separations

MDPI

Article

Effect of Extraction Methods on Essential Oil Composition: A Case Study of Irish Bog Myrtle-*Myrica gale* L.

Shipra Nagar [1,*], Maria Pigott [1], Sophie Whyms [1], Apolline Berlemont [1,2] and Helen Sheridan [1,*]

[1] NatPro Centre, School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 02, D02 PN40 Dublin, Ireland
[2] Faculty of Medicine and Pharmacy, University of Mons (UMONS), Place du Parc, 20, 7000 Mons, Belgium
* Correspondence: nagars@tcd.ie or shhipranagar@gmail.com (S.N.); hsheridn@tcd.ie (H.S.)

Abstract: *Myrica gale* is an aromatic peatland shrub that has reported traditional use as an insect repellent. Different extraction methodologies were used in this study to isolate the essential oil of *Myrica gale* L., including Clevenger hydrodistillation (CH) and microwave-assisted hydrodistillation (MAH). The oils, isolated from different plant parts (leaves, fruit and branches) collected in summer and autumn, were analysed by GC-MS and the volatiles from plant tissue were directly analysed by headspace-GC-MS. A total of 58 components were identified, including 15 monoterpene hydrocarbons (22.78–98.98%), 14 oxygenated monoterpenes (0.91–43.02%), 13 sesquiterpene hydrocarbons (0.05–24.98%), 3 oxygenated sesquiterpenes (0.07–13.16%) and 13 other compounds (0.05–5.21%). Headspace sampling furnished monoterpenes, while CH and MAH extracted monoterpenes and sesquiterpenes, with α-pinene (6.04–70.45%), eucalyptol (0.61–33.80%), limonene (2.27–20.73%) and α-phellandrene (2.33–15.61%) as major components in all plant parts. Quantitative differences occurred between extraction methodologies, with MAH yielding higher quantities of monoterpene and sesquiterpene hydrocarbons and CH targeting oxygenated counterparts. Leaves gave more complex chemical fingerprints than branches and fruit, and the summer collection yielded more components than the autumn collections. An OPLS-DA model was applied to the GC-MS data to compare the chemical profiles based on the extraction techniques and plant parts, and molecular networks were obtained for monoterpenes and sesquiterpenes connected via biosynthetic pathways. The essential oil profile of *Myrica gale* was influenced by the season of collection, plant part and extraction method.

Keywords: Clevenger; microwave; headspace; OPLS-DA; molecular networking

Citation: Nagar, S.; Pigott, M.; Whyms, S.; Berlemont, A.; Sheridan, H. Effect of Extraction Methods on Essential Oil Composition: A Case Study of Irish Bog Myrtle-*Myrica gale* L. *Separations* **2023**, *10*, 128. https://doi.org/10.3390/separations10020128

Academic Editor: Stefania Garzoli

Received: 23 January 2023
Revised: 10 February 2023
Accepted: 13 February 2023
Published: 14 February 2023

1. Introduction

Myrica gale, commonly called bog myrtle or Roideóg in the Irish language [1], is an acidic-soil-loving deciduous shrub from the *Myricaceae* family. It occurs on lakeshores and in bogs, growing from approximately 0.6 m to up to 2 m tall, and with a distribution spanning the northern hemisphere. It flowers in spring, with catkins appearing before the leaves from April to May, and fruiting later in the autumn season. It is usually dioecious, rarely monoecious. On pollination, female flowers produce clusters of yellowish-green knobbly fruits. *Myrica gale* has an aromatic sweet scent known historically to deter insects, such as midges, moths and fleas. Traditionally, in Ireland, branches were left in bedroom cupboards to keep moths away, and branches were incorporated into beds to deter bed bugs and vermin, whilst, in Scotland, fishermen would wear a twig of bog myrtle in their shirt buttonholes to repel midges [1–3].

In ancient Ireland, bog myrtle was thought to be a sacred plant and was used not only as a natural insect repellent but also as a herbal medicine. Traditionally, it was used for the treatment of measles, coughs, sore throats and kidney and urinary complaints [3,4]. In herbal veterinary medicine, *M. gale* was used to remedy liver fluke and worms in cattle; red water fever, gravel and collar sores in horses; and to treat fits in dogs [1]. A report by

Williams, 1993 also stated that "the ashes of burnt leaves were put in the eyes of sheep with a goose feather, to treat blindness" [3].

Many of the purported biological activities of bog myrtle can be attributed to its essential oil, which is especially prevalent in the leaves and reproductive structures, such as fruits and catkins. Essential oils are characteristic of aromatic plants, and these volatile compound mixtures give scent to the reproductive organs and fruits, attracting pollinators and facilitating seed dispersal via animals. Further, essential oils offer protection to plants against herbivores and pathogens, facilitate plant communication and can increase a plant's tolerance to changes in the abiotic environment [5,6]. Essential oil constituents, such as monoterpenes and sesquiterpenes, are produced as plant secondary metabolites and can be acquired from the aerial and subterranean parts of plants. They have been shown to possess a wide range of medicinal properties, such as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and anaesthetic activities, and, since antiquity, have been consistently used in traditional and herbal medicine [7].

Extraction methods strongly influence the phytochemical profiles of plant extracts, in combination with a host of other factors, including the location, plant part(s), season of harvesting, post-harvesting storage and pre-extraction treatment, and, in turn, the biological activity of a medicinal plant. Essential oils have been extracted by steam or hydrodistillation since the Middle Ages [8,9]. Hydrodistillation carried out via Clevenger apparatus is traditionally the most common method used to extract volatile oils from plants and has been used industrially and in multiple studies to extract the essential oils of *Myrica gale* [10–14]. The emergence of new extraction methodologies such as microwave-assisted extraction, ultrasound-assisted extraction and supercritical fluid extraction has facilitated extractions in shorter timeframes, using less energy, and generally with comparable or greater yields in comparison to the conventional hydrodistillation approach [15]. The adoption of a new extraction method can be expected to affect the phytochemical profile of an extract, qualitatively and/or quantitatively. In this study, we aim to investigate the influence of the extraction technique, plant morphology and harvesting season on the chemical profile of volatiles from *Myrica gale*. Aerial plant parts were harvested in summer and autumn seasons and their chemical profiles were obtained by (a) headspace-GC-MS analysis of fresh plant tissue and (b) GC-MS analysis of essential oils extracted by Clevenger hydrodistillation (CH) and microwave-assisted hydrodistillation (MAH) of the leaves, branches and fruit. The data obtained were analysed by multivariate data analysis to determine the variability in the chemical profiles of volatiles obtained from *Myrica gale* with respect to the aforementioned factors. Molecular networking was applied to determine the essential oil components connected through biosynthetic pathways.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and Reagents

If not stated otherwise, all chemicals were of analytical grade, purchased from Merck, and n-alkane standard solution C8–C20 was purchased from Fluka (product number 04070-5ML).

2.1.2. *Myrica gale* Plant Material

Myrica gale aerial parts (leaves, branches and fruit) were collected from a wetland site in Clonlisk, County Offaly, in the midlands of Ireland (52.9444840, −79161840). The leaves were collected in summer on 27 June 2022 and in autumn on 3 October 2022. Branches were also collected on 3 October 2022 and fruit was collected on 21 October 2022. The plant material was stored at 4–8 °C from the date of collection until extraction. For each collection, a plant voucher sample was prepared and deposited in the herbarium at the National Botanic Gardens of Ireland. Headspace analysis and essential oil extraction from plant samples were performed within 1–3 days of collection and extracted oils were stored at 4–8 °C until GC-MS analysis.

2.2. Methods

From each collection, the plant part(s) of interest were separated, and any extraneous material removed. For each resulting sample, plant volatiles were analysed by headspace-GC-MS of the plant material, and essential oil extraction from fresh (not dried) material was carried out by two methods, CH and MAH. For convenience, the term 'volatiles' has been used for the component mixture sampled in headspace analysis, while 'essential oils' refers to the component mixtures extracted by MAH and CH. Sample details, methods of extraction and codes for the corresponding results are outlined in Table 1.

Table 1. *Myrica gale* collections, extraction methods and corresponding codes for results; MAH—microwave-assisted hydrodistillation; CH—Clevenger hydrodistillation.

Sample ID	Collection Date	Collection Season	Plant Part	Extraction Method	Essential Oil Code or Headspace Result Code
NTP0371	27 June 2022	Summer	Leaves	MAH	4M
NTP0371	27 June 2022	Summer	Leaves	CH	4C
NTP0371	27 June 2022	Summer	Leaves	Direct (headspace)	4H
NTP0379A	3 October 2022	Autumn	Leaves	MAH	12M
NTP0379A	3 October 2022	Autumn	Leaves	CH	12C
NTP0379A	3 October 2022	Autumn	Leaves	Direct (headspace)	12H
NTP0379C	3 October 2022	Autumn	Branches	MAH	14M
NTP0379C	3 October 2022	Autumn	Branches	CH	14C
NTP0379C	3 October 2022	Autumn	Branches	Direct (headspace)	14H
NTP0380	21 October 2022	Autumn	Fruit	MAH	15M
NTP0380	21 October 2022	Autumn	Fruit	CH	15C
NTP0380	21 October 2022	Autumn	Fruit	Direct (headspace)	15H

2.2.1. Microwave-Assisted Hydrodistillation

MAH was carried out using the ETHOS X Microwave Extraction System (Milestone Srl, Sorisole, Italy). Leaves (635 g) and fruit (398 g) were extracted whole, while branches (233 g) were coarsely chopped. A moistening pre-treatment was carried out on the plant material by mixing with Milli-Q® purified water (2–3 mL/g of material) and allowing it to soak for 30 min prior to extraction. The instrument extraction programme was a heating step at 600 W for 15 min to heat the sample to reflux, followed by a holding step of 500 W for 40 min. Extracted oil accumulated on top of the water layer in the Clevenger arm and was drained from the collection burette through a stopcock with water from the Clevenger arm. The collection burette was rinsed with diethyl ether to collect the trace oil. The oil was extracted into diethyl ether and the ether layers combined and dried over anhydrous sodium sulphate. The diethyl ether was allowed to evaporate at room temperature and the essential oils were then stored at 4–8 °C.

2.2.2. Clevenger Hydrodistillation

The extraction was performed using a conventional extraction assembly comprising a Clevenger apparatus and a heating mantle. To extract the essential oil, fresh (not dried) plant material was suspended in Milli-Q® purified water (6 mL/g) and heated to reflux. Leaves (633 g) and fruit (376 g) were extracted whole, while branches (576 g) were coarsely chopped. After reflux for 3 h and cooling at room temperature for 30 min, the extracted oil accumulated on top of the water layer in the Clevenger arm and was drained from the collection burette through a stopcock with water from the Clevenger arm. The collection burette was rinsed with diethyl ether to collect the trace oil. The oil was extracted into diethyl ether and the ether layers combined and dried over anhydrous sodium sulphate. The diethyl ether was allowed to evaporate at room temperature and the essential oils were then stored at 4–8 °C.

2.2.3. Gas Chromatography–Mass Spectrometry (GC-MS)

A Shimadzu GC2010 gas chromatograph, connected to an autosampler AOC-5000 with direct injection and headspace modules and a Shimadzu QP2010SE mass spectrometer, operating at 70 eV, was used to analyse the plant volatiles and extracted essential oils. The components were separated on a 5% phenylmethylsiloxane capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 µm). Essential oil samples extracted by CH and MAH were prepared at a dilution of 10 µL of essential oil in 990 µL of hexane (LC-MS grade) and filtered through a 0.22 µm filter prior to GC-MS analysis. The column temperature method comprised four ramps: the first ramp involved raising the oven temperature from 40 °C to 85 °C at a heating rate of 7 °C/min and holding it isothermally for 2 min; the second ramp involved the oven temperature rising from 85 °C to 95 °C at a heating rate of 2 °C/min and holding it isothermally for 1 min; then, a third ramp increased the oven temperature from 95 °C to 200 °C at a rate of 4 °C/min, holding isothermally for 4 min; and, finally, the fourth ramp involved the oven temperature rising from 200 °C to 300 °C at a rate of 15 °C/min and holding it isothermally for 3 min; injector temperature, 250 °C; carrier gas, He (1.0 mL/min) with a split ratio of 5:1; interface temperature, 310 °C; scanning mass range of 50–550 amu.

For headspace measurements, sampling conditions included sample incubation in the headspace chamber at 95 °C for 15 min with continuous agitation at 250 rpm and syringe temperature at 110 °C. The column temperature method used was a single ramp, wherein the oven temperature, initially set at 40 °C for 1 min, was raised to 290 °C at a rate of 5 °C/min and held isothermally for 5 min; carrier gas, He (1.0 mL/min) with a split ratio of 20:1; interface temperature, 300 °C; and mass spectrometer scanning at a mass range of 40–750 amu. Fresh (not dried) plant material (3–5 g) was used for headspace-GC-MS analysis. The leaves and fruits were examined whole, while the branches were coarsely chopped immediately before sample analysis.

2.2.4. Data Analysis and Component Identification

Data analysis was performed on the Global Natural Products Social Molecular Networking (GNPS) online server via the GC-MS EI Data Analysis module [https://gnps.ucsd.edu (accessed on 31 October 2022 and thereafter); [16]]. Data were analysed as two separate datasets for plant volatiles and extracted essential oils due to different instrument methods. All the raw data files were converted from the .qgd format to the .cdf format in the Shimadzu GCMSsolution software and then submitted to the online server for processing and data deconvolution. The deconvoluted data, along with other input files (carbon marker file, metadata, library), were then fed to the server for the library search and molecular networking.

Plant tissue volatiles and components of the extracted essential oils were identified based on Kovat's retention indices (RI) and MS fragmentation patterns and comparison of both with the NIST Standard Reference Database (webbook.nist.gov), NIST 17, and other open-access GC-MS libraries available at the website of GNPS. For RI calculation, a series of n-alkanes in a standard solution (C8–C20) was injected and run in GC-MS with the same column method as used for essential oil samples. For headspace analysis, alkane standards were injected as liquid samples, but run using the column conditions used for headspace analysis. The RI was calculated by the server using the formula Ix = 100n + 100 $(t_x - t_n)/(t_{n+1} - t_n)$, where Ix is the RI value for compound X, t_n and t_{n+1} are the retention times of the hydrocarbons from the n-alkane standard solutions eluted before and after X from the column and t_x is the retention time of X [17]. For the quantification of compounds, the samples were run in duplicate and quantitative analysis was performed by peak area normalisation and calculation of the mean value of two injections for each compound per sample [18].

2.2.5. Multivariate Data Analysis and Molecular Networking

The GC-MS raw data obtained were deconvoluted and processed further to generate molecular networks using the Library Search/Molecular Networking GC workflow at GNPS [https://gnps.ucsd.edu (accessed on 31 October 2022 and thereafter); [16]]. The data generated after deconvolution were used for targeted multivariate data analysis employing the orthogonal partial least square–discriminant analysis (OPLS-DA) model in SIMCA 16.0 to compare the essential oil profiles based on extraction method and plant morphology. For molecular networking, edges were set at cosine score >0.7 and minimum six peaks per match. The number of edges allowed per node was set to 10 and the maximum size of a network was set to 100. Cytoscape version 3.9.1 was used for visualising molecular networks.

3. Results and Discussion

Aerial parts of bog myrtle were collected from Clonlisk during summer and autumn and processed to study volatiles via headspace-GC-MS, and essential oils extracted by MAH and CH via GC-MS analysis. In brief, four samples including leaves collected in the summer (NTP0371, sample code 4) and leaves (NTP0379A, sample code 12), branches (NTP0379C, sample code 14) and fruit (NTP0380, sample code 15) collected in the autumn were analysed in duplicate by (a) headspace-GC-MS, (b) CH and GC-MS and (c) MAH and GC-MS, to yield a total of 24 samples (Table 1). GC-MS data were analysed [18] and the area percentages of compounds obtained for duplicate samples after peak area normalisation were averaged, and their mean values are given in Table 2 for 12 samples. The two hydrodistillation methods require the removal of trace water from the extracted oil. Yields of essential oil are often low, precluding water removal with a drying reagent without first extracting into a suitable solvent in the laboratory-scale work-up [19]. The yield of essential oil by CH of the summer leaf collection was 0.02%, while yields of 0.13%, 0.21% and 1.54% were obtained for the leaves, branches and fruit of the autumn collections, respectively. MAH yielded 0.02%, 0.08% and 0.45% for leaves, branches and fruit, respectively. While the statistical comparison of yields between methods or plant parts was not part of this study, it was evident that the fruit was the highest yielding plant part. In the MAH of essential oils, there are several parameters affecting the extraction efficiency, including the nature and quantity of material, the quantity of water, the microwave power and the extraction time. The initial energy input of 600 W for 15 min was selected to bring the infrared temperature reading of the plant material to 94–98 °C and the vessel contents to reflux, as recommended by the instrument manufacturer. Reducing the energy input to 500 W maintained the desired temperature and adequate reflux over 40 min, a typical runtime for the instrument for essential oil extraction. The parameters were not optimised for extraction efficiency. Essential oil extraction times by CH are typically several hours. In the case of *M. gale*, Wawrzyńczak et al., 2019, extracted dried plant material by hydrodistillation for 4 h in a study on the composition of leaf and flower essential oil [20], and Sylvestre et al., 2005, found that increasing the hydrodistillation time from 30 min to 60 min enriched the sesquiterpene content of the essential oil obtained from freshly harvested leaves [21]. Based on the available literature and previous experience in extracting the material in the lab, the CH extractions were refluxed for 3 h.

Table 2. Table showing composition profiles of essential oils extracted by Clevenger and microwave-assisted hydrodistillation and volatiles through headspace from *M. gale*; values denote averaged peak area % calculated from duplicates; RI*—retention indices calculated experimentally based on retention time of n-alkane standard mix (C8–C20); n.d.—not detected; tr—trace (<0.05%); A and S in superscript denote autumn and summer collection seasons, respectively; OC—other compounds, MH—monoterpene hydrocarbon, OM—oxygenated monoterpene, SH—sesquiterpene hydrocarbon and OS—oxygenated sesquiterpene.

			Clevenger				Microwave				Headspace				
			Leaf ^{S}	Leaf ^{A}	Branch ^{A}	Fruit ^{A}	Leaf ^{S}	Leaf ^{A}	Branch ^{A}	Fruit ^{A}	Leaf ^{S}	Leaf ^{A}	Branch ^{A}	Fruit ^{A}	
S.No	**Compound Name**	**RI***	**4C**	**12C**	**14C**	**15C**	**4M**	**12M**	**14M**	**15M**	**4H**	**12H**	**14H**	**15H**	**Compound Type**
1	E-hex-3-enol	853	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.07	0.64	n.d.	n.d.	OC
2	hexanol	869	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	OC
3	α-thujene	927	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.48	0.47	0.52	0.96	MH
4	α-pinene	937	6.04	28.16	35.03	30.69	17.37	19.93	25.47	22.82	48.56	70.45	60.80	52.96	MH
5	camphene	946	0.24	1.14	1.06	1.29	0.69	1.04	1.62	1.22	1.35	2.03	2.47	3.36	MH
6	benzaldehyde	957	2.09	0.61	n.d.	n.d.	0.21	0.07	n.d.	n.d.	0.23	n.d.	n.d.	n.d.	OC
7	β-pinene	974	1.04	2.86	3.67	3.81	2.78	3.26	5.13	3.19	n.d.	n.d.	n.d.	n.d.	MH
8	sabinene	976	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.17	3.96	7.17	7.49	MH
9	β-myrcene	987	1.67	1.81	n.d.	2.91	2.45	2.13	2.84	3.32	4.34	1.98	3.10	8.42	MH
10	α-phellandrene	1004	2.33	6.00	13.95	10.74	5.47	9.05	14.55	15.61	8.39	3.87	15.05	n.d.	MH
11	δ-3-carene	1015	0.23	0.73	0.95	1.89	0.54	1.22	2.58	2.81	n.d.	0.50	0.56	4.37	MH
12	*p*-cymene	1022	3.22	6.17	1.14	1.74	6.62	4.93	1.28	2.42	n.d.	n.d.	n.d.	n.d.	MH
13	limonene	1026	5.88	14.09	6.37	12.67	14.90	12.97	6.19	2.27	20.73	10.79	8.67	n.d.	MH
14	eucalyptol	1030	25.32	16.05	33.80	19.36	9.36	10.72	13.09	28.02	8.12	3.51	0.61	16.01	OM
15	cis-β-ocimene	1034	0.52	0.61	n.d.	0.59	1.26	1.21	2.67	1.51	n.d.	n.d.	n.d.	n.d.	MH
16	β-trans-ocimene	1047	0.25	0.32	n.d.	0.26	0.56	0.53	0.83	0.60	n.d.	0.22	0.06	0.89	MH
17	γ-terpinene	1055	1.01	2.03	0.55	3.32	1.70	3.29	4.17	4.75	n.d.	1.31	0.58	5.38	MH
18	acetophenone	1067	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	OC
19	α-terpinolene	1087	0.34	0.43	n.d.	0.91	0.44	0.68	1.28	1.30	n.d.	n.d.	n.d.	n.d.	MH
20	linalool	1099	1.20	n.d.	n.d.	n.d.	0.25	0.18	0.15	0.23	n.d.	0.15	0.30	n.d.	OM
21	nonanal	1105	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr	tr	OC
22	allo-ocimene	1130	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr	n.d.	n.d.	tr	MH
23	borneol	1161	1.87	0.60	n.d.	0.37	0.23	0.20	0.18	0.28	n.d.	n.d.	n.d.	0.05	OM
24	4-terpineol	1173	6.72	3.17	0.08	2.51	1.35	1.36	1.51	2.89	n.d.	n.d.	n.d.	n.d.	OM
25	α-terpineol	1188	4.58	1.90	n.d.	1.34	0.72	0.60	0.37	0.85	n.d.	n.d.	n.d.	n.d.	OM
26	methyl salicylate	1192	0.06	0.12	n.d.	n.d.	0.10	0.09	tr	n.d.	0.11	n.d.	n.d.	n.d.	OC
27	α-fenchyl acetate	1220	0.08	n.d.	n.d.	n.d.	tr	0.10	0.11	0.07	n.d.	n.d.	n.d.	n.d.	OM
28	citronellol	1229	0.94	0.51	n.d.	n.d.	0.29	0.49	0.05	n.d.	n.d.	n.d.	n.d.	n.d.	OM
29	bornyl acetate	1287	0.67	0.80	n.d.	0.16	0.57	1.05	1.05	0.50	n.d.	n.d.	n.d.	n.d.	OM
30	2-undecanone	1293	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.16	n.d.	n.d.	n.d.	n.d.	OC
31	methyl geranate	1326	n.d.	n.d.	n.d.	n.d.	tr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	OM
32	carveol acetate	1338	tr	tr	n.d.	tr	tr	tr	tr	tr	n.d.	n.d.	n.d.	n.d.	OM
33	α-terpinyl acetate	1349	1.51	1.80	tr	1.75	1.58	3.26	3.38	2.53	n.d.	n.d.	n.d.	n.d.	OM
34	citronellol acetate	1354	0.13	0.61	n.d.	0.61	0.28	0.82	0.27	0.15	n.d.	n.d.	n.d.	n.d.	OM
35	eugenol	1356	1.84	0.22	n.d.	n.d.	0.18	0.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	OC

Table 2. *Cont.*

			Clevenger				Microwave				Headspace				
			Leaf ^{S}	Leaf ^{A}	Branch ^{A}	Fruit ^{A}	Leaf ^{S}	Leaf ^{A}	Branch ^{A}	Fruit ^{A}	Leaf ^{S}	Leaf ^{A}	Branch ^{A}	Fruit ^{A}	
S.No	**Compound Name**	**RI***	**4C**	**12C**	**14C**	**15C**	**4M**	**12M**	**14M**	**15M**	**4H**	**12H**	**14H**	**15H**	**Compound Type**
36	neryl acetate	1365	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	OM
37	α-copaene	1375	0.17	0.20	0.06	n.d.	0.68	0.83	0.26	0.08	n.d.	n.d.	n.d.	n.d.	SH
38	methyl cinnamate	1381	0.67	0.34	n.d.	n.d.	0.21	0.25	0.15	0.09	n.d.	n.d.	n.d.	n.d.	OC
39	geranyl acetate	1383	tr	0.32	n.d.	n.d.	0.15	0.66	0.25	n.d.	n.d.	n.d.	n.d.	n.d.	OM
40	tetradecene	1394	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	tr	n.d.	n.d.	n.d.	OC
41	E-β-damascone	1413	tr	tr	n.d.	n.d.	tr	tr	tr	n.d.	n.d.	n.d.	n.d.	n.d.	OC
42	β-caryophyllene	1418	0.74	0.12	tr	n.d.	1.45	0.76	0.77	0.13	n.d.	n.d.	n.d.	n.d.	SH
43	γ-elemene	1433	4.95	0.26	0.17	0.12	7.43	1.55	1.40	0.19	n.d.	n.d.	n.d.	n.d.	SH
44	β-gurjunene	1443	0.15	0.05	n.d.	n.d.	0.21	0.21	0.14	0.07	n.d.	n.d.	n.d.	n.d.	SH
45	humulene	1453	tr	tr	n.d.	tr	tr	tr	tr	tr	n.d.	n.d.	n.d.	n.d.	SH
46	2,6-di-tert-butyl-1,4-benzoquinone	1465	0.52	tr	n.d.	tr	tr	tr	tr	tr	n.d.	n.d.	n.d.	n.d.	OC
47	β-selinene	1476	0.20	0.08	n.d.	0.08	0.48	0.66	0.13	0.09	n.d.	n.d.	n.d.	n.d.	SH
48	γ-muurolene	1480	n.d.	n.d.	n.d.	n.d.	0.06	0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	SH
49	germacrene-D	1485	1.27	0.21	n.d.	0.33	1.64	1.91	0.82	0.32	n.d.	n.d.	n.d.	n.d.	SH
50	β-guaiene	1494	0.82	0.39	0.06	0.24	1.89	1.43	0.61	0.30	n.d.	n.d.	n.d.	n.d.	SH
51	α-muurolene	1499	0.10	0.08	n.d.	n.d.	0.27	0.34	0.12	n.d.	n.d.	n.d.	n.d.	n.d.	SH
52	δ-guaiene	1507	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06	n.d.	n.d.	n.d.	n.d.	n.d.	SH
53	δ-cadinene	1524	1.41	0.73	0.21	0.15	4.12	3.59	1.52	0.34	0.36	n.d.	tr	n.d.	SH
54	germacrene B	1557	5.94	0.26	0.57	0.27	6.74	1.67	2.41	0.24	n.d.	n.d.	n.d.	n.d.	SH
55	β-elemenone	1604	4.54	2.05	n.d.	0.50	4.28	2.29	0.82	0.14	n.d.	n.d.	n.d.	n.d.	OS
56	germacrone	1696	8.62	4.08	1.93	1.33	0.34	4.40	1.59	0.34	n.d.	n.d.	n.d.	n.d.	OS
57	octyl octanoate	1778	n.d.	tr	tr	n.d.	tr	tr	tr	n.d.	n.d.	n.d.	n.d.	n.d.	OC
58	6,10,14-trimethyl-pentadecan-2-one	1844	tr	tr	tr	n.d.	tr	0.07	tr	n.d.	n.d.	n.d.	n.d.	n.d.	OS
	Monoterpene hydrocarbons		22.77	64.35	62.70	70.81	54.78	60.25	68.63	61.82	90.01	95.58	98.98	83.83	
	Oxygenated monoterpenes		43.02	25.44	33.91	26.09	14.66	18.77	20.17	35.53	8.12	3.65	0.91	16.06	
	Sesquiterpene hydrocarbons		15.76	2.38	1.10	1.18	24.99	13.01	8.24	1.75	0.36	0.00	0.03	0.00	
	Oxygenated sesquiterpenes		13.16	6.13	1.93	1.82	4.63	6.77	2.41	0.48	0.00	0.00	0.00	0.00	
	Other compounds		5.21	1.62	0.00	0.00	0.85	1.13	0.42	0.25	1.42	0.64	0.00	0.00	
	Total		99.92	99.92	99.64	99.90	99.90	99.93	99.86	99.84	99.92	99.87	99.92	99.89	

3.1. Chemical Profiles of Essential Oils and Volatiles from Plant Tissues

The chemical profiles obtained by the headspace-GC-MS analysis of plant material and by the GC-MS analysis of the essential oils extracted by CH and MAH are given in Table 2. A total of 58 components have been identified in the aerial parts of bog myrtle, comprising 15 monoterpene hydrocarbons, 14 oxygenated monoterpenes, 13 sesquiterpene hydrocarbons, 3 oxygenated sesquiterpenes and 13 other compounds, including carbonyl compounds, unsaturated alcohols and hydrocarbons. The components present at less than 0.05% are mentioned as trace (tr.). A heatmap (Figure 1) was generated to compare the chemical profiles. To visualise major components, heatmap entries were restricted to the components present in at least one sample at >1% of content. Major components in *M. gale* were found to be α-pinene (6.04–70.45%), eucalyptol (0.61–33.80%), limonene (2.27–20.73%) and α-phellandrene (2.33–15.61%), which is in accordance with the literature [10–12]. These components were present in every essential oil examined, from leaves, branches or fruit, obtained by both oil extraction methods. These metabolites have been reported in the literature as inhibitors of acetylcholinesterase [22–25] and indeed we have reported Irish *Myrica gale* essential oil to be an effective inhibitor of acetylcholinesterase in vitro, albeit with low potency [26]. This activity is observed for the essential oils of all aerial parts of *Myrica gale*. Many insecticides, both natural and synthetic, exert their action by disrupting the insect's nervous system transmission through modulation of cholinesterases, causing paralysis and death. The acetylcholinesterase activity of *Myrica gale* provides support for the plant's numerous traditional applications in the control of insects and parasitic worms [26].

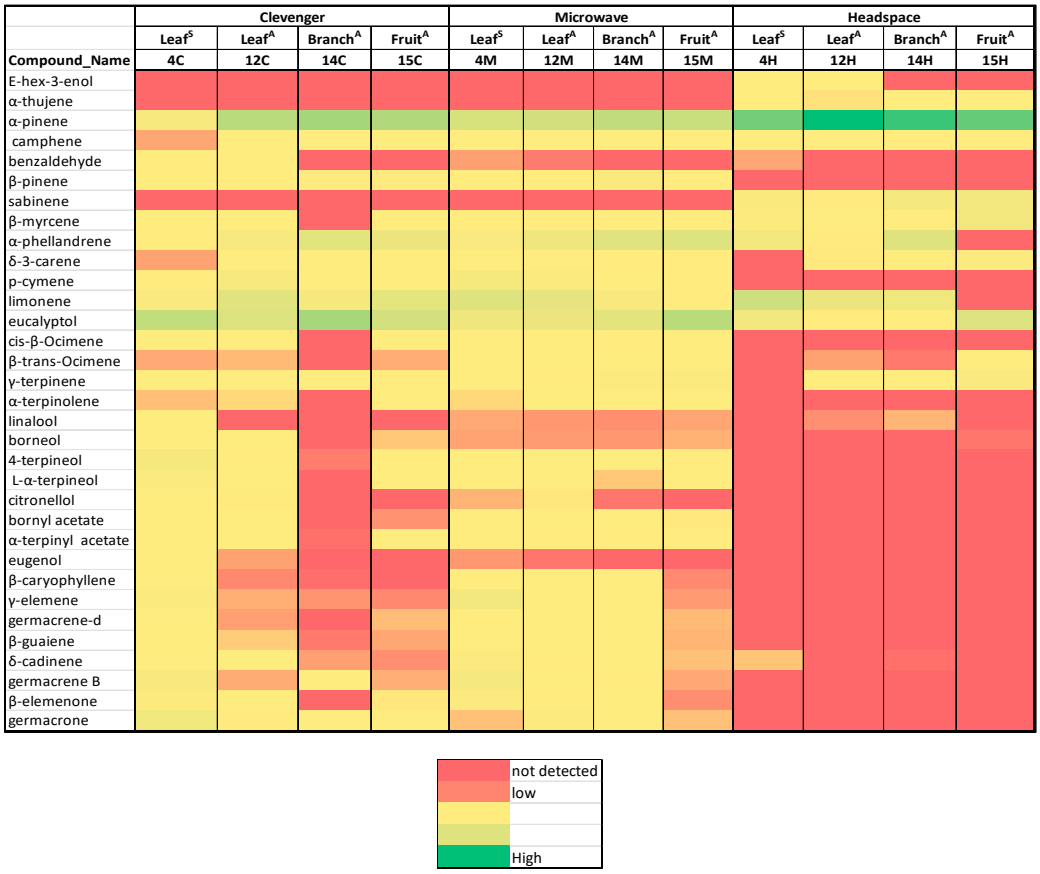

Figure 1. Heatmap showing the relative compositions of major components in extracted essential oils and volatiles from different plant parts of bog myrtle; A and S in superscript denote autumn and summer collection seasons, respectively; components with peak area percentage > 1% in at least one sample have been included.

The chemical profiles were further evaluated using multivariate data analysis to compare the influence of the extraction method and plant morphology. In the beginning, principal component analysis was applied as an untargeted model on the GC-MS data to see any grouping of data points (score plot not shown). When the clustering of data points was observed, the OPLS-DA model was applied as a targeted analysis to observe any discrimination in the data points based on extraction methods and plant tissue. For OPLS-DA modelling, GC-MS data were taken as the X variable, while extraction methods and plant parts were taken as the Y variables.

3.2. Comparison of Extraction Methods

Application of the OPLS-DA model to determine the variability in the composition profiles with respect to the extraction method generated a score plot (Figure 2A) with R2x(cum), R2y(cum) and Q2(cum) values of 0.878, 0.967 and 0.896, respectively. R2x(cum) and R2y(cum) indicate the cumulative fraction of the variance in the X and Y variables depicted by the model, respectively, while Q2(cum) denotes the cumulative predictive competence of the model. In other words, R2x shows that the model fits well to the data and Q2 > 0.5 shows the high predictability of the model [27]. The score plot (Figure 2A) exhibits three significantly separated groups—blue, green and yellow—with clustered data points. Each data point on the plot represents a sample, and clustering refers to the similarity in the datasets that could be qualitative and/or quantitative. Blue data points represent chemical profiles of CH extracted oils from all plant parts (leaf, branch and fruit). Similarly, green corresponds to MAH and yellow corresponds to the chemical profiles of volatiles released during headspace analysis. Well-separated groups indicate the influence of the extraction conditions on the volatile components isolated from the bog myrtle samples. This is corroborated by the heatmap visualisation (Figure 2), where the three treatments (CH, MAH and headspace) display a similar colour pattern (with the exception of 14C) within each treatment among different plant parts. Unlike CH and MAH, direct headspace sampling accessed mainly monoterpene hydrocarbons, including α-pinene, α-thujene, sabinene, eucalyptol, β-myrcene and camphene, regardless of plant part, though volatiles from fruit lacked α-phellandrene and limonene, observed in high amounts in the leaf and branch. In headspace analysis, the plant parts are directly exposed without any pre-treatment and the volatiles are released from the plant matrix at a high temperature and pass to the instrument for detection [28]. On the other hand, CH and MAH employ steam and hot water to extract the molecules from the plant matrix and carry over less volatile components, that are not detected by headspace. Disadvantages of CH are the long heating and extraction times required and the potential for chemical changes to terpene components. MAH offers reduced extraction times, i.e., 40 min in comparison to 3 h reflux, thus utilising less energy, and it is a greener, alternative extraction method. Additionally, microwave heating of the water within the samples distends its cells, leading to rupture of the glands and oleiferous receptacles [29]. This can result in the extraction of greater quantities of specific components and the extraction of larger-molecular-weight volatiles such as sesquiterpenes. A high temperature makes the plant tissue softer and more accessible to steam, which aids in extracting both monoterpenes and sesquiterpenes [30]. It was observed that direct headspace analysis detected much higher relative quantities of α-pinene than was detected in the hydrodistilled oils from all plant parts (Table 2). This is because the chromatograms obtained for essential oils were more crowded compared to their headspace counterparts (Figure 3). As the data were normalised for comparison, α-pinene, being a principal component in the headspace sample chromatograms, gained a higher area percentage owing to the low number of peaks compared to CH and MAH.

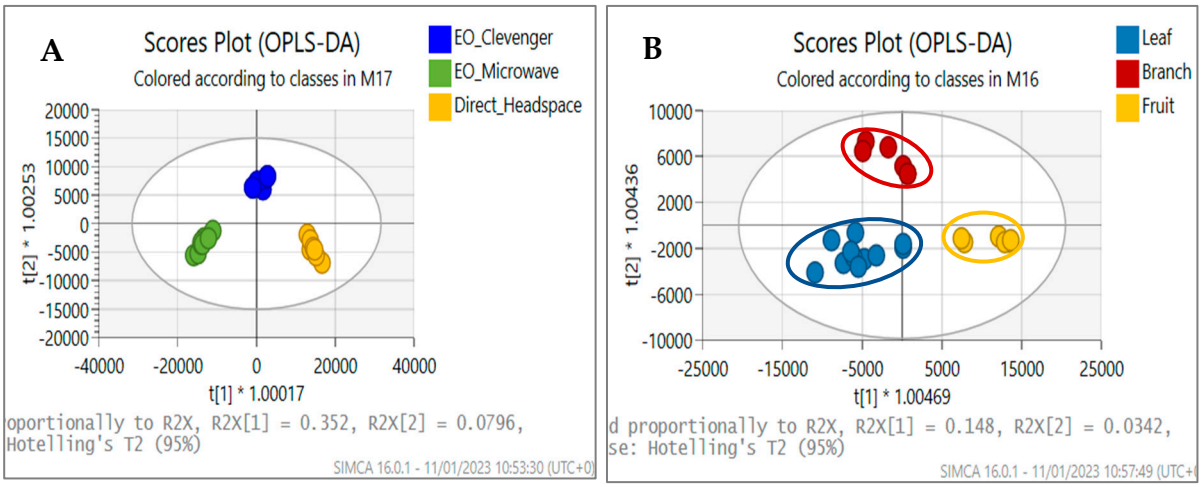

Figure 2. Orthogonal partial least square–discriminant analysis (OPLS–DA) score plots generated from GC-MS data of the essential oils and volatiles from *M. gale*. (**A**) Distribution of data points based on the treatment, i.e., blue and green denote essential oil (EO) samples obtained by Clevenger and microwave–assisted hydrodistillation, respectively, while yellow denotes volatiles produced by headspace; $R^2x(cum) = 0.878$, $R^2y(cum) = 0.967$, $Q^2(cum) = 0.896$. (**B**) Distribution of data points based on plant parts, i.e., blue, red and yellow denote leaf, branch and fruit, respectively, $R^2x(cum) = 0.937$, $R^2y(cum) = 0.639$, $Q^2(cum) = 0.377$.

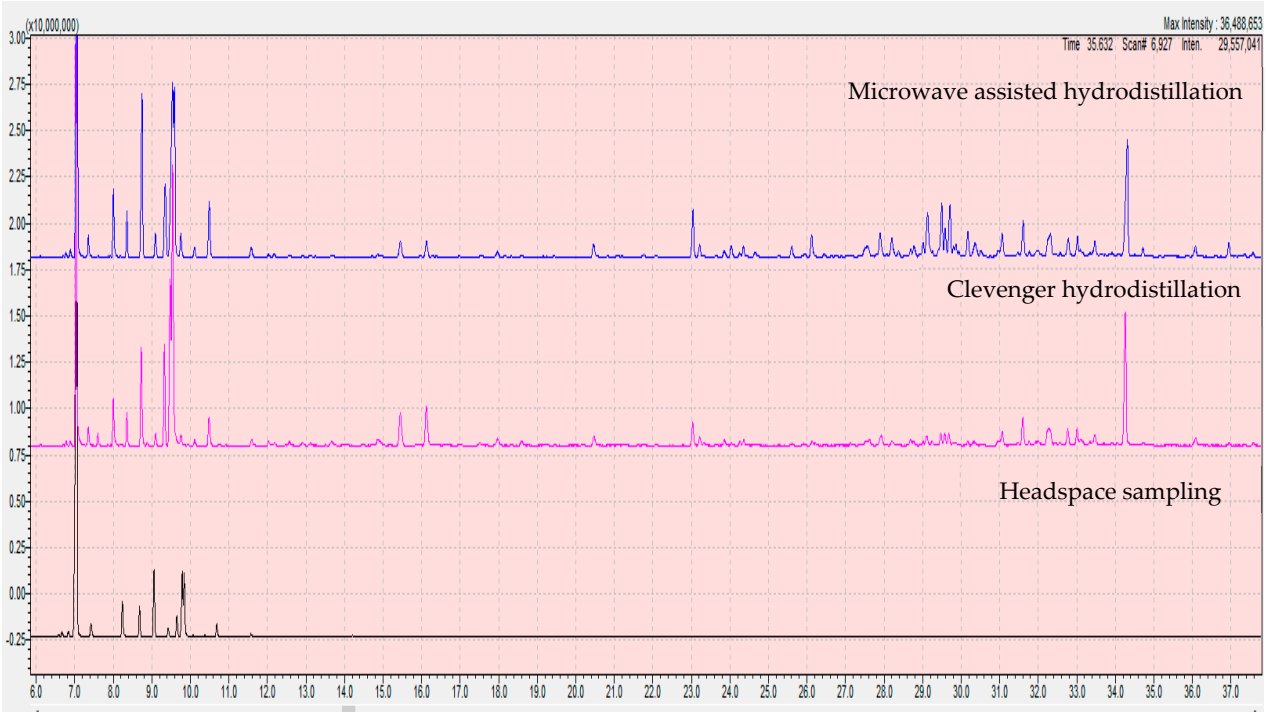

Figure 3. GC-MS chromatograms of leaves (sample code 12) collected in autumn season; blue and pink chromatograms denote essential oils extracted by microwave and Clevenger hydrodistillation, respectively, while black represents the headspace-GC-MS chromatogram.

The chemical profiles of essential oils isolated by CH and MAH are found to be qualitatively similar but quantitatively somewhat diverse. For instance, MAH extracted relatively similar or higher amounts of monoterpene hydrocarbons—α-phellandrene [4M (5.47%) > 4C (2.33%); 12M (9.05%) > 12C (6.00%); 14M (14.55%)~14C (13.95%); 15M (15.61%) > 15C (10.74%)]; γ-terpinene [4M (1.70%)~4C (1.01%); 12M (3.29%) > 12C (2.03%); 14M

(4.17%) > 14C (0.55%); 15M (4.75%) > 15C (3.32%)]; δ-3-carene [4M (0.54%)~4C (0.23%); 12M (1.22%)~12C (0.73%); 14M (2.58%) > 14C (0.95%); 15M (2.81%) > 15C (1.89%)]; β-pinene [4M (2.78%) > 4C (1.04%); 12M (3.26%) > 12C (2.86%); 14M (5.13%) > 14C (3.67%); 15M (3.19%)~15C (3.81%)]; α-terpinolene [4M (0.44%)~4C (0.34%); 12M (0.68%)~12C (0.43%); 14M (1.28%) > 14C (not detected); 15M (1.30%)~15C (0.91%)]; cis-β-ocimene [4M (1.26%) > 4C (0.52%); 12M (1.21%) > 12C (0.61%); 14M (2.67%) > 14C (not detected); 15M (1.51%) > 15C (0.59%)]; β-trans-ocimene [4M (0.56%)~4C (0.25%); 12M (0.53%)~12C (0.32%); 14M (0.83%) > 14C (not detected); 15M (0.60%)~15C (0.26%)]; and sesquiterpene hydrocarbons—γ-elemene [4M (7.43%) > 4C (4.95%); 12M (1.55%) > 12C (0.26%); 14M (1.40%) > 14C (0.17%); 15M (0.19%)~15C (0.12%)]; β-guaiene [4M (1.89%) > 4C (0.82%); 12M (1.43%) > 12C (0.39%); 14M (0.61%)~14C (0.06%); 15M (0.30%)~15C (0.24%)]; δ-cadinene [4M (4.12%) > 4C (1.41%); 12M (3.59%) > 12C (0.73%); 14M (1.52%) > 14C (0.21%); 15M (0.34%)~15C (0.15%)]—over CH per plant part, though some of the components were present in minimal amounts. The pattern was reversed for their oxygenated monoterpenes, i.e., eucalyptol [4M (9.36%) < 4C (25.32%); 12M (10.72%) < 12C (16.05%); 14M (13.09%) < 14C (33.80%); except fruit, 15M (28.02%) > 15C (19.36%)]; 4-terpineol [4M (1.35%) < 4C (6.72%); 12M (1.36%) < 12C (3.17%); except branch, 14M (1.51%) > 14C (0.08%); 15M (2.89%)~15C (2.51%)]; germacrone [4M (0.34%) < 4C (8.62%); 12M (4.40%)~12C (4.08%); 14M (1.59%)~14C (1.93%); 15M (0.34%) < 15C (1.33%)]. Moreover, CH furnished higher amounts of α-pinene over MAH for all autumn samples but not leaves from the summer collection, where it was 6.04% compared to 17.37% extracted by MAH. To summarise, MAH extracted relatively higher amounts of monoterpene (54.78%) and sesquiterpene hydrocarbons (24.98%) compared to CH which extracted thethe former at 22.78% and the latter at 15.77%. Additionally, CH yielded higher amounts of oxygenated monoterpenes than MAH for the leaf from summer [4C (43.02%) > 4M (14.66%)]; leaf from autumn [12C (25.44%) > 12M (18.77%)]; and branches [14C (33.91%) > 14M (20.17%)]. Overall, the CH and MAH extraction processes resulted in sesquiterpene enrichment compared to the headspace sampling technique. MAH facilitated the enrichment of monoterpene and sesquiterpene hydrocarbons, while CH furnished higher content of oxygenated derivatives. While hydrodistillation is suited to laboratory-scale applications, it should be considered that steam distillation is often employed on an industrial scale. Wawrzyńczak et al., 2021, reported that the compositions of *M. gale* leaf and flower essential oils obtained by industrial steam distillation were largely consistent with the compositions of essential oils obtained by the hydrodistillation of material of the same origin, but with quantitative differences. Limonene, α-pinene and *p*-cymene were major components of the steam-distilled oils, which were mostly composed of monoterpene and sesquiterpene hydrocarbons. However, the content of oxygenated monoterpenes, such as eucalyptol, was much less in the steam-distilled oils compared to oils obtained by laboratory-scale hydrodistillation [13].

3.3. Comparison of Plant Tissues and Collection Season

An OPLS-DA model to determine the variability in composition profile with respect to plant morphology revealed R2x(cum), R2y(cum) and Q2(cum) values of 0.937, 0.639 and 0.377, respectively. In the score plot (Figure 2B), blue, red and yellow data points denote the chemical profiles of volatiles and essential oils from the leaf, branch and fruit, respectively. The plot displays scattered data points within each ring, which refers to the variability in the chemical profiles within the same tissue.

Branch: The cluster of red data points denotes all the samples from the branch extracted by CH, MAH and headspace. Unlike Figure 2A, the cluster is not tight and the lack of spacing between the data points reveals little variability in the chemical profiles. It is evident from the heatmap (Figure 2) that the essential oil composition of branches from CH (14C) is closer to headspace (14H) than MAH (14M), as both techniques have extracted monoterpenes as major components. Moreover, 14C contains α-pinene (35.03%), eucalyptol (33.8%), α-phellandrene (13.95%), limonene (6.37%) and β-pinene (3.67%) as major components, followed by camphene (1.06%), *p*-cymene (1.14%), germacrone (1.93%)

and other components in minimal amounts, while headspace sampling furnished α-pinene (60.80%), α-phellandrene (15.05%), limonene (8.67%) and camphene (2.47%), along with additional components—sabinene (7.17%) and β-myrcene (3.1%)—that were absent or below the detection limit for the CH extract. On the contrary, MAH was found to be the most efficient technique as it extracted most of the monoterpenes and sesquiterpenes, as well as their oxygenated counterparts.

Fruit: In the score plot, yellow data points are split into two clusters, with one cluster corresponding to the similar chemical profiles of fruit obtained for two techniques i.e., CH and MAH, whereas the other refers to headspace data that appear different (Table 2). The same is observed in the heatmap (Figure 2), where 15C and 15M have similar results but are different from those of 15H. As discussed before, headspace sampling focussed on monoterpenes—α-pinene (52.96%), eucalyptol (16.01%), β-myrcene (8.42%), sabinene (7.49%), γ-terpinene (5.38%), δ-3-carene (4.37%) and camphene (3.36%). On the contrary, CH and MAH were able to isolate monoterpene and sesquiterpenes, with MAH furnishing relatively higher amounts (Table 2).

Leaf: In the score plot, blue data points collectively represent the leaf samples from the summer (sample 4) and autumn collections (sample 12). Following the trend, headspace data were different to CH and MAH, revealing only monoterpenes, with α-pinene (48.56% in 4H; 70.45% in 12H) as a principal component, followed by limonene (20.73% in 4H; 10.79% in 12H), α-phellandrene (8.39% in 4H; 3.89% in 12H), eucalyptol (8.12% in 4H; 3.51% in 12H), sabinene (5.17% in 4H; 3.96% in 12H), β-myrcene (4.34% in 4H; 1.98% in 12H), camphene (1.35% in 4H; 2.03% in 12H) and other components. CH and MAH revealed similar profiles, extracting monoterpenes and sesquiterpenes from leaves. Hence, it has been seen that data points obtained for the same plant tissue but using different extraction methods have greater disparity compared to the data points obtained for the same extraction method but from different plant parts. In other words, extraction methods have a higher influence on the chemical profiles than plant morphology.

Comparing the leaf samples from the summer and autumn collections using the CH technique, sample 4C from the summer, over 12C from the autumn, has higher amounts of oxygenated monoterpenes (43.02% over 25.44%), sesquiterpene hydrocarbons (15.76% over 2.38%), oxygenated sesquiterpenes (13.16% over 6.12%) and other compounds (5.21% over 1.62%), while 12C has higher monoterpene hydrocarbon content (64.35% over 22.78% in 4C). For MAH, monoterpene hydrocarbons (60.25% in 12M, 54.78% in 4M), oxygenated monoterpenes (18.77% in 12M, 14.66% in 4M) and oxygenated sesquiterpenes (6.77% in 12M, 4.63% in 4M) were higher in the autumn collection, while sesquiterpene hydrocarbons (13.01% in 12M, 24.98% in 4M) were higher in the summer collection. The headspace results of 4H and 12H appear similar, with 12H containing γ-terpinene, β-trans-ocimene, δ-3-carene and linalool as additional components.

3.4. Molecular Networking

Deconvoluted GC-MS data were processed for the library search and molecular networking on the GNPS platform. Molecular networks were generated based on spectral similarities between the components. Since the GC-MS method used for isolated essential oils was different from that of headspace-GC-MS analysis, two separate sets of data analyses were performed for extracted oils and volatiles, which generated separate networks. Figure 4 displays three molecular networks—4A and 4B correspond to the networks of monoterpenes and sesquiterpenes, respectively, reported from CH and MAH essential oils, while the network in 4C refers to the monoterpenes reported in volatiles in headspace sampling. The network does not include all the detected components, but rather those that have a biosynthetic relationship. The components (nodes) are connected based on the spectral similarity of a minimum of six peaks per MS spectrum, which refers to similar fragmentation patterns and hence the similar chemical structures. These compounds, with similar chemical structures in a plant tissue, are usually the products or precursors produced by the same biosynthetic pathways. For instance, α-terpinyl acetate (node 300)

in Figure 4A is not directly connected structurally to γ-terpinene (node 115), but rather via α-terpinolene (node 95). This is a remarkable advantage of the molecular networking approach, as the method checks and prevents the possibility of reporting false molecules from the plant that are not a part of its biosynthetic pathways [31]. Further, the number on each node is a cluster index that refers to an individual chemical compound. Each node displays a pie chart, comprising red, green and blue segments, which represents abundance of the component in the branch, leaf and fruit, respectively. For instance, node 300 represents α-terpinyl acetate and it is present in almost similar content in essential oils isolated from the branch (0.03–3.38%), fruit (1.75–2.53%) and leaf (1.51–3.26%). Similarly, the network of sesquiterpenes and their distribution within the plant tissue, are given in 4B. Overall, monoterpenes were found to be well distributed in all aerial parts, while sesquiterpenes were found in a relatively higher proportion in the leaves of bog myrtle (Figure 4B). The Figure 4C network was obtained for headspace analysis, which was able to extract only monoterpenes, as evidenced by the heatmap (Figure 2). Further, there are two nodes with cluster indexes 183 and 184 that refer to α-pinene. This is because the cluster index directly corresponds to the scan numbers, and both scans 183 and 184 belong to the peak of α-pinene. It is pertinent to mention that pie charts in every node do not represent the absolute but only a relative distribution of components within the given attribute (plant part) based on the area abundance of its peak in the feature table, which is not normalised. In other words, the pie charts give an approximation of their relative distribution.

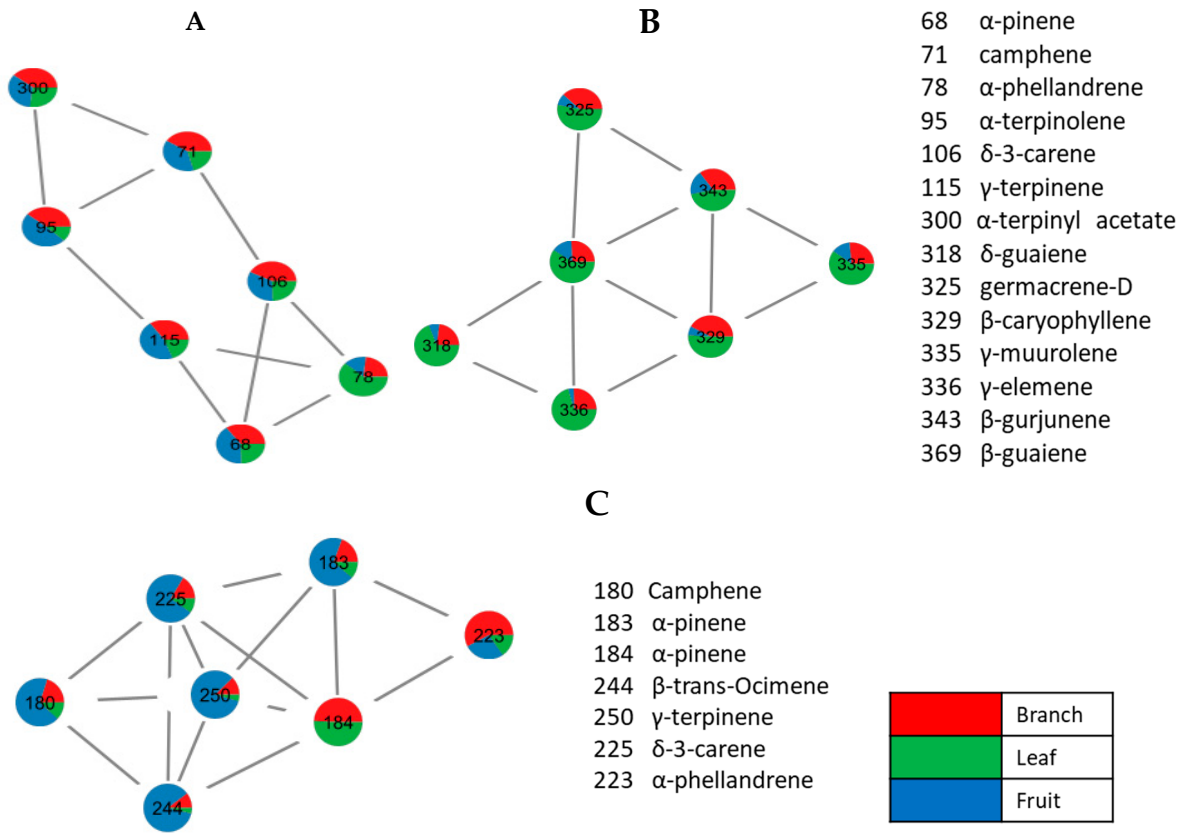

Figure 4. Molecular networks of monoterpenes and sesquiterpenes predicted based on spectral similarity. Each node represents a compound and the pie chart within each node, showing red, green and blue segments, represent its relative abundance determined in branch, leaf and fruit, respectively. (**A**) Molecular network of monoterpenes obtained for essential oils extracted by microwave and Clevenger hydrodistillation; (**B**) molecular network of sesquiterpenes obtained for extracted essential oils using microwave and Clevenger hydrodistillation; (**C**) molecular network of monoterpenes obtained for volatiles produced during headspace sampling.

4. Conclusions

Sampling techniques and extraction methods have a greater influence on the chemical profiles of volatiles and essential oils than the plant morphology and harvesting season. In these experiments, headspace sampling was only able to extract and detect monoterpenes, while the CH and MAH methods gave additional sesquiterpene enrichment. Amongst conventional and modern extraction techniques, both were successful in producing similar essential oil profiles qualitatively. However, MAH yielded higher content of monoterpene and sesquiterpene hydrocarbons, while CH could access more of the oxygenated counterparts. OPLS-DA was proven to be a robust model that illustrated well the relationships between the data. Molecular networking was found to be an effective tool in utilising the spectral fragmentation information of secondary metabolites and linking those molecules that are connected via biosynthetic pathways. While the reported anticholinesterase activity of *Myrica gale* essential oil can be attributed to several monoterpene inhibitor components, there may be synergistic and/or antagonistic effects between them and other minor components. Components with known anticholinesterase activity support the traditional uses of *Myrica gale* in repelling insects. Further work exploring the influence of extraction techniques on the anti-cholinesterase activity of different plant parts is in progress. The potential applications of these essential oils, or indeed an essential oil of the total aerial parts of *Myrica gale*, in insect repellent products or insecticides from a natural source warrant further study.

Author Contributions: Conceptualisation, S.N. and H.S.; Data curation, S.N.; Formal analysis, M.P., S.W. and A.B.; Investigation, M.P. and S.W.; Methodology, S.N. and H.S.; Writing—original draft, S.N., M.P. and S.W.; Writing—review and editing, S.N., M.P. and H.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research and the APC were funded by the Unlocking Nature's Pharmacy from Bogland Species (UNPBS) Project under grant number DOJProject209825, funded by the Department of Justice, Ireland.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Peter O'Connell, NatPro Centre, for assisting in microwave extraction; Olga Knutova, School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin, Ireland, for performing all GC-MS measurements; and the Department of Justice of Ireland for the financial support.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Fay, M.F. *Ireland's Generous Nature by Peter Wyse Jackson*; Missouri Botanical Garden Press: St. Louis, MO, USA, 2014; pp. 19–410.
2. Hart, H.C. *Flora of the County Donegal: List of the Flowering Plants and Ferns with Their Localities and Distribution*; Sealy, Bryers and Walker: Dublin, Ireland, 1898.
3. Williams, N. *Díolaim Luibheanna*; Sáirséal-Ó Marcaigh: Dublin, Ireland, 1993; p. 195.
4. Allen, D.E.; Hatfield, G. *Medicinal Plants in Folk Tradition: An Ethnobotany of Britain & Ireland*; Timber Press: Portland, OR, USA, 2004.
5. Sharifi-Rad, J.; Sureda, A.; Tenore, G.C.; Daglia, M.; Sharifi-Rad, M.; Valussi, M.; Tundis, R.; Sharifi-Rad, M.; Loizzo, M.R.; Oluwaseun Ademiluyi, A.; et al. Biological activities of essential oils: From plant chemoecology to traditional healing systems. *Molecules* **2017**, *22*, 70. [CrossRef]
6. Pavela, R.; Benelli, G. Essential Oils as Ecofriendly Biopesticides? Challenges and Constraints. *Trends Plant Sci.* **2016**, *21*, 1000–1007. [CrossRef]
7. Al-Maharik, N.; Jaradat, N.; Hawash, M.; Al-Lahham, S.; Qadi, M.; Shoman, I.; Jaber, S.; Rahem, R.A.; Hussein, F.; Issa, L. Chemical Composition, Antioxidant, Antimicrobial and Anti-Proliferative Activities of Essential Oils of Rosmarinus officinalis from five Different Sites in Palestine. *Separations* **2022**, *9*, 339. [CrossRef]
8. Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological effects of essential oils—A review. *Food Chem. Toxicol.* **2008**, *46*, 446–475. [CrossRef]
9. Tandon, S. 7 Distillation Technology for Essential Oils. In *Extraction Technologies for Medicinal and Aromatic Plants*; International Center for Science and High Technology: Trieste, Italy, 2008; p. 115.

10. Ložienė, K.; Labokas, J.; Vaičiulytė, V.; Švedienė, J.; Raudonienė, V.; Paškevičius, A.; Šveistytė, L.; Apšegaitė, V. Chemical composition and antimicrobial activity of fruit essential oils of Myrica gale, a neglected non-wood forest product. *Balt. For.* **2020**, *26*, 1–8. [CrossRef]
11. Carlton, R.R.; Waterman, P.G.; Gray, A.I. Variation of leaf gland volatile oil within a population of sweet gale (*Myrica gale*) (Myricaceae). *Chemoecology* **1992**, *3*, 45–54. [CrossRef]
12. Skene, K.R.; Sprent, J.I.; Raven, J.A.; Herdman, L. *Myrica gale* L. *J. Ecol.* **2000**, *88*, 1079–1094. [CrossRef]
13. Wawrzynczak, K.; Sadowska, B.; Więckowska-Szakiel, M.; Kalemba, D. Composition and Antimicrobial Activity of *Myrica gale* L. Leaf and Flower Essential Oils and Hydrolates. *Rec. Nat. Prod.* **2021**, *15*, 35–45. [CrossRef]
14. Jaenson, T.G.; Pålsson, K.; Borg-Karlson, A.K. Evaluation of extracts and oils of tick-repellent plants from Sweden. *Med. Vet. Entomol.* **2005**, *19*, 345–352. [CrossRef]
15. Azwanida, N.N. A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Med. Aromat. Plants* **2015**, *4*, 1–6.
16. Wang, M.; Carver, J.J.; Phelan, V.V.; Sanchez, L.M.; Garg, N.; Peng, Y.; Nguyen, D.D.; Watrous, J.; Kapono, C.A.; Luzzatto-Knaan, T.; et al. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat. Biotechnol.* **2016**, *34*, 828–837. [CrossRef] [PubMed]
17. Van den Dool, H.; Kratz, P.D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* **1963**, *11*, 463–471. [CrossRef] [PubMed]
18. Li, Y.-Q.; Kong, D.-X.; Wu, H. Analysis and evaluation of essential oil components of cinnamon barks using GC–MS and FTIR spectroscopy. *Ind. Crop. Prod.* **2013**, *41*, 269–278. [CrossRef]
19. Luque de Castro, M.D.; Jimenez-Carmona, M.M.; Fernandez-Perez, V. Towards more rational techniques for the isolation of valuable essential oils from plants. *TrAC Trends Anal. Chem.* **1999**, *18*, 708–716. [CrossRef]
20. Wawrzyńczak, K.; Jakiel, A.; Kalemba, D. Composition of leaf and flower essential oil of *Myrica gale* L. *Biotechnol. Food Sci.* **2019**, *83*, 87–96.
21. Sylvestre, M.; Legault, J.; Dufour, D.; Pichette, A. Chemical composition and anticancer activity of leaf essential oil of *Myrica gale* L. *Phytomedicine* **2005**, *12*, 299–304. [CrossRef]
22. Hung, N.H.; Quan, P.M.; Satyal, P.; Dai, D.N.; Van Hoa, V.; Huy, N.G.; Giang, L.D.; Ha, N.T.; Huong, L.T.; Hien, V.T.; et al. Acetylcholinesterase Inhibitory Activities of Essential Oils from Vietnamese Traditional Medicinal Plants. *Molecules* **2022**, *27*, 7092. [CrossRef]
23. Miyazawa, M.; Yamafuji, C. Inhibition of Acetylcholinesterase Activity by Bicyclic Monoterpenoids. *J. Agric. Food Chem.* **2005**, *53*, 1765–1768. [CrossRef] [PubMed]
24. Perry, N.S.; Houghton, P.J.; Theobald, A.; Jenner, P.; Perry, E.K. In-vitro inhibition of human erythrocyte acetyl-cholinesterase by *Salvia lavandulaefolia* essential oil and constituent terpenes. *J. Pharm. Pharmacol.* **2000**, *52*, 895–902. [CrossRef]
25. Seo, S.-M.; Jung, C.-S.; Kang, J.; Lee, H.-R.; Kim, S.-W.; Hyun, J.; Park, I.-K. Larvicidal and Acetylcholinesterase Inhibitory Activities of Apiaceae Plant Essential Oils and Their Constituents against *Aedes albopictus* and Formulation Development. *J. Agric. Food Chem.* **2015**, *63*, 9977–9986. [CrossRef] [PubMed]
26. Pigott, M.; Nagar, S.; Woulfe, I.; Scalabrino, G.; Sheridan, H. Unlocking Nature's Pharmacy: Composition and bioactivity of essential oil of bog-myrtle (*Myrica gale*) grown on Irish boglands. *Planta Med.* **2022**, *88*, 236.
27. Triba, M.N.; Le Moyec, L.; Amathieu, R.; Goossens, C.; Bouchemal, N.; Nahon, P.; Rutledge, D.N.; Savarin, P. PLS/OPLS models in metabolomics: The impact of permutation of dataset rows on the K-fold cross-validation quality parameters. *Mol. Biosyst.* **2014**, *11*, 13–19. [CrossRef] [PubMed]
28. Sithersingh, M.J.; Snow, N.H. Chapter 9—Headspace gas chromatography. In *Handbooks in Separation Science*; Elsevier: Amsterdam, The Netherlands, 2021; Volume 2, pp. 251–265.
29. Bousbia, N.; Abert Vian, M.; Ferhat, M.A.; Petitcolas, E.; Meklati, B.Y.; Chemat, F. Comparison of two isolation methods for essential oil for rosemary leaves. Hydrodistillation and micro hydrodiffusion and gravity. *Food Chem.* **2009**, *114*, 355–362. [CrossRef]
30. Handa, S.S. An Overview of Extraction Techniques for Medicinal and Aromatic Plants. *Extr. Tech. Med. Aromat. Plants* **2008**, *1*, 21–54.
31. Trautman, E.P.; Crawford, J.M. Linking biosynthetic gene clusters to their metabolites via pathway-targeted molecular networking. *Curr. Top. Med. Chem.* **2016**, *16*, 1705–1716. [CrossRef] [PubMed]