Analysis of Volatiles in Palestinian Honey by HS- SPME/GCMS to Determine its Botanical Origin

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Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed: ……

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Date: ……

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ABSTRACT

Nine different unifloral Palestinian honey samples (Citrus, Thymus Capitatus, Thymelea Hirsuta, Ziziphus Spinachristi, Eucalptus Camalydensis, Goffypium Officinalis, Centaurea Italica, Tolpis Virgata, and Wild Flower) were analyzed by Head Space Solid Phase Micro Gas Chromatography Mass Spectrometry Extraction (HS-SPME/GCMS). The analysis performed exhibited TIC profiles characteristics for each type of honey. This procedure demonstrated a rapid characterization method for the analysis of different types of honey, by revealing the presence or absence of certain organic constituents.

The GCMS Chromatographic profiles show that honey contains a variety of volatile compounds particularly phenols, aldehydes, ketones, acids, and alcohols. In addition, the compound present in a specific honey offer a marker profile that can be utilized in relating a given honey to its floral origin.

Citrus honey was characterized by the presence of three volatile compounds: 2-amino, benzoic acid methylester, 1-Hydroxylinalool, and phenol,2-methoxy-4-(1-propanol), but *Thymus Capitatus* honey showed six marker compounds: 3,4-Dimethoxy benzaldehyde; Benzene-3-methylbutyl; Thymol, Vanilline; 2-prpanone-1,3-diphenyl, and 3,4,5-Trimethoxy benzaldehyde. On the other hand, *Thymelea Hirsuta* honey is characterized by the presence of a group of alcohols and phenols particularly Hexanol; Nonanol; Benzylalcohol; Benzene propanol; and 4-Methoxyphenol .

Ziziphus Spinachristi honey has three volatile markers: Benzeneacetanitrile; 3-Methoxy benzladehyde and Phenol-2-methoxy-4-(1-propanol)-6-acetate. *Eucalyptus Camalydensis* honey contains specifically: 2-propyl,1-pentanol and pentadecane. *Goffypium Officinalis* honey was characterized by the presence of 2-Methyl decanol, Eicosane and 2-Furanomethanol, while *Centaurea Italica* has only one volatile marker compound: 2,4,6-Trimethylphenol. On the other hand, *Tolpis Virgata* honey has two markers: Tridecane and 3,5-Dihydroxytoluene. The *Wild Flower* honey is distinguished from other floral honey by the presence of three volatile markers: 3,4-Dimethyl benzaldehyde; Hexadecane and Heptadecane.

To My
Parents and my family

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CHAPTER 3. RESULTS AND DISCUSSION

LIST OF ABBRIVIATIONS

GC	Gas Chromatography
MS	Mass Spectrometry
GCMS	Gas Chromatography Mass Spectrometry
HS	Head Space
SPME	Solid Phase Micro Extraction
RSD	Relative Standard Deviation
NIST	National Institute of Standards and Technology
TIC	Total Ion Chromatogram
t_R	Retention Time
ng	Nano Gram
µl	Micro Liter
ml	Millie Liter
mm	Millie Meter
n	# of Replicates
$M^{+\cdot}$	Molecular ion
EI	Electron Impact
CI	Chemical Ionization
HPLC	High Performance Liquid Chromatography
ppb	Part per billion

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Deanship of Graduate studies

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1.1 Introduction

Recently, interest in the analysis of honey has increased dramatically with the aim of meeting the regulations required by authorities for marketing requirements [1]. Particularly important in this respect is the floral and geographical origin. The botanical origin of honey, which greatly influences consumer preference, remains difficult to determine [2]. Therefore, particular stress has been put on the need to develop suitable analytical methods to determine the botanical and geographical origin of honey in order to prevent fraud and to authenticate honey.

Honey is produced by honey bees from nectar and honey dew. It is composed of a mixture of carbohydrates, water, traces of organic acids, enzymes, amino acids, pigments, pollen, wax, and other organic compounds responsible for its flavors [3].

Unifloral honeys possess highly characteristic aromas, indicating the presence of various volatile components. Some of these are probably derived from the original sources of nectar, some are dependent on the physiology of the bee, and others arise in the processing stage after harvest [3].

The colors of honey form a continuous range from very pale yellow through amber to a darkish red amber to nearly black. The variations are almost entirely due to the plant source of the honey, although climate may modify the color.

The flavor and aroma of honey vary even more than the color. Although there seems to be a characteristic "honey flavor", almost an infinite number of aroma and flavor variations can exist. As with color, the variations appear to be governed by the floral source. In general, light-colored honey is mild in flavor and darker honey has a more pronounced flavor [4].

1.2 Uses of Honey

Because of honey's complex and unusual composition, it has several interesting attributes. It is used primarily as an enjoyable food and secondly in folk medicine due to its significant antibacterial activity.

1.2.1 Antibacterial Activity

An ancient use of honey was in medicine as a dressing for wounds and inflammations. Today, medicinal uses of honey are largely confined to folk medicine. On the other hand, since milk can be a carrier of some diseases, it was once thought that honey might likewise be such a carrier. Some years ago this idea was examined by adding nine common pathogenic bacteria to honey. All the bacteria died within a few hours or days [5]. Honey is not a suitable growth medium for bacteria for two reasons, it is fairly acidic, which makes it particularly effective as antimicrobial agents in media of low pH (3.9). It is too high in sugar content for growth to occur. This killing of bacteria by the osmotic

effect seems to function by literally drying out the bacteria. The inhibition effect was due to the hydrogen peroxide produced and accumulated in diluted honey [5]. This material, well known for its antiseptic properties, is a by product of the formation of gluconic acid by the enzyme glucose oxidase which is present in honey. The peroxide can inhibit the growth of certain bacteria in the diluted honey. Since it is destroyed by other honey constituents, an equilibrium level of peroxides will occur in a diluted honey, its magnitude depends on many factors such as enzyme activity, oxygen availability, and amount of peroxide-destroying materials. The amount of inhibine (peroxide accumulation) in honey depends on floral type, age, and heating [5] .

1.2.2 Food Value

Honey is primarily a high-energy carbohydrate food. Because its distinct flavors cannot be found elsewhere, it is an enjoyable treat. The honey sugars are largely the easily digestible "simple sugars," similar to those in many fruits. It can be regarded as a good food for both infants and adults. The proteins and enzymes of honey, though used as indicators of heating history and hence table quality in some countries, are not present in sufficient quantities to be considered nutritionally significant. Several of the essential vitamins are present in honey, but in insignificant levels. The mineral content of honey is variable, but darker honeys have significant quantities of minerals [5] .

1.3 Composition of Honey

Honey is essentially a highly concentrated water solution of two sugars, glucose and fructose, with small amounts of other complex sugars. Many other substances also occur in honey, but the sugars are by far the major components. The principal physical characteristics and behavior of honey are due to its sugars, but the minor constituents such as flavoring materials, pigments, acids, and minerals are largely responsible for the differences among individual honey types [4].

1.3.1 Water Content

The natural moisture of honey in the comb is that remaining from the nectar after ripening. The amount of moisture is a function of the factors involved in ripening, including weather conditions and original moisture of the nectar. After extraction of the honey, its moisture content may change, depending on conditions of storage. Normally the moisture content varies between 15-17% [5].

1.3.2 Sugars

Sugars represent the main components of honey. Besides the two main constituents, monosaccharides glucose and fructose, other more complex sugars

have been found in honey [6]. The sugars account for 95 to 99.9 % of the solids honey [5]. The identity of these sugars has been studied using a thin layer chromatography, ion chromatography with an amperometric pulsed detector, and high performance liquid chromatography [6]. They are classified according to their size or the complexity of the molecules of which they are made. Glucose and fructose account for about 85 percent of the solids in honey, and they are still by far the major sugars in honey. Four disaccharides have been identified: sucrose, maltose, isomaltose, turanose, and ten trisaccharides are present: melezitose, 3-α -isomaltosylglucose, maltotriose, l-kestose, panose, isomaltotriose, erlose, theanderose, centose, and isopanose. Two more complex sugars, isomaltotetraose and isomaltopentaose, have been also identified. Most of these sugars are present in quite small quantities. They do not occur in nectar, but are formed either as a result of enzymes added by the honeybee during the ripening of honey or by chemical action in the concentrated, somewhat acid sugar mixture of honey [5].

1.3.3 Acids

The acids in honey account for less than 0.5 percent of the solids, this level contributes not only to the flavor, but is in part responsible for the excellent stability of honey against microorganisms [3]. Several acids have been found in honey, gluconic acid being the major one. It is produced from glucose through the action of an enzyme called glucose oxidase. A method based on HLPC was described in order to characterize organic acids in honey the average recoveries of the acids ranged from 89 to 104% [6]. Many acids were found particularly: acetic, butyric, lactic, oxalic, succinic, tartaric, and maleic acid as shown in figure (1.1). Also many acids can be detected at volatile temperature using GCMS particularly: nonanoic acid, octanoic acid and decanoic acid [6].

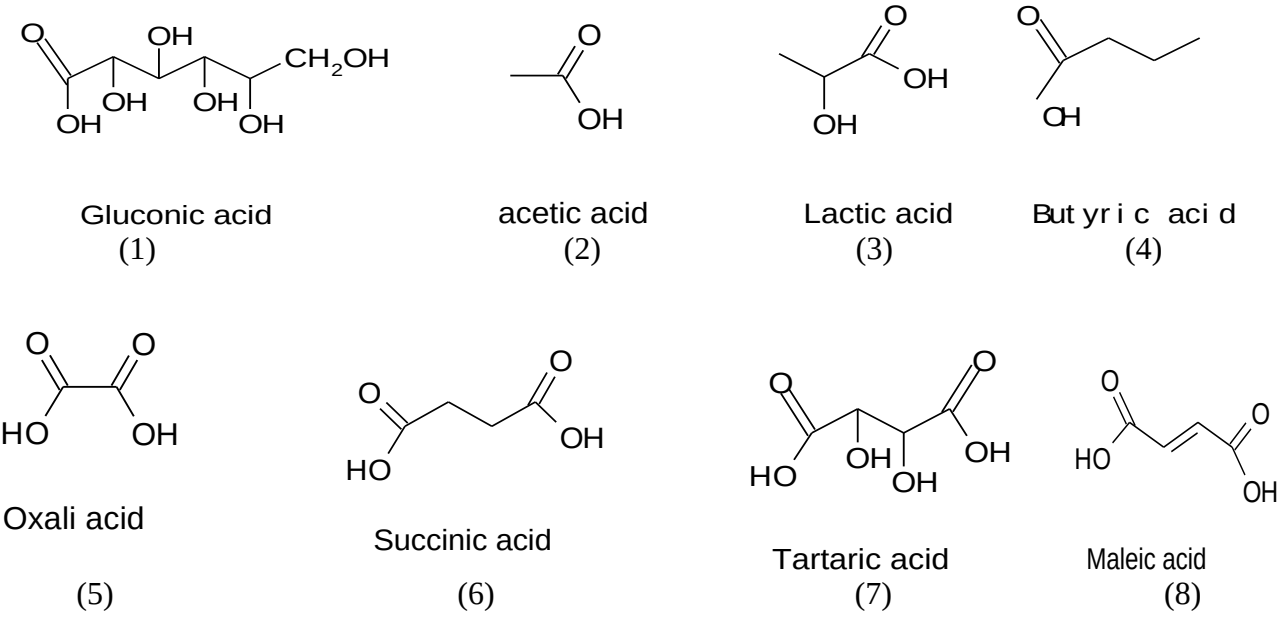


Figure (1.1): Some common organic acids in honey

1.3.4 Proteins and Amino Acids

The protein content of honey is normally less than 0.5%, but the amount of nitrogen in honey is low, 0.04 % on the average [6]. Recent work has shown that only 40 to 65 percent of the total nitrogen in honey is in proteins, and amino acids [4]. Of the 8 to 11 proteins found in various honeys, 4 are common to all, and appear to originate in the bee rather than the nectar. These various honeys contain 11 to 21 free amino acids; Proline, glutamic acid, alanine, phenylalanine, tyrosine, leucine, and isoleucine are the most common, with proline predominating. Amino acids are known to react slowly, or more rapidly by heating, with sugars to produce yellow or brown materials. Part of the darkening of honey with age or heating may be due to this [5].

1.3.5 Minerals

When honey is dried and burned, a small residue of ash invariably remains, which is the mineral content. It varies from about 0.04% in pale honey to 0.02% in some dark honey sample [6]. Schuette and his colleagues at the University of Wisconsin have examined the mineral content of light and dark honey [5]. An ion chromatography (IC) method was applied for the analysis of inorganic anions (chloride, sulphate) [6]. The mineral and trace element content in honey samples could give an indication of the environmental pollution and also an

indication of the geographical origin of honey. In general, the honey investigated has shown higher mineral contents: calcium, magnesium, copper, iron, potassium, manganese and phosphorus [6].

1.3.6 Enzymes

One of the characteristics that sets honey apart from all other sweetening agents is the presence of enzymes. These conceivably arise from the bee, pollen, nectar, or even yeasts or micro-organisms in the honey. Those most prominent are added by the bee during the conversion of nectar to honey.

Enzymes are complex protein materials that under mild conditions bring about chemical changes, which may be very difficult to accomplish in a chemical laboratory without their aid. The changes that enzymes bring about throughout nature are essential to life. Some of the most important honey enzymes are invertase, diastase, and glucose oxidase. Invertase, also known as sucrase or saccharase splits sucrose into its constituent simple sugars, glucose and fructose. Other more complex sugars have been found recently to form in small amounts during this action and in part explain the complexity of the minor sugars of honey. Although the work of invertase is completed when honey is ripened, the enzyme remains in the honey and retains its activity for some time. Since the enzyme also synthesizes sucrose, its thought that final low value for the sucrose content of honey represents an equilibrium between splitting and forming

sucrose. Diastase (amylase) digests starch to simpler compounds but no starch is found in nectar. What its function is in honey is not clear. Diastase appears to be present in varying amounts in nearly all honey and it can be measured. It has probably had the greatest attention in the past, because it has been used as a measure of honey quality in several European countries [7].

1.3.7 Flavonoids

Flavonoids constitute a large family of plant phenolic pigments. Many plant systems contain a large number of flavonoids and each plant tends to have a distinctive profile. The flavonoid content reaches about 0.5% in pollen, 10% in propolis and about 6000 ppb in honey. Only flavonoid aglycones seem to be present in propolis and honey. The flavonoids in honey and propolis have been identified as flavonones and flavonols. The antimicrobially active flavanone was found to be present in 11 of 12 honey samples of different origins as shown in figure (1.2) [5].

Flavonone (11)

Flavonol (12)

Figure (1.2): Some common flavonoids

1.3.8 Phenolic compounds

During the second metabolism of plants, various hydroxybenzoic, and hydroxycinnamic acids are formed. It has been shown that concentrations of such substances differ in various plants [6]. Aromatic carbonic acids (phenolic acids) arise from the phenyl-propanoid metabolism in plants. Various honeys from different floral sources have been analysed regarding their phenolic acid contents. The evaluation of the patterns concerning phenolic acids, phenolic esters and aromatic carbonyl compounds could probably give an indication of the botanical origin of honeys [6].

1.3.9 Aroma compounds

Flavour/fragrance qualities of honey are very much dependent on the volatile and semivolatile organic compounds present in both the sample matrix and the headspace aroma. Volatiles contribute significantly to the honey flavour and to its variation with floral origin and the method of handling. The identification of volatile components is of importance to the understanding of flavour. Their isolation and quantification though from a complex mixture such as honey is very difficult. Such knowledge would be helpful and is essential in ascertaining a honey's floral origin [6]. The simultaneous distillation extraction (SDE) which

developed in 1964, was used for the isolation of most volatile compounds from matrix

1.4 Methods for testing authenticity with respect to botanical origin

1.4.1 Pollen analysis

Pollen analysis (melissopalynology) with light microscopy was the first method used to determine the botanical origin of honey .It has the advantage that it needs only inexpensive instrumentation. The drawback is that it needs highly specialized personnel and cannot for the time being be aided by computers for more efficient assessment of data. Pollen analysis can be qualitative and quantitative. Generally , for the determination of the botanical and geographical origin of honey qualitative pollen analysis is carried out. In this analysis the percentage representation of different pollen types is determined. For example citrus honey should have at least 10% citrus pollen, rape honey is expected to contain more than 45% rape pollen, while chestnut honey must contain more than 90% of chestnut pollen [6].

1.4.2 Determination of aroma and phenolic compounds

Phenolics are another class of compounds that have been used for proof of the botanical origin. Recently, the use of these compounds for the determination of the botanical origin of honey has been extensively reviewed [8]. There seems to be

some typical markers for some unifloral honeys. Methyl Anthranilate and Kaempferol for rosemary honey, this is not the case with many unifloral which honeys which do not have specific markers. Aroma compounds and flavonids have been used as specific markers for unifloral honeys [9]. Extraction of honey aroma by organic solvent for qualitative analysis and subsequent determination of the honey aroma spectrum have shown differences between unifloral honeys [10]. However the extraction of volatiles is not suitable for routine testing of the botanical origin of honey because it is time consuming. Dynamic headspace analysis of honey aroma can be useful for routine authenticity testing of the botanical origin of honey and it has been used with promising results [11,12]. Another relatively new technique is the use of Solid Phase Micro Extraction (SPME) enrichment technique for the analysis of aroma compounds. It has an advantage over dynamic headspace since it has an increased sensitivity and that less volatile aroma compounds can also be analyzed [13,14].

1.5 Methods of sampling of honey volatiles

Two methods are utilized to extract volatile compounds from honey matrix. The first is the well know solvent extraction technique. The second is relatively a new method which involves solid phase micro extraction technique (SPME).

5.1 Solvent extraction

The essential oil components are extracted first by diluting the honey with water, then homogenization with mechanical stirring followed by refluxing with a suitable organic solvent. The organic phase is then collected, dried with anhydrous sodium sulfate and concentrated. The residue is diluted and analyzed by gas chromatography [15].

1.5 Solid Phase Micro Extraction (SPME)

SPME is a solventless extraction technique based on the exposure of an immobilized stationary phase in the headspace over a honey matrix, followed by thermal desorption in the GCMS injection port for analysis [16].

This technique is based on equilibrating the analytes among the three phases of the system. These phases are the polymeric liquid coating of the SPME fiber, the headspace, and the aqueous solution, as shown in figure (1.3) [17].

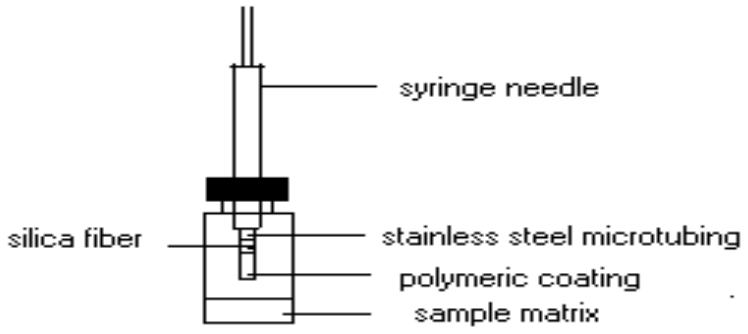


Figure (1.3): Method of sampling of honey volatiles using SPME technique

1.6 Methods of analysis of volatiles of honey

1.6.1 Gas Chromatography (GC)

Honey volatiles sampled in a suitable method were analyzed by gas chromatography. Usually the gas chromatograph used is equipped with an autosampler, autoinjector, a capillary column and a flame ionization detector, with helium gas as a mobile phase. The compounds were identified by comparison of chromatographic retention times with those of authentic standards [18].

1.6.2 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography system equipped with a refractive index detector and two columns was used. Aqueous phosphoric acid was used as the mobile phase at constant flow rate. Diluted honey samples were injected for analysis [19]. HPLC was used to characterize organic aliphatic acids, and phenolic acids [6]. The compounds were identified by comparison of chromatographic retention times with those of authentic standards [18].

1.6.3 Gas Chromatography / Mass Spectrometry (GC/MS)

The GC/MS separates at the beginning the chemical mixtures and then identifies the components at the molecular level. It is one of the most accurate tools for analyzing volatile samples. The GC works on the principle that a mixture will vaporize immediately after injection into the injection port which is kept at a

temperature normally above 200 ^{o}C. The mixture will separate into individual substances as it passes through the capillary column. As the various sample components emerge from the column opening carried by helium, they flow through the interface to the MS [20].

In the Mass spectrometer molecules of each component are ionized using two methods; Electron impact (EI) or chemical ionization (CI) which creates positively charged species called molecular ions. Some of the ions fragment into smaller daughter ions and neutral fragments. The molecular ion ($M^{+\cdot}$) and all positively charged fragments are separated according to their masses, as they move towards the detector where their intensity is recorded.

Identification of a compound is based on its mass spectrum, since each has a unique fragmentation pattern. A library of mass spectra, of hundred of thousands of compounds, is stored in the instrument and may be searched to assist in identifying the unknowns eluted [21].

There are many other methods used and some of these methods are summarized in table (1.1).

Table (1.1) : Different methods used for analysis of honey volatiles

Sample name	**Some detected Compounds**	**Method of analysis**	**References No.**
1)Rhus succedanea	Furancarboxaldehyde(18 ng/g); Benzeneacetaldehyde ((49ng/g	Adsorptive column chromatography	28
2)Lavendula stoechs (Portuguese honey)	2–Furaldehyde(192ng/g); 2-Furanomethnol(12 ng/g); 2-Furanocaboxaldehyde-5-hydroxymethyl (17 ng/g)	Likens Nickerson steam distillation/solvent extraction followed by Gas chromatography-FID	23
3) Acacia	Methyl butanal; Heptanal ; 5-Hepten-2-one-6-methyl	Solvent Extraction followed by Gas chromatography-mass spectrometry	11
4)Heather (From England)	2-Ethyl furan; 1-Pentene-3-ol		

These methods should give high concentrations if we compare them with SPME method, but their concentrations appear to be law for example in Lavendula stoechs honey we can find (192, 12, 17ng/g) for 2-Furaldehyde, 2-Furanomethanol and 2-Furancarboxaldehyde-5-hydroxymethyl respectively. On the hand the concentrations for this compounds that found by SPME method is higher. Also in Rhus succedanea we can find Furancarboxaldehyde (18ng/g) and

Benzeneacetaldehyde (49 ng/g), but the concentration of these compounds appear to be higher using the SPME method analysis, so this is makes our method more efficient table (3.12-3.20).

1.7 Previous work

Studies have been done on the analysis of volatiles in honey by SPME/GCMS, in different places all over the world, and many research groups have been involved in these investigation. Perez *et.al.* had conducted investigation on Spanish honey, in which a large number of organic volatiles have been found. The main volatiles belong to hydrocarbons, benzene derivatives, branched aldehydes, organic acids, and other compounds like alcohol. Difference in the composition of volatiles from various unifloral Spanish honey was observed, and the comparative analysis of the volatiles showed that some compounds can be used for characterization of their floral source [16].

Radovic *et. al* analyzed forty three authentic honey samples of different botanical origins taken from England and Denmark by gas chromatography mass spectrometry. A qualitative analysis of the volatile compounds identified was performed in order to assess the marker compounds. The results seemed to indicate the existence of certain marker compounds. In general, linear and branched aldehydes, ketones, short chain alcohol, acetone, furfural and

benzaldehyde. Qualitative data analysis showed that the presence of these volatile organic compounds was characteristic for each honey sample [8].

In another work, SPME/GCMS method has been developed to characterize the floral origin of different honeys. The analysis of four different unifloral honeys (Wild flower, Eucalyptus, sulla, and Citrus) has been performed and qualitative data showed that each honey present some characteristic profile. The SPME sampling was performed by using a septumless device, in order to minimize the presence of extraneous components [22].

Nozal *et al* has used the SPME/GCMS method to determine thymol, ecualyptol and menthol residues in five samples of honey. The result seemed to indicate the existence of a certain amount of thymol and ecualyptol in some honey samples, while menthol was found in another honey sample of different floral origin [15].

In another study Christine *et. al.* was carried out an investigation to determined the floral origin markers of Heather honeys, Calluna Vulgaris and Erica Arborea. Many compounds were separated by GC/MS. In comparison with 11 other honey types, four of the separated compounds proved to be markers of the Ericaceae family, three were specific for Calluna Vulgaris and 3 others discriminate for the Erica Aborea samples [2].

Collin *et. al.* has studied the floral origin of Portuguese Lavender honey derived from Lavandula angustifolia and Lavandula latifolia. Several samples was

analyzed, the result indicated the existence of specific compounds. In general aldehydes, acids, and alcohol were present [23].

1.8 Aim of the work

The aim of this work is to investigate the presence of volatiles that can be utilized as markers in the Palestinian unifloral honeys specifically: *Thymus capitatus, Zizipus spinachristi, Thymelea hirsuta, Tolpis virgata, Goffypium officinalis, Citrus, Centaurea italica, Eucalyptus camalydensis*, *and Wild flower* The amount of volatiles present will also be determined to support quantitatively the marker profiles of the selected type of honey. HS-SPME/GCMS will be utilized for this purpose.

3. Results and Discussion

3.1 Optimization of the HS-SPME/GCMS method

The analysis of volatiles in honey was done on nine Palestinian honey samples of different floral origin (Citrus, Thymus Capitatus, Thymelea Hirsuta, Ziziphus Spinachristi, Eucalyptus Camalydensis, Goffypium Officinalis, Centaurea Italica, Tolpis Virgata, and Wild Flower). The honey were samples analyzed by HS-SPME /GCMS technique. Since honey is a complex mixture that contains a high amount of sugar, different factors were considered to optimize HS- absorption. Several parameters have been proven to affect the sensitivity of the SPME technique particularly sampling temperature, SPME fiber exposure time, fiber polarity, and matrix weight. Upon closely studying our optimized method appears to reveal more polar compounds (alcohol and acid) than other method. We think that this is due to the polarity of both the column (Omegawax) and the fiber (carbowax divinylbenzene) which generate higher system sensitivity to these compounds [24]. Sample temperature equilibration was done using five different temperatures (40, 50, 60, 70, and 80^0C) at fixed time. The results showed that the 70^0C is the temperature at which the highest concentration of volatiles was obtained as shown in figure (3.1). A decomposition was also noticed when the temperature was raised to 80 ^{0}C (figure (3.1)).

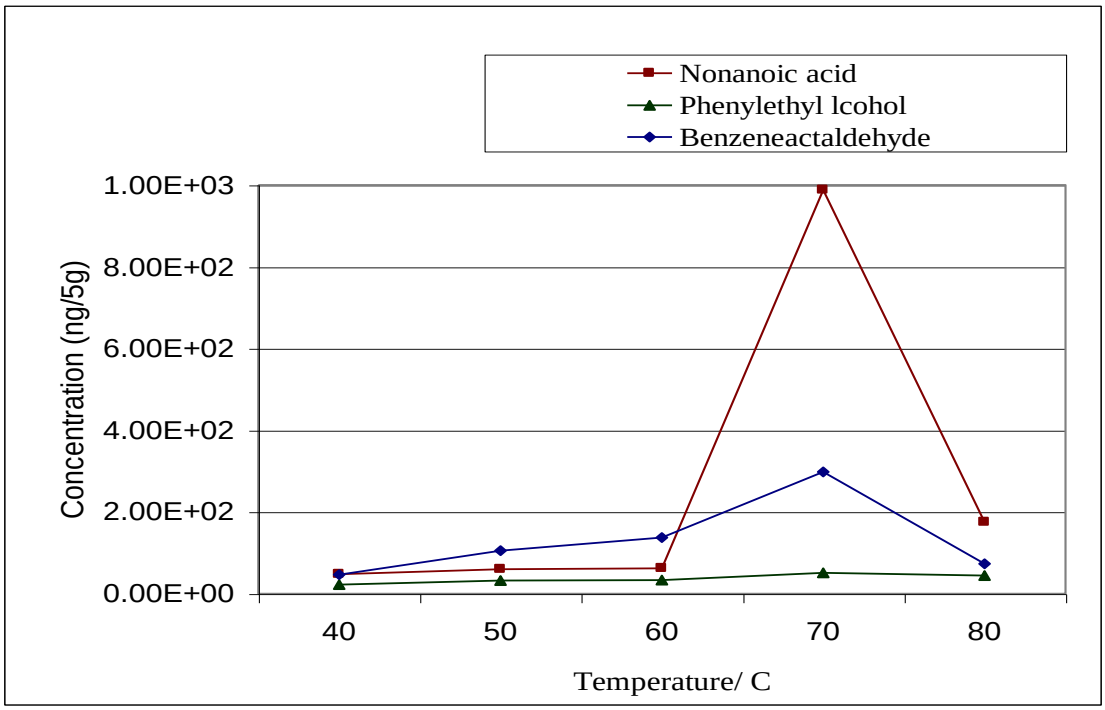


Figure (3.1): The concentration of selected Citrus honey volatiles at different equilibration temperature.

The equilibration time of the SPME fiber was determined using different sampling times (40, 50, 60, 70, and 80 minutes), at fixed temperature (70 ^{0}C). The result showed that the 50 minute sampling time, is the time at which the highest concentration of volatiles was obtained as shown in figure (3.2).

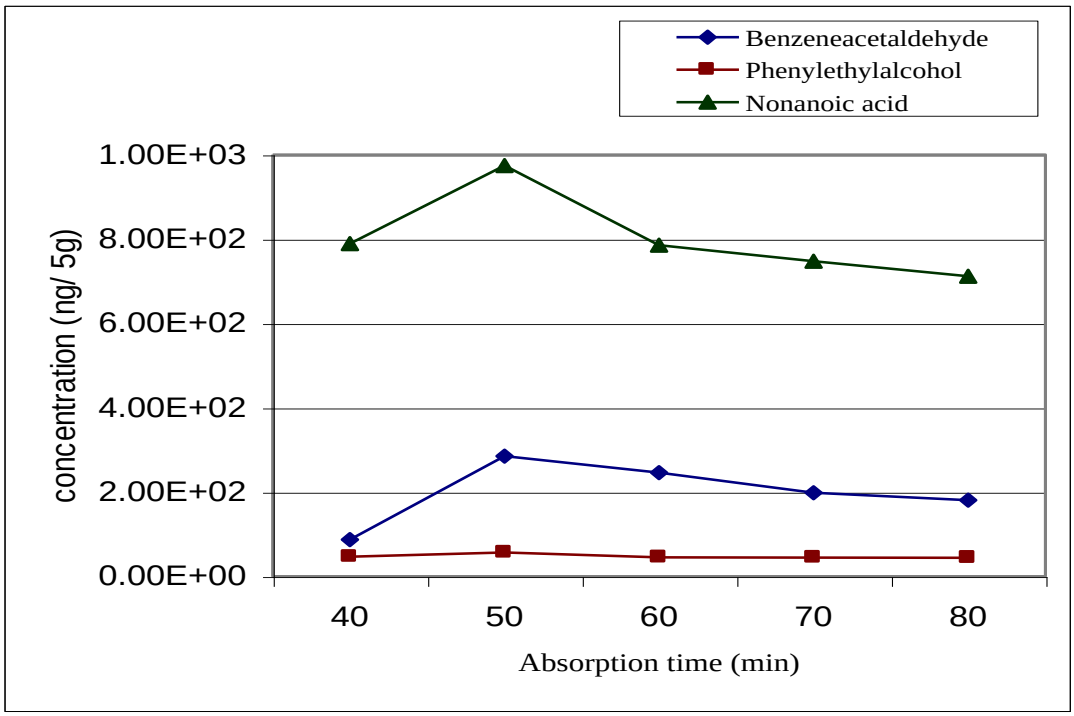


Figure (3.2): The concentration of selected Citrus honey volatiles at different SPME fiber exposure times

Therefore, we choose 70^{o}C and 50 minutes as the optimized condition for carrying out the analysis for all the honey samples under investigation. In order to address the optimized

method reproducibility, three successive injections of the same honey were made using identical experimental conditions (sampling time and temperature, fiber)

3.2 Identification of the Palestinian honey constituents

Forty six volatile compounds present in the Palestinian honey have been detected and identified using HS-SPME/GCMS analysis. The GCMS developed method through this investigation provides very good selectivity and sensitivity. A baseline separation was seen almost in all the volatiles separated as shown in the TIC generated (figure (3.3)-(3.11)). Among the volatiles detected were compounds that belong to the phenol, ketone, ester, acid and aldehyde families as shown in table (3.1). A total of thirty six signals were shown in the Citrus honey as shown in figure (3.3). Fourteen of these compounds were identified as shown in table (3.2). In general they are of low molecular weight, linear and branched aldehydes, phenols and acids.

Figure ((3.4)-(3.11)) presents the TIC of the rest of unifloral honey used in this study. The identified volatiles are presented in table ((3.3)-(3.10)).

3.3 Determination of the amounts of volatile components in honey

Three replicates of each individual honey were analyzed to check the reproducibility of the optimized GCMS method. Relative standard deviation (RSD) values of the retention time and the volatile peak areas of Citrus honey are shown in table (3.11). Reproducibility of other honey samples used in this study are shown in Appendix I.

These results demonstrate excellent reproducibility in retention time (0.02- 0.39 %) while the peak area is fairly acceptable. (1.766-22.8 %)

Table (3.11): Reproducibility of Citrus honey volatile constituents using the optimized HS-SPME/GCMS conditions

* No.	Average of t_R (min)	Average Area	RSD of t_R *(n=3)*	RSD of Area *(n=3)*
1	7.342	260645.1	0.31	4.351
2	8.460	223161.5	0.39	3.584
3	9.201	696163.6	0.09	13.20
4	9.425	214158.8	0.15	2.537
5	10.89	6654729	0.05	6.638
6	14.12	464113.7	0.03	15.99
7	14.63	2034598	0.06	7.466
8	15.58	172539.5	0.07	9.284
9	16.33	415795.5	0.02	22.80
10	17.70	7255635	0.09	1.766
11	18.60	1152364	0.09	6.325
12	19.64	4745624	0.12	5.324
13	20.39	3806550	0.25	6.325
14	23.29	3919349	0.37	21.91

*1) Nonanal; 2) 2-Furancarboxaldehyde; 3) Benzaldehyde; 4) 1,6-octadien-3-ol,3,7-dimethyl;

5) Benzeneacetaldehyde; 6) Phenylethyl alcohol ; 7) 2-Ethyl hexanoic acid

8) Pantolactone; 9) Octanoic acid; 10) Nonanoic acid; 11) 2-Amino benzoic acid methylester;

12) 1-Hydroxylinalool; 13)Phenol,2-methoxy,4-(1-propanol); 14) 2-Furancarboxaldehyde-5-hydroxymethyl

The concentration of the unifloral honey volatiles that desorbed from the SPME fiber to the GC was determined by comparing their average peak areas of the sample with a known concentration of the corresponding compounds in the external standard mixture. An external standard curves were prepared for all the standards as shown (3.12-3.14).

Using excel software the concentrations of the volatile compounds were calculated and are represented in tables (3.12-3.20). The correlation coefficients (R^2) values were always > 0.99, for all the detected compounds. The HS-SPME/GCMS method proved to be sensitive as reflected from the limits of detection (LOD) (signal to noise ratio S/N=3) and limits of quantitation (LOQ) (signal to noise ratio S/N=12). Three examples of the calibration curve are shown in (figure (3.12-3.14)

Depending on the results shown in tables (3.12-3.20), the following observation can be made:

1) All the samples analyzed contain between two or three carboxylic acids of molecular weight ranging between 120 and 200 g /mole. The concentration of these acids is higher than 150ng/5g in each honey sample. These acids appear in the chromatograms as broad peaks as expected.

2) All but two contains 2-furancarboxaldehyde-5-hydroxymethyl with concentrations higher than 200 ng/5g in each sample.

3) All but one contains benzeneacetaldehyde with concentrations ranging between 270 ng/5g and 850 ng/5g. In addition six honey samples contain small amount of nonanal and four samples contain decanal, and four honey samples contain benzaldehyde with different amounts.

4) All but one contains alcohols in different amount and concentrations ranging between 17-250 ng/5g. Some of these alcohols have a high molecular weight such as phenylethyl alcohol.

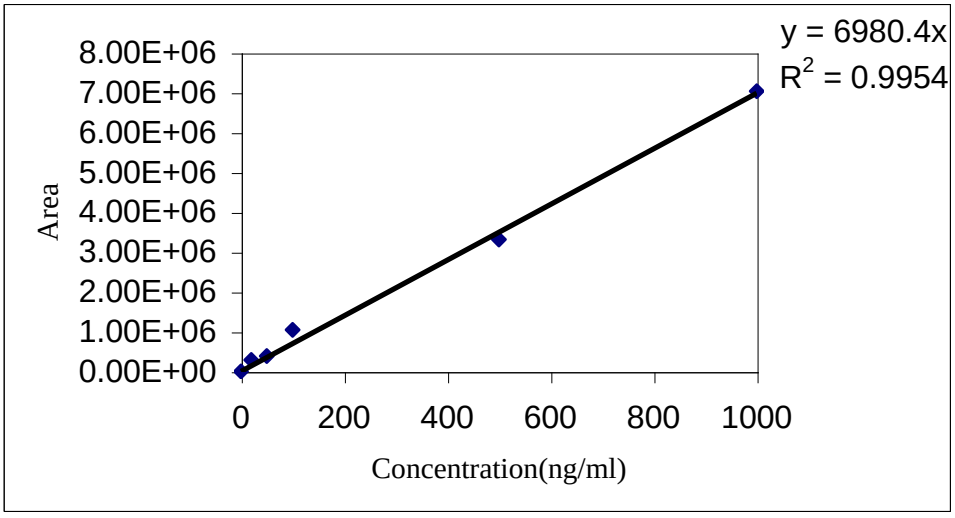


Figure (3.12): Calibration curve for Nonanal volatile compound

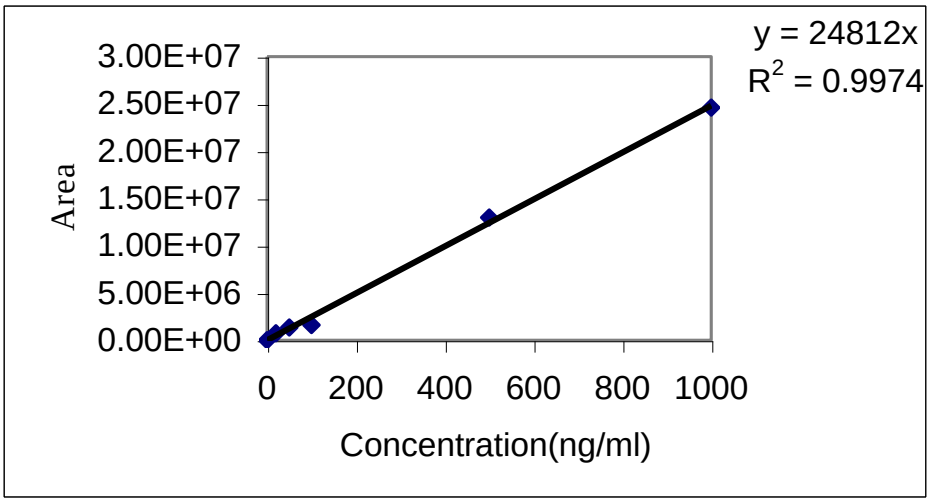


Figure (3.13): Calibration curve for Benzaldehyde volatile compound

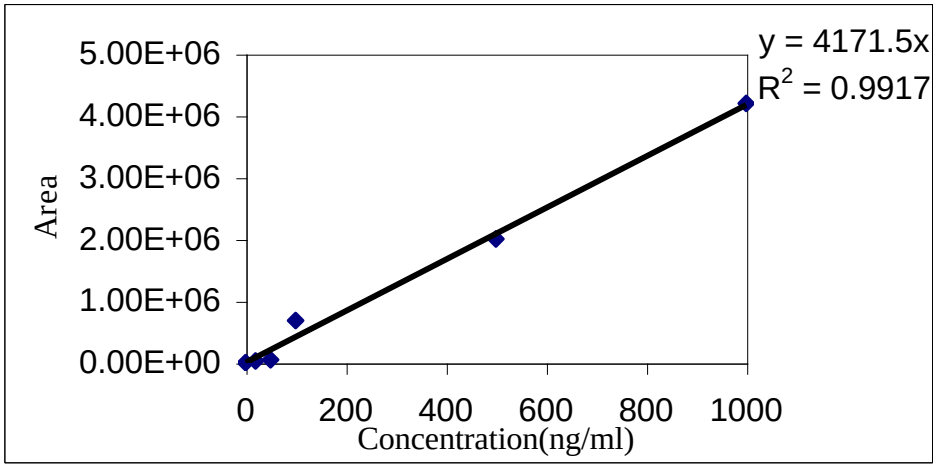


Figure (3.14): Calibration curve for 2-Ethyl hexanoic acid volatile compound

Table (3.12): Quantitation parameters of Citrus Honey volatiles using GCMS; (*n=3*)

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Nonanal	0.9954	7.777	31.08	37.33
2-Furancarboxaldehyde	0.9974	9.615	38.46	99.96
Benzaldehyde	0.9974	2.021	8.086	28.05
1,6-Octadien-3-ol,3,7-dimethyl	0.9979	3.482	13.95	17.55
Benzeneacetaldehyde	0.9944	32.60	130.4	280.1
Phenylethyl alcohol	0.9903	3.386	13.54	52.42
2-Ethyl hexanoic acid	0.9917	28.88	115.3	487.7
Pantolactone	0.9921	5.244	20.97	27.55
Octanoic acid	0.9929	37.50	150.3	157.6
Nonanoic acid	0.9990	41.66	166.6	972.2
2-Furancarboxaldehyde-5-hydroxymethyl	0.9989	16.21	64.86	289.1

Table (3.13): Quantitation parameters of Thymus Capitatus honey volatiles using GCMS;(*n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Benzaldehyde	0.9964	2.021	8.086	24.77
Benzeneacetaldehyde	0.9944	32.60	130.4	342.3
Phenylethyl alcohol	0.9903	3.386	13.54	221.2
Hexanoic acid, 2-ethyl	0.9917	28.88	115.3	468.6
Thymol	0.9957	8.721	34.88	232.1
Nonanoic acid	0.9990	41.66	166.6	201.2
2- Furancarboxaldehyde-5-(hydroxymethyl)	0.9989	16.21	64.86	222.4
Vanillin	0.9927	8.955	35.82	166.3

***** R^2 : Correlation coefficient

****** Limit of detection ; signal- to- noise ratio equals 3

******* Limit of quantitation ; signal- to- noise ratio equals 12

Table (3.14): Quantitation parameters of Thymelea Hirsuta Honey volatiles using GCMS; (*n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Nonanal	0.9954	7.777	31.08	32.87
Decanal	0.9952	3.865	15.46	33.65
Benzaldehyde	0.9974	2.021	8.086	22.83
Dodecanal	0.9936	8.253	33.01	38.07
1,6-Octadien-ol,3,7-dimethyl	0.9979	3.488	13.95	37.07
Benzyl alcohol	0.9964	3.073	12.95	47.80
Phenylethyl alcohol	0.9903	3.386	13.54	978.3
Hexanoic acid, 2-ethyl	0.9917	28.88	115.3	443.6

Nonanoic acid	0.9990	41.66	166.6	983.7
Decanoic acid	0.9998	31.25	132.5	160.8
2-Furancarboxaldehyde-5-hydroxymethyl	0.9974	16.21	64.86	515.7

Table (3.15): Quantitation parameters of Ziziphus Spinachristi Honey volatiles using GCMS; (n=3)

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Benzaldehyde	0.9974	2.021	8.086	48.24
Benzeneacetaldehyde	0.9944	32.60	130.4	804.1
Phenylethyl alcohol	0.9903	3.386	13.54	150.4
Dodecanal	0.9936	8.253	33.01	60.34
2-Ethyl hexanoic acid	0.9917	28.88	115.3	227.8
Octanoic acid	0.9929	37.50	150.1	241.2
Nonanoic acid	0.9990	41.66	166.6	793.9
Decanoic acid	0.9998	31.25	132.5	718.1
2-Furancarboxaldehyde,5-hydroxymethyl	0.9989	16.21	64.86	804.5

Table(3.16): Quantitation parameters of Eucalyptus Camalydensis Honey volatiles using GCMS; *(n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Nonanal	0.9954	7.777	31.08	35.84
Decanal	0.9952	3.865	15.46	20.01
Benzeneacetaldehyde	0.9944	32.60	130.4	693.3
2-Ethyl hexanoic acid	0.9917	28.88	115.3	152.9

Octanoic acid	0.9929	37.50	150.1	232.6
Nonanoic acid	0.9999	41.66	166.6	601.1
2-Furancarboxaldehyde-5-hydroxymethyl	0.9989	16.21	64.86	230.5

Table (3.17): Quantitation parameters of Goffypium Officinalis Honey volatiles using GCMS; *(n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Nonanal	0.9954	7.777	31.08	34.12
2-Furancarboxaldehyde	0.9974	9.615	38.46	40.88
Benzeneacetaldehyde	0.9944	32.60	130.4	363.8
2-Furanomethanol	0.9953	9.740	38.96	60.08
Phenylethyl alcohol	0.9903	3.386	13.54	146.4
2-Ethyl hexanoic acid	0.9917	28.88	115.3	252.7
Pantolactone	0.9921	5.244	20.97	90.35
Octanoic acid	0.9929	37.53	150.1	537.9
Nonanoic acid	0.9990	41.66	166.6	931.3
Decanoic acid	0.9998	31.25	132.5	243.8
2-Furancarboxaldehyde-5-(hydroxymethyl)	0.9989	16.21	64.86	777.7

Table (3.18): Quantitation parameters of Centaurea Italica Honey volatiles using GCMS;*(n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Benzaldehyde	0.9974	2.021	8.086	37.13
Benzeneacetaldehyde	0.9944	32.60	130.4	749.2
Phenylethyl alcohol	0.9903	3.386	13.54	96.03
2-Ethyl hexanoic acid	0.99179	28.88	115.3	160.8
2,4,6-Trimethyl phenol	0.9959	3.441	13.76	185.7
Nonanoic acid	0.9990	41.66	166.6	785.1
Decanoic acid	0.9998	31.25	132.5	890.1

Table (3.19): Quantitation parameters of Tolpis Virgata Honey volatiles using GCMS; *(n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Nonanal	0.9954	7.777	31.08	86.11
Decanal	0.9952	3.865	15.46	19.11
Benzeneacetaldehyde	0.9944	32.61	130.4	270.7
2-Ethyl hexanoic acid	0.9917	28.88	115.3	216.7
Nonanoic acid	0.9990	41.66	166.6	815.1
2-Furancarboxaldehyde-5-(hydroxymethyl)	0.9989	16.21	64.86	206.8

Table (3.20): Quantitation parameters of Wild Flower Honey volatiles using GCMS; *(n=3)*

Target Volatile Compounds	* R^2	** LOD(ng)	*** LOQ(ng)	Amount (ng)
Nonanal	0.9954	7.777	31.08	180.9
Decanal	0.9952	3.865	15.46	42.47
1,6-Octadien,3-ol-3,7-dimethyl	0.9979	3.488	13.95	21.66
Benzeneacetaldehyde	0.9944	32.61	130.4	848.1
2-Ethyl hexanoic acid	0.9917	28.88	115.3	256.7
Octanoic acid	0.9929	37.51	150.1	271.5
Nonanoic acid	0.9990	41.66	166.6	961.3
Decanoic acid	0.9998	31.25	132.5	887.4

Our investigation showed that most of the components identified are presents in all honey samples but in different amounts. The ratio between some component of different honey could be used to distinguish the different floral origin. These results are shown in figure ((3.15)- (3.26)).

Figure (3.15) showed that nonanal is present in six honey types, but in different concentrations. In the Wild Flower honey for example, the concentration of nonanal is noticeably high (180.9 ng/5g), compared with other types of honey. This aliphatic aldehyde (nonanal), contributes partly to the particular fruity odor [25-26], of the six honey types indicated.

Another example is the 2-Furancarboxaldehyde as shown in figure (3.16). It present in all the honey types with a higher extent in Citrus, and Goffpium Officinalis (99.9, 40 ng/5g). Benzaldehyde however which is presented in figure (3.17), is absent in nearly half of the samples, but it found in Citrus, Thymus Capitatus, Thymelea Hirsuta, Ziziphus Spinachristi, and Centaurea Italica with concentrations ranging between (28- 48 ng/5g).

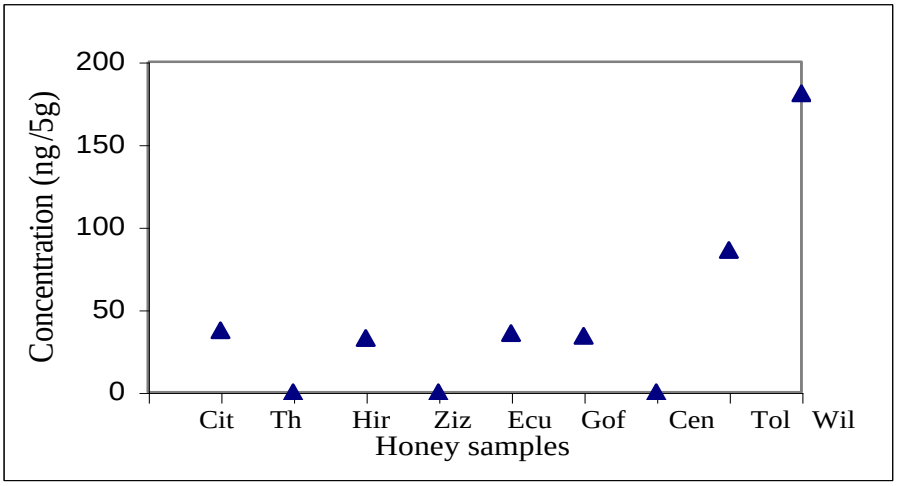


Figure (3.15): Concentrations of Nonanal in honey samples

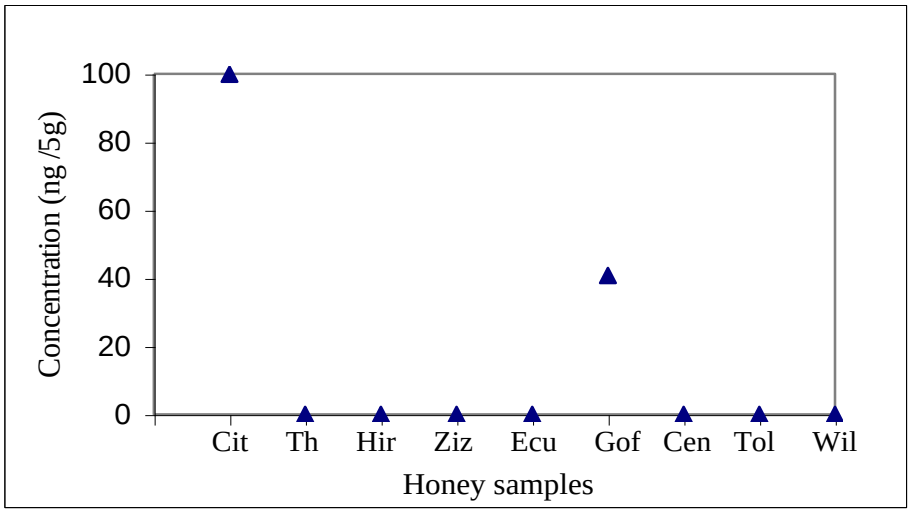


Figure (3.16): Concentrations of 2-Furancaboxaldehyde in honey samples

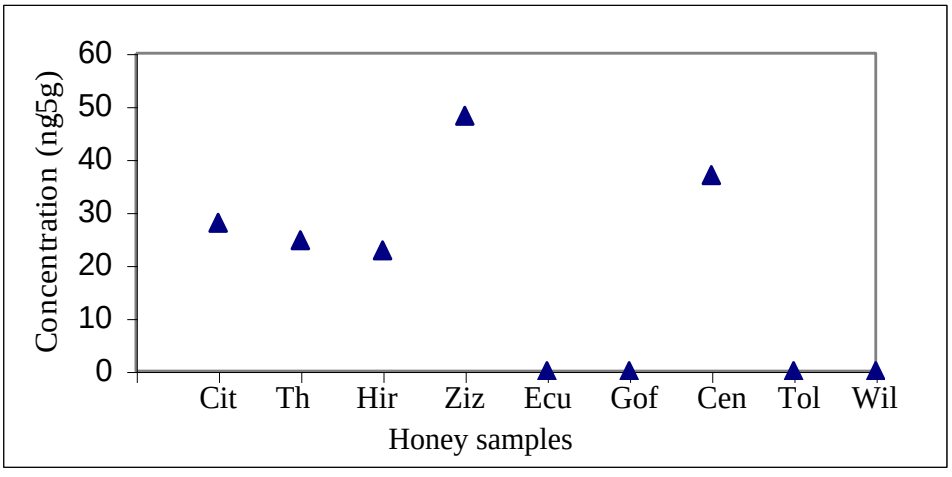


Figure (3.17): Concentrations of the Benzaldehyde in honey samples

Cit: Citrus; Th: Thymus Capitatus; Hir:Thymelea Hirsuta; Ziz: Ziziphus Spinachristi;
Ecu: Eculptus Camalydensis; Gof: Goffypium Officinalis; Cen: Centaurea Italica;
Tol: Tolpis Virgata; Wil: Wild Flower

Compared to other honey types, Citrus, Thymelea Hirsuta, Ziziphus Spinachristi, Goffypium Officinalis, and Centaurea Italica contain relatively high concentrations of 2-furancaboxaldehyde-5-hydroxymethyl (289, 515, 804, 777 and 890 ng/5g respectively). This high concentration is attributed to the fructose decomposition at high temperature [16]. Since our analysis is performed at reasonably low temperature, we tend to accept Bonveehi's insistence on considering this compound to be inherent to the honey and its presence being considered as an indication of the freshness of the honey samples [27]. Figure (3.19), shows no existence of benzeneacetaldehyde in Thymelea Hirsuta, and this absence can be considered as a marker for this specific type of honey. However phenylethyl alcohol compound seems to be characteristic marker for Thymelea Hirsuta honey since it exist found in high concentration (978 ng/5g) compared to other honey types as shown in figure (3.20). Phenylethyl alcohol has an intense floral and fragrant odor [26-28].

Hexanoic acid was found to have a high concentration in Citrus, Thymus Capitatus, and Thymelea Hirsuta (487, 468, 443 ng/5g respectively) and this is nearly twice the amount which was found in the other honey samples (152, 252, 160, 216, 256 ng/5g), as shown in figure (3.21). On the other hand, nonanoic acid can be seen in all honey types with high

concentration as revealed in figure (3.22). Figure (3.23), showed that 2-furanomethanol present exclusively in Goffypium Officinalis (60 ng/5g).

The presence of thymol and vanillin in a noticeable concentration (232,166 ng/5g respectively), in Thymus Capitatus is obvious as shown in figures (3.24),(3.25). In addition, figure (3.26) showed the noticeable concentration (185ng/5g) of 2,4,6-Trimethylphenol that seems to be characteristics for the Centaurea Italica since it was absence from the other honey samples.

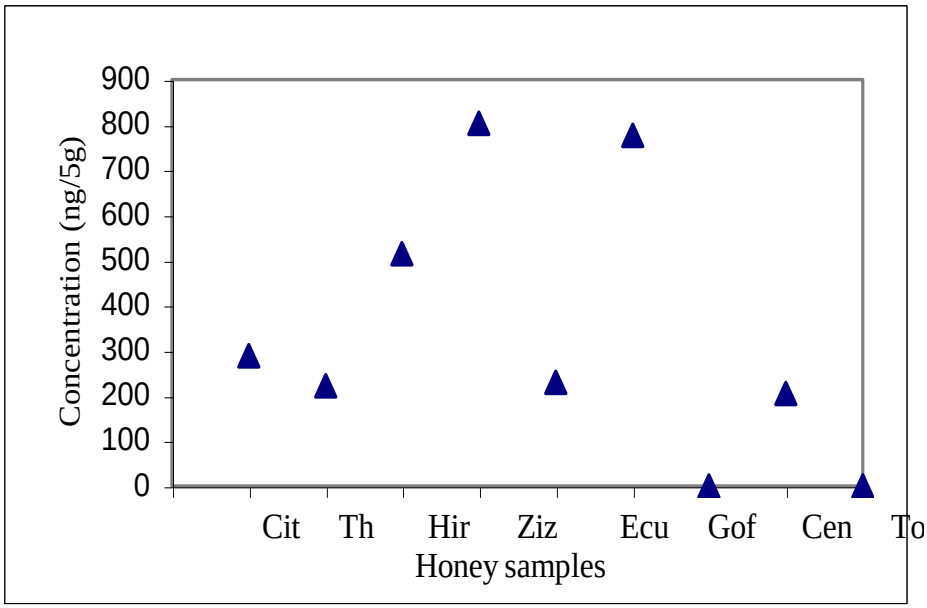


Figure (3.18): Concentrations of 2-Furancarboxaldehyde- 5-hydroxymethyl in honey samples

Cit: Citrus; Th: Thymus Capitatus; Hir:Thymelea Hirsuta; Ziz: Ziziphus Spinachristi; Ecu: Eculptus Camalydensis; Gof: Goffypium Officinalis; Cen: Centaurea Italica; Tol: Tolpis Virgata; Wil: Wild Flower

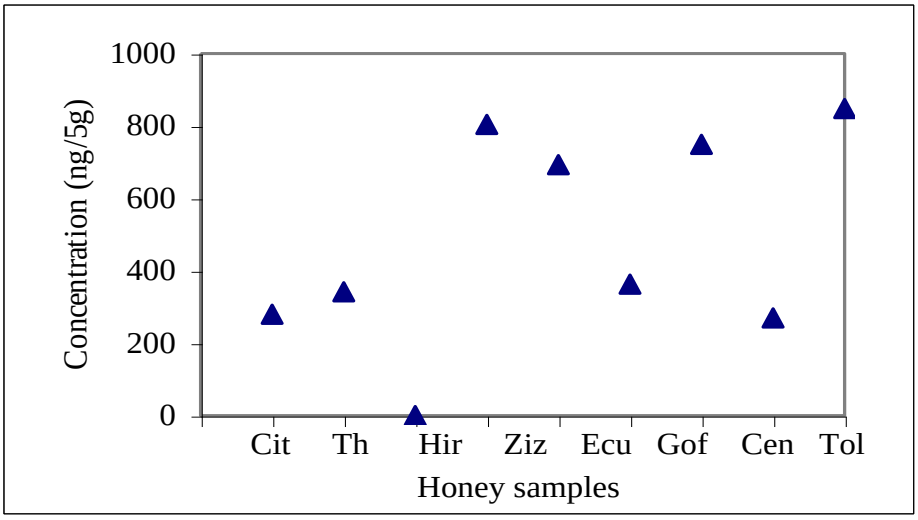


Figure (3.19): Concentrations of Benzeneacetaldehyde in different honey samples

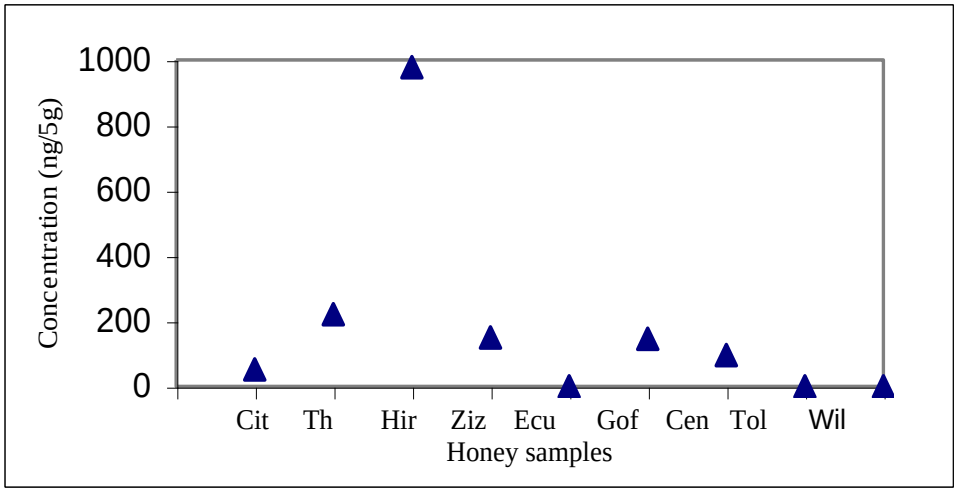
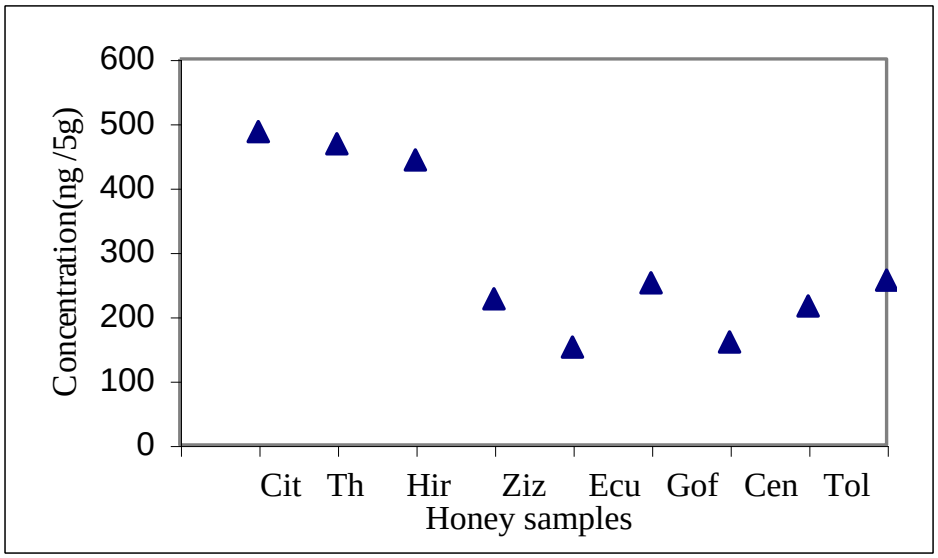


Figure (3.20): Concentrations of Phenylethyl alcohol in different honey samples

Cit: Citrus; Th: Thymus Capitatus; Hir:Thymelea Hirsuta; Ziz: Ziziphus Spinachristi;
Ecu: Eculptus Camalydensis; Gof: Goffypium Officinalis; Cen: Centaurea Italica;
Tol: Tolpis Virgata; Wil: Wild Flower



Figure(3.21): Concentration of 2-Ethyl hexanoic acid in different honey samples

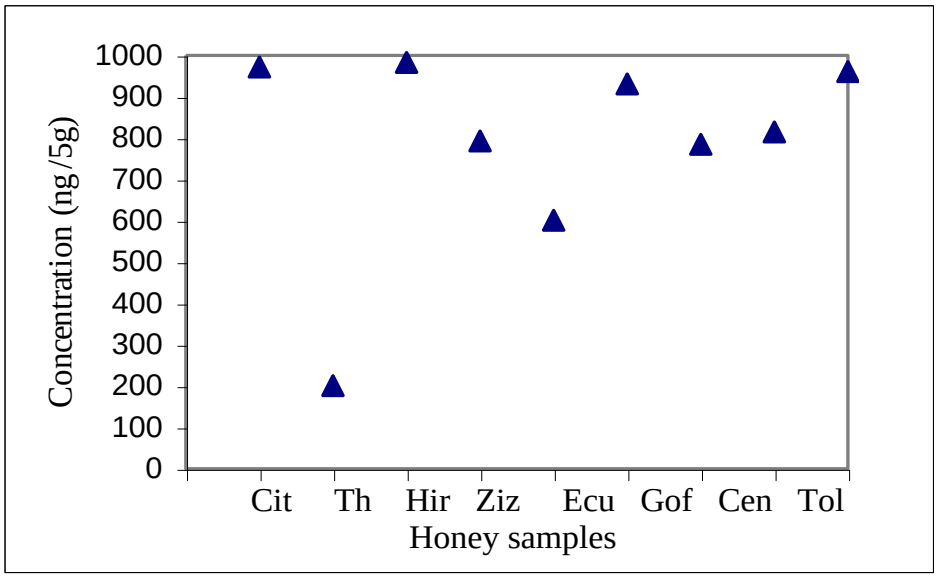


Figure (3.22): Concentrations of Nonanoic acid in different honey samples

Cit: Citrus; Th: Thymus Capitatus; Hir:Thymelea Hirsuta; Ziz: Ziziphus Spinachristi; Ecu: Eculptus Camalydensis; Gof: Goffypium Officinalis; Cen: Centaurea Italica; Tol: Tolpis Virgata; Wil: Wild Flower

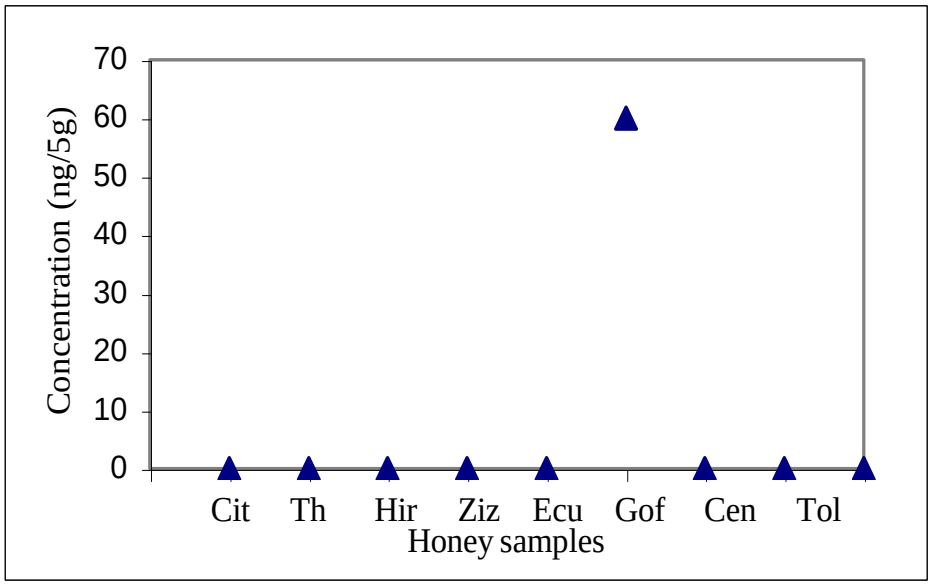


Figure (3.23): Concentration 2-Furanomethanol in different honey samples

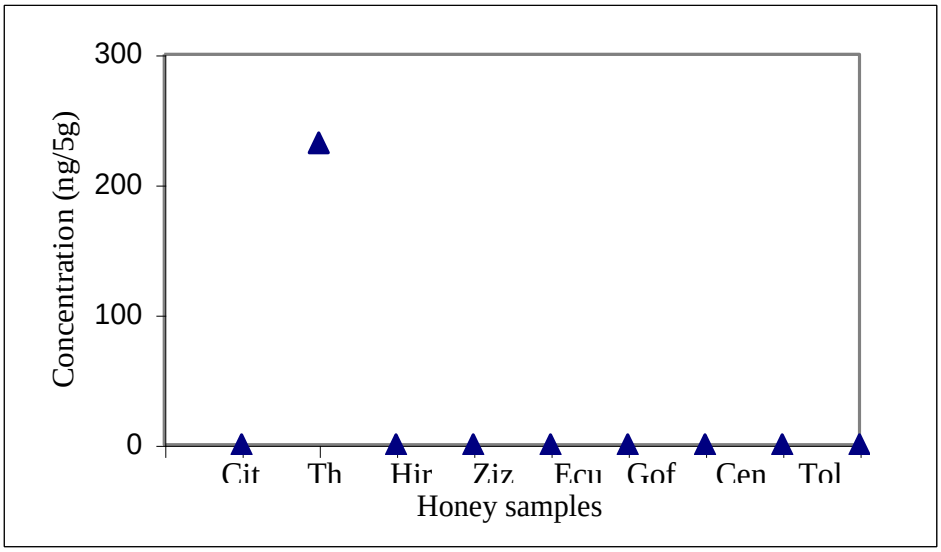


Figure (3.24): Concentration of Thymol in different honey samples

Cit: Citrus; Th: Thymus Capitatus; Hir:Thymelea Hirsuta; Ziz: Ziziphus Spinachristi;
Ecu: Eculptus Camalydensis; Gof: Goffypium Officinalis; Cen: Centaurea Italica;
Tol: Tolpis Virgata; Wil: Wild Flower

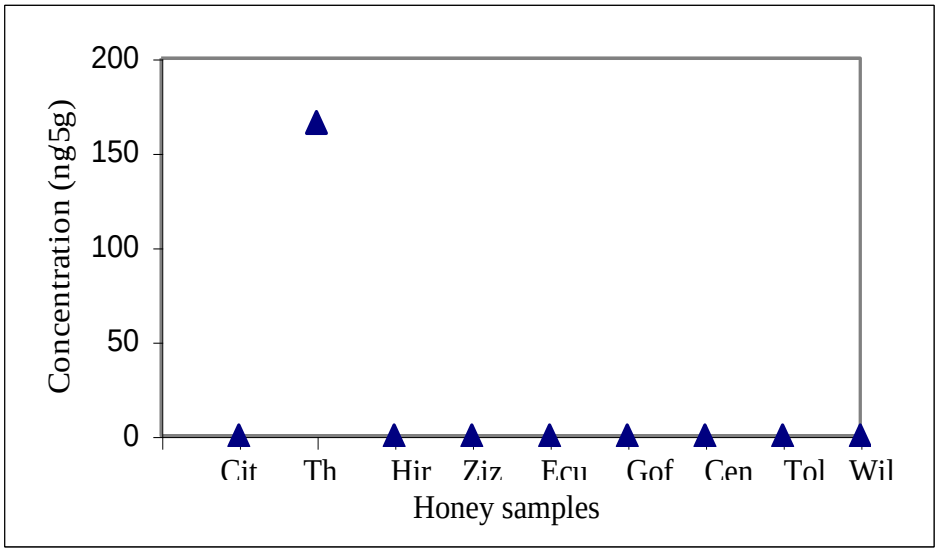


Figure (3.25): Concentration of Vanillin in different honey samples

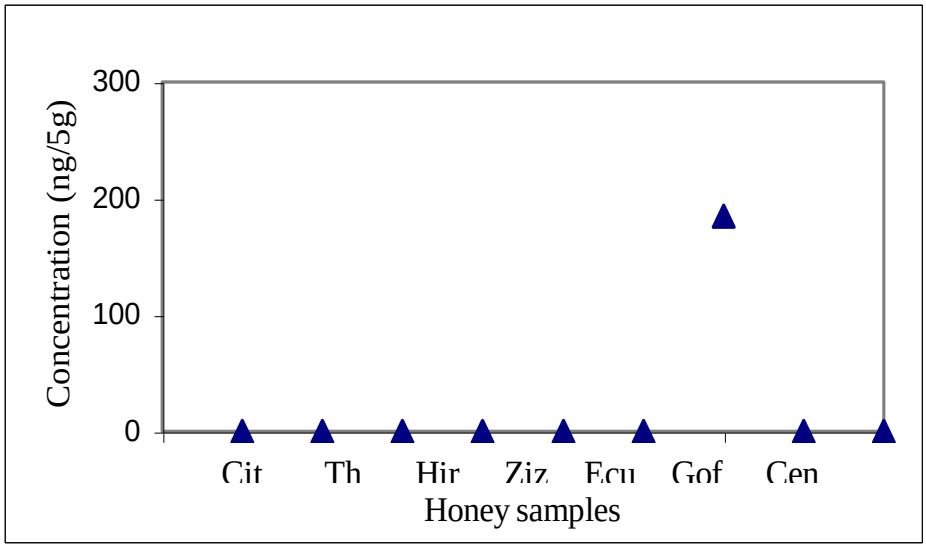


Figure (3.26): Concentration of 2,4,6-Trimethylphenol in different honey samples

Cit: Citrus; Th: Thymus Capitatus; Hir:Thymelea Hirsuta; Ziz: Ziziphus Spinachristi; Ecu: Eculptus Camalydensis; Gof: Goffypium Officinalis; Cen: Centaurea Italica; Tol: Tolpis Virgata; Wil: Wild Flower

3.4 Honeys markers for the characterization of different unifloral honey

From the previous results, it is possible to ascertain that the study of volatile fraction of honey using HS-SPME/GCMS provides useful information for the determination of the unifloral origin of honey. This technique is simple, rapid and fairly reproducible. Table (3.21) summarizes typical proposed markers that could be utilized to characterize the unifloral origin of Palestinian honey.

Table (3.21): Typical Marker compounds suggested for certain unifloral honeys from Palestine

No.	Botanical origin of honey	Marker compound	
		Presence of	Absence of
1	Citrus	2-amino benzoic acid methylester	
		1-Hydroxylinalool	
		Phenol,2-methoxy,4-(1-propenyl)	
2	Thymus Capitatus	Thymol	
		Vanillin	
		3,4-Dimethoxy benzaldehyde	
		3,4,5-Trimethoxy benzaldehyde	
		3-Methylbutyl benzene	
		2-Propanone,1,3-diphenyl	
3	Thymelea Hirsuta		Benzeneacetaldehyde
		Hexanol	
		Nonanol	
		Benzyl alcohol	
		Benzenepropanol	
		4-methoxyphenol	
4	Ziziphus Spinachristi	Phenol,2-methoxy-4-(1-propanol)-6-acetate	
		3-Methoxy benzaldehyde	
		Benzeneacetanitrile	
5	Oculptus Camalydensis	2-Propyl,1-pentanol	
		Pentadecane	
6	Goffypium Officinalis	2-Furanomethanol	
		Eicosane	
		2-Methyl decanol	
7	Centaurea Italica	2,4,6-Trimethylphenol	
8	Tolpis Virgata	Tridecane	

		3,5-Dihydroxytoluene	
9	Wild Flower	Hexadecane	
		Heptadecane	
		3,4-Dimethyl benzaldehyde	

In general, linear and aromatic aldehydes, short chain alcohols, phenols, and alkanes were found in almost all of the analyzed honey samples. According to the data obtained, Citrus honey can be characterized by the presence of Phenol, 2-methoxy-4 (1-propanol), 1-Hydroxylinalool, and 2-Amino benzoic acid methylester. In addition, these compounds are absent from all other honey samples figure (3.27).

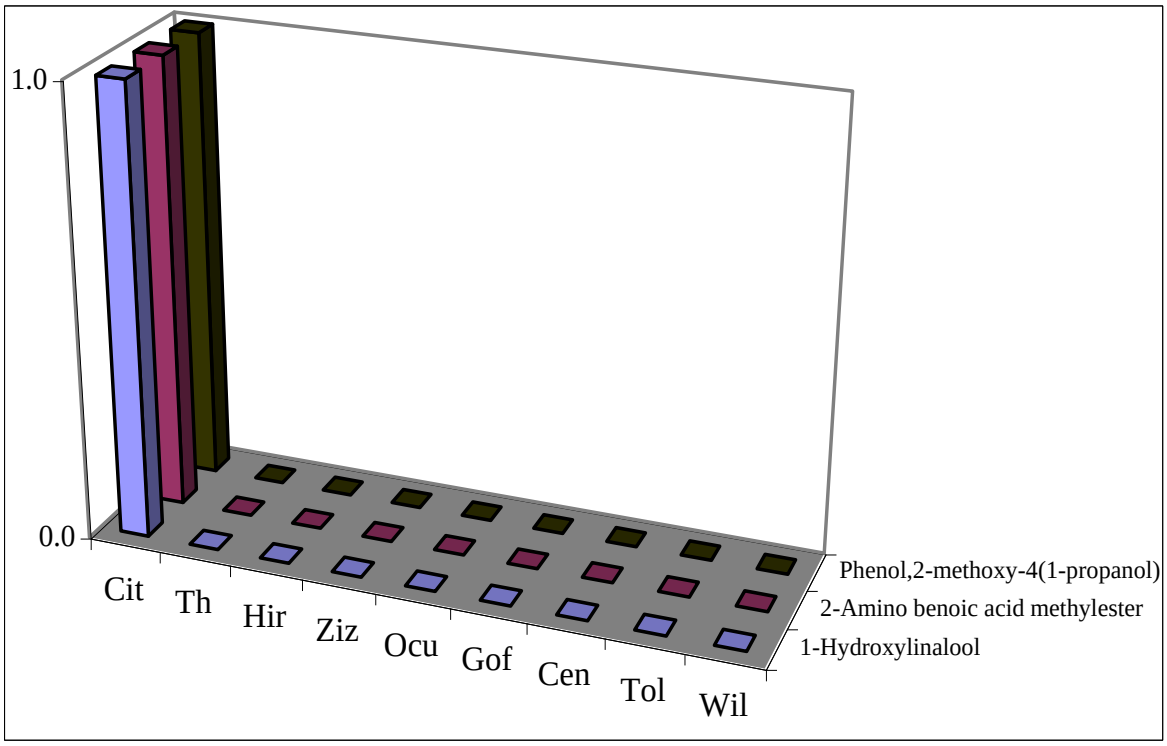


Figure (3.27): Marker compounds for Citrus honey

Zero and one at the Y-axis scale represent the absence and presence of volatile Compound respectively

Thymol, vanillin, 3,4-Dimethoxy benzaldehyde, 3,4,5-Trimethoxy benzaldehyde, 3-methylbutyl benzene, and 1,3-Diphenyl propanone, are compounds that may be used as marker compounds for Thymus Capitatus honey as shown in figure (3.28). These volatile compounds are absent from the other eight honey type samples.

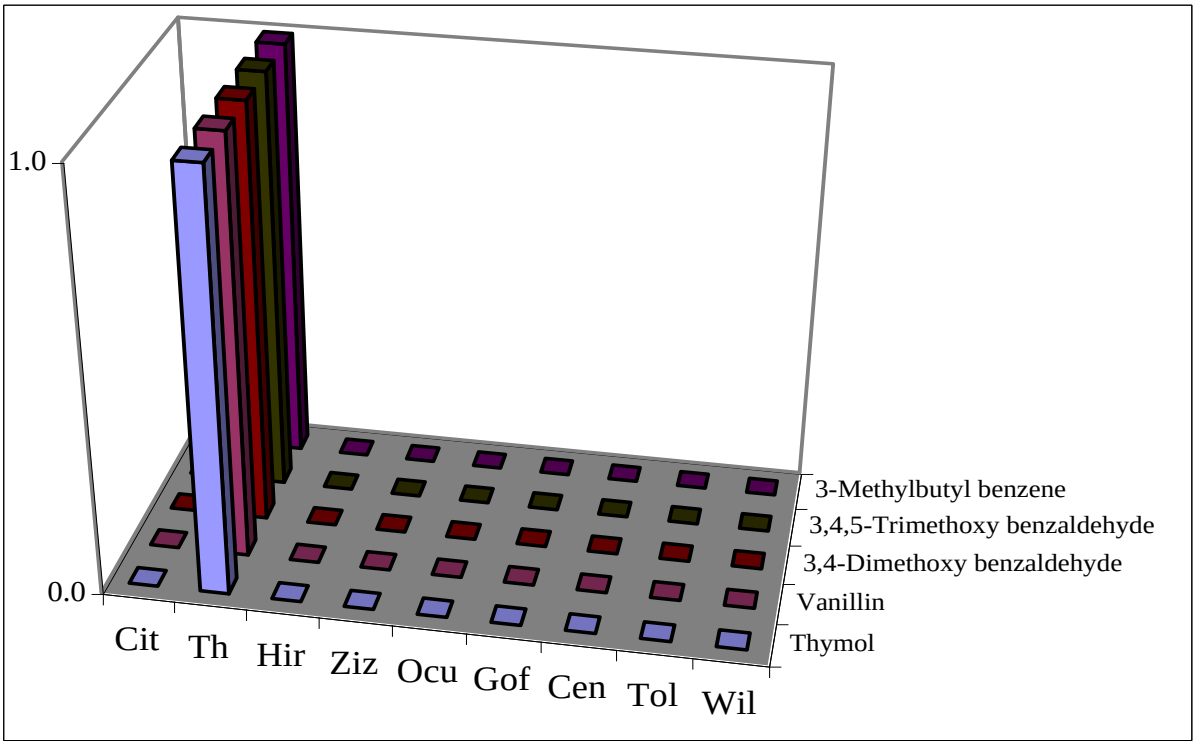


Figure (3.28): Marker compounds for Thymus Capitatus honey

Zero and one at the Y-axis scale represent the absence and presence of volatile Compound respectively

Thymelea Hirsuta honey is characterized by the presence of linear chains and aromatic alcohols contrary to the other honey types as figure (3.29) revealed. Five of these compounds are Hexanol, Nonanol, Benzyl alcohol, Benzenepropanol, and 4-Methoxyphenol. Therefore, these compounds are markers for this type of honey. In addition, Benzeneacetaldehyde, which is present in all honey samples, is absent in Thymelea Hirsuta. Together, the absence and presence of the latter and the former compounds characterize this type of honey.

On the other hand Phenol, 2-methoxy, 4-(1-propanol)-6-acetate (which contribute to the estery and fruity and odor)[30], 3-Methoxy benzaldehyde, and Benzeneacetonitrile, are characteristic for the Ziziphus Spinachristi, figure (3.30). While Oculptus Camalydensis has two markers one is 2-Propyl- 1-pentanol, and the other is Pentadecane as shown in figure (3.31).

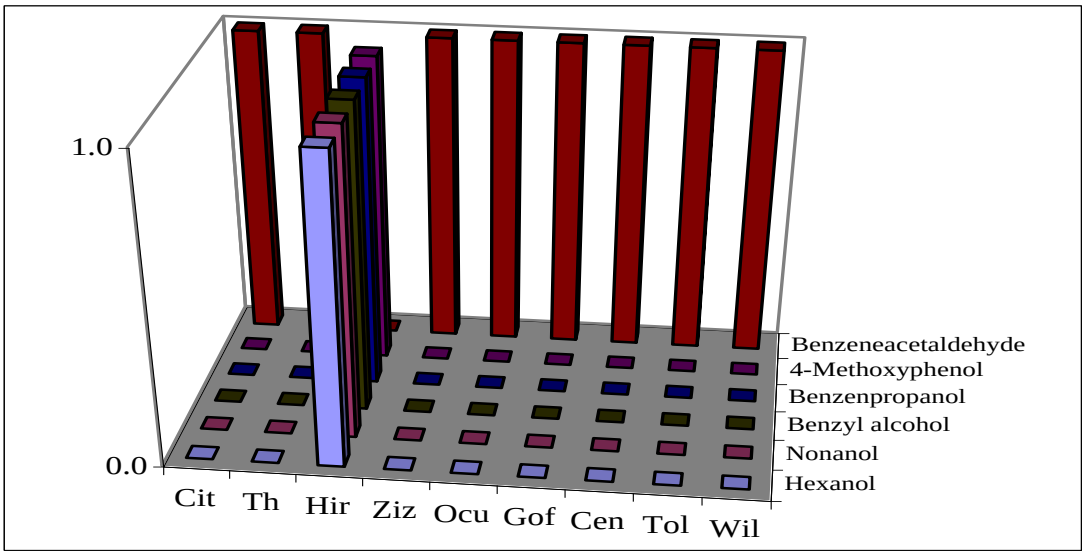


Figure (3.29): Marker compounds for Thymelea Hirsuta honey

Zero and one at the Y-axis scale represent the absence and presence of volatile Compound respectively

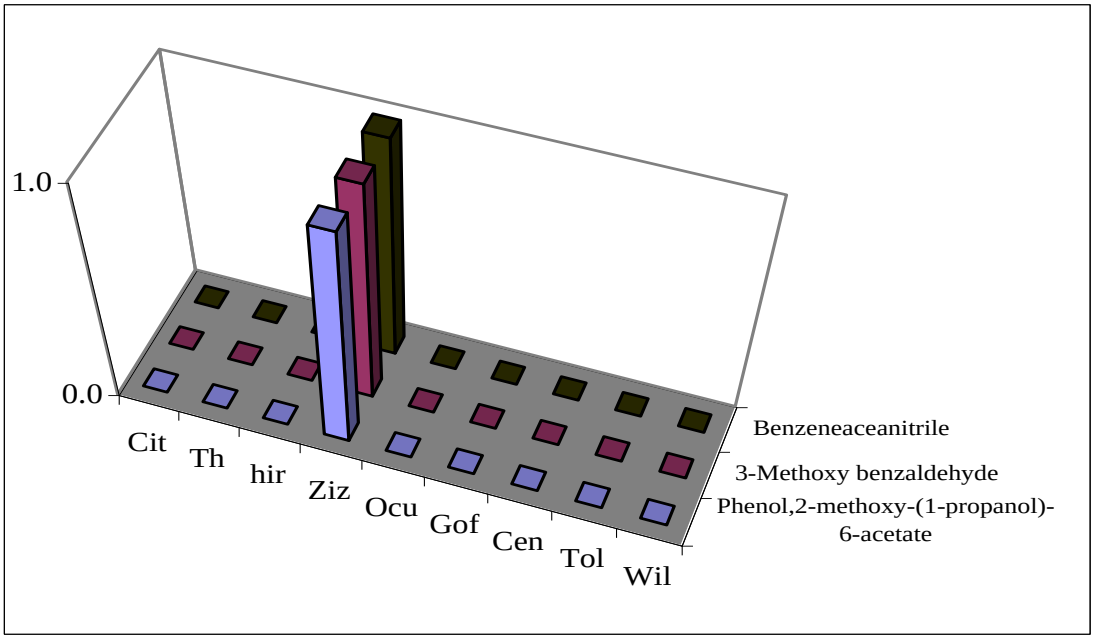
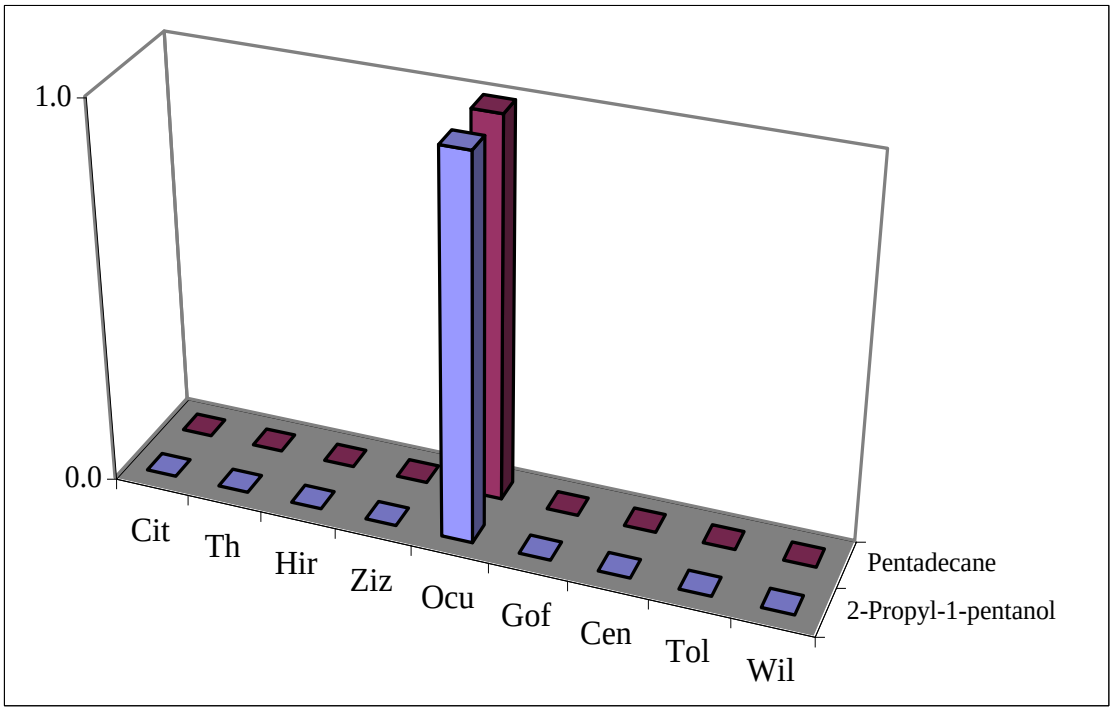


Figure (3.30): Marker compounds for Ziziphus Spinachristri honey



Figure(3.31):Marker compounds for Eucalyptus Camalydensis honey

Zero and one at the Y-axis scale represent the absence and presence of volatile compound respectively

Figure (3.32), shows the characteristic volatile compounds for the Goffypium Officinalis. These are 2-Furanomethanol, Eicosane and 2-Methyl decanol. These compounds are present in this type of honey and absent in the other honey samples. Therefore Centaurea Italica can be characterize by the presence of one marker compound, as shown in figure (3.33), that is 2,4,6-Trimethylphenol, which is absent in the other type of honey.

On the other hand the Tolpis Virgata honey contains two volatile compounds that do not exist in the other types of honey figure (3.34). These are Tridecane, and 3,5-Dihydroxytoluene. Also, according to the data obtained, the Wild Flower honey characterized by the presence of Hexadecane, Heptadecane and 3,4-Dimethyl benzaldehyde as shown in figure (3.35).

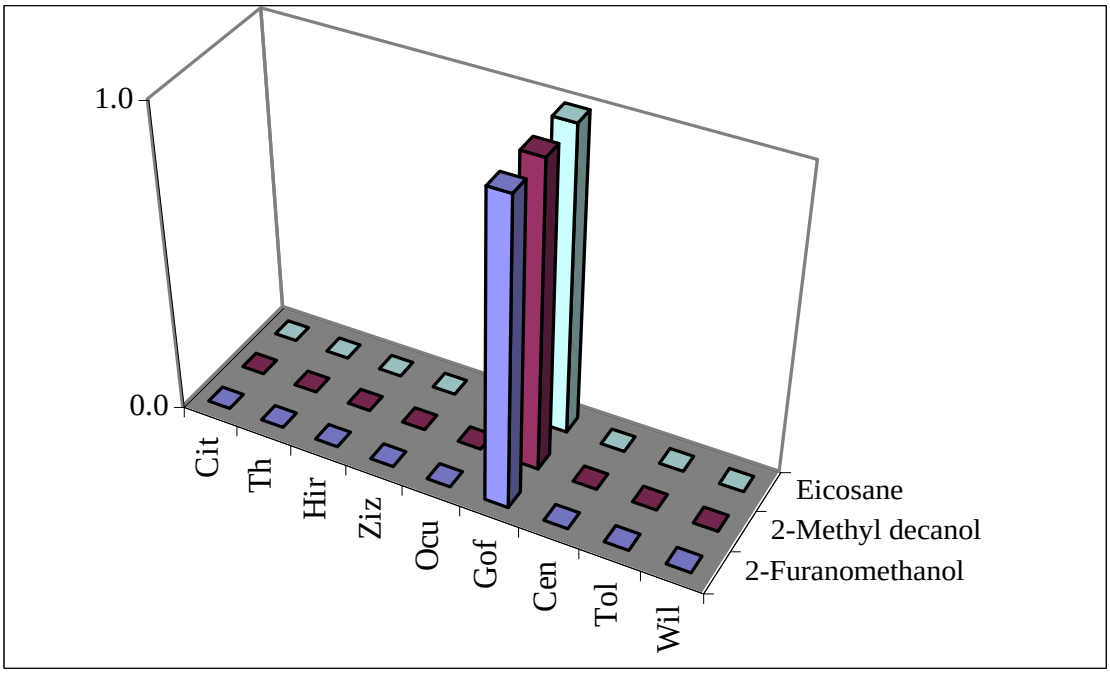


Figure (3.32): Marker compounds for Goffypium Officinalis honey

Zero and one at the Y-axis scale represent the absence and presence of volatile Compound respectively

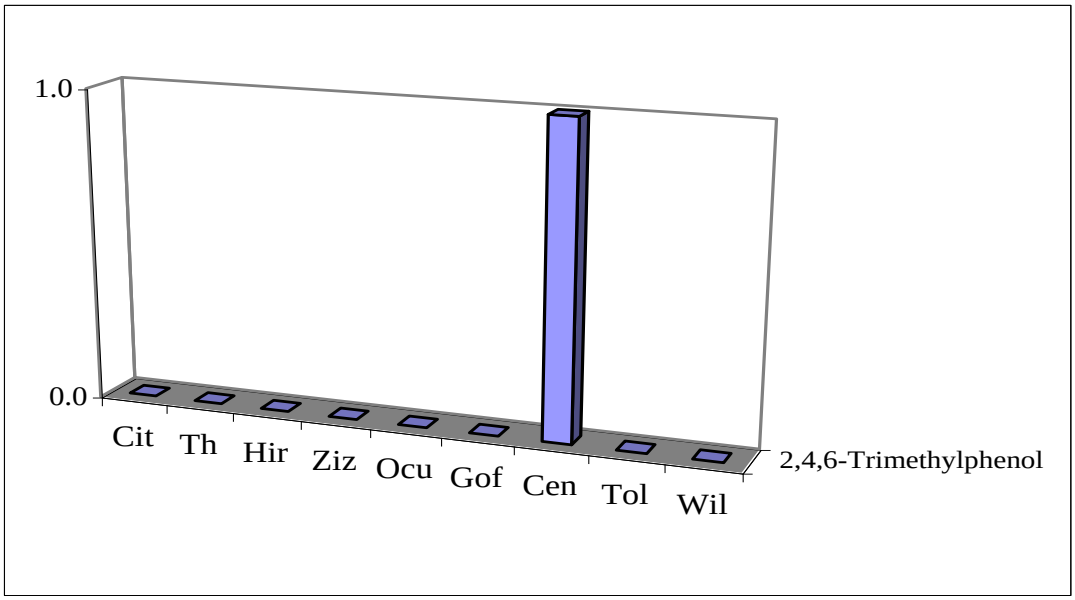


Figure (3.33): Marker compounds for Centaurea Italica honey

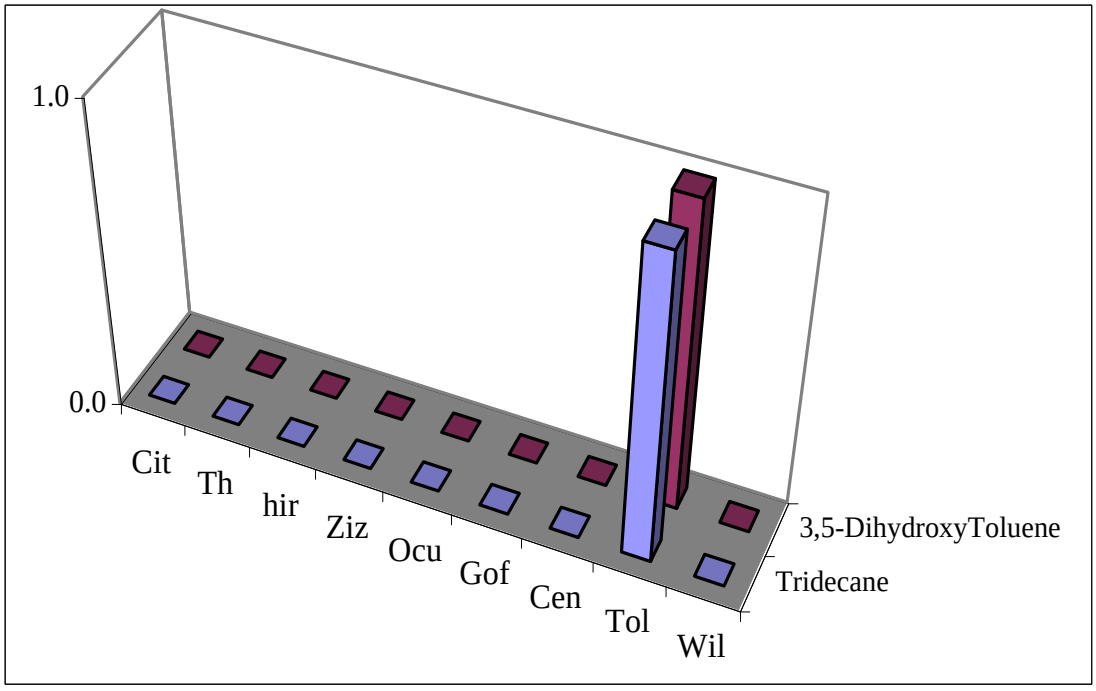


Figure (3.34): Marker compounds for Tolpis Virgata honey

Zero and one at the Y-axis scale represent the absence and presence of volatile Compound respectively

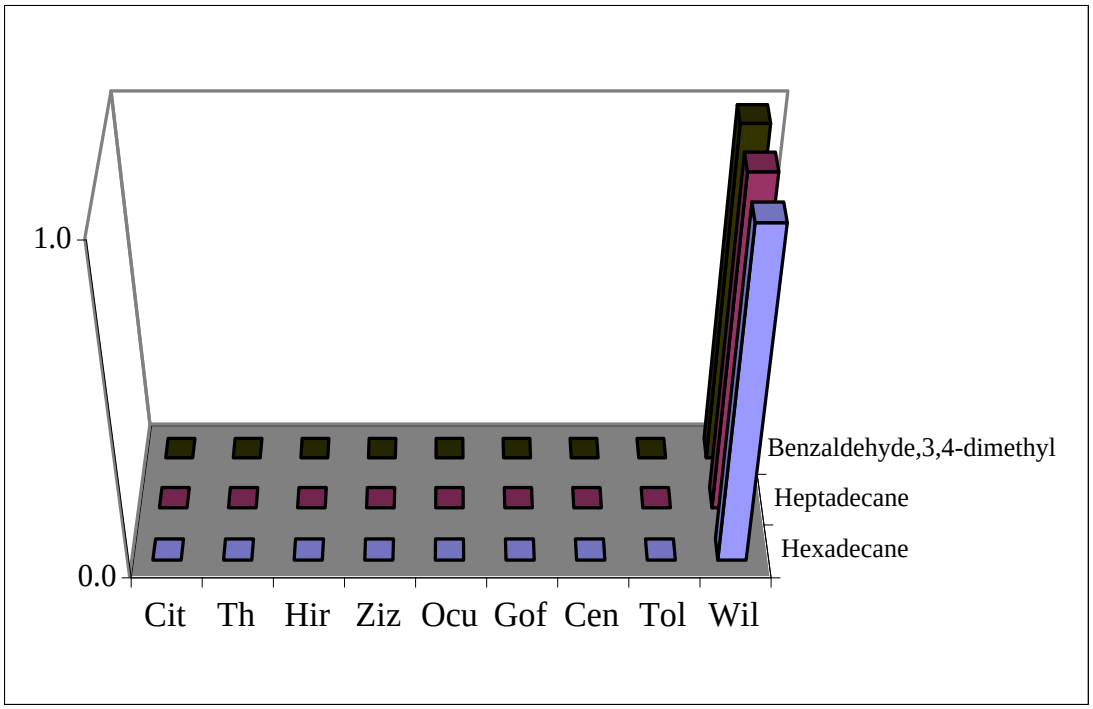


Figure (3.35): Marker compounds for Wild Flower honey

Zero and one at the Y-axis scale represent the absence and presence of volatile Compound respectively

Conclusion

Due to the presence of different volatile compounds in honey, the HS- SPME/GCMS technology had been chosen as analytical method to characterize the origin of unifloral Palestinian honey. This method also allows the identification and quantitation of all the separated volatile from honey. The optimal results were obtained using 65µm carbowax divinylbenzene (DVB), was found to absorb most of the target volatile than the commonly used polydimethyl siloxane (PDMS) coated fiber. The relative standard deviation, percentage of the retention time and the peak area was an indication of the precision of the proposed method.

In all honey samples the most abundant compounds were aldehydes, organic acids, phenols and alcohols. These compounds found in different percentages and in different concentrations due to the floral origin of the honey. This result was used to assess certain markers for the types of honey selected.

Citrus honey was characterized by the presence of three volatile compounds, but Thymus Capitatus honey showed six marker compounds including Thymol, Vanilline. Thymelea Hirsuta honey however is characterized by the presence of a group of alcohols and phenols particularly Hexanol; Nonanol; Benzylalcohol; Benzene propanol; and 4-Methoxyphenol. Ziziphus Spinachristi honey has three volatile markers, but Eucalyptus Camalydensis honey contains specifically: 2-propyl-1-pentanol and pentadecane. Goffypium Officinalis honey was characterized by the presence of three markers while Centaurea Italica has only one volatile marker compound.

Tolpis Virgata honey has two markers, while the Wild Flower honey is distinguished from other floral honey by the presence of three volatile marker compounds.

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