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Composition and Efficacy of a Natural Phytotherapeutic Blend against Nosemosis in Honey Bees

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Abstract: Honey bees are essential to sustaining ecosystems, contributing to the stability of biodiversity through pollination. Today, it is known that the failure of pollination leads irremediably to the loss of plant cultures and, as a consequence, inducing food security issues. Bees can be affected by various factors, one of these being *Nosema* spp. which are protozoans specifically affecting adult honey bees and a threat to bee populations around the world. The composition of the phytotherapeutic product (*Protofil*®) for treating nosemosis was analyzed from a biochemical point of view. The most concentrated soluble parts in the phytotherapeutic association were the flavonoids, most frequently rutin, but quercetin was also detected. Additionally, the main volatile compounds identified were eucalyptol (1.8-cineol) and chavicol-methyl-ether. To evaluate the samples' similarity–dissimilarity, the PCA multivariate statistical analysis, of the gas-chromatographic data (centered relative percentages of the volatile compounds), was applied. Statistical analysis revealed a significant similarity of *Protofil*® with the *Achillea millefolium* (Yarrow) samples and more limited with *Thymus vulgaris* (Thyme) and *Ocimum basilicum* (Basil), and, respectively, a meaningful dissimilarity with *Taraxacum officinale* (Dandelion). The results have shown a high and beneficial active compounds concentration in the analyzed herbs. High similarity with investigated product recommending the *Protofil*®, as the treatment compatible with producing organic honey.

Keywords: *Apis mellifera*; *Nosema* spp.; *Protofil*®; biochemical analysis

1. Introduction

Bees are necessary for maintaining ecosystems, contributing to biodiversity through pollination, a vital factor for a wide range of crops and wild plants. Today, it is known that the failure of pollination will lead irremediably to the loss of plant cultures and, as a consequence, food security concerns [1].

Worldwide, 75% of the crops are pollinated by insects with 57 species (mostly bees) as crucial pollinators for approximately 107 plants [1,2].

Honey bees (*Apis mellifera L.*) are affected by many diseases, the most important being of fungal and viral origin. The main factors affecting disease are small colony population size, extended winter, reduction of cleaning flights, feed supplements, and the hive's excessive humidity [3–5].

Under these circumstances, nosemosis caused by *Nosema apis* Zander and *Nosema ceranae* Fries protozoa became the principal threat and the most commonly found in honey bee populations [6–12].

During the last decade, allopathic drugs against nosemosis were restricted to a few active substances such as Fumagillin (fumidil), an antibiotic obtained from *Aspergillus fumigatus.* Unfortunately, although an efficient product, due to the risk of residues, EMA has excluded this product from use in Europe in February 2016 [6,13–18].

In the given circumstances in the treatment of nosemosis, a reliable backup could be ecologic phytotherapy, the usage of whole herbs or parts, with recognized antiprotozoal activity (like flowers of *Matricaria chamomilla*, *Hypericum perforatum* or *Achillea millefolium*, leaves of *Mentha piperita,* or leaves and flowers of *Ocimum basilicum*) currently being viewed as a great opportunity [19–22].

The Research and Development Institute for Beekeeping has developed an herbal product that presents the blend of essential oils highly efficient against *Nosema* spp [23]. Essential oils used in this product are derived from herbs found in spontaneous flora, which include different cyclic and aliphatic hydrocarbons, triterpenes and sesquiterpenes, phenolic structures, oleanolic acid, flavones, microelements, and the vitamins of B group [24].

This study aimed to analyze the composition of *Protofil*® as commercial product suggested was the usage in honey bees' production, as well as to analyze basil, thyme, yarrow, and dandelion, and to compare them to the aforementioned product, respectively.

2. Materials and Methods

The product *Protofil*® plant association is a brownish solution, with a characteristic aromatic odor and taste, designed to combat *Nosema* spp., and unique advantage is that it has no contraindications (no intoxication or any side effects) to honey bees [25,26].

The sample of the product *Protofil*® was chemically investigated, directly from the producer, the ICDA (Research and Development Institute for Beekeeping, Bucharest, Romania).

Besides *Protofil*®, samples of *Achillea millefolium*, *Thymus vulgaris*, *Ocimum basilicum*, and *Taraxacum officinale*, were chemically investigated as well.

The physicochemical methods used to investigate *Protofil*® and plants were: Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) of the filtered undiluted or diluted hydro-alcoholic extracts and Mass-Spectrometry (GC-MS) coupled with Gas Chromatography of volatile compounds separated by hydro-distillation-extraction in an organic solvent (SDE).

2.1. RP-HPLC Investigation

RP-HPLC investigation of the flavonoid standards and hydro-alcoholic extract samples was performed on a Jasco apparatus (*Abbl&e-Jasco, Bucharest, Romania*) equipped with: quaternary pump (PU-2080 Plus); mixing unit (LG-2080-04 Quaternary Gradient); degasser (DG-2080-54 4); spectrophotometric detector (UV-2070 Plus Intelligent UV/VIS Detector); acquire and process computer data (JASCO ChromPass Chromatography Data System, Version 1.7.403.1), through an LC-Net II/ADC interface.

The conditions of analysis were:

- Column: Nucleosil 100 C18, 250 × 4.6 mm × mm, 5 μm particle diameter;
- UV wavelength: 254 nm; Mobile phase: Acetonitrile: Water = 50:50; Temperature: 25 °C; Flow rate: 1.0 mL/min; Injected volume: 20 μL.

For flavonoids, evaluation of their concentration in hydro-alcoholic extracts were performed using the obtained HPLC calibration curves. The flavonoids' identification correlated the detection of retention times with the standards matching. Therefore, before analysis, the samples were filtered, and, in most cases, they were diluted (1:100).

The samples' bioactive compounds concentration was measured using the calibration curves for the available flavonoids, results being expressed as mg of flavonoid compound, separated at the retention times corresponding to the standard/mL of sample.

For the *RP-HPLC*, the following standards were used:

- Rutin (≥94%) (Sigma-Aldrich, Taufkirchen, Germany),
- Quercetin (≥95%) (Sigma-Aldrich, Taufkirchen, Germany),
- Chrysene (>98%) (Sigma-Aldrich, Taufkirchen, Germany),
- Flavone (≥99%) (Sigma-Aldrich, Taufkirchen, Germany).

Standard solutions were obtained by dilution in 96% ethanol (*Chimopar*, *Bucharest, Romania*) also HPLC purity solvents being used for the chromatographic analysis: acetonitrile (HPLC grade) (*Fluka Chemie, Mūnchen, Germany*) and bidistilled water HPLC (*Fluka Chemie, Mūnchen, Germany*).

2.2. GC-MS Analysis

The *GC-MS analysis* of SDE-separated volatile compounds implied the use of hexane (GC grade) (*Fluka)* for the extraction of volatile compounds separated, and anhydrous sodium sulfate (>99%) (*Merck*) to dry the hexane extract. The Kovats retention indices were calculated based on GC-MS assays performed under the same conditions for a mixture of linear C8–C20 alkanes (*Fluka Chemie*).

2.3. Separation of Volatile Compounds by Hydrodistillation-Extraction (SDE)

The GC-MS analysis of the separated volatile compounds from hydro-alcoholic extracts by hydrodynamic extraction in hexane (SDE) allowed the relative percentage concentrations of the components to be evaluated using the area method (Equation (1)):

$$\text{Relative concentration } (\%) = \frac{\text{Area (compound)}}{\sum \text{ Area}} \times 100 \quad (1)$$

For the analysis of the separated volatile compounds, an HP 6890 Series GC (*Hewlett Packard*), coupled with an HP 5973 Mass Selective Detector mass spectrometer was used.

The GC assay conditions were: Column: HP-5MS, L = 30 m, inner diameter 0.25 mm, film thickness 0.25 μm; Temperature program: 50 to 250 °C at a speed of 6 °C/min; Injector temperature: 280 °C; Detector temperature: 280 °C; Injection volume: 2 μL; Carrier gas: He.

For the MS detector, an EE energy of 70 eV was used, at a source temperature of 150 °C, scanning range of 50–300 amu, with the speed of 1 s^{-1} for mass spectrometry, and the obtained spectra, compared with a NIST/EPA/NIH Mass Spectral Library 2.0 database (2002). For data acquisition, version B.01.00/98, of HP Enhanced Chem Station G1701BA software was used, the data processing, being completed utilizing the HP Enhanced Data Analysis program. Hydro-alcoholic samples (~800 mL) were prepared and the condensed volatile compounds, extracted in an SDE system, in 20 mL hexane. The method lasted four hours, and the separated hexane extract was dried. Dry hexane extracts were then GC-MS analyzed, determining the relative percentage concentration of the volatile compounds.

2.4. Statistical Multivariate Principal Component Analysis (PCA) of GC Data

Multivariate analysis of gas chromatography data for hexane extracts of volatile compounds, allowed a classification of samples based on volatile compounds and their relative concentrations, identifying the similarity of these samples. To assess the investigated samples similarity–dissimilarity, the multivariate statistical data analysis—Principal component analysis (PCA), of gas-chromatographic data, was used, the GC data being used for analysis, and validated by *cross-validation* method.

3. Results

3.1. HPLC Curve Calibration for Standard Compounds

To evaluate the concentration of the flavonoid compounds in hydro-alcoholic extracts, calibration curves for the available flavonoids, rutin, quercetin, chrysene, and flavone, were determined. In the case of *rutin*, the HPLC analysis of the standard solutions indicated the chromatographic peak presence in the retention time range of 2–3 min (most probably, a mixture of isomers due to the presence of two chromatographic peaks that were analyzed together). The quercetin chromatographic peak was detected to 4.2 min, the HPLC examination of *chrysene* and *flavone*, assigning peaks, after 9.8 and, respectively, 15.8 min. The HPLC results for standards are presented in Figure 1.

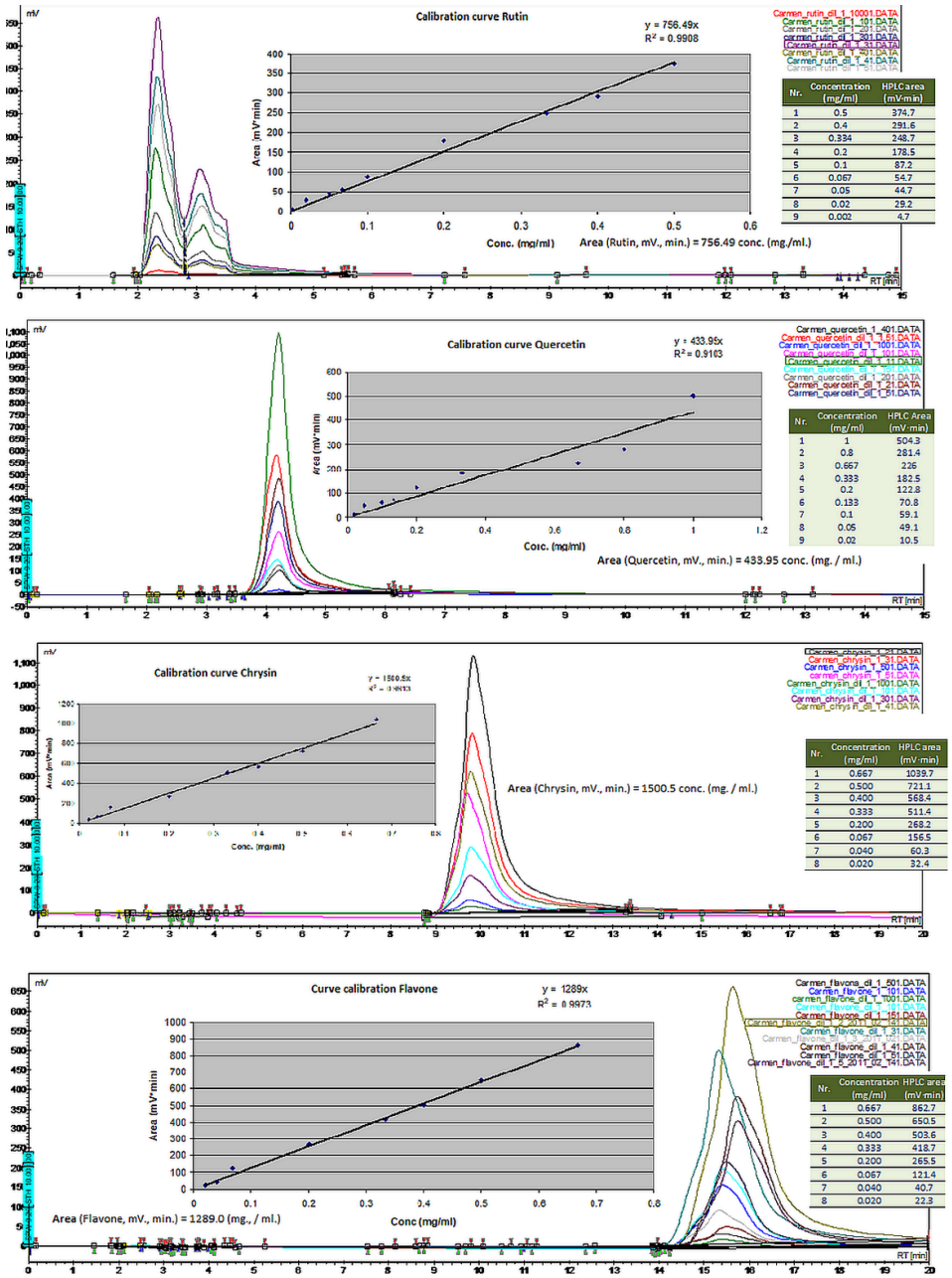

Figure 1. Calibration curves for rutin, quercetin, chrysene, and flavone.

Evaluation of the Flavonoids' Concentration

Concentrations of the studied samples (mean of four replicates, expressed as mg flavonoid available/mL sample) are shown in Table 1.

Table 1. Concentrations of compounds (mean of four replicates), expressed as mg flavonoids/mL, determined from HPLC analyzes.

Nr.	Compound	RT (min)	Conc. (Ba) (mg/mL)	Conc. (Th) (mg/mL)	Conc. (Ya) (mg/mL)	Conc. (Da) (mg/mL)	Conc. (PF) (mg/mL)
1	*Rutin*	2–3.6	1.843	3.437	2.543	1.049	1.540
2	*Quercetin*	4.2	0.017	9.379	0.232	0.029	0.061
3	*Chrysene*	9.8	0.027	0.012	0.004	0.000	0.007
4	*Flavone*	15.8	0.000	0.000	0.010	0.016	0.002

RT-retention time; Conc.-concentration; Ba-basil; Th-thyme; Ya-yarrow; Da-dandelion; PF-*Protofil*®.

HPLC chromatograms of undiluted samples and etalons, for *Ocimum basilicum*, *Thymus vulgaris*, *Achillea millefolium*, and *Taraxacum officinale* are presented in Figure 2, and the chromatogram for the associated conditioning *Protofil*®, in Figure 3.

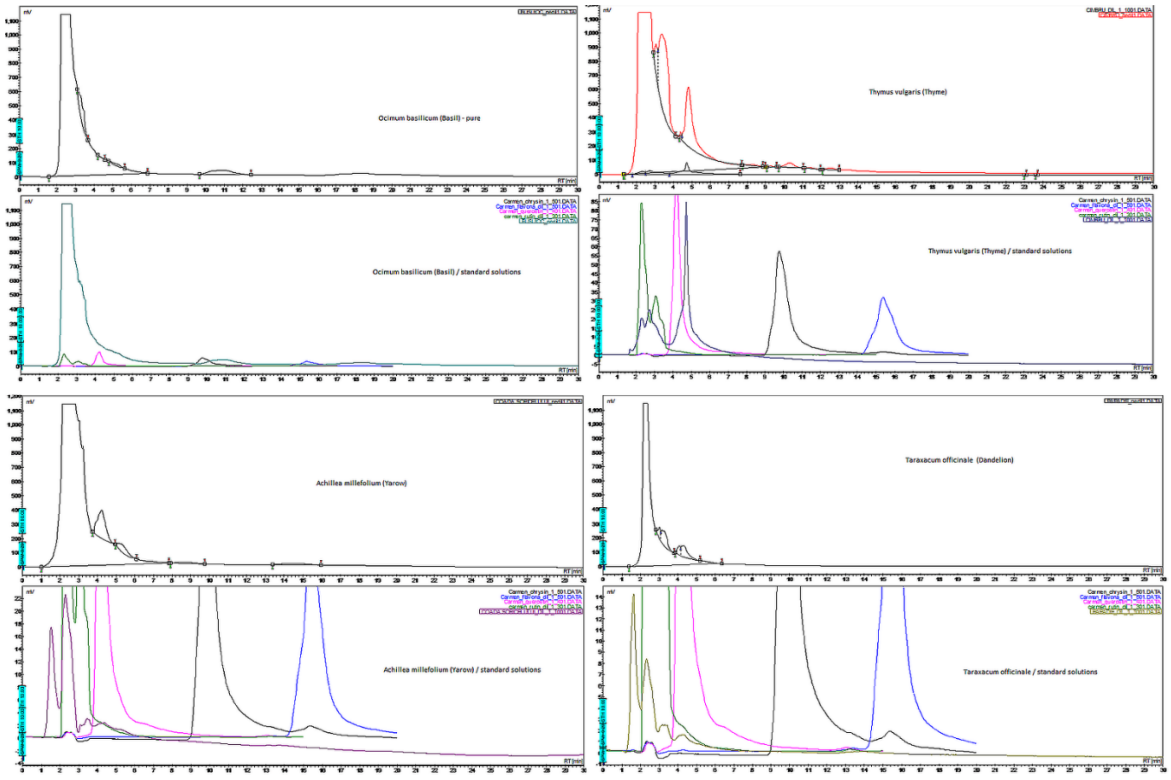

Figure 2. The HPLC chromatograms obtained for the pure samples undiluted (up) and ethalons overlayed (down) for Basil (*Ocimum basilicum*), Thyme (*Thymus vulgaris*), Yarrow (*Achillea millefolium*) and Dandelion (*Taraxacum officinale*).

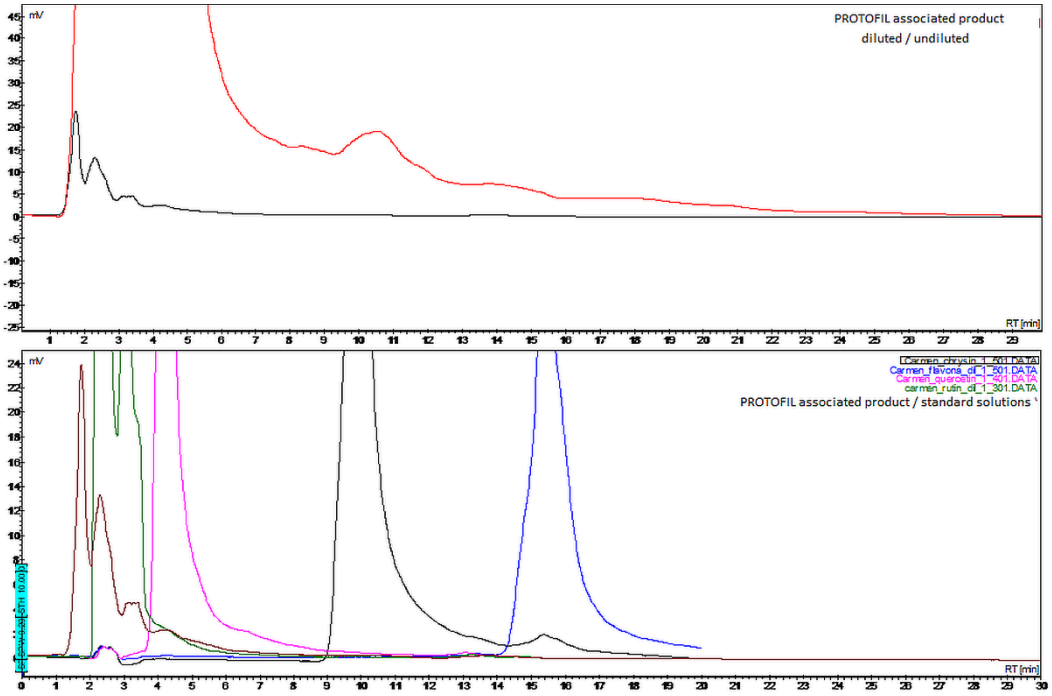

Figure 3. Overlap chromatograms from HPLC analysis for samples/standards (undiluted and diluted) for *Protofil*®.

The most concentrated were the flavonoids (expressed as *rutin*) separated at the beginning of the chromatogram due to the higher hydrophilicity of these compounds, containing saccharide residues, followed by polyphenolic flavonoids of the quercetin type.

Chrysene, a *bis*-phenolic compound, and similar structures separated at high retention times were detected in medium–low concentrations, while flavone a non-phenolic compound were detect in extremely low concentrations. Analyzing the data for the four herb samples leads to results close to the *Protofil's* obtained data, except in the case of quercetin (probably due to inappropriate *rutin* separation).

The HPLC separation of the flavonoid compounds studied, on the C18 nonpolar column, correlates well with their hydrophobicity, with retention times increasing with hydrophobicity, expressed as the logarithm of the octanol/water partition coefficient, calculated with the QSAR Properties program in the *HyperChem* 5.1 package (log P_{rutin} = 1.61, log $P_{quercetin}$ = 0.28, log $P_{chrysin}$ = 1.75 and log $P_{flavone}$ = 2.32). The best correlation is polynomial of order 2 (r^2 = 0.98).

3.2. GC-MS Analysis of Volatile Compounds' Relative Concentration

For basil extract, (the most significant from the set of analyses), 56 components (expressed as *abundance* of 10,000) were separated, the most concentrated compound identified being *chavicol-methyl-ether* (55%) (Table 2).

In the case of volatile compounds in the GC-MS of *Thymus vulgaris* (Thyme), 43 chromatographic peaks were identified, the most concentrated being *eucalyptol* and *γ-terpinene* (Table 3).

Table 2. Results of GC-MS analysis for *Ocimum basilicum* (Basil) samples.

No.	A Compound Identified by GC-MS	RT (min)	Kovats Index	Relative Concentration (%)
1	*Column*	5.198	948	0.00
2	*alpha-Pinene*	5.686	974	1.29
3	*Column*	5.944	987	0.00
4	*Column*	6.173	999	0.00
5	*Camphene*	6.250	1002	0.15
6	*beta-Phellandrene*	6.796	1028	0.50
7	*beta-Pinene*	6.955	1035	1.42
8	*Sabinen/beta-pinene*	7.037	1039	0.64
9	*Bicyclo [3.1.0]hexane, 4-methyl-1-(1-methylethyl)-, didehydro*	7.584	1063	0.05
10	*Terpinolen*	7.830	1074	0.06
11	*Limonene*	8.101	1086	1.10
12	*Dihydrocarveol*	8.195	1090	0.08
13	*3-Carene/alpha-pinene*	8.318	1095	0.27
14	*Eucalyptol*	8.582	1106	7.31
15	*gamma-Terpinen*	9.005	1124	0.14
16	*Terpinolen*	9.740	1155	0.13
17	*2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-*	10.116	1171	0.09
18	*Linalool*	10.192	1174	0.31
19	*Fenchone*	10.739	1198	0.08
20	*Tetrahydroactinidiolide*	11.015	1210	0.05
21	*Camphor*	12.607	1280	0.08
22	*Caprylyl acetate*	12.683	1283	0.02
23	*Fenchyl acetate*	13.136	1303	0.05
24	*Chavicol methyl ether*	13.870	1337	54.91
25	*Bornyl acetate*	15.034	1391	0.04
26	*alpha-Cubebene*	15.239	1400	0.04
27	*Copaene*	16.015	1437	0.30
28	*Di-epi-alpha-cedrene*	16.138	1443	0.28
29	*beta-Bourbonene*	16.362	1454	0.24
30	*beta-Elemene*	16.479	1459	0.58
31	*alpha-Bergamotene*	16.984	1484	0.15
32	*alpha-Bergamotene*	17.290	1498	5.79
33	*Caryophyllene*	17.401	1504	6.75
34	*trans-Caryophyllene/Isocaryophyllene*	17.684	1518	0.25
35	*beta-Farnesene*	17.778	1522	1.74
36	*Humulene*	18.300	1548	1.88
37	*gamma-Muurolene*	18.436	1555	0.22
38	*(Z)-beta-Farnesene*	18.706	1568	0.48
39	*Germacrene D*	18.906	1578	3.41
40	*alpha-Himachalene*	19.088	1587	0.54
41	*Eremophilene*	19.293	1598	0.11
42	*Elixene*	19.358	1601	0.23
43	*beta-Cedrene*	19.599	1613	0.10
44	*gamma-Cadinene*	19.658	1616	0.75
45	*beta-Cadinene*	19.758	1621	0.19
46	*1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-*	19.928	1630	6.33
47	*Calamenene*	20.275	1648	0.09
48	*Caryophyllene oxide*	21.790	1728	0.02
49	*Palmitic acid*	27.519	2068	0.15
50	*Ethyl palmitate*	27.584	2072	0.41
51	*Column*	30.163	2262	0.00
52	*Arachidic acid*	30.598	2299	0.10
53	*Ethyl linolenate*	30.980	2333	0.08
54	*Column*	31.755	2407	0.00
55	*Column*	32.214	2456	0.00
56	*Column*	33.230	2577	0.00

RT-retention time.

Table 3. Results of GC-MS analysis for *Thymus vulgaris* (Thyme) samples.

No.	A Compound Identified by GC-MS	RT (min)	Kovats Index	Relative Concentration (%)
1	*solvent*	5.254	951	0.00
2	*alpha-Thujene*	5.454	962	2.06
3	*alpha-Pinene*	5.683	974	2.94
4	*Camphene*	6.247	1002	1.39
5	*beta-Terpinen*	6.8	1028	0.05
6	*beta-Pinene*	6.952	1035	0.58
7	*beta-Pinene*	7.035	1039	2.51
8	*alpha-Thujene*	7.581	1063	0.43
9	*alpha-Terpinen*	7.828	1074	2.14
10	*Limonene*	8.104	1086	0.99
11	*beta-Phellandrene*	8.321	1095	0.45
12	*Eucalyptol*	8.556	1105	57.63
13	*gamma-Terpinen*	9.015	1124	15.03
14	*Terpinolen*	9.743	1155	0.19
15	*2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-*	10.113	1171	0.11
16	*Dehydro-p-cymene*	10.583	1191	0.11
17	*2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-*	11.018	1210	0.06
18	*2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-*	12.616	1280	0.18
19	*Methyl chavicol*	13.874	1337	0.14
20	*Thymol methyl ether*	13.991	1342	1.68
21	*2-Isopropyl-1-methoxy-4-methylbenzene*	14.15	1350	1.61
22	*Bornyl acetate*	15.031	1391	0.06
23	*Ylangene*	15.883	1431	0.06
24	*Copaene*	16.012	1437	0.20
25	*beta-Bourbonene*	16.359	1453	0.14
26	*Ylangene*	17.293	1499	0.07
27	*Caryophyllene*	17.387	1503	5.10
28	*Alloaromadendren*	17.716	1519	0.15
29	*Humulene*	18.292	1548	0.20
30	*gamma-Muurolene*	18.686	1567	0.48
31	*alpha-Muurolene*	18.803	1573	0.07
32	*Germacrene D*	18.903	1578	0.07
33	*alpha-Muurolene*	19.22	1594	0.57
34	*gamma-Cadinene*	19.661	1617	0.61
35	*beta-Cadinene*	19.761	1622	0.76
36	*alpha-Muurolene*	20.107	1639	0.07
37	*Calamenene*	20.272	1648	0.30
38	*Caryophyllene oxide*	21.788	1728	0.15
39	*5,9,9-Trimethyl-spiro[3.5]non-5-en-1-one*	27.029	2035	0.06
40	*Palmitic acid, ethyl ester*	27.605	2073	0.49
41	*Cholesterol, trifluoroacetate*	30.677	2306	0.00
42	*Linolenic acid, methyl ester*	31.006	2335	0.11
43	*2,4,4,6,6,8,8-Heptamethyl-1-nonene*	32.699	2511	0.00

RT-retention time.

The most concentrated volatile components in the *Achillea millefolium* (Yarrow) specimens were: *camphor* (relative concentration of 37.5%) and *eucalyptol* (25%), the total GC-separated compounds, in this case, being 44, some of which derived from the column (especially at the high separation temperatures cases) (Table 4).

Table 4. Results of GC-MS analysis for *Achillea millefolium* (Yarrow) samples.

No.	A Compound Identified by GC-MS	RT (min)	Kovats Index	Relative Concentration (%)
1	*alpha-Pinene*	5.681	974	0.22
2	*Camphene*	6.251	1002	0.78
3	*Yomogi alcohol*	7.42	1056	0.26
4	*3-Thujene*	7.59	1063	0.17
5	*Terpinolen*	7.837	1074	0.46
6	*4-Oxo-beta-isodamascol*	7.913	1077	0.62
7	*beta-Terpinen*	8.319	1095	0.17
8	*Eucalyptol*	8.577	1106	25.14
9	*2-Carene*	9.012	1124	1.12
10	*2-Norpinanol, 3,6,6-trimethyl-*	9.652	1151	0.43
11	*3-Thujanone*	10.91	1205	3.17
12	*Isopulegol*	11.021	1210	0.69
13	*alpha-Thujone*	11.133	1215	0.79
14	*4-Oxo-beta-isodamascol*	11.679	1239	0.85
15	*cis-Sabinol*	11.779	1243	0.37
16	*Verbenyl ethyl ether*	11.867	1247	0.79
17	*Lavandulol*	12.061	1255	0.57
18	*Lavandulol*	12.208	1262	0.79
19	*4-Oxo-beta-isodamascol*	12.42	1271	0.58
20	*Camphor*	12.596	1279	37.52
21	*E-3,5-Dimethylhex-2-en-1,2-dicarboxylic acid*	13.031	1299	1.25
22	*Isobornyl formate*	13.142	1304	1.01
23	*Chavicol methyl ether*	13.877	1337	3.27
24	*trans-Chrysanthenyl Acetate*	14.288	1356	0.21
25	*Isobornyl acetate*	15.028	1390	0.35
26	*cis-Carvyl Acetate*	16.345	1453	0.36
27	*Capric acid, ethyl ester*	16.82	1476	1.13
28	*9-Cedranone*	19.811	1624	0.34
29	*Spathulenol*	21.656	1721	0.45
30	*Caryophyllene oxide*	21.785	1728	1.95
31	*2-Cyclohexene-1-carboxaldehyde,2,6-dimethyl-6-(4-methyl-3-pentenyl)*	22.437	1763	0.16
32	*4(equatorial)-n-Propyl-trans-3-oxabicyclo[4.4.0]decane*	22.496	1766	0.21
33	*gamma-Eudesmol*	22.614	1773	1.94
34	*beta-Guaiene*	22.772	1782	0.58
35	*alpha-Eudesmol*	23.148	1803	2.47
36	*Humulane-1,6-dien-3-ol*	23.425	1818	0.78
37	*Aristolone*	23.742	1836	0.24
38	*Ethyl myristate*	24.347	1871	0.16
39	*Palmitic acid, ethyl ester*	27.602	2073	3.37
40	*Ethyl Oleate*	30.504	2291	0.28
41	*Linoleic acid ethyl ester*	30.663	2305	2.19
42	*Ethyl linolenate*	30.992	2334	1.79
43	*Column*	31.962	2429	0.00
44	*Column*	32.584	2498	0.00

RT-retention time.

The total concentration of active compounds in the hexane extracts of dandelion was identified. Upon identification of total active compounds, relative concentration was determined. The highest concentration of 5.7% *eucalyptol*, and 62.5% *ethyl palmitate* (Table 5) was recorded, respectively.

Table 5. Results of GC-MS analysis for *Taraxacum officinalis* (Dandelion) samples.

No.	A Compound Identified by GC-MS	RT (min)	Kovats Index	Relative Concentration (%)
1	*Bicyclo[2.1.1]hexan-2-ol, 2-ethenyl-*	5.146	945	0.59
2	*p-Xylene*	5.252	951	2.42
3	*Octane, 1-chloro-*	5.458	962	0.43
4	*alpha-Thujene*	5.693	975	0.53
5	*Isovaleraldehyde, diethyl acetal*	5.945	987	1.72
6	*Linalyl propionate*	6.239	1002	0.64
7	*Pentane, 1,1-diethoxy-*	7.003	1037	1.41
8	*Eucalyptol*	8.572	1106	5.72
9	*trans-Verbenol*	9.03	1125	0.40
10	*Chavicol methyl ether*	14.012	1343	0.46
11	*Bicyclo[2.2.1]heptane, 2-cyclopropylidene-1,7,7-trimethyl-*	17.385	1503	0.66
12	*2,3-Dehydro-4-oxo-beta-ionone*	19.688	1618	0.50
13	*Ethyl laurate*	20.863	1679	1.15
14	*Ethyl myristate*	24.371	1872	2.17
15	*Oxirane, 2-methyl-2-(1-methylethyl)-*	25.117	1916	0.93
16	*Methyl 2-methylhexanoate*	26.016	1971	0.71
17	*Ethyl palmitate*	27.614	2074	62.49
18	*Eicosane*	28.084	2106	1.31
19	*2,6-Pyrazinediamine*	30.522	2292	0.55
20	*Ethyl stearate*	30.622	2301	1.02
21	*Ethyl linolate*	30.687	2307	8.13
22	*Methyl linolenate*	31.016	2336	6.06

RT-retention time.

The volatile compounds GC-MS analysis is presented in Figures 4 and 5.

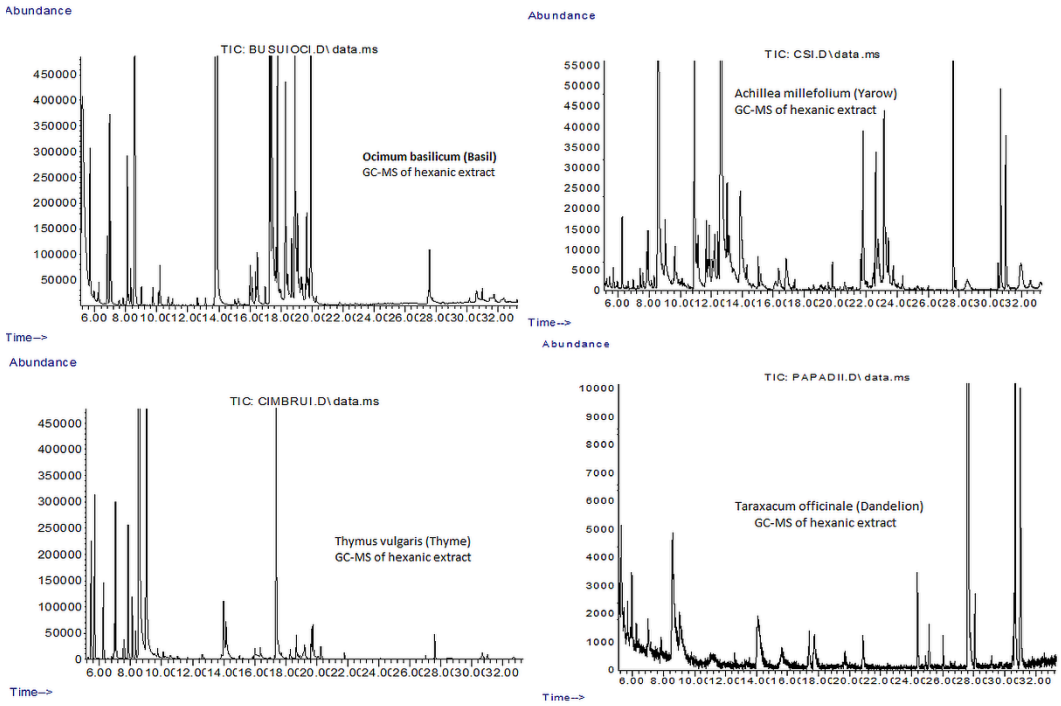

Figure 4. Chromatograms of GC-MS analysis of hexane extract obtained from basil, thyme, yarrow, and dandelion.

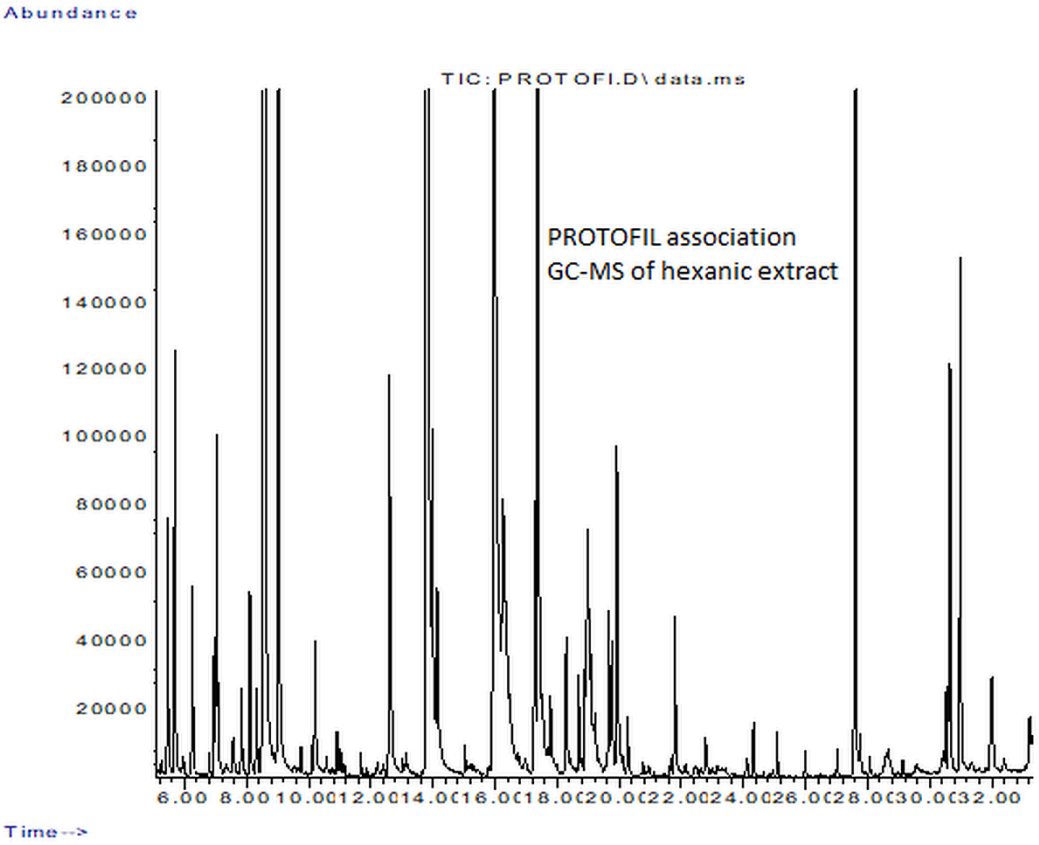

Figure 5. Chromatograms of GC-MS analysis of hexane extract obtained from *Protofil*®.

The *Protofil*® analysis, described the most relevant absolute concentration, totaling 74 different components, as well as their absolute abundance in the hexane extract. The highest recorded concentration of active ingredients was *eucalyptol* (28.6%), followed by *chavicol-methyl-ether* (28.1%), while the lover concentration of *thymol* (7.19%), and *gamma-Terpinen* (5.86%), was also present in the investigated sample (Table 6).

Table 6. Results of GC-MS analysis for *Protofil*® samples.

No.	A Compound Identified by GC-MS	RT (min)	Kovats Index	Relative Concentration (%)
1	*p-Xylene*	5.253	951	0.05
2	*1-Isopropyl-4-methylbicyclo[3.1.0]hex-2-ene/alpha-Phellandrene*	5.458	962	0.72
3	*alpha-Pinene*	5.687	974	1.20
4	*Valeraldehyde, diethyl acetal*	5.946	988	0.07
5	*Camphene*	6.252	1003	0.56
6	*beta-Terpinen*	6.798	1028	0.07
7	*beta-Pinene*	6.957	1035	0.39
8	*beta-Pinene*	7.039	1039	0.95
9	*3-Carene*	7.585	1063	0.15
10	*alpha-Terpinen*	7.832	1074	0.24
11	*Limonene*	8.102	1086	0.55
12	*beta-Phellandrene*	8.32	1095	0.24
13	*Eucalyptol*	8.531	1104	28.61
14	*gamma-Terpinen*	9.013	1124	5.86
15	*Terpinolene*	9.747	1155	0.10
16	*4-Isopropyl-1-methyl-2-cyclohexene-1-ol*	10.118	1171	0.08
17	*Linalool*	10.2	1175	0.44
18	*3,4-Dimethylstyrene*	10.57	1190	0.08

Table 6. *Cont.*

No.	A Compound Identified by GC-MS	RT (min)	Kovats Index	Relative Concentration (%)
19	*Thujone*	10.911	1205	0.12
20	*4-Isopropyl-1-methyl-2-cyclohexene-1-ol*	11.022	1210	0.07
21	*4-Oxo-beta-isodamascol*	11.68	1239	0.06
22	*1,3-Dioxolane, 2,2-dimethyl-4,5-bis(1-methyl phenyl)-*	12.209	1262	0.05
23	*Tetrahydroactinidiolide*	12.415	1271	0.04
24	*Camphor*	12.597	1279	1.73
25	*E-3,5-Dimethylhex-2-en-1,2-dicarboxylic acid*	13.026	1298	0.04
26	*alpha-Terpineol*	13.138	1304	0.07
27	*Chavicol methyl ether*	13.784	1333	28.09
28	*Thymol methyl ether*	13.978	1342	1.43
29	*2-Isopropyl-1-methoxy-4-methylbenzene*	14.136	1349	0.80
30	*Bornyl acetate*	15.035	1391	0.09
31	*Thymol*	15.799	1427	0.05
32	*Thymol*	15.958	1434	7.19
33	*Carvacrol*	16.252	1448	0.77
34	*Carvacrol*	16.739	1472	0.06
35	*1-Cyclopropene-1-pentanol, à,î,î,2-tetramethyl-3-(1-methyl phenyl)-*	16.992	1484	0.07
36	*alpha-Bergamotene*	17.274	1498	0.89
37	*Caryophyllene*	17.386	1503	3.29
38	*beta-Farnesene*	17.785	1523	0.27
39	*Humulene*	18.296	1548	0.49
40	*gamma-Muurolene*	18.696	1568	0.34
41	*Germacrene D*	18.901	1578	0.35
42	*Eugenol methyl ether*	18.978	1582	0.83
43	*gamma-Cadinene*	19.659	1616	0.48
44	*beta-Cadinene*	19.759	1622	0.40
45	*cis-alpha-Bisabolene*	19.924	1630	1.21
46	*Calamenene*	20.27	1648	0.22
47	*Ethyl laurate*	20.764	1674	0.05
48	*Cadala-1(10),3,8-triene*	20.952	1683	0.04
49	*Spathulenol*	21.657	1721	0.07
50	*Caryophyllene oxide*	21.792	1728	0.55
51	*delta-Cadinol*	22.127	1746	0.06
52	*12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*	22.444	1764	0.05
53	*Cubenol*	22.626	1774	0.04
54	*tau-Cadinol*	22.779	1782	0.16
55	*Cadalene*	24.113	1858	0.08
56	*Ethyl myristate*	24.324	1870	0.15
57	*Hexahydrofarnesyl acetone*	25.082	1914	0.12
58	*Ethyl pentadecanoate*	25.987	1969	0.08
59	*Naphthalene, 1,2,3,4,4a,5,6,7-octahydro-4a-methyl-*	27.027	2035	0.08
60	*Ethyl palmitate*	27.591	2072	4.45
61	*Ethyl (9E)-9-hexadecenoate*	27.762	2084	0.13
62	*Nonadecane*	28.061	2104	0.05
63	*cyclopentane carboxylic acid, 4-hexadecyl ester*	28.672	2147	0.08
64	*Ethyl heptadecanoate*	29.125	2181	0.04
65	*2-Piperidinone, N-[4-bromo-n-butyl]-*	29.565	2214	0.06
66	*4-Butoxy-2,4-dimethyl-2-pentene*	30.411	2283	0.08
67	*Ethyl Oleate*	30.494	2290	0.20
68	*Ethyl stearate*	30.594	2298	0.20
69	*9,12-Octadecadienoic acid, ethyl ester*	30.652	2304	1.06
70	*Linolenic acid, ethyl ester*	30.981	2333	1.37
71	*Linolenic acid, ethyl ester*	31.357	2368	0.05
72	*2,4,4,6,6,8,8-Heptamethyl-2-nonene*	31.998	2433	0.57
73	*(Z)-7-Hexadecenal*	32.403	2477	0.04
74	*2,4,4,6,6,8,8-Heptamethyl-1-nonene*	33.226	2577	0.26

RT-retention time.

3.3. Statistical Multivariate Principal Component Analysis (PCA) of GC Data

PCA reveal that *Protofil*® had a significant similarity with yarrow, more limited similarity with thyme and basil, and little similarity with dandelion. Data variation described 53% for PC1, and 34% for PC2 and, for this classification, the *chavicol-methyl-ether*, and *α-muurolen* were essential, as a first main component. Eucalyptol concentration had significance, as a following main component (Figure 6).

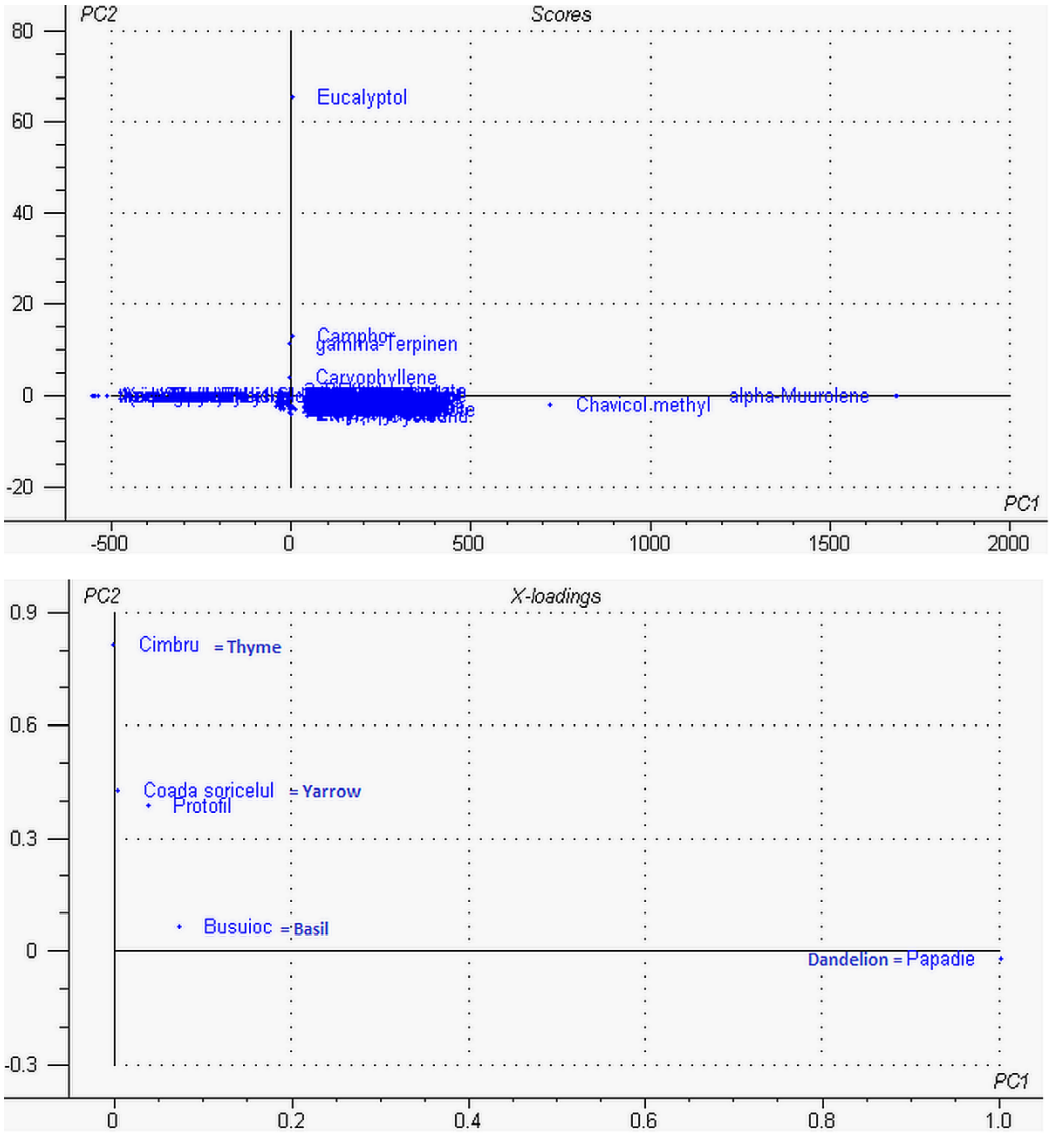

Figure 6. PCA analysis—Graph of records for volatile compounds (**up**) and chart of PCA analysis scores of GC data (**down**), for volatile compounds in the studied samples.

Analyzing the outcome of the chromatographic analyses used in this study, HPLC and GC-MS, an approximately equal proportion for the four distinct studied components in the *Protofil*® association it was ascertained.

4. Discussion

Considering treatment with antibiotics is now forbidden in European countries, control of nosemosis has to be completed mainly by employing defensive and alternative measures. Additionally, if a beehive is critically impaired by nosemosis, the strategy, from an economic point of view

and in many countries, is to destroy those colonies, although losses could become sizeable. Nevertheless, in the literature, there are presented efforts to combat nosemosis, original phytotherapeutic conditionings being proposed, a present study trying to be part of this cause by proposing this phytotherapeutic approach.

Research shows that food supplements are common in beekeeping [24]. Research was conducted to evaluate the brood development from colonies, which were fed with different naturals supplement added in supplementary food compared to product *Protofil*®. According to result of this research, after the winter period and during the period of preparation for principal honey harvest, the best results were obtained for *Protofil*® and *Echinacea* [24].

For instance, thymol was among the first natural substances studied in the beehive infections [27–29] as well as various thymol links [30]. In our results, the volatile compounds analyzed in *T. vulgaris* were *eucalyptol* and *γ-terpinene*.

For example, Maistrello et al. [19], had evaluated the effectiveness of different phyto compounds, like resveratrol, thymol, vetiver essential oil, and lysozyme, to control nosema in honeybees. The results revealed that bees, fed especially with thymol, which is also identified in our study, and resveratrol considerably reduced infection rates and extending longevity. Thymol and resveratrol have therefore been shown to be effects for control of nosemosis [19].

Mărghitaş et al. [23] investigated the influence of nettle, thyme and *Echinacea*, fresh juice of onion and garlic, and *Protofil*® as supplementary feed in artificially weakened bee colonies. The most effective results in this field experiment were recorded in bees supplemented with nettle [23].

In another study, *N. ceranae* infection was stopped with the use of oxalic acid syrup, in laboratory and field studies, being proposed by authors, as an alternative control strategy [16].

Yucel and Dogaroglu [31] studied comparatively, for three years, the activity of Fumagillin, and thymol in *N. apis* infection, in 208 honey bee colonies. The results confirm the present investigation with the aim of phytotherapy efficiency and underlining the importance of alternative treatments in honey bees [31].

The observed low mortality, as well as the honey production, which also brings the organic honey's benefits, does validate the *Protofil*® use judiciousness, as a reliable phytotherapeutic choice. This observation is significant from the organic product consumers and the beekeepers' economic point of view because research has shown consumers' higher willingness to pay for organic honey [32]. The efficacy of *Protofil*® for treating nosemosis was demonstrated on 15 colonies. The mortality values compared to the honey production/categories/total quantity, confirmed the judiciousness of treatments with *Protofil*® [33].

Cola [34] tested to caraway, *Protofil*®, fresh juice of onions, garlic, stinging nettle, thyme, *Echinacea*, and selenium on the bee families artificially weakened by removing the existing population of 3/4 from initial. It was found that the most significant influence in this research had a stinging nettle, which was in agreement with earlier findings [24].

5. Conclusions

The chromatographic analyzes completed on plant extracts from different botanical families revealed that the most concentrated soluble components in the alcohol–water mixture were flavonoids, most often rutin, identified in high concentrations in most of the studied samples (except the thyme), but also its corresponding aglycons. The most significant volatile compounds identified were *eucalyptol* (1,8-cineol) and *chavicol-methyl ether,* for *Lamiaceae* (basil and thyme) samples and *camphor* for *Asteraceae* (yarrow) family. Representatives of the *Compositae* family were less concentrated in the volatile compounds (except thyme, significant from this point of view).

The results of our study revealed a considerable similarity of *Protofil*® with with *A. millefolium*, less so with *T. vulgaris* and *O. basilicum*, while they were significantly different from *T. officinale*. The results revealed a high concentration of beneficial active components of herbs in *Protofil*®, and the promised

benefits of organic honey, with no residues, plus the lack of undesirable effects, but the further research are still necessary.

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