Geographical Prediction of Parsley Origins Based on Chromatographic Fingerprinting and Quantitative Analysis: Application to Mediterranean Cultivars

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Abstract   In this study, for the first time, quantitative and qualitative chromatographic analysis for the content measurements of parsley varieties in nine cities of three big producers/consumers countries in the Mediterranean region: France, Lebanon and Jordan, were carried out. Parsley oils were extracted by hydrodistillation then examined by GC-MS to reveal their complex chemical profiles for plants clustering. Results showed that 29 compounds were quantified and identified in all parsley plant oil extracts. Among organics; apiol, myristicin, adamantane and benzenamine,N,N-4-trimethyl, not only displayed the highest levels in overall contents, but also were the responsible compounds for parsley-origin classification. Among the countries, France has clearly distinct content of benzenamine,N,N-4-trimethyl. GC-MS data were exposed to PCA and HCA to reveal: the variations of parsley chemical profiles as a result of geographical areas, to show the compounds that were responsible for clustering cultivars, and finally, to facilitate the future profile prediction and countries clustering of unknown parsley based on their chemical profile. Nine different cities of parsley samples from different origins were divided into two clusters based on benzenamine,N,N,4-trimethyl, apiol, myristicin, adamantane contents. Classification of parsley based on GC-MS profile will meet the practical requirements of parsley applications in clinical researches, industrial production, patients' self-production, and contribute to the standardization of commercially available parsley cultivars in the Mediterranean countries. The results of this work will accelerate the future profile prediction and origin-clustering of unknown parsley based on their profile.

Keywords   Medicinal parsley, Gas Chromatography, Principal Component Analysis, Cluster Analysis, Geographical Prediction

1. Introduction

The huge amount of data in natural products science has been increased over the last decade due to the application of modern instruments and chemometrics. This development performed a better understanding in the chemistry of natural products, especially for those used in medical applications [1]. Using natural products, especially herbal plants as traditional medicine and their molecules with therapeutic ability have been extended worldwide [2]. The World Health Organization (WHO) classified the traditional medicine as a complementary or alternative [3] since this medicine has preserve its popularity in the developing countries and its use is rapidly distributing in industrialized regions [4].

The essential   oils extracted from   herbal plants contain different kinds of volatile compounds such as phenol derived aromatic and aliphatic, mono and sesquiterpenes compounds [5]. Different studies show that many essential oils and extracts obtained from plants are widely produced in the Mediterranean region [6]. Different varieties and large number of herbal plants are grown in the Mediterranean, for example; sage, savory, thyme, lavender, clove, oregano, rosemary, salvia judaica boiss [7-8], salvia fruticosa, origanum syriacum, melissa officinalis, althaea damascena, cyclotrichium origanifolium, origanum ehrenbergii, viola libanotica, clinopodium libanoticum, thymbra spicata and satureja thymbra [9-13], chamomile, menthe, achillea, artemisia, coriander and parsley. These plants showed their benefits in the biological activity tests for their antioxidant power, inhibitors to the growth of cancer cells [14], antispasmodic, antiemetic, stimulant, analgesic, and emmenagogue [15]. In addition to the medical usage, these essential compounds are used in food, drugs and perfumery industries [16]. For examples, in France, Lebanon and Jordan, many studies showed that herbal plants are used

in cosmetic, food, pharmaceutical and perfumery applications, treatment of abdominal pains and disorders, flatulence, diabetes, common cold, arthritis, parasitic worms, neurodegenerative diseases, urinary tract infections and kidney stones [9-10,13,17-20].

One of the most famous herbs produced and consumed in the Mediterranean Region is parsley (Petroselinum crispum) [21]. Parsley is a leafy, green herb, aromatic, culinary, and medicinal plant [22]. There are three types of parsley: the curly leaf parsley (P. crispum var. crispum) that is frequently used as a trimming; the flat leaf parsley (P. crispum var. neapolitanum) which is used in Mediterranean dishes such as tabbouleh and salad decoration; and the third kind is called root parsley (P. crispum var. tuberosum) that is developed as a root vegetable [23]. Fresh parsley is considered as a rich source of vitamin B, vitamin C, β-carotene, zinc, boron, fluorine, iron and calcium in an absorbable form [24].

Parsley is grown wildly outdoors and it is seasonally harvested in the Mediterranean, Middle East and North Africa (MENA) regions. The perfect environmental conditions to parsley growth are sunny seasons, soil humidity, and pH between 5.3 and 7.3 [24]. Parsley normal average height is 60 -120 cm and its chemical composition mainly depends on water stress, so that, it must be provided with continuously water source to get the highest quality and maximum amount [24]. Great differences reveal in the chemical composition of essential oils and the quality obtained from the parsley plant from different origins, and these differences are related to many factors: plant quality, geographical location, soil type, day length, irradiance, water supply, season of harvesting, climatic and soil properties, cultivation process, distillation technique and rain levels [6,25].

Regarding the extraction techniques of essential oils [19,26-33]; hydrodistillation by Clevenger type apparatus [26], liquid–liquid extraction [27], solvent extraction [28], solid phase micro extraction [32], ultrasound extraction [19] and microwave [33] have been implemented to extract the maximum amount of essential oils from plant. However, hydrodistillation by Clevenger type apparatus is the most famous technique since it is fast and showed high efficient extraction yield with low cost [26]. Regarding the analysis, GC-MS is the most popular technique used for analyzing the essential oils since it could separate complex mixtures into their individual components, identify and quantify them easily [25,34].

Hierarchical cluster analysis (HCA) and Principal component analysis (PCA) are beneficial tools in analytical chemistry for data classifications. They were implemented in analytical studies for many purposes; to fingerprint the most relevant compounds in recognizing plant varieties, to detect the variation on chemical profiles as a result of growing plants in different areas and with variations in growth time, to ensure whether the cultivars in the cluster analysis could also be grouped together and to show the compounds that were responsible for grouping cultivars between clusters [35].

There is currently no available systematic clustering for the parsley-origin samples between Mediterranean countries that are considered as big cultivars and consumers, which is indispensable to explore the similarities/differences if any between plants-origin batches. Hence, this study was implemented to examine the chemical profiles of essential oils in parsley grown in Mediterranean countries. For the first time, parsley plants obtained from nine cities of three countries; France, Jordan and Lebanon were collected, extracted and analyzed using GC-MS. The parsley samples were scanned to reveal all possible compounds necessary for plants clustering. The validated method was evaluated for selectivity and precision (repeatability and day to day intermediate precision). Grouping the plants samples from different countries was accomplished using unsupervised clustering methods (i.e. PCA and HCA). Furthermore, the importance of the chemical contents for samples classification has been revealed. To the best of our knowledge, this study is the first to classify the Mediterranean parsley samples and to use the PCA and HCA to predict the location for future samples analyzed in blind.

2. Material and Method

2.1. Parsley Samples

Fresh parsley plants were collected from local cultivators during August/September 2019. Samples were selected from three countries France, Jordan and Lebanon. From each country, three different cities were selected to collect parsley from their cultivators, and from each city; 5 bundles of parsley were taken. French cultivated samples were obtained from the following areas and regions: Paris (F1), Dijon (F2) and Picardie (F3), the Jordanian samples were provided from Southern Jordan Valley (J1), Amman airport region (J2) and Mafraq (J3), and finally the Lebanese ones were collected from Tripoli (L1), Beka'a Valley (L2) and Kherbet Eljord (L3).

2.2. Reagents

The chemicals used in this work were of HPLC ultra-grade: n-Hexane 95% obtained from TEDIA, USA, and sodium sulphate anhydrous Na_2SO_4 were purchased from Biochem Chemopharma, France. Ultra pure water (18 MΩ cm^{-1}) was produced by Milli-Q plus water purification instrument (Millipore, Molsheim, France).

2.3. Essential Oils Extraction from Parsley

The extraction of parsley essential oils was carried out by hydrodistillation using a Clevenger type apparatus according to the following procedure: after collecting the plants samples from the cultivars, the leaves of the 5 bundles had been cut down by plant scissors to ensure a homogenous mixture of leaf particles with no seeds or stem, and inserted into stainless steel blender container. Then, 1.5 L of ultra pure water had been added. The contents had been mixed for about 15 min, after that, immediately, the mixture was

transferred into 5.0 L Clevenger apparatus (Chitransh borosilicate, India). Finally, the mixture was heated under temperature regulation. After the operating temperature reached the boiling point of the mixture in the Clevenger apparatus distillation flask, the heating was continued for extra 2 hrs. Parsley essential oils were collected in 10.0 mL beaker, and then a small amount of anhydrous sodium sulphate was added to eliminate any remaining water in the extracted oil. A concentration of 0.02% (v/v) extracted essential oil/ n-hexane was prepared for the GC-MS injection. Finally, Acrodisc syringe (PAU-Gelman Lab, 0.45 μm, 25 mm diameter) membrane micro-filter was used to filter the extract, and then the extract was collected in a screw-capped vial. Samples were reserved in the dark in a freezer (at least -10℃) until time of analysis.

The extractions and the GC-MS injections were carried out in triplicate. The injection volume was 10.0 μL. The content of each essential oil was reported as an average of the three injections. Quantification of parsley chemical content for each compound was achieved by internal normalization, i.e. by comparing the ratio of peak area of the component in the sample with the summation of all peak areas in the parsley plant sample.

2.4. Gas Chromatography – Mass Spectrometer Detector

The chemical profiles of the parsley extracts obtained by GC-MS were all generated using a VARIAN Model CP-3800 Gas Chromatographic system coupled with Saturn 2200 GC/MS/MS, CP-8400 autosampler. The GC column was SLB-5MS fused silica capillary column, 30 m length × 0.25 mm internal diameter, 0.25-μm film thickness, (Sigma-Aldrich, USA). The front injector temperature was set at 250℃.

The temperature was programmed in a linear generic gradient mode from 60℃ to 250℃ at a rate of 3℃/min with a holding time of 1 min. The carrier gas was helium of (99.999% purity) at constant column flow rate of 1.0 mL.min^{-1}. Solutes detection was achieved using mass spectrometer with scan range from 40-450 m/z, and samples were injected in a split ratio of 1:10 with ionization energy of 70 eV. The overall runtime was 65 min/sample, and the injection volume was 10.0 μL/sample.

2.5. Chromatographic Identification and Quantification of Extracted Essential Oils

To evaluate the selectivity, solvent blank was injected to ensure that no artifact signal peaks were appeared at the targeted retention time. The quantitative analysis for each essential oil component expressed in a content% was calculated according to the following estimation:

$$Percent = \frac{Peak\ area\ of\ the\ component}{Summation\ \ of\ all\ peak\ areas} x\ 100\% \quad (1)$$

The identification of each compound (i.e. name, molecular weight and structure) was carried out based on matching the spectrum of each compound with the MS computer library using the database of National Institute Standard and Technology (NIST 05 MS Library and AMDIS 2.6).

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single matrix. Herein, the precision was evaluated as intra-day and inter-day precision and expressed by the relative standard deviation (RSD) of the peak area variations of pinene as probe analyte in repeated samples. Interday precision was calculated from the measurements of 12 replicates of consecutive days, where intraday precision was measured by the analysis of five replicates in a single day.

3. Statistical Analysis

3.1. Multivariate Analysis of Parsley Samples Using Unsupervised Clustering Methods

Arrangement of chromatographic data

At first, the GC data was organized in a data matrix $X_{n \times l}$, where n is the number of plants (i.e. cities) and l is the number of solutes. In this study, matrix X had the size of 9×29. Matrix X was subjected to clustering methods (as will be explained below). All the collected samples were priori assigned to class membership; hence, unsupervised clustering methods will be applied [35-36].

PCA and HCA

The most common unsupervised methods in analytical chemistry for grouping and clustering samples are PCA and HCA [37]. In this study, HCA and PCA were implemented to confirm whether the parsley obtained from different cities would be grouped together based on the GC-MS analysis outcomes of the 29 compounds in each plant sample. In fact, PCA reveals the compounds that are responsible for grouping the samples [35,38-39]. The classification outcomes might be necessary for clinical researchers in the selection and discrimination of plant samples in certain cities. The results may help to show the similarities and the differences between parsley profiles cross the big producers and consumers of parsley (i.e. Lebanon, Jordan and France).

In PCA, matrix X is divided into two matrices; score matrix T and loading matrix L using non-linear iterative partial least-squares or singular value decomposition. Therefore, $X_{9 \times 29}$ is divided into $T_{9 \times h}$ and $L_{h \times 29}$ (h is the number of optimum factors required for matrix decomposition). The value of h is estimated by leave-one-out cross validation method. When h is computed, scores and loadings vectors are appeared to estimate the clusters and the significance of variables for clustering plants. Using HCA in GC data is important to emphasize their natural clusters and patterns in a two dimensional space. The results are showed in the form of a dendrogram to allow rapid figure out of clusters and correlations between the studied samples [36,38-39].
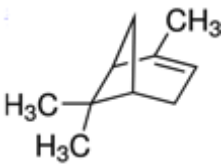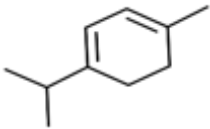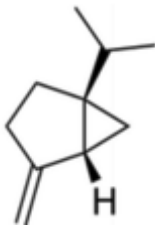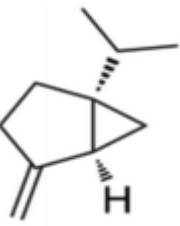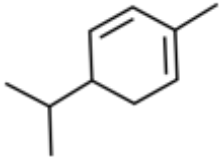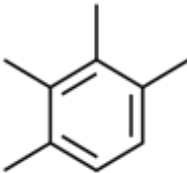
3.2. Statistical Software

Data acquisition and processing were achieved using MS Workstation software (version 6.6 SP1). PCA and HCA statistical analysis were performed by Chemoface 1.61 software which work under Matlab® (Mathworks, 8.6, USA) and *XLS*TAT software (Excel, Microsoft®). The peak area ratio was measured for each solute as following: peak ratio = peak area of solute /summation of peak areas of the sample) and used to quantify the content of the each solute.
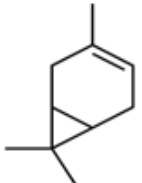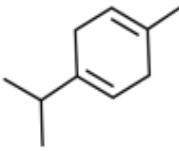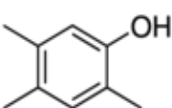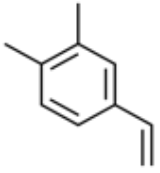
4. Results and Discussion
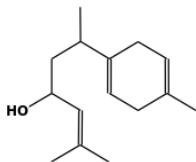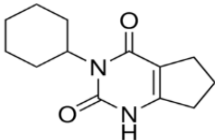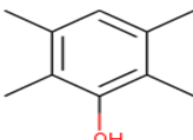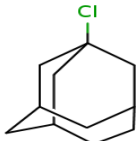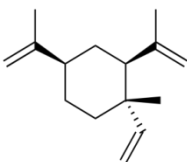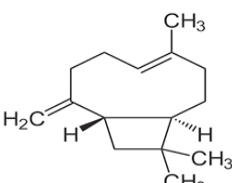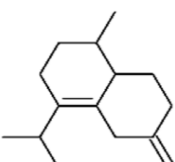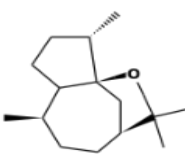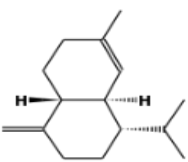
4.1. GC Analysis and Variability of Parsley Contents in Different Samples

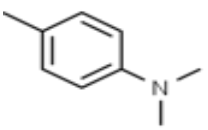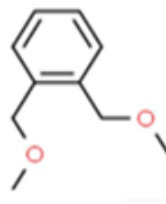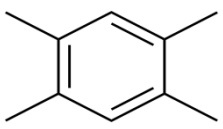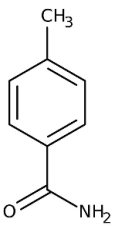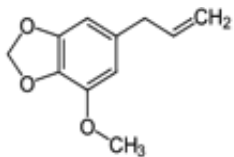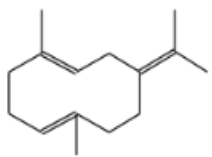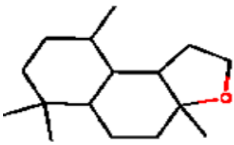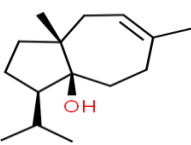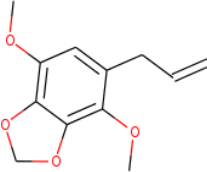Twenty nine compounds were detected in parsley samples in this study, and identified in the plant samples over the 9 cities in Lebanon, Jordan and France as shown in Table 1.

Table 1. The 29 solutes identified in parsley samples obtained from the nine cities in France, Jordan and Lebanon

Name of compound	Chemical structure	Chemical formula	M.wt (g/mol) [40]	Retention time (min)	pKa, LogP values [40]
α-Pinene		$C_{10}H_{16}$	136.23	6.27	LogP = 2.8
α- Terpinene		$C_{10}H_{16}$	136.23	7.42	LogP = 3.16
(+)-Sabinene		$C_{10}H_{16}$	136.23	7.60	LogP = 2.86
(-)-Sabinene		$C_{10}H_{16}$	136.23	7.93	LogP = 2.86
α-Phellandrene		$C_{10}H_{16}$	136.23	8.52	LogP = 3.21
Benzene-1,2,3,4-tetramethyl		$C_{10}H_{14}$	134.22	9.24	LogP = 4.1

Adamantane		$C_{10}H_{16}$	136.23	9.50	LogP = 4.24
3-Carene		$C_{10}H_{16}$	136.23	9.94	LogP = 4.38
γ-Terpinene		$C_{10}H_{16}$	136.23	10.43	LogP = 3.16
Phenol 2,4,5 trimethyl		$C_9H_{12}O$	136.19	11.58	pKa = 10.57 LogP = 3.15
Benzene,4-ethenyl-1,2-dimethyl		$C_{10}H_{12}$	132.20	11.81	LogP = 4.43
Benzenamine,N,N,4-trimethyl		$C_9H_{13}N$	135.21	12.94	pKa = 5.34 LogP = 2.59
Benzen,1,2-bis(methoxymethyl)		$C_{10}H_{14}O_2$	166.22	13.62	LogP = 1.09
Durene		$C_{10}H_{14}$	134.22	14.28	LogP = 4.00
p-Toluamide		C_8H_9NO	135.16	16.23	pKa = 16.21

Turmerol		$C_{15}H_{24}O$	220.35	16.77	LogP = 4.81
Lenacil		$C_{13}H_{18}N_2O_2$	234.29	17.87	pKa = 10.66
Durenol		$C_{10}H_{14}O$	150.21	18.25	pKa = 10.88
Tricyclo[4.3.1.1(3,8)]undecane, 3-chloro		$C_{11}H_{17}Cl$	184.70	18.40	LogP = 3.44
β-Elemene		$C_{15}H_{24}$	204.35	24.44	LogP = 4.74
β-Caryophyllene		$C_{15}H_{24}$	204.35	25.68	LogP = 4.52
Bicyclo[4.4.0]dec-1-ene-2-isopropyl-5-methyl-9-methylene		$C_{15}H_{24}$	204.35	28.21	LogP = 4.51
Liguloxide		$C_{15}H_{26}O$	222.37	28.55	pKa = -4.2 LogP = 3.87
γ-Cadinene		$C_{15}H_{24}$	204.35	29.91	LogP = 4.51

Myristicin		$C_{11}H_{12}O_3$	192.21	30.28	pKa = -4.5 LogP = 2.54
Germacrene B		$C_{15}H_{24}$	204.35	31.33	LogP = 4.78
Guaioxide		$C_{15}H_{26}O$	222.37	31.47	pKa = -4.2 LogP = 3.87
(+)-Carotol		$C_{15}H_{26}O$	222.37	33.05	pKa = 0.26 LogP = 3.79
Apiol		$C_{12}H_{14}O_4$	222.24	36.01	pKa = -4.40 LogP= 2.34

The same procedure was applied for all samples, so the comparison between all samples is valid. The detected compounds had variable proportions and were appeared in 40 min. Figure 1, showed a model example of GC-MS chromatogram of Picardy-origin/France parsley. GC-MS analysis in Figure 1 showed that benzenamine,N,N, 4-trimethyl, apiol, myristicin, adamantane have high intensity at retention times of 12.94, 36.01, 30.28 and 9.50 min, respectively, where apiol and myristicin separated at longer retention times in comparison to others which would be related to their high boiling point (i.e. 187℃, 211℃, 276℃ and 294℃ for adamantane, benzenamine,N,N, 4-trimethyl, myristicin and apiol, respectively).

The precision was evaluated as intra-day and inter-day precision and expressed by the relative standard deviation (RSD) of the peak area variations of pinene as tested analyte in repeated samples. Intraday precision was measured by the analysis of five replicates in a single day, while interday precision was evaluated from the measurements of 12 replicates of consecutive days. The intra and inter-day precisions (%RSD) were 2.70% and 3.57%, respectively. The chemical composition % of parsley samples (%peak area) are shown in Table 2.

Twenty nine compounds were identified in parsley essential oils. The detected compounds were related to ten different classes: monoterpene hydrocarbons (eight compounds), aromatic amine (one compound), aromatic hydrocarbons (4 compounds), amino acid (one compound), sesquiterpenes hydrocarbons (five compounds), oxygenated sesquiterpenes (three compound), monoterpenoids (two compounds), and phenylpropanoids (one compound), terpenes (three compounds) and phenol (one compound) as shown in Figure 2.

The main four solutes of the parsley essential oil leaves that contained the highest contents levels as shown in Table 2 were: benzenamine,N,N,4-trimethyl (15.19% - 41.16%), apiol (6.96% -36.27%), myristicin (3.72 - 20.44%) and adamantane (4.05% - 19.96%). Regarding apiol and myristicin, our results agree quite well with those reported in another Mediterranean country, Greece and Estonia/Europe [5,41] who applied hydro distillation method for essential oil separation and GC for identifying the extracts. They found that the main contents of essential oil of the leaves of parsley were myristicin and apiol. On the other hand, El-Zaeddi et al., [42] found 1,3,8-p-menthatriene and β-phellandrene as the main components that represented 68%–72% of the total

concentration of essential oil of parsley in Spain. As noticed, the quantities of terpenes and other contents within plants vary significantly from one region to another [35-37]. Moreover, in Table 2, looking to the three cities from each country, terpene's and other contents levels vary significantly from one growing countries to another and from

plant to plant within the same growing area. This might be related to the mineral contents of the groundwater, soil, growing medium and air pollution [43].

The variations in terpenes and other contents in the studied plants would be necessary to obtain good clustering objectives as will be appeared in the next sections.
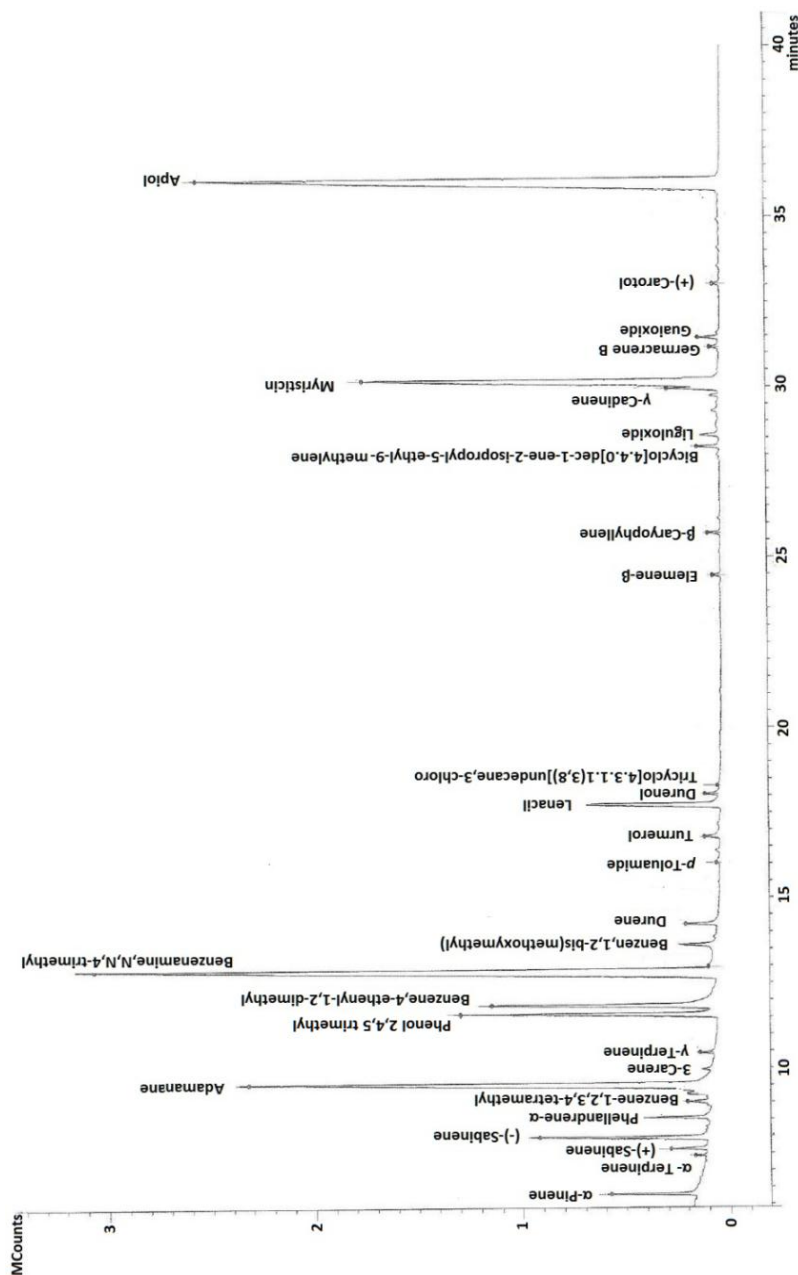
Figure 1.   GC-MS of Picardy-origin/France parsley

Table 2.   Chemical composition of parsley plant samples obtained from different cities in the Mediterranean Region. French samples (F) are: (F1) Paris, (F2) Dijon, (F3) Picardy. Jordanian samples (J) are: (J1) Southern Jordan Valley, (J2) Amman Airport area, (J3) Mafraq. Lebanese samples (L) are: (L1) Tripoli, (L2) Beka'a Valley, (L3) Kherbet Eljord

Name of solute	F1%	F2%	F3%	J1%	J2%	J3%	L1%	L2%	L3%
α-Pinene	1.070	0.180	1.020	1.730	4.080	2.130	1.600	3.100	2.370
α- Terpinene	0.080	0.000	0.000	0.260	0.450	0.260	0.000	0.390	0.270
(+)-Sabinene	0.500	0.280	0.480	0.270	2.410	1.520	0.900	1.840	1.410
(-)-Sabinene	1.960	0.970	2.760	5.210	7.150	4.690	3.280	4.860	4.130

| Name of solute	F1%	F2%	F3%	J1%	J2%	J3%	L1%	L2%	L3%
α- Phellandrene	0.860	0.420	0.830	1.440	1.780	1.460	0.780	1.260	1.430
Benzene-1,2,3,4-tetramethyl	1.030	0.870	0.670	1.060	1.450	1.360	0.970	0.000	0.400
Adamantine	11.730	4.050	12.800	17.030	19.960	15.120	11.670	18.170	12.200
3-Carene	0.120	0.000	0.140	0.700	0.640	0.450	0.510	0.500	0.450
γ-Terpinene	0.300	0.450	0.200	1.010	1.410	1.240	0.930	0.720	0.850
Phenol 2,4,5 trimethyl	5.660	1.930	4.620	6.470	6.790	6.360	3.980	4.500	4.150
Benzene,4-ethenyl-1,2-dimethyl	5.180	4.290	5.080	7.990	10.190	9.210	4.270	5.570	2.590
Benzenamine,N,N,4-trimethyl	41.160	38.630	21.890	12.780	17.510	30.640	12.420	15.190	36.430
Benzen,1,2-bis(methoxymethyl)	0.670	0.840	0.470	1.600	2.020	2.100	0.000	0.000	0.000
Durene	0.000	0.000	0.000	1.030	1.450	1.620	0.000	0.000	0.000
p-Toluamide	0.160	0.000	0.000	0.320	0.240	0.240	0.000	0.000	0.000
Turmerol	0.300	0.220	0.300	0.690	0.000	0.420	0.000	0.000	0.000
Lenacil	0.500	0.000	3.360	0.170	0.200	0.100	0.000	1.160	1.730
Durenol	0.080	0.190	0.230	0.640	0.370	0.540	0.000	0.000	0.000
Tricyclo[4.3.1.1(3,8)]undecane,3chloro	0.060	0.070	0.090	0.150	0.180	0.000	0.000	2.920	0.510
Elemene-β	0.060	0.000	0.140	0.580	0.190	0.000	0.000	1.330	0.510
β-Caryophyllene	0.160	0.440	0.220	0.700	0.870	0.430	1.110	0.790	0.280
Bicyclo[4.4.0]dec-1-ene-2-isopropyl -5-methyl-9-methylene	0.260	0.440	0.370	0.660	1.180	0.700	0.650	1.160	1.000
Liguloxide	0.090	0.000	0.330	0.190	0.760	0.520	0.890	0.560	0.340
γ-Cadinene	0.130	0.130	0.790	0.150	0.740	0.610	0.510	1.030	0.980
Myristicin	20.440	12.300	12.670	13.690	3.720	8.480	15.580	16.980	11.600
Germacrene B	0.190	0.000	0.000	0.520	0.490	0.410	0.770	0.490	0.410
Guaioxide	0.080	0.110	0.310	0.630	0.690	0.370	0.810	0.540	0.400
(+)-Carotol	0.210	0.010	0.120	1.280	0.680	0.710	2.100	1.050	0.810
Apiol	6.960	33.180	30.110	21.050	12.400	8.310	36.270	25.890	14.760
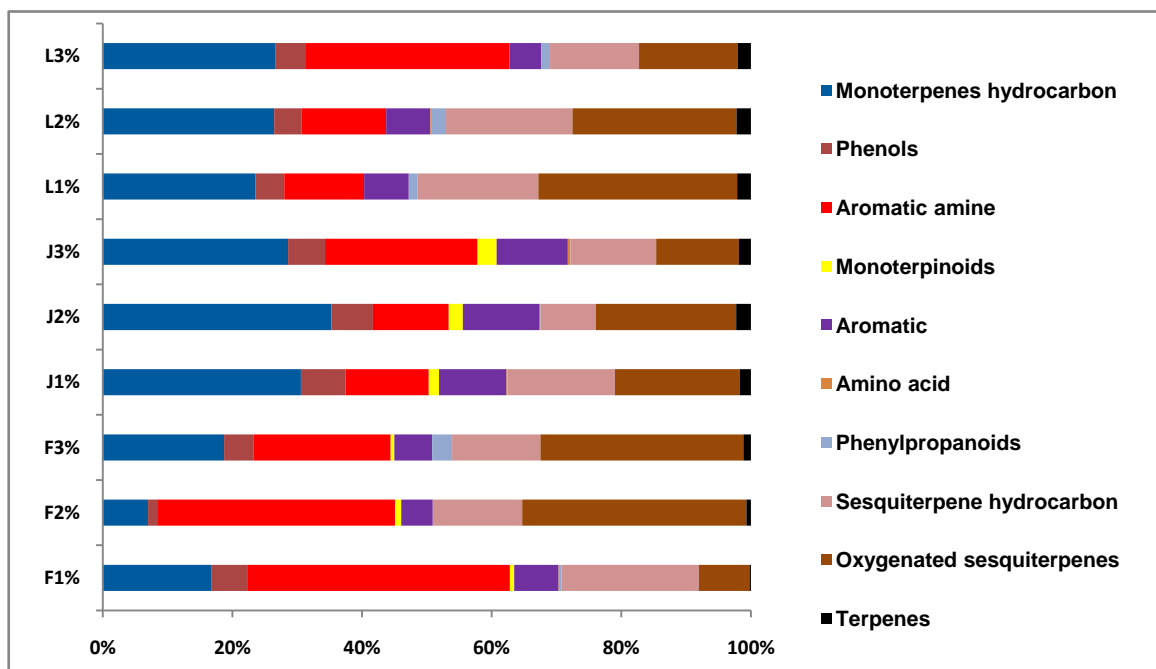
Figure 2. Major volatile classes in different parsley plant samples-origins. French samples (F) are from: (F1) Paris, (F2) Dijon, (F3) Picardy. Jordanian samples (J) are from: (J1) Southern Jordan Valley, (J2) Amman Airport area, (J3) Mafraq. Lebanese samples (L) are from: (L1) Tripoli, (L2) Beka'a Valley, and (L3) Kherbet Eljord

4.2. Comparison between Parsley Chemical Contents of French, Jordanian and Lebanese Samples

It was clear that all parsley plant samples collected from the 9 areas have the same major four abundant components: adamantane, benzenamine,N,N-4-trimethyl, myristicin and apiol, with different order from one sample to another. There are variations between one content to another in the same sample, this result is referred to overlapping or interfering of other peaks due to some physical changes like solubilization and co-dissolution in the process of decoction when heat is applied to distillate essential oil compositions [44].

Our results were in agreement with other studies from Spain, Egypt, Brazil and Saudi Arabia which found that both myristicin and apiol were the major components [45-47]. Adamantane was also mentioned in previous study from Turkey as a main component [48], where, benzenamine,N,N-4-trimethyl was not found in the parsley from Spain, Egypt, Turkey, Brazil and Saudi Arabia [45-48]; which provide the Mediteranian parsley a special characteristic to facilitate distinguishing it from other parsley-type origin.

A quick look at the distribution of the compounds in our study showed retention time was mainly affected by the volatility of compounds. Any peak before 6 min was mainly the solvent n-hexane which is very volatile. The compounds detected between 6-10 min had the highest volatility (α-pinene, α-terpinene, (+) sabinene, (-) sabinene, α-phellandrene, benzene-,1,2,3,4-tetramethyl, adamantane, 3-carene). Compounds identified between 10-20 min were moderately volatile (γ-terpinene, phenol 2,4,5 trimethyl, benzene,4-ethenyl-1,2-dimethyl, benzeneamine,N,N,4-trime thyl, benzene,1,2-bis(methoxymethyl, durene, ρ-toluamide, turmerol, lenacil, durenol, tricyclo[4.3.1.1(3,8)]undecane, 3-chloro). Finally, late detected compounds had the lowest volatility (β-elemene, β-caryophyllene, bicyclo[4.4.0] dec-1-ene-2-isopropyl-5-methyl-9-methylene, liguloxide, γ-cadinene, myristicin, germacrene, guaioxide, (+)-carotol and apiol). Regarding the three Mediterranean countries in this study, in general, the same compounds showed the highest, modest and lowest concentration, which means that they have similar content for the main compounds regardless the small differences in their level.

The quantitative and qualitative results showed variations in the percentage at each compound; this is related to: 1) geographic variations; climate, seasonal changes, amount of time of exposure to sunlight during the day, humidity, 2) environmental variations; soil composition, pH of the soil, 3) type of original seeds of plant [35,49]. Jordan and Lebanon share common temperature's variations during the four seasons of the year with main differences in humidity percentage (i.e. humidity in the Eastern Mediterranean side of Lebanon is high due to the topography of the narrow coastal plain close to a long parallel and high mountains chain). The evaporation from the sea, return back to the crowded cities of the plain producing humidity up to 85% between July-September. Where France has different

temperature variations during the year (lower temperature than Jordan and Lebanon during the whole year, especially in summer and spring seasons). Moreover, chemical variation could be explained to genetic factors impacting the secondary metabolism, harvesting time of the biological material in different development phases, interaction with microorganisms and insects, and different post-harvest techniques [45,50]. In addition, the growth of parsley in hot weathers such as Mediterranean regions (in late spring and summer), make it easily open to stress because of water deficiency in warm countries. This stress causes a decrease in biomass, leaf number per plant that resulted from a disruption of photosynthesis, transpiration and metabolic processes [41,51]. The effect of water stress is not only impacted the plant growth and essential oil yield, but it may change the quality of the oil. To unravel the differences/similarities between parsley samples from France, Jordan and Lebanon, multivariate analysis has been applied to the chromatographic data in the next section.

4.3. Parsley Characterization by PCA and HCA

Both PCA and HCA are performed to confirm whether the parsley obtained from different cities would be grouped together based on the GC-MS measured active ingredients, including volatile oils with a total number of 29 solutes (29 detected peak). PCA can reveal the compounds that are responsible in grouping parsley samples. Parsley classification will be helpful in the industrial production, and informative map for individual cultivars. Indeed, these results would help to show the similarities and the differences between parsley-origin samples of some Mediterranean countries that are considered as big producers and consumers of parsley.

Arrangement of chromatographic data: Chromatographic data can be arranged as a data matrix X of n samples or location rows and l variables or compounds. In this study, matrix X has the size of 29 (solutes) × 9 cities. Matrix X was subjected into HCA and PCA as discussed below. Data was preprocessed using normalizing methodology which allowed for better and interpretably PCA outputs.

Quantitative parsley classification by PCA and HCA

In this study, two data matrices where built. The data matrix X (29×9) (i.e. 29 solutes from 9 cities) is divided to two matrices, score matrix T and loading matrix L using PCA algorithm. In PCA at first, the loadings are computed. In math, loadings are the Eigen vector of the matrix (XXT) [37]. The loadings are grouped into a matrix L. The gathered loadings are orthogonal and normalized. The relationship between the original matrix X, the loading matrix L and the score matrix T is described as:

$$X = TLT \qquad (2)$$

Mathematically, matrix X is decomposed in the product of two matrices, T and L, on the condition that L is formed by orthonormal columns. T is the obtained as: T = XTL. In this

work, size of X is 29×29 while size T is 29×h and L is h×29, where h is the factors required to decompose matrix X. The optimum number of factors (h) is needed to create maximum number of loadings and scores and produce informative discrimination among samples/parsley.

In order to classify parsley-origin samples and to determine the chemical contents responsible for grouping, the GC-MS data were exposed to HCA and PCA analysis, since these tools can reduce the complexity of multivariate data without losing the major information, reveal the variance, and visualize data grouping [35]. Herein, 29 chemical contents are the original dimensions in PCA. By measuring the covariance matrix between the 29 dimensions, PCA can produce 29 orthogonal PCs to explain 100% of the total variance of the orthogonal data. Each PC is correlated with the original 29 variables (dimensions).

All detected chemical contents were important for parsley-origin grouping. Therefore, the variable numbers implemented in clustering was 29 identified compounds × 9 cities. The HCA is appeared in Figure 3 based on average linkage clustering.

As indicated in Figure 3, two main clusters collect the 9 areas of the 3 countries. Cluster A collects the three French parsley-origins and cluster B collects the 6 Jordanian and Lebanese parsley-origins. HCA analysis revealed the same and/or comparable constituents of parsley plants. The French plant samples (3 cities) were grouped alone in Cluster A; accordingly, parsley samples collected from France are dramatically different from the other samples of other countries. This result is related to the distinct contents of benzenamine,N,N-4-trimethyl than other countries as shown in Figure 4 and Figure 5.

Regarding the PCA outcomes, PC1 describes 84.63% of original information (Figure 4), PC1 has negative loading for the 6 areas in Jordan and Lebanon. Where, PC2, has 6.78% of the variance of data set has a major contribution for the 3-French regions only. These 2 PCs collect 91.41% of the total variance in data.

Figure 4. revealed the major contents for grouping. Adamantane, benzenamine,N,N-4-trimethyl, myristicin and apiol were not correlated with other compounds and more important for origin-parsley grouping. This outcome is proved by the ones obtained before for the highest content marker compounds in the studied parsley samples.

As depicted in the biplot outcome, benzenamine, N,N-4-trimethyl was dominant to separate the French parsley-origin (F1, F2, and F3) far from the other samples collected from other regions. Apiol and myristicin were responsible constituents to isolate one sample from Jordan and one from Lebanon (J3 and L3). Adamantane was of large impact to isolate 4 parsley-origin samples (i.e. 2 Jordanian and 2 Lebanese) than other samples (J1, J2, L1, and L2). On the other hand, apiol is a common constituent with levels varying depending on the contents of the plant sample (i.e. leaves vs. bud, vs. mixture and the ratio of small to large leaves). Therefore, the difference in apiol level between samples is expected.
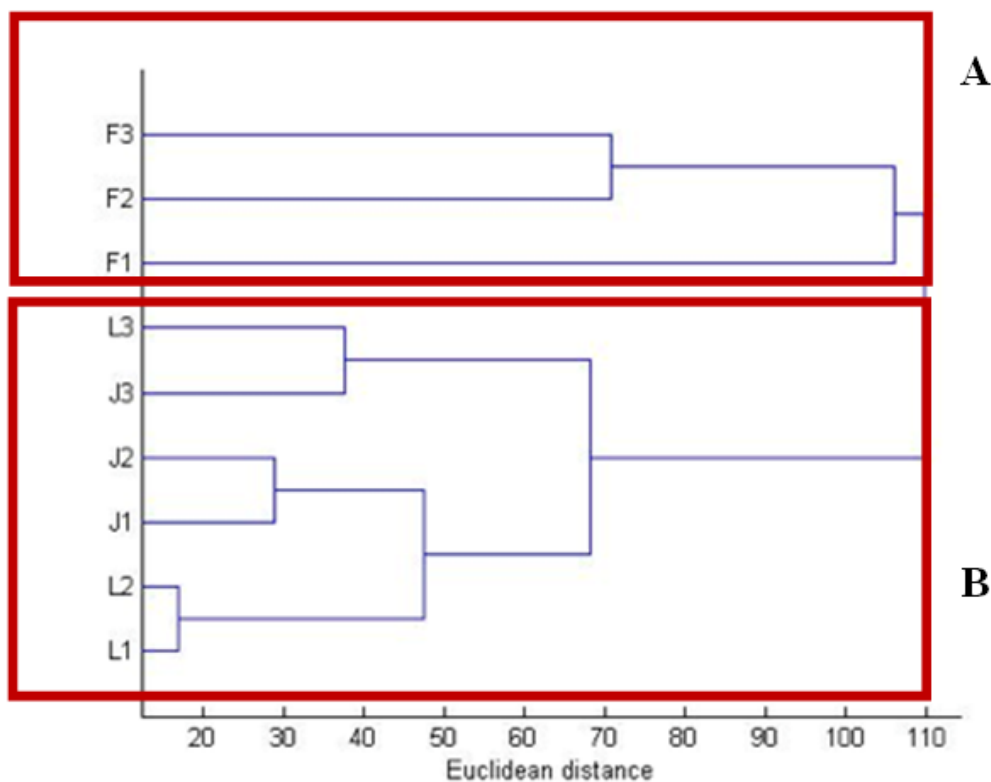

Figure 3. Dendogram of the 9 parsley-origins samples; Paris (F1), Dijon (F2), Picardie (F3), Southern Jordan Valley (J1), Amman airport region (J2), Mafraq (J3), Tripoli (L1), Beka'a Valley (L2) and Kherbet Eljord (L3)
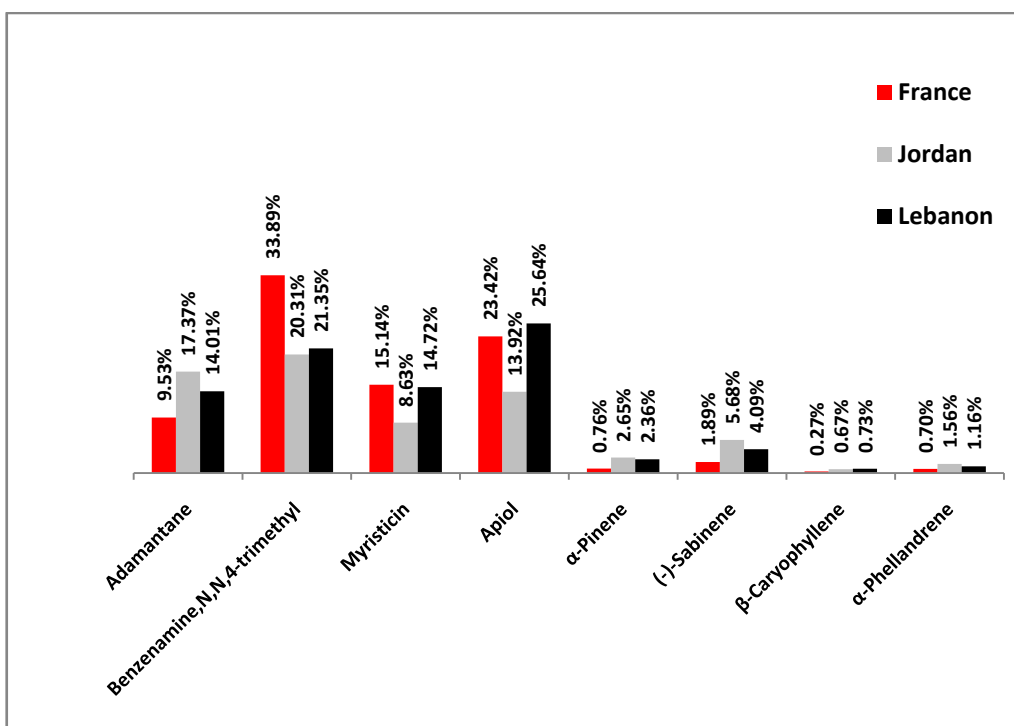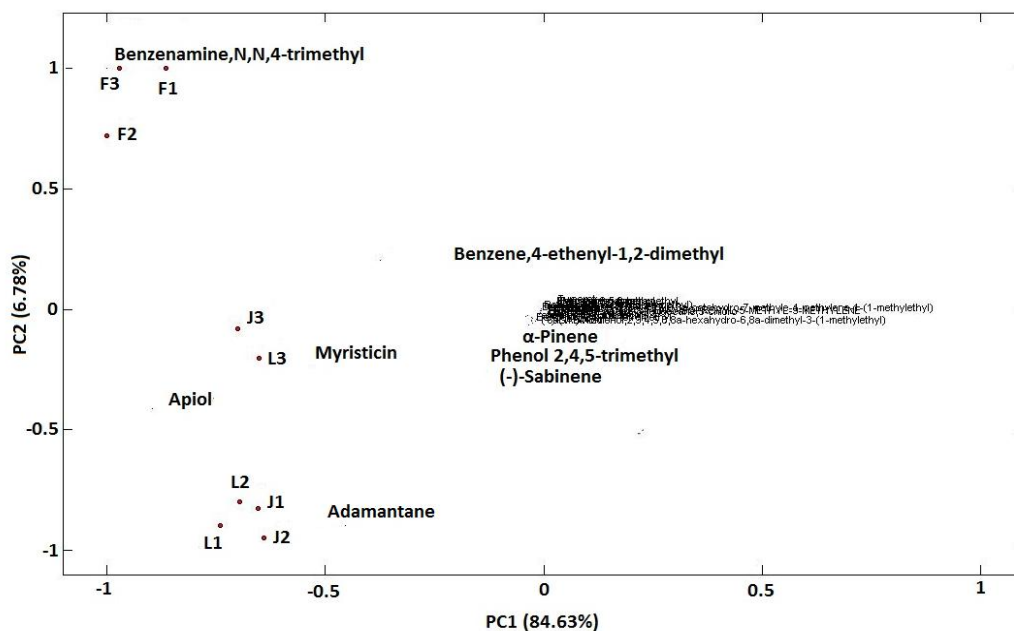
Figure 4. PCA output: bi-plot obtained from the 9 origin- parsley oil components of France, Jordan and Lebanon

Figure 5. Average abundance of the four main compounds selected by PCA model in the three Mediterranean countries samples in comparison to some other parsley contents

PCA explains where to find the longer radial separation of the content from the center (i.e. most important content is in distinctive countries). In our study, adamantane and benzenamine,N,N-4-trimethyl were the most significant contents followed by myristicin and apiol.

5. Conclusions

GC-MS was used to scan the chemical contents of 29 contents in 9 cities/regions of 3 Mediterranean countries: France, Jordan and Lebanon-parsley plants. The chemical contents were used to group parsley samples with the help of PCA and HCA. The grouping outputs uncovered the geographical origin of parsley plants. By HCA and PCA, the 9 parsley-origin plants were classified: first cluster consisted of the Lebanese and Jordanian cities, the second cluster had only the French plants that revealed distinct constituents of benzenamine,N,N-4-trimethyl. Multivariate analysis

revealed also which constituents are major in differentiating cultivars since plants were clustered into 2 clusters; cluster A is benzenamine,N,N-4-trimethyl dominant, cluster B is apiol, myristicin and adamantane dominant. The results were fairly efficient since classifications based intensively on all contents considering all medically relevant compounds in parsley. Further studies will be carried out in the future to include parsley from all the Mediterranean countries in the same period in order to fill up the missing data in this area.

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Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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