EOs and four other *Melaleuca* EOs. Sample codes refer to Table 1. The color box indicated the abundance of each compound. Red represents high density and blue represents low density.

**Table 4.** Factor loadings, contributions (%), and squared cosine ( $\cos^2$ ) values. The positive important contributions are highlighted in blue, and the negative important contributions are highlighted in pink. The highest  $\cos^2$  values are highlighted in green.

#	Compounds	Factor Loadings and Contributions (%)		Squared Cosines ( $\cos^2$ )	
		F1	F2	F1	F2
1	$\alpha$ -Thujene	0.138 (0.129)	−0.649 (4.945)	0.019	<b>0.425</b>
2	$\alpha$ -Pinene	−0.546 (2.024)	−0.759 (6.695)	0.302	<b>0.575</b>
5	Sabinene	0.563 (2.191)	−0.055 (0.042)	<b>0.327</b>	0.004
6	$\beta$ -Pinene	−0.517 (1.799)	−0.601 (4.208)	0.268	<b>0.362</b>
7	Myrcene	−0.341 (0.824)	−0.317 (1.311)	<b>0.123</b>	0.113
9	$\alpha$ -Phellandrene	0.525 (1.832)	−0.237 (0.681)	0.273	0.059
13	$\alpha$ -Terpinene	0.853 (4.851)	0.033 (0.010)	<b>0.724</b>	0.001
14	<i>p</i> -Cymene	0.621 (2.579)	0.033 (0.011)	0.385	0.001
15	Limonene	−0.157 (0.163)	−0.357 (1.474)	0.024	0.127
16	$\beta$ -Phellandrene	0.539 (1.963)	0.052 (0.036)	0.293	0.003
17	1,8-Cineole	−0.685 (3.144)	−0.667 (5.139)	<b>0.469</b>	0.442
18	( <i>Z</i> )- $\beta$ -Ocimene	−0.561 (2.106)	0.794 (7.323)	0.314	<b>0.629</b>
19	( <i>E</i> )- $\beta$ -Ocimene	−0.527 (1.866)	0.628 (4.594)	0.278	<b>0.395</b>
20	$\gamma$ -Terpinene	0.947 (6.044)	0.013 (0.002)	<b>0.902</b>	0.000
23	Terpinolene	0.943 (5.940)	−0.001 (0.000)	<b>0.886</b>	0.000
24	Linalool	−0.502 (1.693)	0.605 (4.263)	0.253	<b>0.366</b>
29	Terpinene-4-ol	0.948 (6.034)	0.047 (0.024)	<b>0.900</b>	0.002
31	$\alpha$ -Terpineol	0.074 (0.037)	−0.923 (9.987)	0.006	<b>0.858</b>
49	$\alpha$ -Terpinyl acetate	−0.637 (2.725)	−0.708 (5.793)	0.407	<b>0.498</b>
60	$\alpha$ -Gurjunene	0.862 (4.918)	−0.023 (0.008)	<b>0.734</b>	0.001
63	$\beta$ -Caryophyllene	−0.833 (4.665)	−0.247 (0.703)	<b>0.696</b>	0.060
67	Aromadendrene	0.483 (1.556)	0.015 (0.003)	0.232	0.000
71	$\alpha$ -Humulene	−0.586 (2.293)	−0.338 (1.318)	<b>0.342</b>	0.113
72	( <i>E</i> )- $\beta$ -Farnesene	−0.582 (2.268)	0.753 (6.598)	0.338	<b>0.567</b>
73	Alloaromadendrene	0.373 (0.904)	−0.237 (0.654)	0.135	0.056
77	<i>trans</i> -Cadina-1(6),4-diene	0.911 (5.546)	0.050 (0.026)	<b>0.828</b>	0.002
84	$\beta$ -Selinene	−0.434 (1.261)	−0.469 (2.534)	0.188	0.218
88	Ledene	0.431 (1.232)	−0.422 (2.043)	0.184	0.176
89	$\alpha$ -Selinene	−0.407 (1.106)	−0.369 (1.568)	0.165	0.135
90	( <i>Z,E</i> )- $\alpha$ -Farnesene	−0.549 (2.022)	0.786 (7.179)	0.302	<b>0.617</b>
91	Bicyclogermacrene	0.894 (5.351)	0.067 (0.053)	<b>0.798</b>	0.005
96	( <i>E,E</i> )- $\alpha$ -Farnesene	−0.584 (2.283)	0.751 (6.563)	0.341	<b>0.564</b>
102	$\delta$ -Cadinene	0.917 (5.655)	−0.024 (0.005)	<b>0.844</b>	0.000
103	Zonarene	0.873 (5.105)	0.073 (0.065)	<b>0.762</b>	0.006
111	( <i>E</i> )-Nerolidol	−0.620 (2.580)	0.707 (5.811)	0.385	<b>0.499</b>
116	Globulol	0.016 (0.001)	0.005 (0.000)	0.000	0.000
117	Viridiflorol	−0.486 (1.593)	−0.602 (4.188)	0.238	<b>0.360</b>
121	Ledol	−0.506 (1.718)	−0.599 (4.143)	0.256	<b>0.356</b>

## 2.2. HPTLC Analysis of *Melaleuca* EOs

The less-polar components of the UHM, labeled (e) through (h) according to Do et al. [38], separated well under the selected HPTLC development conditions, as shown in Figure 3A. This indicated that the initial method involving Hex/EtOAc 90:10 (*v/v*) as the solvent system was appropriate for the separation of non-polar components of interest in our samples. However, the  $R_f$  of some *Melaleuca* oil components appear to exceed that of the highest UHM component. For future purposes, an additional component with higher  $R_f$  may need to be added to the UHM to better encompass the range of our samples.

In general, the HPTLC results complemented the findings obtained from the GC-MS analysis. The oils of *M. alternifolia* (TTOs) (Figure 3B), despite their wide variety of sources, exhibited a very similar separation pattern dominated by terpinen-4-ol as the primary component. When developed with Hex/EtOAc 90:10 (*v/v*), this main constituent

of TTO appeared at an  $R_f$  value of  $0.278 \pm 0.067$ . A group of mono- and sesquiterpenes, including  $\alpha$ - and  $\beta$ -pinene ( $R_f = 0.767 \pm 0.031$ ) and (+)-aromadendrene ( $R_f = 0.738 \pm 0.062$ ), was showcased as the second most prominent band, with a collective  $R_f$  of  $0.747 \pm 0.065$ . Included in this group were ledene and  $\delta$ -cadinene, identified by GC-MS. This band was followed in intensity by  $\alpha$ -terpineol ( $R_f = 0.147 \pm 0.052$ ) and 1,8-cineole ( $R_f = 0.504 \pm 0.081$ ), respectively.

In cases where oil components were merged, as observed by the overlapping or blending of colors, such components exhibited a shift in  $R_f$  values compared to those of the corresponding reference standards. This may be caused by interactions due to large amounts of components competing for the limited silica surface area. The  $R_f$  of the merged oil constituents, as well as the presence of additional constituents, was established by GC-MS.

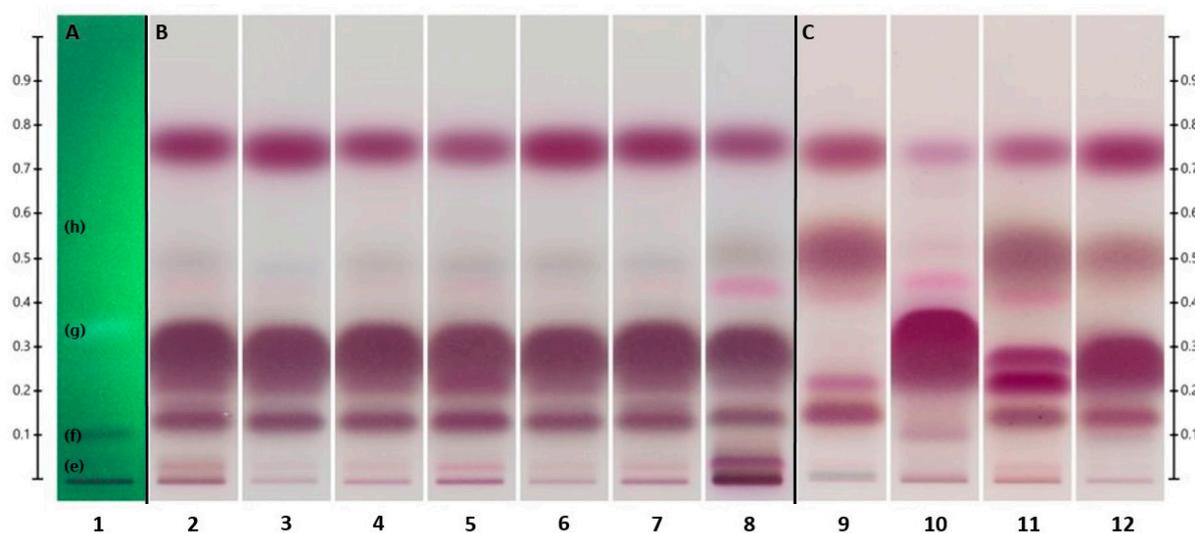
The other four *Melaleuca* EOs (Figure 3C) displayed a diverse pattern compared to each other, as well as in comparison to the TTOs. 1,8-cineole could be observed as the most characteristic component in  $M_{Ca}$  (track 9) and  $M_{Ni}$  (track 11), merged with small amounts of  $\alpha$ -terpinyl acetate ( $R_f = 0.539 \pm 0.050$ ) and limonene ( $R_f = 0.486 \pm 0.047$ ). In contrast with  $M_{Ca}$  and  $M_{Ni}$ , cineole was less prominent in  $M_{Ro}$  (track 12) and almost insignificant in  $M_{Ne}$  (track 10). No terpinyl acetate was observed in either  $M_{Ne}$  or  $M_{Ro}$ , yet limonene was still present in  $M_{Ro}$ .

The second most noticeable band of  $M_{Ca}$  was a group of mono- and sesquiterpenes merged at  $R_f = 0.743 \pm 0.052$ . These were identified by GC-MS as  $\alpha$ - and  $\beta$ -pinene,  $\beta$ -caryophyllene ( $R_f = 0.727 \pm 0.059$ ),  $\alpha$ -humulene ( $R_f = 0.727 \pm 0.062$ ), and  $\alpha$ -phellandrene ( $R_f = 0.759 \pm 0.031$ ). Also identified by GC-MS in small amounts were  $\alpha$ - and  $\beta$ -selinene. Two other chemicals present at a lower intensity, yet highly identifiable on  $M_{Ca}$ , were  $\alpha$ -terpineol and linalool ( $R_f = 0.243 \pm 0.056$ ).

The two most prominent bands in  $M_{Ne}$ , combined near 0.3  $R_f$ , were identified as nerolidol ( $R_f = 0.279 \pm 0.056$ ) and linalool, respectively. They were followed in intensity by the mono- and sesquiterpene band near 0.75  $R_f$ , comprised of  $\beta$ -caryophyllene,  $\beta$ -pinene,  $\beta$ -ocimene ( $R_f = 0.747 \pm 0.047$ ), and farnesene ( $R_f = 0.737 \pm 0.060$ ), as identified by GC-MS. Small, yet still characteristic of  $M_{Ne}$ , was geraniol at  $R_f = 0.124 \pm 0.067$ . A bright-pink band at  $R_f = 0.432 \pm 0.045$  was recognized as caryophyllene oxide by isolating the component on a TLC preparation plate (Section 3.4.2) and confirmed by GC-MS analysis against its reference standard and available GC-MS libraries. Caryophyllene oxide appeared to be a byproduct of  $\beta$ -caryophyllene since it was present in both TLC and TIC reference standard chromatograms.

The  $M_{Ni}$  oil was characterized by a large amount of 1,8-cineole. As with  $M_{Ca}$ , this large band was mixed with a small amount of terpinyl acetate and limonene. The second-largest band was composed of mono- and sesquiterpenes, confirmed by GC-MS as  $\alpha$ - and  $\beta$ -pinene,  $\beta$ -caryophyllene, and ledene. Other main components were nerolidol,  $\alpha$ -terpineol, and viridiflorol ( $R_f = 0.213 \pm 0.021$ ).

In  $M_{Ro}$ , the signature component was linalool, with a small amount of terpinen-4-ol merging into it. Mono- and sesquiterpenes composed the second-strongest band near 0.76  $R_f$ , followed by cineole as the third characteristic band. Constituents of the second band were recognized as  $\alpha$ -pinene, aromadendrene, alloaromadendrene, and ledene by GC-MS. Merged into a fourth band was  $\alpha$ -terpineol, followed by globulol a bit below, at  $R_f = 0.137 \pm 0.022$ . System suitability as well as  $R_f$  values for the major components were established by SSTs (Figure 4A) and reference standards (Figure 4B), analyzed under the same HPTLC conditions.

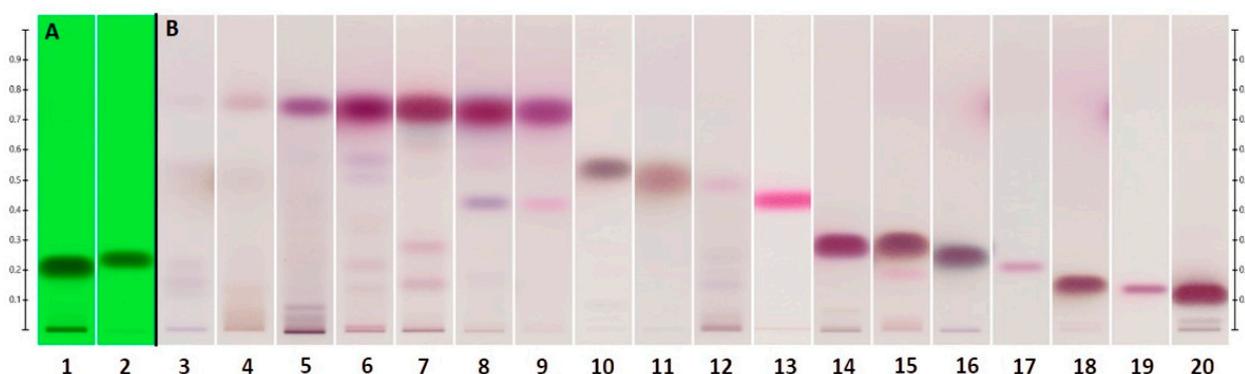


**Figure 3.** Separation of the UHM under UV<sub>254</sub> (A) (see Table 5), tea tree oils (B), and other *Melaleuca* oils (C) (see Table 1) under visible light, developed with Hex/EtOAc 90:10 (*v/v*) on Silica gel 60 F<sub>254</sub>.

**Table 5.** List of UHM \* components used in this study.

Label	Name	CAS#
(e)	Phthalamide	85-41-6
(f)	9-Hydroxyfluorene	1689-64-1
(g)	Thioxanthen-9-one	492-22-8
(h)	2-(2H-Benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl) phenol	3147-75-9

\* UHM components visible under the established analytical conditions.



**Figure 4.** SSTs (A) are shown under UV<sub>254</sub> light. Reference standards (B) (see Table 6) are shown under visible light. Developed with Hex/EtOAc 90:10 (*v/v*) on Silica gel 60 F<sub>254</sub>.

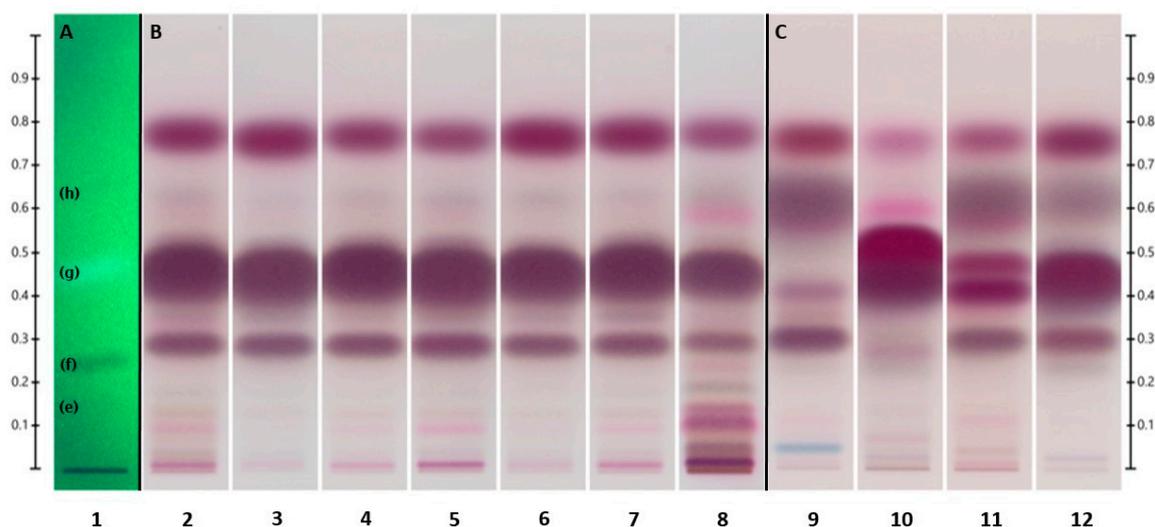
An enhanced separation of more polar constituents in the oils (lower  $R_f$  range) was obtained when developed with Hex/EtOAc at a ratio of 80:20 (*v/v*). Figure 5A emphasizes an increased distance among UHM constituents. Additionally, target oil components that merged at low  $R_f$  values under the previous solvent system were now appearing mid-range and better separated.

The most significant improvement with a more polar solvent system was an increased separation of terpinen-4-ol ( $R_f = 0.532 \pm 0.049$ ) and  $\alpha$ -terpineol ( $R_f = 0.385 \pm 0.054$ ) in TTOs (Figure 5B). With the other *Melaleuca* oils (Figure 5C), where the differences among samples relied more on the polar region, a greater distinction could be made among nerolidol ( $R_f = 0.549 \pm 0.052$ ), linalool ( $R_f = 0.514 \pm 0.055$ ), viridiflorol ( $R_f = 0.494 \pm 0.027$ ), and terpinen-4-ol ( $R_f = 0.532 \pm 0.049$ ), as well as between  $\alpha$ -terpineol ( $R_f = 0.385 \pm 0.054$ ), globulol ( $R_f = 0.376 \pm 0.021$ ), and geraniol ( $R_f = 0.263 \pm 0.052$ ). These differences in  $R_f$

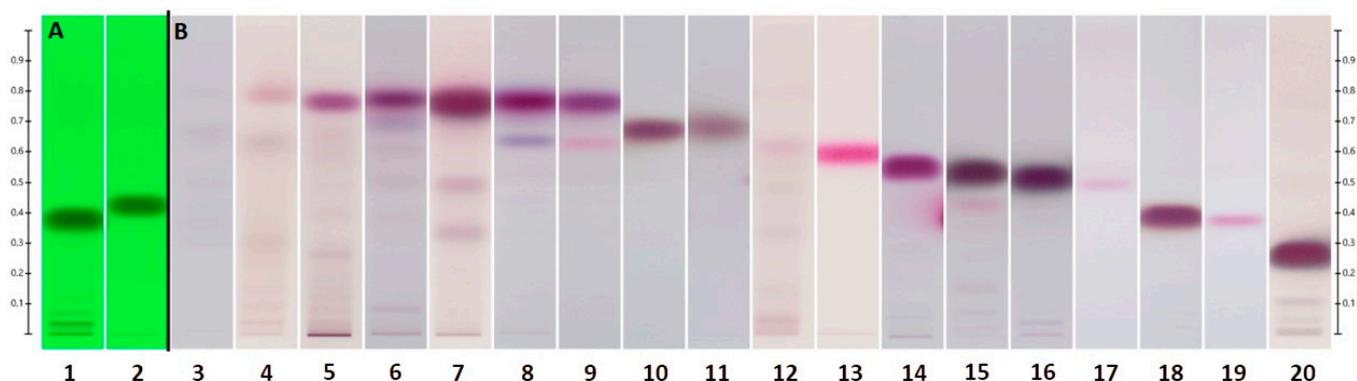
were more evident at lower concentrations, as demonstrated by the SSTs (Figure 6A) and the individual reference standards (Figure 6B) analyzed under exact HPTLC developing conditions.

**Table 6.** System suitability standards (SSTs) and reference standards for *Melaleuca* EOs.

Track	Name	CAS#	Track	Name	CAS#
1	Isoeugenol	97-54-1	11	1,8-Cineole	470-82-6
2	Isoeugenyl acetate	93-29-8	12	(S)-(-)-Limonene	5989-54-8
3	(1R)-(+)- $\alpha$ -Pinene	7785-70-8	13	(-)-Caryophyllene oxide	1139-30-6
4	$\alpha$ -Phellandrene	99-83-2	14	Nerolidol	7212-44-4
5	Ocimene mix	13877-91-3	15	(-)-Terpinen-4-ol	20126-76-5
6	(+)-Aromadendrene	489-39-4	16	(-)-Linalool	1126-91-0
7	Farnesene mix	502-61-4	17	Viridiflorol	0552-02-03
8	$\alpha$ -Humulene	6753-98-6	18	(-)- $\alpha$ -Terpineol	10482-56-1
9	$\beta$ -Caryophyllene	87-44-5	19	(-)-Globulol	489-41-8
10	$\alpha$ -Terpinyl acetate	80-26-2	20	Geraniol	106-24-1



**Figure 5.** Separation of the UHM under UV<sub>254</sub> (A) (see Table 5), tea tree oils (B), and other *Melaleuca* oils (C) (see Table 1) under visible light, developed with Hex/EtOAc 80:20 (*v/v*) on Silica gel 60 F<sub>254</sub>.



**Figure 6.** SSTs (A) are shown under UV<sub>254</sub> light. Reference standards (B) (see Table 6) are shown under visible light. Developed with Hex/EtOAc 80:20 (*v/v*) on Silica gel 60 F<sub>254</sub>.

### 3. Materials and Methods

#### 3.1. Sample Selection and Preparation

TTOs from *M. alternifolia* were selected based on their previously established biological activity as a potential attractant for the male Mediterranean fruit fly [23,24,26,27]. Essential oils from other *Melaleuca* species were also included for the purpose of comparison (Table 1). Each sample was diluted to 20% of its original purity using methylene chloride, ACS Reagent, CAS# 75-09-2 (J.T. Baker-Avantor, Center Valley, PA, USA). If necessary, concentration and application volume were adjusted for optimum HPTLC separation.

#### 3.2. Standard Selection and Preparation

A universal HPTLC calibration mix (UHM) (Sigma-Aldrich, St. Louis, MO, USA) was used as a reference standard for HPTLC separations. It contains eight different compounds diluted in methanol at ready-to-use concentrations [38], four of which were observed under our HPTLC conditions using a UV<sub>254</sub> light source (Table 5).

Stock solutions of isoeugenol, CAS# 97-54-1, and isoeugenyl acetate, CAS# 93-29-8 (Sigma-Aldrich, St. Louis, MO, USA), were prepared in methylene chloride at a concentration of 100 µL/mL and 100 µg/mL, respectively. A 20 µL/mL (21.6 mg/mL;  $\delta = 1.08$  g/mL) methylene chloride dilution of isoeugenol, as well as a 20 mg/mL dilution of isoeugenyl acetate, were prepared from their corresponding stock and used as system suitability standards (SST1 and SST2).

A series of reference standards (Table 6) were obtained from Sigma-Aldrich (St. Louis, MO, USA), and were prepared and analyzed under the same conditions as the EOs to confirm the R<sub>f</sub> values of the oil components on HPTLC.

#### 3.3. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

*Melaleuca* EO samples were analyzed on an Agilent 7890B GC coupled with a 5977B mass selective detector (GC-MS) (Agilent Technologies, Santa Clara, CA, USA). A DB-5 column (30 m × 0.25 mm inner diameter with 0.25 µm film thickness) was used with an electron ionization source set at 70 eV. The temperatures of the ion source and quadrupole were 230 °C and 150 °C, respectively. The mass spectrometry transmission line was 250 °C. Injector and detector temperatures were kept at 220 °C and 230 °C, respectively. The oven temperature program was set at 60 °C for 1.3 min and increased to 246 °C at 3 °C/min. A constant helium flow of 1.3 mL/min was applied [41]. The selected mass range was *m/z* 35 to 450 Da and scan rate was 2.8 scans/s. Mass Hunter B.07.06 software (Agilent Technologies) was used for data acquisition and processing. One µL of diluted samples was injected into the GC–MS on splitless mode.

Linear retention indices (RIs) were calculated using the van Den Dool and Kratz [42] equation in relation to a homologous series of *n*-alkanes (C<sub>9</sub>–C<sub>21</sub>). Compound identification was achieved by comparison of their corresponding mass spectra and RIs to those reported in a mass spectral library developed at the USDA-ARS-SHRS laboratory with authentic compounds and with the commercial libraries MassFinder [43], Adams Library [41], Flavours and Fragrances of Natural and Synthetic Compounds 3 (FFNSC-3) [44], Wiley 12/NIST 2020 [45], and an in-house library “SHRS Essential Oil Constituents-DB-5 Column”. Retention indices were also verified with data reported in the specific literature [46–54] and internet sources [55–57]. Each oil was analyzed in triplicate. Relative percentages were directly obtained from peak total ion current (TIC) areas. All these standards were purchased from the following sources:  $\alpha$ -pinene (CAS# 80-56-8), camphene (CAS# 79-92-5), benzaldehyde (CAS# 100-52-7), sabinene (CAS# 3387-41-5),  $\beta$ -pinene (CAS# 127-91-3), myrcene (CAS# 123-35-3), 6-methyl-5-hepten-2-ol (CAS# 1569-60-4),  $\alpha$ -phellandrene (CAS# 99-792-5),  $\delta$ -3-carene (CAS# 13466-78-9), 1,4-cineole (CAS# 470-67-7),  $\alpha$ -terpinene (CAS# 99-86-5), *p*-cymene (CAS# 99-87-6), limonene (CAS# 5989-27-5), 1,8-cineole (CAS# 470-82-6), ocimene mixture (CAS# 13877-91-3),  $\gamma$ -terpinene (CAS# 99-85-4), linalool oxide (CAS# 60047-17-8), terpinolene (CAS# 586-62-9), linalool (CAS# 78-70-6), terpinen-4-ol (CAS# 20126-76-5),  $\alpha$ -terpineol (CAS# 10482-56-1), citronellol (CAS# 106-22-9), geraniol (CAS#

106-24-1), thymol (CAS# 89-83-8), carvacrol (CAS# 499-75-2),  $\alpha$ -terpinyl acetate (CAS# 80-26-2), eugenol (CAS# 97-53-0), geranyl acetate (CAS# 105-87-3), methyl eugenol (CAS# 93-15-2),  $\beta$ -caryophyllene (CAS# 87-44-5), aromadendrene (CAS# 489-39-4),  $\alpha$ -humulene (CAS# 6753-98-6), (*E*)- $\beta$ -farnesene (CAS# 18797-84-8), farnesene, mixture of isomers (product number W383902), alloaromadendrene (CAS# 25246-27-9), nerolidol (CAS# 7212-44-4), caryophyllene oxide (CAS# 1139-30-6), globulol (CAS# 489-41-8), viridiflorol (CAS# 552-02-3),  $\alpha$ -bisabolol (CAS# 23089-26-2), farnesol mixture (CAS# 4602-84-0) from Sigma-Aldrich, St. Louis, MO, USA;  $\beta$ -phellandrene (CAS# 555-10-2) from Toronto Research Chemicals (Toronto, ON, Canada);  $\alpha$ -copaene (CAS# 3856-25-5) and  $\beta$ -elemene (CAS# 515-13-9) from Fluka Chemical Co., Buchs, SG, Switzerland; and (+)-*ar*-curcumene (CAS# 4176-06-1) from BOC Sciences Shirley, NY, USA.

### 3.4. Thin-Layer Chromatography Analysis

#### 3.4.1. Automated High-Performance Thin-Layer Chromatography (HPTLC) Analysis

Chromatography was performed using a CAMAG HPTLC system equipped with VisionCATS 3.1 software (CAMAG, Muttenz, Switzerland). Initial conditions were set following the established HPTLC/TLC protocol for essential oils [37,63]. An HPTLC Silica gel 60 F<sub>254</sub> glass-backed plate, 20 × 10 cm (Supelco Merck KGaA, Darmstadt, Germany, operating as Millipore-Sigma in St. Louis, MO, USA), was activated by heat using a TLC Plate Heater III (CAMAG, Muttenz, Switzerland) for 10 min. at 65 °C prior to analysis. Toluene/ethyl acetate 93:7 (*v/v*) was used as the mobile phase. However, previous bioassays (P.E.K. unpublished data) had indicated that sterile male medflies were repelled by toluene residue, prompting the search for an alternative mobile phase. Hexane was selected due to its similar polarity to toluene.

*Melaleuca* EO constituents appear in different amounts and cover a relatively wide polarity range when separated by TLC. Tabanca et al. [27] used hexane/acetone 90:10 (*v/v*) and obtained a good separation that produced two TTO fractions attractive to sterile male medflies, yet these fractions still contained a mixture of chemicals. Further separation was necessary to identify possible individual attractants. Various ratios of hexane/ethyl acetate were then attempted in preliminary experiments and it was decided that two separate solvent combinations provided improved resolution of the fractions of interest.

For the separation of monoterpenes and other non-polar oil constituents, a solution of 45 mL hexane (Hex), Certified ACS, CAS# 92112-69-1 (Fisher Chemical, Thermo Fisher Scientific, Waltham, MA, USA), and 5 mL ethyl acetate (EtOAc), HPLC grade, ≥99.7%, CAS#141-78-6 (Sigma-Aldrich, St. Louis, MO, USA), was prepared for a ratio of 90:10 (*v/v*). To favor the separation of more polar compounds, 40 mL Hex and 10 mL EtOAc were mixed for an 80:20 (*v/v*) ratio.

An aliquot of each oil sample was dispensed into a 1.5 mL screw-cap vial, covered with TFE/SIL septum cap (J.G. Finneran Associates, Inc., Vineland, NJ, USA), and placed into an Automatic TLC Sampler (ATS4) (CAMAG, Muttenz, Switzerland). An activated silica gel plate was placed in its corresponding holder. Samples were applied as thin bands (8 mm long, 8 mm from the bottom edge of the plate) using a 25  $\mu$ L Hamilton syringe with spray application needle and nozzle. Syringe and needle were automatically rinsed 5 times with methanol, ACS grade, CAS# 67-56-1 (Supelco Merck KGaA, Darmstadt, Germany, as EMD Millipore Corporation, Burlington, MA, USA), between samples.

The HPTLC plate was developed in an Automatic Developing Chamber (ADC2) (CAMAG, Muttenz, Switzerland). The chamber containing a saturation pad was saturated for 20 min with 25 mL of the selected mobile phase. To remove as much moisture as possible, the system was also activated for 10 min with a saturated magnesium chloride aqueous solution prepared from magnesium chloride hexahydrate, (MgCl<sub>2</sub>·6H<sub>2</sub>O), CAS# 7791-18-6 (Sigma-Aldrich, St. Louis, MO, USA). Development was automatically started and stopped once the solvent front reached a preset height of 85 mm. After development, the plate was allowed to dry for 1 min at room temperature in the fume hood.

A vanillin/sulfuric acid derivatizing reagent was prepared following Wagner and Bladt [63], by adding 0.4 g of vanillin Reagent Plus, 99%, CAS# 121-33-5 (Sigma-Aldrich, St. Louis, MO, USA), to 100 mL of 190 proof ethanol, USP, CAS# 64-17-5 (Decon Labs, Inc., King of Prussia, PA, USA). The ethanolic solution was kept refrigerated until use. Concentrated sulfuric acid, Certified ACS Plus, CAS# 7664-93-9 (Fisher Chemical, Thermo Fisher Scientific, Waltham, MA, USA), was added shortly before use at a proportion of 20  $\mu$ L acid per mL of vanillin solution.

Automatic derivatization of the plate to generate color occurred inside a Derivatizer chamber (CAMAG, Muttenz, Switzerland) with 2 mL of vanillin/H<sub>2</sub>SO<sub>4</sub> reagent. The derivatizing reagent was applied by spraying through a yellow nozzle at spray level 3. Colors were observed after heating the plate for 1.5 to 3.0 min at 100 °C on a CAMAG Plate Heater III, depending on color intensity. Images of the plate were taken at various stages of the process using a Visualizer 2 (CAMAG, Muttenz, Switzerland) with a 16 mm lens under RT White, UV<sub>254</sub>, and UV<sub>366</sub> light. The retention factors ( $R_f$ ) values were calculated by VisionCATS software version 3.1. Profiles and comparisons were also generated using VisionCATS.

#### 3.4.2. Automated Preparative Thin-Layer Chromatography Analysis

Preparative TLC was used to isolate unknown bands for identification in cases where a component was not readily identified, and a standard could not be easily referenced for confirmation. An HPTLC Silica gel 60 F<sub>254</sub> glass-backed plate, 20 × 10 cm (Supelco Merck KGaA, Darmstadt, Germany, operating as Millipore-Sigma in St. Louis, MO, US), was used to separate and collect a reasonable amount of the unknown chemical for further identification. The plate was initially activated by heating at 100 °C for 15 min on a CAMAG TLC Plate Heater III.

Sample application was conducted in a CAMAG ATS4 autosampler, where 2  $\mu$ L oil was applied in 20 consecutive bands, 8 mm long each, making a solid horizontal line 8 mm from the bottom edge of the plate. Once the sample was applied, the plate was developed in a previously saturated CAMAG ADC2 chamber. A total of 35 mL mobile phase was used, 25 mL for saturation and 10 for development. Development stopped when solvent front reached 85 mm.

In the case of nerolina oil, a bright pink band at  $R_f = 0.451 \pm 0.022$  (40–45 mm from the bottom) was our target chemical. A 5 × 10 cm strip was cut out of the developed plate and was sprayed with vanillin reagent in a CAMAG Derivatizer. The bright pink band on the derivatized strip provided the measurements of the area to scrape to obtain our unknown from the remaining (non-derivatized) portion of the plate. Scraped silica containing our compound of interest was extracted with 1 mL methylene chloride and filtered through a 0.2  $\mu$ m Whatman AUTOVIAL™ 5 syringeless filter (Global Life Sciences Solutions USA LLC–Cytiva, Marlborough, MA, USA) for GC-MS analysis.

#### 3.5. Statistical Analysis

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were applied to TTOs and other *Melaleuca* EOs and their chemical constituents, using the XLSTAT 2021 (Addinsoft, New York, NY, USA) for PCA and JMP (JMP® Pro 17.0.0, SAS Institute Inc. Cary, NC, USA) for HCA. Both PCA and HCA were performed on the means of those volatile constituents higher than 0.5%; the covariance data matrix was 38 × 11 (418 data). Pearson's correlation model was used for PCA, Euclidean distance for measure, and Ward's method for HCA analysis.

## 4. Conclusions

The results of this study demonstrate that HPTLC serves as a quick and effective analytical technique for the screening of selected *Melaleuca* oils. Its automated steps eliminate most human error and provide better reproducibility. A wide variety of samples may be analyzed by combining the most suitable mobile and stationary phases for the

target analytes. This allows for the selection of less toxic solvents, such as hexane instead of toluene, while maintaining comparable retention factors to those in the Pharmacopeia. It also provides a fast detection tool for more polar additives or contaminants that may not be detected under GC-MS conditions. Samples can be applied as a long, narrow band, allowing multiple samples to be simultaneously analyzed and a cleaner separation of individual components.

An advantage over other analytical techniques is that multiple samples and standards may be analyzed at the same time and under true identical conditions using HPTLC. Moreover, the development process is nondestructive, which allows samples to be scraped and extracted from the plate for further studies. For this purpose, a template may be created from a prior plate derivatized with color reagent.

On the other hand, there are some disadvantages to this procedure. In the case of highly volatile constituents, there is a high probability of evaporation during the process. In addition, some oil components do not react with the derivatizing reagent and therefore do not emit a visible color. While some may be seen under UV light, others may not be visible at all. Another complicating factor is that compounds found in trace amounts may fall under the detection limit of the HPTLC instrument. A more complex mixture of coeluting chemicals also represents a challenge. Two or more developments may be required for better separation of these target constituents.

New studies are currently in process, involving two-dimensional and multigradient developments to address the above-mentioned challenges. Future work prospects include the addition of a densitometry module and a TLC-MS interface to achieve a more accurate quantification and precise recovery of individual oil components for further analysis. With so many favorable features and few obstacles, this technique proved to be an efficient and reliable screening tool for the selected *Melaleuca* oils.

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