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**STUDY OF STATIC HEADSPACE AND HEADSPACE SOLID
PHASE MICROEXTRACTION (HS-SPME) AS A TOOL FOR
GREEK HONEY CHARACTERIZATION AND AUTHENTICITY
ASSESSMENT**

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ΧΑΡΑΚΤΗΡΙΣΜΟ ΚΑΙ ΤΗΝ ΑΥΘΕΝΤΙΚΟΤΗΤΑ ΕΛΛΗΝΙΚΩΝ ΜΕΛΙΩΝ

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ABSTRACT

Volatile compounds are a vital aspect of honey as they provide its unique aroma. Thus, they substantially influence honey's quality, along with consumers' preferences, in both national and worldwide markets.

This study focuses on determining honey's volatile compounds for Greek honeys' characterization and botanical classification based on their volatile profile. For this purpose, two methods, a static HS-GC-MS and an HS-SPME-GC-MS were developed, optimized, validated, and compared to evaluate their suitability for analyzing the volatile composition of honey. 36 Greek honey samples from 12 different botanical origins (pine, fir, oak, thyme, heather, orange, chestnut, cotton, arbutus, carob, sage and vanilla) were collected from various geographical regions of Greece during the harvesting period of 2019, and they were all analyzed with both methods. Compared to static HS, HS-SPME proved to be more efficient, recovering a wider variety of analytes with higher sensitivity. Target and suspect screening workflows were then applied for the detection and quantification of volatile compounds. As a result, several potential biomarkers were identified and were evaluated as indicative of honey's botanical origin, such as Isophorone, which can be characterized as a potential marker of Arbutus honey.

SUBJECT AREA: Honey Authenticity and Characterization

KEYWORDS: authenticity, volatile compounds, honey, gas chromatography, mass spectrometry

ΠΕΡΙΛΗΨΗ

Το πτητικό κλάσμα είναι κύριος παράγοντας του μελιού καθώς παρέχει το μοναδικό του άρωμα. Συνεπώς επηρεάζει μερικώς την ποιότητα του μελιού καθώς και τις προτιμήσεις των καταναλωτών τόσο σε εγχώριο όσο και σε διεθνές επίπεδο. Η μελέτη αυτή εστιάζει στον προσδιορισμό των πτητικών αναλυτών ελληνικού μελιού με στόχο τον χαρακτηρισμό και την βοτανολογική τους κατάταξη με βάση το αρωματικό τους κλάσμα. Για τον σκοπό αυτό δύο μέθοδοι, μία static HS-GC-MS και μία HS-SPME-GC-MS αναπτύχθηκαν, βελτιστοποιήθηκαν, επικυρώθηκαν και συγκρίθηκαν προκειμένου να αξιολογηθεί η καταλληλότητά τους για την ανάλυση του πτητικού κλάσματος του μελιού. Τριάντα έξι ελληνικά δείγματα από δώδεκα διαφορετικές βοτανικές προελεύσεις (πεύκο, έλατο, βελανιδιά, θυμάρι, ερείκι, πορτοκάλι, καστανιά, βαμβάκι, κουμαριά, χαρουπιά, φασκόμηλο και βανίλια) συλλέχθηκαν από διάφορες περιοχές της Ελλάδας κατά την περίοδο συγκομιδής 2019 και αναλύθηκαν όλα και με τις δύο μεθόδους. Σε σύγκριση με την static HS η HS-SPME φάνηκε να είναι πιο αποδοτική, ανακτώντας μεγαλύτερο εύρος αναλυτών με μεγαλύτερη ευαισθησία. Στην συνέχεια εφαρμόστηκαν πορείες στοχευμένης αλλά και ύποπτης σάρωσης για την ανίχνευση και τον ποσοτικό προσδιορισμό των πτητικών αναλυτών. Ως αποτέλεσμα, διάφοροι πιθανοί βιοδείκτες ταυτοποιήθηκαν και εκτιμήθηκαν ως ενδεικτικοί της βοτανικής προέλευσης του μελιού, όπως η Isophorone που μπορεί να χαρακτηριστεί ως πιθανός δείκτης του μελιού της κουμαριάς.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αυθεντικότητα και χαρακτηρισμός τροφίμων

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: αυθεντικότητα, πτητικοί αναλύτες, μέλι, αέρια Χρωματογραφία, φασματομετρία Μαζών

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PREFACE

This master thesis was conceived and performed at the Laboratory of Analytical Chemistry (Department of Chemistry, National and Kapodistrian University of Athens, Greece) under the supervision of the Professor, Nikolaos Thomaidis.

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CHAPTER 1: Honey an overview

1.1 Introduction to Greek food products

There is no denying that food products are one of the most important aspect of the modern world. Society is affected by them in many ways. Consuming products with a high-nutritional value has been proven essential for human's health as this strengthens the immune system, provides people with a wealth of nutrients and prevents them from diseases. Moreover, both the national and the worldwide economy depends on food industry. In Greece, foods occupied the 14.2% of the total exports during 2019 and the estimated revenue was 4,806,000,000€ according to ELSTAT. It is a well-known fact that in our Mediterranean country a wide variety of foods are produced and Greece should emphasize on them. Honey is one of the most important representatives of our national production. It is widely consumed with an average of 2.47 Kg per person, ranking Greece second among the European countries with the highest levels of honey per capita consumption in 2019. Moreover, the estimated annually production of Greece fluctuates between 15,000–20,000 tons [1]. A further study in this product provides us with significant details and leads to essential conclusions.

1.2 Definition of honey

Honey is a natural, viscous, stable sweetener, consisting mainly of fructose, glucose and water. It is produced by honeybees "*Apis mellifera*" from nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants. Apart from sugars, honey contains a noteworthy number of other various components, such as enzymes, vitamins, minerals, amino acids, phenolic compounds, organic acids, flavonoids, pollen, wax and volatile compounds [2]. The precise composition of honey depends on many factors. First and foremost it is affected by the botanical and the geographical origin of the product. However, other aspects, like the climate, honeybees, manipulation, processing, packaging and storage time, also play an important role [3]



Figure 1: Packaged honey sample

1.3 Types of honey

Honey is classified in two types according to its origin. These are nectar or blossom honey and honeydew honey [4]. Nectar honey, as it can be understood from its name, is made from nectar of the plants which is collected by the honeybees. The quantity of collected nectar is related to either internal factors such as the nature of the plant or external factors like humidity, temperature, soil conditions and the amount of wind. On the contrary, the honeydew one comes mostly from plants secretion or the secretion of insects feeding on plants juices. There are existed differences between honeydew and blossom honey. Honeydew honey has stronger flavor and higher antioxidant properties than those of nectar honeys. Moreover, honeydew honey includes a greater number of oligosaccharides, which present a potential prebiotic activity. On the other hand, nectar honey has much more sweet taste than that of honeydew honey [5]. The simplest way to determine the botanical origin of a honey is to measure its electric conductivity. According to the Council Directive 2001/101/EC, honeydew has electric conductivity more than 0.80 mS/cm while the correspondence value of a nectar honey is below this limit. Nevertheless, exceptions can be noticed. For instance, some honey samples like those from chestnuts, strawberry plants, heather, eucalyptus, lime, manuka, tea tree or jelly bush honeys are regarded as blossom honeys, but they often have electric conductivity values above 0.8 ms/cm [6]. In addition to these, honey can

be classified as monofloral or polyfloral. Monofloral honeys are produced by one plant species while the polyfloral one has several plant sources [7].

In Greece there is a considerable reputation and a long tradition in bee-keeping. Greek honey is rich in vitamins, minerals, antioxidants and aromatic substances and as it has already been mentioned, approximately 15,000–20,000 tons are produced annually from different parts of the territory. Thyme plants are cultivated in Aegean sea, Ionian sea and in Central Greece. Pine honey encounters in Crete, Thasos, Evia, Samos, Chalkidiki, fir trees are found in Peloponnese while other types such as orange, cotton and chestnut honey are produced in fewer amounts [8]. Menalon vanilla fir honey from Arkadia and thyme-pine honey of Crete are two honey products with a protected designation of origin (POD). Some categories of Greek honey, which were analyzed during this project are described below [9].

- Thyme honey, which is the most famous honey of Greece. It is light in color, tends to crystallize 6-18 months after its production and Antimicrobial and anti-istrogenic effect are included in its advantages. The plant by which is made from grows on dry slopes or coastal stony places
- Pine honey, which is the basis of Greek production, estimated in 65% of the total honey production. It is rich in minerals and antioxidants, but poor in sugars and this blocks its crystallization
- Cotton honey, a brown honey which will have crystallized by few months after the extraction. It is mainly linked to extrafloral nectars.
- Heather honey, which is located on all hills and slopes covered by deadly forest vegetation. It offers plenty of nectar and pollen to the bees and especially the autumn heather is ranked in first places among the preferences of beekeepers.
- Fir honey, a rare type of honey which never crystallizes. Its color depends on the geographical origin and the Fir honey of Vytina is declared as POD
- Oak honey, whose color is dark and taste is spicy. It is rich in antioxidants and shows an anti-inflammatory and antimicrobial action
- Citrus honey: This type of honey is very light to amber in colour. It is characterized by a delicate floral odour and aroma and crystallized soon after extraction, whereas

it possesses high content of zinc element. A characteristic example of citrus is the orange honey.

1.4 Chemical composition of honey

Honey is a complex matrix in which different chemical compounds are present. Sugars and moisture form honey's main part in a percentage of 95% [10]. However other micro components such as phenolic compounds, minerals, proteins, vitamins, amino acids and volatile organic compounds are also determined [11]. Sterols, pigments, oils and phospholipids are also found. Properties of these categories will be described in the upcoming paragraphs. Volatile compounds, which are the main subject of this work, can be used as a fingerprint for the authentication of honeys, their botanical origin and their organoleptic qualities [12]

1.4.1 Sugars

Essentially, a concentrated water solution of glucose and fructose along with other minor amounts of different sugars form honey. Monosaccharides, in which group glucose and fructose belong to, account for the 70% of sugar content while disaccharides are responsible for the 10-15% respectively [13]. Sugars do play a crucial role in the required time which needs honey to crystallize. This time depends mostly on the ratio of fructose to glucose (F/G). Honey samples, which do not crystallize for a long time have F/G ratio greater than 1.33, and if the ratio is less than 1.11, honey crystallizes quickly. The rate at which glucose crystallizes also depends on the glucose/water ratio (G/W). A slow crystallization of honey occurs when G/W ratio is less than 1.7, while this phenomenon becomes fast and complete when this ratio is greater than 2.0 [14]. Disaccharides and trisaccharides, which are also found in honey, can be hydrolyzed to monosaccharides. This hydrolysis is helped by enzymatic activity. A significant example is the hydrolysis of sucrose to glucose and fructose by the enzyme invertase [15]. Sugar content is affected by storage and processing and is dependent on geographical and botanical origin of honeys. Considering this, sugars could be indicators of authenticity.

1.4.2 Minerals

Honey has a low mineral content ranging from 0,1-0,2 % in nectar honeys to 1% in honeydew honeys [16]. In recent years, the number of studies who focus on the determination of minerals in honey samples is on an increase. Honey is made without human influence, so the determined minerals can be used as a marker of the mineral content of the soil and vegetation in the area from which raw material for honey is collected. In this purpose, different methods, such as ICP-AES and ICP-MS have been applied, determining a wide variety of metals in honey. K, Na, Cd, Pb, Fe, Mn, Cu, Ni, Cr, Zn, Al, Mg, Ca, B and Se are some of them [17], [18]

1.4.3 Vitamins [19]

Honey contains several vitamins. Almost all of them are water soluble. The determination of vitamins in honey and generally in foods is a demanding challenge, because of their instability. Vitamins are affected by many parameters, such as oxygen, temperature, light, pH and moisture. In regard with honey, the removal of pollen during the commercial filtration causes reduction of vitamin content. Oxidation of ascorbic acid by hydrogen peroxide, which produced by glucose oxidase, is another reason of vitamins loss. Both chromatographic and titrimetric methods have been developed for the determination of vitamins. Among them, the B-complex vitamin such as thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B8 or H) and folic acid (B9) as well as ascorbic acid have been identified and quantified.

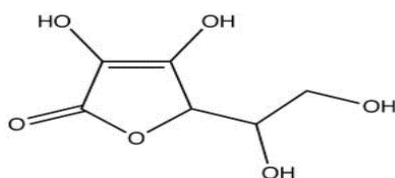


Figure 2: Structure of Vitamin C

1.4.4 Organic acids [20]

Honey acids represent less than 0.5% of total solids. Despite of this minor quantity, they are so important for taste, color, aroma and honey preservation, making it difficult for microorganisms to grow. The majority of these compounds are produced by the action of enzymes secreted by bees during ripeness and storage. Gluconic acid is the

main honey organic acid, representing the 70–90% of the total organic acids. It comes from glucose by the action of glucose oxidase. Apart from gluconic acid, other organic acids have been identified in honeys, some of which are markers of the botanical origin of a sample. For instance, high quantities of quinic acid are related to Erica honey, while high concentrations of citric acid can be used as a marker of thyme honey.

1.4.5 Proteins, amino acids and enzymes

Twenty proteins have been identified in honey [20]. Some of them, for instance globulins, albumins, nucleoproteins and proteases are present in every type of honey. Protein content in majority varies between 0.1 and 0.5 %. A reduction or absence of protein content is a potential indicator of adulteration, overheating or/and long storage of the sample.

Enzymes are found in small amounts in honeys [20]. The most important of them are invertase, glucose-oxidase and diastase. In case of an overheating or during the time, enzymes activity is decreased. The enzyme content depends on several factors, such as temperature, nectar abundance flow, honey botanical origin, transformation degree and the bee

Amino acids in honey are either of a plant or an animal origin. Pollen is the main source of amino acids, so its profile can be proved useful to characterize the botanical origin of honey [21]. Nevertheless free amino acids are added by bees themselves resulting in a high variability of the amino acid content within honeys from the same botanical source. The major amino acid in honey is proline, which derives mainly from bees during the conversion of nectar into honey. Generally, amino acids appears in honey in a ratio of 0.3-1.0 % w/w [22]. Several methods are applied for the determination of amino acids in honey, such as HPLC-MS.

1.4.6 Phenolic compounds

Not only honey includes phenolic compounds but it is rich in them. These chemical substances are secondary metabolites of plants. They are divided into two groups, flavonoids and phenolic acids [23]. Both of them are responsible of the antioxidant action of honeys. However, their role is not limited in this, as they show other beneficial results, such as wound healing, skin cells and tissues protection from oxidative

damage and food preservation. Their role in human health is crucial as they present anticarcinogenic, antiatherogenic, antiviral, immune-modulating, anti-inflammatory, antithrombotic, and analgesic functions [24]. The actual composition of phenolic compounds depends on floral and geographical origin. Phenolic acids are separated into two groups, the hydroxycinnamic acid derivatives and the hydroxybenzoic derivatives. The first group has a characteristic C6-C3 structure and caffeic acid, ferulic acid and p coumaric acid belong to it. The last has a characteristic structure of C6-C1 and the most representative examples are vanillic acid and syringic acid

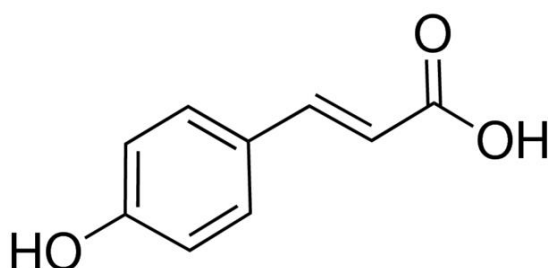


Figure 3: The structure of p-Coumaric acid

Flavonoids share a common carbon skeleton of diphenyl propanes, two benzene rings (ring A and B) joined by a linear three-carbon chain. The central three-carbon chain may form a closed pyran ring (ring C) with one of the benzene rings. Flavonoids generally appears as glycosides with glucose as major sugar in order to form a flavonoid less reactive and more soluble to water which has a protective effect on plant. According to the degree of oxidation of the pyran ring C and the position of the secondary ring B, flavonoids are ranked into several classes, flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols.

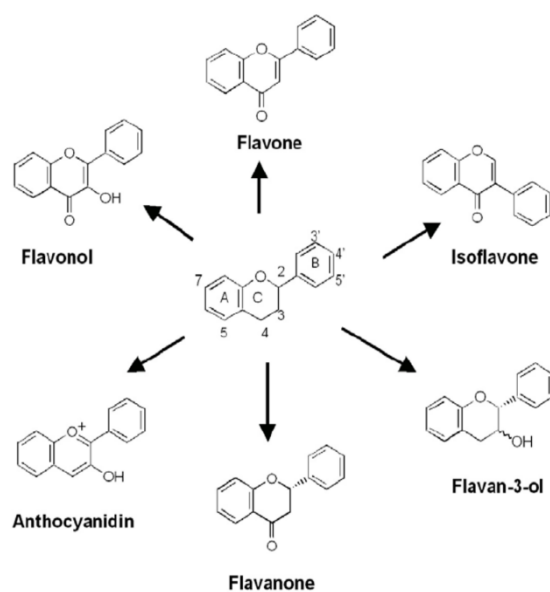


Figure 4: The structure of the more common flavonoids in honeys

[25]

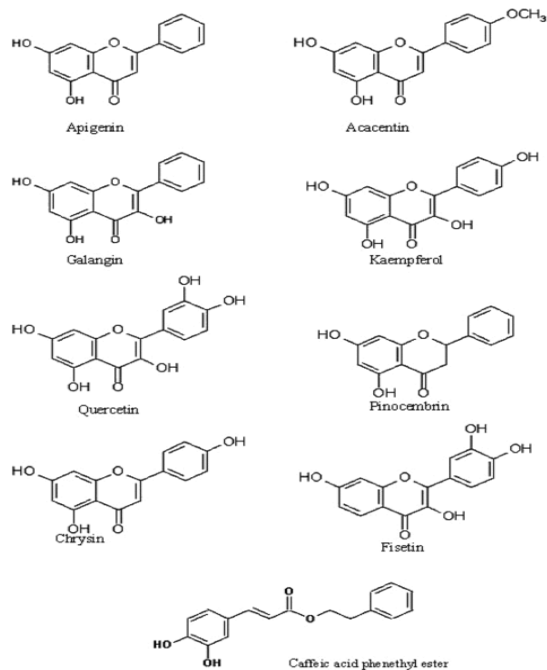


Figure 5: Chemical structures of the most important structures found in honey and propolis

[26]

For the determination of phenols, a lot of analytical techniques have been used, such as HPLC-MS [27] and HPLC-DAD [28]

1.4.7 Volatile compounds

Volatile organic compounds (VOCs) are a highly important group of analytes which are identified in honey. Honey's aroma is formed by them. Flavor constituents are related to the botanical origin of a honey sample and depend on honeybees, thermal processing and the storage of honey[29]. The main classes of volatile compounds which have been reported in honey are furans, aldehyde, esters, ethers, ketones, alcohols, carboxylic acids, alkanes, terpenes, nor-isoprenoids and pyrene derivatives and are presented in Tables 1,2 and 3 [30]. Considering that the identification of honey origin is a challenging area, several studies in honey samples have confirmed that volatile composition and a wide variety of these components can be referred as floral markers of honey samples. In other words, honey sample of different botanical origin can be distinguished by the determination of one or more characteristic volatile compounds. For instance, hexanal and heptanal are markers of lavender honey, while lilac aldehyde and hotrienol are used as markers of citrus honey [31]. Moreover, regardless of aroma distribution, volatile compounds play an key role in the commercial field. They are linked to the freshness of honey and influence consumers on their food selection [32].

In terms of the identification of volatile compounds in honey samples several methods have been applied, such as HeadSpace(HS) SPME GC-MS[33], purge and trap GC-MS[34] and static HS-GC-MS. SPME has the advantage of being a flexible, simple method that is used for a wide range of analytes and a relatively economic extraction technique.

Table 1: Some Volatile compounds from several chemical classes, which have been identified in honey samples worldwide

Name	Formula	CAS number	Category
Hexanoic acid	C ₆ H ₁₂ O ₂	142-62-1	Acids
Heptanoic acid	C ₇ H ₁₄ O ₂	111-14-8	Acids
Octanoic acid	C ₈ H ₁₆ O ₂	124-07-2	Acids
Nonanoic acid	C ₉ H ₁₈ O ₂	112-05-0	Acids
Decanoic acid	C ₁₀ H ₂₀ O ₂	334-48-5	Acids
phenylacetic acid	C ₈ H ₈ O ₂	103-82-2	Acids
2-methylbutanoic acid	C ₅ H ₁₀ O ₂	600-07-7	Acids
p-Anisealdehyde	C ₃ H ₈ O ₇	123-11-5	Aldehydes
Nonanal	C ₉ H ₁₈ O	124-19-6	Aldehydes
Decanal	C ₁₀ H ₂₀ O	112-31-2	Aldehydes
Benzaldehyde	C ₇ H ₆ O	100-52-7	Aldehydes
Carvacrol	C ₁₀ H ₁₄ O	499-75-2	Alcohols
2-Ethyl-1-Hexanol	C ₈ H ₁₈ O	104-76-7	Alcohols
Benzenemethanol	C ₇ H ₈ O	100-51-6	Alcohols
2,3 butanediol	C ₄ H ₁₀ O ₂	513-85-9	Alcohols
3-Methyl-2-butanol	C ₅ H ₁₂ O	598-75-4	Alcohols
1-Hexanol	C ₆ H ₁₄ O	111-27-3	Alcohols
1-Octanol	C ₈ H ₁₈ O	111-87-5	Alcohols

Table 2: Some Volatile compounds from several chemical classes, which have been identified in honey samples worldwide (Continued)

Name	Formula	CAS number	Category
1-Nonanol	C ₉ H ₂₀ O	143-08-8	Alcohols
1-Decanol	C ₁₀ H ₂₂ O	112-30-1	Alcohols
phenylethyl alcohol	C ₈ H ₁₀ O	60-12-8	Alcohols
3-Hexen-1-ol	C ₆ H ₁₂ O	544-12-7	Alcohols
Acetone	C ₃ H ₆ O	67-64-1	Alcohols
2,3 Butanedione	C ₄ H ₆ O ₂	625-34-3	Ketones
3-Hepten-2-one	C ₇ H ₁₂ O	1119-44-4	Ketones
2-Heptanone	C ₇ H ₁₄ O	110-43-0	Ketones
Beta damascenone	C ₁₃ H ₂₀ O	35044-68-9	Ketones
Beta-Thujone	C ₁₀ H ₁₆ O	1125-12-8	Ketones
4-Ketoisophorone	C ₉ H ₁₂ O ₂	1125-21-9	Ketones
Isophorone	C ₉ H ₁₄ O	78-59-1	Ketones
3-oxoionol	C ₁₃ H ₂₀ O ₂	34318-21-3	Ketones
1-Octene	C ₈ H ₁₆	111-66-0	Hydrocarbons
Octane	C ₈ H ₁₈	111-65-9	Hydrocarbons
Nonane	C ₉ H ₂₀	111-84-2	Hydrocarbons
Decane	C ₁₀ H ₂₂	124-18-5	Hydrocarbons
Limonene	C ₁₀ H ₁₆	138-86-3	Hydrocarbons

Table 3: Some Volatile compounds from several chemical classes, which have been identified in honey samples worldwide (Continued)

Name	Formula	CAS number	Category
Ethyl Benzoate	C ₉ H ₁₀ O ₂	93-89-0	Esters
Ethyl Hexanoate	C ₈ H ₁₆ O ₂	123-66-0	Esters
Ethyl Heptanoate	C ₉ H ₁₈ O ₂	106-30-9	Esters
Ethyl Octanoate	C ₁₀ H ₂₀ O ₂	106-32-1	Esters
Ethyl Nonanoate	C ₁₁ H ₂₂ O ₂	123-29-5	Esters
Dimethyl disulfide	C ₂ H ₆ S ₂	624-92-0	Sulfur compounds
Methional	C ₄ H ₈ OS	3268-49-3	Sulfur compounds
2,3-butanethiol	C ₄ H ₁₀ O ₂	513-85-9	Sulfur compounds
Furanmethanol	C ₅ H ₆ O ₂	98-00-0	Furanes
Furfural	C ₅ H ₄ O ₂	98-01-1	Furanes
Furaneol	C ₆ H ₈ O ₃	3658-77-3	Furanes
Furan, 2,5-dimethyl-	C ₆ H ₈ O	625-86-5	Furanes
2- acetylfuran	C ₆ H ₆ O ₂	1192-62-7	Furanes
Carvacrol	C ₁₀ H ₁₄ O	499-75-2	Terpenes
α-Terpinene	C ₁₀ H ₁₆	99-86-5	Terpenes
α-Pinene	C ₁₀ H ₁₆	80-56-8	Terpenes
β-Linalool	C ₁₀ H ₁₈ O	78-70-6	Terpenes
Hotrienol	C ₁₀ H ₁₆ O	53834-70-1	Terpenes

In Table 4, the odor description of some volatile compounds which are present in honey is described

Table 4: Odor characteristics of some volatile compounds identified as odorants in honey [35][36][37]

Compound	Odor description
Dimethyl Disulfide	Garlic, sweaty
1-Hexanol	Fruity, Soft, Aromatic, Alcoholic
Ethyl Hexanoate	Fruity, anise
Octanal	Lemon
Linalool	Floral, muscat
β -Damascenone	Baked apple
Phenyl ethyl alcohol	Rose
Pentanal	Malt, Almond, Pungent
Hexanal	Fatty, green, grassy, powerful, penetrating
Heptanal	Fatty, Citrus
Octanal	Lemon
Nonanal	Fatty, Citrus, Green
Butanoic acid	Rancid, Soy, Pungent
Pentanoic acid	Sweat
Octane	Solvent

1.5 Quality criteria of honey

Assuring honey quality is an important issue. It is verified by several quality criteria, which are described below, whose value is priceless as they can determine a potential adulteration and guarantee honey botanical origin

1.5.1 pH and free acidity

Microbiological contamination is related to pH and free acidity. Conclusions about the stability of honey is provided by this quality factor. The presence of organic acids, particularly gluconic acid, as well as inorganic ions, such as phosphate and chloride make pH acid, most of the times inside the range of 3.5-5.5. According to EU Directive 110/2001, the free acidity of honey must be bellow of 50 milli-equivalents/kg [5]. In general, Honeydew honeys show a higher mean pH value and acidity than blossom honey. For the determination of free acidity, a titrimetric method can be applied [38]

1.5.2 Electrical conductivity

Electrical conductivity expresses the ability of a material to conduct an electric current. It is relevant to the botanical origin [20]. Mineral content, organic acids, inorganic ions, proteins and other components such as sugars, polyols and pollen grains, which can act as electrolytes, are also related to electrical conductivity. According to European legislation (EU Directive 110/2001), the electrical conductivity of blossom honeys must be below the limit of 0.8 mS/cm, whereas honeydew and chestnut honeys and blends of these with blossom honey must present values of electrical conductivity higher than this number. Exceptions to this rule are found in honeys whoso origin derives from strawberry tree (*Arbutus unedo*), eucalyptus, bell heather (*Erica*), lime (*Tilia* spp.), manuka, ling heather (*Calluna vulgaris*), or jelly bush (*leptospermum*) and tea tree (*Melaleuca* spp.). [5]

1.5.3 Ash content

Another quality criterion of the botanical origin of honeys is ash content. Ash content shows a considerable difference between blossom and honeydew samples. In general, ash content of honeydew honeys is higher than the correspondent one of blossom honeys. In honeydew samples, its value is below 1.2% w/w while in blossom

samples it is below 0.6% w/w. Ash content is a measure of quality that evaluates the mineral content present in honey.

1.5.4 Moisture content and water activity

Moisture is one of the most important characteristics of honey. Some of its physical properties, such as crystallization and viscosity are affected by this parameter [3]. Water content is affected by soil and climatic conditions, intensity of nectar flux, season of harvesting, manipulation by beekeepers during period of harvest, extraction, processing and storage conditions, degree of maturation and botanical and geographical origin [20]. Honeys' capability to remain stable and to resist spoilage by yeast fermentation is determined by moisture, whose content cannot exceed 20%. [5]

1.5.5 Proline

The main amino acid of honey is proline. It is useful in detection sugar adulteration and is related to honey ripeness. Levels of proline are significantly lower in adulterated honeys and a value less than 180 mg/Kg characterizes a honey as either non-ripe or adulterated. [5]

1.5.6 Sugars

As it has already been mentioned in paragraph 1.4.1.1, sugars are one of the main constituents of honey. According to the standards of the Codex Alimentarius Committee on Sugars (2001), for floral honey the minimum amount of reducing sugars is 60 g/100 g^{±1} for floral honey, while sucrose can be presented in maximum value of 5g in 100 g of floral honey. [3]

1.5.7 5-Hydroxymethylfurfural (HMF)

HMF is connected with honey's freshness. Long storage and excessive heating as well as the adulteration with commercial sugars cause an increase in the levels of HMF. This furanic compound, which is produced through the Maillard reaction, can not

be used without an extra factor in the determination of long storage and heat treatment, as other factors, such as sugar profile, pH and moisture affects the levels of HMF. However, European Union (EU Directive 110/2001) has established a limit of 40 mg/Kg HMF in honey samples. Nevertheless, there are two exceptions. Honeys from tropical areas, whose limit is 80 mg/Kg and honeys with low enzymatic action, whose limit is 15mg/Kg. [5]. In Figure 6 the proposed reaction for the formation of HMF is presented

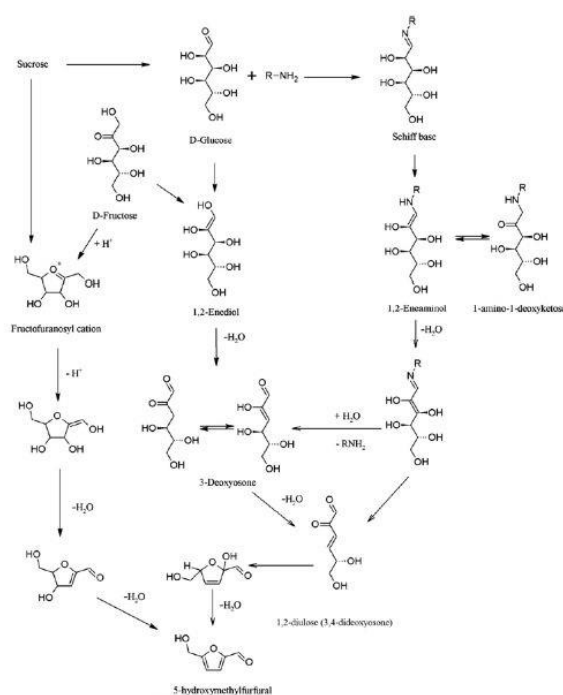


Figure 6: Proposed reaction scheme for the formation of 5-hydroxymethylfurfural [39]

1.5.8 Enzymatic activity

Enzymatic activity is also related to freshness of honey. Activity of diastase and invertase is reduced during heating and storage. A minimum value of 8 on the Schade scale for the activity of diastase has been established by European legislation. In honeys, such as citrus, which have low natural enzyme content, a minimum value of 3 is allowed, unless the content of HMF is higher than 15 mg/kg. [5]

1.5.9 Water insoluble solids

Water insoluble solids demonstrates honey's impurities. Their major source is wax and according to European standards, the permitted values of water insoluble solids have been defined as 0.1 g/100 g honey. [40]

1.5.10 Organoleptic characteristics

Aroma, flavor and color form the organoleptic characteristics of honey. The first two were discussed in the previous units, especially in the chapter about volatile compounds which attribute honey's aroma. In terms of color, researchers have found its relevance to geographical origin of honeys. For example, honeydew honeys are darker than nectars. However, ash, mineral and phenolic content as well as temperature and time of storage also affect the shade of honey. [5]

Chapter 2: Honey authenticity, literature review

2.1 Food Authenticity

Authenticity of food products is a decisive factor in terms of both commercial and health levels. Cases of adulteration, misleading characterization, mislabeling, and false origin are all related to authenticity [41]. All these issues most of the times aim to the commercial profit of producers as they substitute an original ingredient of the product with a cheaper one. Consumers are not only cheated but they face serious health dangers by this action. Issues, such as poisoning of Chinese children in 2008 by melamine, which was added in milk in order to falsely increase the protein content or a methanol poisoning in the Czech Republic, Poland, and Slovakia due to adulteration of “on top” liquor of unknown origin in 2012 are some examples which have taken place in recent years. In this way, many regulations have been enforced globally in order to avoid food adulteration and mislabeling. Authentication of the origin of the food product, tracing the product to a specific region, has also attracted researchers to verify products of certified origin and the differentiation of cultivars and varieties. In this sense regional origin or traditional production are supporting by created labelling systems, such as the quality labels created by the European Union: Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI). PDO certification verifies that a product originates from a specific region or country. Uniqueness of a product is defined by its geographical environment and the related natural characteristics. Finally, also the production itself has to occur in a specific region. PGI includes similar requirements, however the production of a product is more flexible and can be only partially performed in the protected region [42].

Honey authenticity is monitored by Codex Alimentarius standard, EU Honey Directive and other authorities. International standards which certify the origin of honeys and/or the mode of production have been established [43]. The origin of the honey is related to both the geographical and the botanical origin, while production includes the harvesting of honey hives and processing. The botanical source, for example pine, fir etc, by which a honey derives from is the purpose of the determination of a honey's botanical origin. Determination of the geographical origin verifies the specific region where a honey is produced. In order to achieve the above, different methods have to be applied and chemical markers need to be identified.

Volatile organic compounds which attribute to aroma is an essential factor in determining their origin. These analytes are mostly identified by Gas Chromatography methods. In Tables 5-16 Several analytical techniques, results and conclusions based on previous scientific researches about the volatile compounds of honey are presented.

2.2 Volatile Organic Compounds in honey, literature review

Having made an extended literature research, the most important parameters and conclusions of selected researches are attached in Tables 5-16.

Table 5: Literature review of previous researches about the volatile compounds of honey

Samples	Sample preparation	Technique	Results	Statistics	Ref
119 unifloral honeys from 14 regions of Greece	HS- SPME Fibre: DVB/CAR/PDMS 50/30 μ m Equilibration: 15 min, 45°C in a water bath at 600 rpm Sampling time: 30 min sample volume: 4mL (1:1 honey: water) Extraction temperature: 45 °C	HS-SPME-GC/MS Injector T: 250°C Column: B-5MS Oven T: 40°C (3 min) to 260 °C at 8 °C/ min (6 min held) Flow rate He: 1,5 mL/min MS-transfer line T: 270 °C EI: 70 eV	55 volatile compounds were identified Voc's concentration: hyme > fir > pine > orange blossom Potential chemical markers: 30 volatile compounds	MANOVA, LDA (Wilks'Lambda = 0.002, $X^2=$ 665.719, df = 93, $p=0.000 < 0.05$ for the first function and Wilks' Lambda = 0.157, $X^2=$ 185.768, df = 60, $p = 0.000 < 0.05$ for the second)	[33]

**Table 6: Literature review of previous researches about the volatile compounds of honey
(Continued)**

Samples	Sample preparation	Technique	Results	Statistics	Ref
8 samples of Polish honey (rape, acacia, linden, buckwheat, heather, honey-dew and polyfloral honey)	Dilution: 5:1 w/w honey to water, NaCl addition SPME: Fibre: DVB/CAR/PDMS 30/50µm Extraction: 60°C, 40 min Thermostating time: 10 min	GC-FID Injector: 260 °C (split splitless) Column: Stabilwax-DA Oven T: 50 °C (for 1 min), at 5 °C/min from to 200 °C and then to 240 °C at 10 °C/min (total 50 min) Flow rate He: 2,2 mL/min Transfer line T: 240°C Detector T: 240°C / 250°C	Presence of more than 300 compounds and identification of 178 Markers for Heather honey: benzoic acid, phenylacetic acid, dehydrovomifoliol and isophorone. Markers for Buckwheat honey: furfural, 2-methylbutyraldehyde and 3-methylbutyraldehyde.		[4]
80 samples with different botanical origin, acacia, sunflower, tilia or lime from Spain, Romania, and Czech Republic	Purge and trap Extraction: 45 °C, 20 min Thermal desorption: 220 °C, 10 min at 10 mL/min He flow Cryofocused: cold trap at -30 °C heating to 250 °C at a rate of 99 °C/s IS: 2-pentanol	GC-MS Column: DB-WAX Oven: 40 °C (held 2 min) to 190 °C (held 11 min) at 4 °C/min and finally to 220 °C at 8 °C/min (held 8 min) Flow rate He: 1.0 mL/min, Inlet T: 230 °C. EI: 70 eV	51 identified compounds. 17 in acacia honey, 9 in sunflower and 8 in tilia showed significant differences between countries. cis-linalool oxide was only found in acacia	ANOVA, LDA ($\alpha=0,05$) PCA, SLDA A correct classification of all the samples was achieved with the exception of 10% of the sunflower honeys from the Czech Republic	[44]

**Table 7: Literature review of previous researches about the volatile compounds of honey
(Continued)**

Samples	Sample preparation	Technique	Results	Statistics	Ref
39 Greek pine honeys	HS- SPME Fibre: DVB/CAR/PDMS 50/30 μm Equilibration: 15 min, 45°C in a water bath at 600 rpm Sampling time: 30 min sample volume: 4mL (1:1 honey: water) Extraction temperature: 45 °C	HS-SPME-GC/MS Injector T: 250°C Column: B-5MS Oven T: 40°C (3 min) to 260°C at 8 °C/ min (6 min held) Flow rate He: 1,5 mL/min MS-transfer line T: 270°C El: 70 eV	55 volatile compounds were identified	MANOVA LDA Samples were satisfactorily classified according to geographical origin (84.6% correct prediction) using 8 selected volatile compounds.	[45]
42 Greek thyme honeys	Dilution in distilled water at 3000 rpm for 10 min. The sediment was dried at 40 °C HS- SPME Fibre: DVB/CAR/PDMS 50/30 μm Equilibration: 15 min, 45°C at 600 rpm Sampling time: 30 min sample volume: 4mL (1:1 honey: water) Extraction T: 45°C.	HS-SPME-GC/MS Injector T: 250°C Column: B-5MS Oven T: 40°C (3 min)to 260 °C at 8 °C/ min (6 min held) Flow rate He: 1,5 mL/min MS-transfer line T: 270 °C El: 70 eV	47 volatile compounds were identified and semi-quantified. classification according to geographical origin (64.3% correct prediction) Using 9 volatile compounds	LDA Pillai's Trace = 3.876 (F = 3.370, p= 0.004b0.05) and Wilks' Lambda =0.002 (F = 2.374, p= 0.001b0.05) LDA(Wilks' Lambda =0.037, X^2= 112.177, df = 36, p= 0.001 <0.05 and Wilks' Lambda = 0.266, X^2= 45.057, df = 24, p= 0.006 <0.05 for the second)	[46]

**Table 8: Literature review of previous researches about the volatile compounds of honey
(Continued)**

Samples	Sample preparation	Technique	Results	Statistics	Ref
19 samples, obtained in 3 different areas of the world (Spain, Malaysia and Bangladesh)	IS: 3-octanol and PCB-30 HS-SPME Sampling time: 40 min Extraction T: 50 °C Equilibration: 30 min. Exposure: 50 °C for 40 min at 600 rpm Fibre: DVB/PDMS 50/30 Desorption: 5 min 260 °C	HS-SPME-GC-MS(Q TOF) Injector T: 260°C Column: B-WAXETR Oven T: 60°C (1 min) to 240°C at 5 °C/min (20 min held) Flow rate He: 1,2 mL/min Source T: 230° for EI and 300°C for PCI Quadrupole T: 150°C MS-transfer line T: 250 °C	Identified compounds classified in 4 major classes: benzene derivatives, non-aromatic carboxylic acids and esters, terpenes and nitrogenated compounds and many of them were used as geographical origin and floral markers	PC1(88% of explained variance) was mainly associated with toluene and it allowed the discrimination of BD honeys from the rest. PC2, 4% C1 discriminated GAL from both groups of tropical sample	[47]
13 honey samples from four botanical origins: heather, (raspberry, rape, alder buckthorn)	Dilution with water NaCl Fibre: DVB/CAR/PDMS 30/50 µm Desorption: 10 min Incubation: 5 min at 60 °C Extraction: 20 min 250 rpm	GC-MS Injectors T: 250°C Column: DB5-MS Oven T: 35°C, 45°C/min to 85°C, 9°C/min to 200°C, 45°C/min to 280°C holding time 1 min Flow rate He: 1.0 mL/min for GC-MS	46 odour compounds were detected Heather honeys markers: Isophorone, 2-methylbutyric acid, absence of linalool	HCA	[48]

**Table 9: Literature review of previous researches about the volatile compounds of honey
(Continued)**

Samples	Sample preparation	Technique	Results	Statistics	Ref
66 honey (Lavender and Thyme) samples from Spain (Valencia)	Purge and Trap: Extraction: 45 ° C, 20 min Desorption: 220 ° C 10 min Cold trap: -30 ° C to 250 ° C (at a rate of 99 ° C/ s) IS: 2-Pentanol	Purge and trap-GC-MS Injector T: 250 ° C Column: DB-WAX Oven T: 40 ° C (2min held) to 194°C(11 min held) with 4 ° C / min and then to 220°C(8 min held) with 8 ° C / min Flow rate He: 1,0 mL/ min	30 volatile compounds were identified and semi-quantified, 1-hexanol: marker of lavender Ketones: more abundant in thyme than labelled honey Linalool and hotrienol the only honey in which terpenes identified	ANOVA($\alpha=0,05$) PCA: PC1(40%), PC2(22,0%) standardised coefficients: PC1 (0.731); PC2(0.819); PC3 (0.323); PC4 (0.227); PC5 (0.586); PC6 (0.363) PC7(0.088) Correct classification (90.9%)	[49]
17 multifloral honey samples from Brazil (Parana)	Dissolution with NaCl Incubation: 60 ° C for 10 min Extraction: 10 min 60 ° C	Static HS-SPME-GC-MS Injector T: 150 ° C (splitless) Column: HP-5 MS Oven T: 40 ° C to 150°C with 8 ° C / min and then to 200°C with 15 ° C / min Flow rate He: 1,0 mL/ min Ion Source T: 230 ° C Transfer line T: 150 ° C	Identification of 44 volatile compounds Honey samples from Guaraqueçaba were characterized by the presence of hotrienol while those from Cambará showed epoxylinolol, benzaldehyde as discriminating compounds	PCA (17×15) 6 PCA were extracted with Eigenvalues ≥ 1 and they explained 91.9% of the total variance. LDA (6 components from PCA with Eigenvalues ≥ 1)	[50]

Table 10: Literature review of previous researches about the volatile compounds of honey (Continued)

Samples	Sample preparation	Technique	Results	Statistics	Ref
16 sugarcane honey samples from Portugal (Madeira)	SPME Dilution: 3:2 w/v, addition NaCl Fibre: DVB/CAR/PDMS 50/30µm Extraction: 60 °C for 60 min	SPME-GC-MS Injector T: 250 °C (splitless) Column: BP-20 Oven: 40°C for 2 min, then at 0.25°C / min to 45°C (2 min held) then at 4 °C/min to 70°C (2 min held), then at 3°C/ min to 130°C (2 min held) and at 3°C/ min to 220°C (7 min held) Flow rate He: 1,0 mL/ min Ion Source T: 230 °C Quadrupole T: 150 °C Transfer line T: 270 °C	Identification of 87 volatile compounds,	PCA (18 VOC's) LDA (Wilk's Lambd = 0.003992, p<0.0000001))	[51]
Monofloral honeys from Brazil	SPME: Fibre: PDMS/DVB Equilibration time: 15 min Exposure time: 15-45 min 700 rpm Desorption time: 5 min Extraction: 45 min, 45 °C	GC-MS Injector T: 250 °C Column: HP-5 Oven T: 40 °C for 2 min, then with 2 °C/min ramp to 60 °C, then with 3 °C/min to 90 °C and with 4 °C/min to 240 °C (held for 10 min) Flow rate: 1,0 mL/ min Ion Source T: 200 °C Detector T: 250 °C	Identification of 114 Volatile compounds Potential markers for the botanical sources: linalool for malícia honey, D-sylvestrene for chanana honey, rose oxide for algaroba honey and benzenethanol for angico honey.	PCA (71% of the variation)	[52]

Table 11: Literature review of previous researches about the volatile compounds of honey (Continued)

Samples	Sample preparation	Technique	Results	Statistics	Ref
72 monofloral honeys from different countries	HeadSpace Incubation: 15 min, 45 °C Syringe T: 80 °C IS: 2-acetylpyridin Mixed with NaCl	GC-IMS Injector T: 150 °C (split 1:10) Column: DB-225 Oven T: 40 °C for 2 min, then with 8 °C/min to 120 °C (held for 10 min) Flow rate Nitrogen: 1,5 mL/ min Transfer line T: 120 °C	3-hydroxy-2-butanone(acetoin), trans-2-pentenal, and 3-methylbutanol: characteristic for honeydew honeys	PCA LDA(k=10)	[53]
7 honey samples from Finland	Dilution with H₂O SPME: Fibre: DVB/CAR/PDMS 30/50µm Pre-incubation time: 5 min, Incubation T: 60 °C Extraction: 20 min, 250 rpm Desorption: 10 min	GC MS, GC-FID Injectors: 250°C Columns: DB5-MS Oven T(GC-MS): 35 °C, 45 °C/min to 85 °C, 9 °C/min to 200 °C, 45 °C/min to 280 °C holding time 1 min (total 16.67 min) Oven T(GC-FID): 35 °C, 17 °C/min to 280 °C, holding time 4 min Flow rate He: 1,0 mL/ min(GC-MS) and 2,0(GC-FID)	C–O: 73 odor-active compounds identified, 37 were common in all samples.	PCA(R²X(1) = 0.7) PLSR (four groups R²Y values with two components varied from 0.82 to 0.97 and Q² value from 0.55 to 0.80 and with 5 components Q² values were between 0.82 and 0.97)	[54]

**Table 12: Literature review of previous researches about the volatile compounds of honey
(Continued)**

Samples	Sample preparation	Technique	Results	Statistics	Ref
21 samples of 3 different unifloral samples from India	Addition of anhydrous sodium sulfate and internal standard (benzophenone) SPME: Fibre: PDMS/DVB 65µm Condition: 250°C for 30min Heated: 40°C for 60min Exposure: 30min	GC-MS Injector: 220 °C (splitless) Column: HP-5MS Oven T: 40 °C (for 5 min), at 5 °C/min to 250 °C Flow rate He: 0,9 mL/min Source T: 230°C Interface T: 150	42 volatiles were detected, 29 of them for the first time, 1,6-anhydro and 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl were present in apple and saffron honey		[55]
28 Thyme honey samples from different locations of Greece	SPME: Fibre: DVB/CAR/PDMS Equilibration: 60°C, 30min Sampling: 60 min	GC-MS Injector T: 220 °C Column: HP-5MS Oven T: 40 °C (for 3 min), at 3 °C/min to 160 °C and then to 200 °C at 10 °C/min Transfer line T: 290°C El: 70 eV	Isolation of 62 Volatile compounds Potential botanical markers of Greek Thyme honey: Phenylacetaldehyde, phenylacetone, 2-phenyl-2-butenal, 2-methylbutyrophene, carvacrol, 3-hydroxy-4-phenyl-2-butanone, 3-hydroxy-1-phenyl-2-butanone, 3-hydroxy-4-phenyl-2-buten-3-one	The average concentration of 7 compounds was significantly different (p<0,05) phenylacetaldehyde, acetophenone, octanoic acid, carvacrol, phenylethyl alcohol, nonanal, and hexadecane. PCA: 85.7% of cross-validated grouped cases correctly classified.	[56]

Table 13: Literature review of previous researches about the volatile compounds of honey (Continued)

Samples	Sample preparation	Technique	Results	Statistics	Ref
7 kinds of honey from different regions of Algeria	Addition of NaCl SPME: Fibre: PDMS/CAR Equilibration: 45°C and 55°C Extraction: 45°C, Conditioning: 5min at 275°C	GC-MS Column: HP-5MS Oven T: 60 °C (for 2 min) to 300°C (hold for 5 min) at 4 °C/min Flow rate He: 1mL/min	124 volatile compounds were identified	AHC	[12]
Pure honey from 4 different floral sources: Citrus, Alfalfa, Marjoram and Black seed from Egypt	Dilution with deionised water with a ratio of 5:1 SPME: Fibre: DVB/CAR/PDMS 50/30µm Precondition: 270°C, 60 min Equilibration: 70°C for 30 min Extraction: 70°C for 60 min	GC-MS and GC-APCI-MS Column: ZB-Wax Oven T: 40 °C (for 3 min), at 4 °C/min to 160 °C then to 200 °C at 10 °C/min then to 230°C (held for 5 min) at 125°C/ min Flow rate He: 1,0 mL/min Ion Source T: 200°C	Identification of 119 volatile compounds (only 62 compounds were found in all four examined honey)	PCA PLS(GC-MS): $R^2p=0.93$ and RMSEP=2.97% PLS(GC-APCI-MS): $R^2p=0.96$ and RMSEP=2.52%# ($R^2p=0.95$ and RMSEP=2.60%)	[57]
Honey samples	Fibre: CAR/PDMS/DVB 50/30µm Extraction: 60°C for 36 min, 40°C for 18 min and 0°C for 6 min (total 60 min) Desorption: 10 min	GC-MS Injector T: 260 °C Column: Rtx-5MS Oven T: 40 °C (Held for 10 min) to 300 °C at 5 °C/ min (Held 20 min) Flow rate He: 1.0 mL/min Detector T: 260°C	Optimization of a method based on the use of three different extraction temperatures		[58]

**Table 14: Literature review of previous researches about the volatile compounds of honey
(Continued)**

Samples	Sample preparation	Technique	Results	Statistics	Ref
Ulmo honey samples from Chile	Extraction of volatiles: addition of ultrapure water Equilibration: 60 °C for 20 min SPME: Fibre: DVB/CAR/PDMS 50/30µm Contitioning: 270 °C, 1 h Exposure: 60 °C for 40 min Extraction of non-volatile/semi-volatile compounds: dissolousion in 300 mL acidified distilled water (pH 2adjusted with 1 M HCl),elution with MeOH, evaporation to dryness	GC-MS Injector T: 240 °C, splitless (volatiles) and 280°C split ratio1:30(non/semi volatiles) Column: Rtx®-5MS Oven T: 40 °C (held for 5 min) to 250 °C at 5 °C/min, then to 290 °C (held for 15 min at 10 °C/min) for a total run time of 47 min(volatiles). 80 °C(held for 1 min) to 200 °C(held for 5 min) at 5 °C/min , then to 250 °C at 5 °C/min, followed by 3 °C/min increase rate to 300 °C (held for 5 min) then to 320 °C at 10 °C/min (held for 10 min) for a total run time of 66 min Flow rate He: 36.8 cm/s Ion Source T: 230 °C(volatiles) and 280°C (/semi volatiles) Quadrupole T: 200 °C El: 70eV	Identification of 50 volatile compounds The most important flavor components: benzaldehyde, octane, nonanal, 4-methoxybenzaldehyde Identification of 27 non/semi volatile compounds.		[59]

Table 15: Literature review of previous researches about the volatile compounds of honey (Continued)

Samples	Sample preparation	Technique	Results	Statistics	Ref
15 honey samples from Lithuania	saturated NaCl solution Equilibration of samples: 20 min at 40°C and magnetically stirred SPME Fibre: CAR/PDMS Exposure: 30 min Desorption: 3 min	GC-MS Injector T: 250 °C (split mode) Column: Elite-5 MS Oven T: 40 °C (held for 5 min) to 250 °C (held for 10 min) at 5 °C/min Flow rate He: 1.0 mL/min	93 compounds in honey were identified, Benzaldehyde and benzenacetaldehyde were the only compounds found in all 15 honey samples. Markers: pentanal, 1,4-pentanediol, 2,3-dihydro-5-methylfuran, 4-pentenal, 2-octene, decane, 2,3-dimethylheptane, limonene, 2,3-dimethylbenzofuran and decanoic acid; heptane, 1-pentanol, butanoic and pentanoic acids, 2-methyl-1-pentanol, p-cresol, p-cymene		[29]
70 honey samples from northern and central Mozambique	Purge and trap: 45 °C for 20 min using nitrogen (100 mL/min) Thermal desorption: 220 °C for 10 min at 10 mL/min He flow rate) Cold trap (-30°C) heating to 50 °C IS: 2-pentanol	GC-MS Column: DB-WAX Oven T: 40 °C (Held for 2 min) to 190 °C at 4 °C/min (Held 11 min) and finally to 220 °C (Held 8 min) at 8 °C/min Flow rate He: 1.0 mL/min EI: 70 eV	Identification of 48 volatile compounds	ANOVA ($\alpha=0,05$) Correct classification 96,7% due to 3-Methylbutan-1-ol and other factors	[60]

2.3 Conclusions about literature related to honey

In SPME method, there is a syringe consisting of the main body and the fiber, in which a liquid static phase is attached. Fibre is exposed to the supernatant liquid space of the sample or it is submerged in the liquid body of the sample in order to absorb the analytes. Simultaneously, the sample can be heated or be stirred. After receiving the compounds, SPME syringe is inserted into the gas chromatograph and is exposed to a carrier gas. A thermal desorption which takes place in the next step leads to the separation of the compounds. SPME has a wide variety of advantages. It is simple, fast, with an increased sensitivity and independent in the use of solvents. All these have made it an appropriate method in regard with the determination of volatile organic compounds in honey. Abdelhamid Neggad et al [12] applied an SPME-GC-MS method and managed to identify 124 volatile organic compounds in Algerian honeys. In another research, after having optimized the SPME extraction parameters in an SPME-GC-MS method, Ana Carolyny Vieira da Costa et al [52] analyzed Brazilian honey samples and determined several volatile compounds, such as linalool, D-Sylvestrene, rose oxide and benzenethanol, which functioned as chemical markers for the determination of the botanical origin of these samples. In a similar way, Ioannis K. Karabagias et al[33], collected 119 honey samples from 14 different regions of Greece, in which 55 different volatile compounds were identified and semi-quantified belonging to various compound classes, like alcohols, esters, aldehydes, ketones, carboxylic acids, hydrocarbons and other compounds. Moreover, these researchers concluded by an application of MANOVA statistics that nonane, octanal, limonene, Octanoic acid ethyl ester Decanal, Beta thujone and others can be considered as chemical markers relevant to the botanical origin of these Greek samples.

Chapter 3: Scope and Objectives

In this study, we decided to construct a target database, including volatile compounds which are present in honey, and then optimize and validate both a static Headspace and a Headspace-SPME-GC-MS methods in order to analyze Greek honey samples from different botanical origins in terms of authenticity and characterization assessment. More specifically we aimed to identify through target and suspect screening the volatile chemical composition of each variety along with potential biomarkers indicative of each botanical origin.

Chapter 4 Analytical Techniques

4.1 Gas Chromatography (GC)

One of the most important analytical techniques in quantity and quality chemical analysis is definitely gas chromatography [61]. In this method, components of a vaporized sample are separated by being distributed between a mobile gaseous phase and a liquid or a solid stationary phase held in a column. Analytes must be volatile from scratch or they need to be converted into volatile substances, by methods such as derivatisation and go instantly into the gas phase without a simultaneous disintegration. Elution depends on the flow of an inert gaseous mobile phase. It must be mentioned that in comparison with other types of chromatography, analytes do not interact with the mobile phase. The only role of the mobile phase is found in transporting the analytes through the column. In most of the times, column is housed in a thermostated oven. Generally speaking, column's temperature must be monitored and is relevant to the degree of separation required as well as to the boiling point of the analytes of the sample. For samples with a broad boiling range, it is more appropriate to use a temperature program in which temperature is increased either continuously or in steps as the separation proceeds. Mobile phase of gas chromatography is always gas. However, static phase can either be solid or liquid. This fact divides gas chromatography into two categories, GSC and GLC. In Gas-solid chromatography (GSC) stationary phase is solid, while the retention of analytes is caused by adsorption/desorption steps with the solid phase. On the other hand, in gas-liquid chromatography (GLC), stationary phase is liquid that is held on a finely-divided inert solid support. The retention of analytes is based on solute partitioning between mobile (gas) and liquid phase. In a GC is comprised of a carrier gas, mainly Helium, Nitrogen or hydrogen compressed in cylinders. A GC column is attached to the injection port and samples are introduced into the carrier gas stream at a suitable temperature in which vaporization of all components is accomplished. A syringe is forced through a rubber septum at the injection port in order to inject the sample. The exit of the column is connected with a detector. Detectors must be sensitive, and selective. There is a wide variety of detectors which can be potentially coupled with gas chromatography, such as Thermal Conductivity Detector (TCD), Flame Ionization Detector (FID) and Mass Spectrometry (MS). A gas chromatography system is

presented in Figure 7. Sample 's injection is a key step in GC. Techniques such as purge and trap, static headspace and SPME can be used in this way.

- Head space (HS): In this technique, a gas tight syringe collects the analytes which are in the supernatant phase in equilibrium with the liquid or solid sample. The vial is heated and stirred for a specific time and the components of the sample are transferred to the gas phase, from which they are sampled. This equilibrium is affected by various aspects, such as extraction time, temperature, sample fluidity, ionic forces in the sample, and the nature of the extraction fiber [12] . In order to shift the equilibrium toward the HS, inorganic salt could be added to increase the ionic strength in the medium. The most effective salts are KCl and NaCl. Pre-concentration can be achieved via HS and an increase sensitivity it's one of their most important advantages [32] .
- Solid Phase Micro Extraction (SPME): In SPME method, there is a syringe consisting of the main body and the fiber, in which a liquid static phase is attached. Fibre is exposed to the supernatant liquid space of the sample or it is submerged in the liquid body of the sample in order to absorb the analytes. Simultaneously, the sample can be heated or be stirred. After receiving the compounds, SPME syringe is inserted into the gas chromatograph and is exposed to a carrier gas. A thermal desorption which takes place in the next step leads to the separation of the compounds. SPME has a wide variety of advantages. It is simple, fast, low cost with an increased sensitivity and an ability to eliminate the use of solvents during sample preparation limits the loss of compounds during the evaporation step, problems that are typically found with solvent extraction methods [35] .

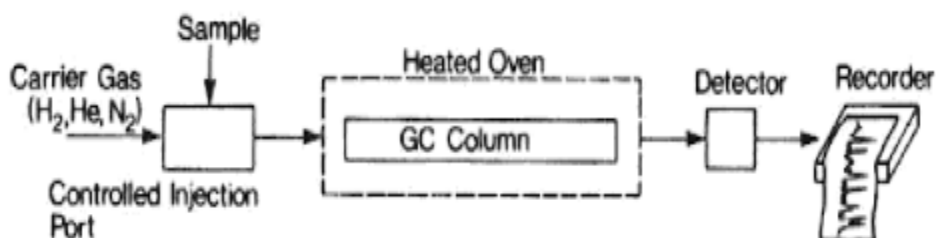


Figure 7: A gas Chromatography system

4.2 Mass Spectrometry (MS)

Mass spectrometry are some innovative chemical techniques which are based on ionization of atoms or molecules or on the production of ion fragments in gas phase and in the recording of the relative intensity of the ionic electricity, which corresponds to mass-to-charge (m/z) ratio. This means that a type of ionization to ionize the substances is necessary. There are two larger types of ionization sources, gas phase sources and desorption sources. On the one hand, in gas phase sources, sample is initially transferred to gas phase and then it is ionized. In this category of ion sources belong chemical ionization (CI) along with electron impact (EI). On the other hand, a liquid or solid sample is converted into gas ions in one step. This type includes electrospray ionization (ESI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI) among others. Ionization source are also characterized either as soft or hard. Hard sources transfer to the molecules of an analytes a great amount of energy which leads them to remain in an extensive stimulated state. A downward transition which follows results in more broken bonds and a production of more ionic fragments with a ratio of m/z less than those of the parent ion. At the other end of the spectrum, soft sources lead to a limited fragmentation [61].

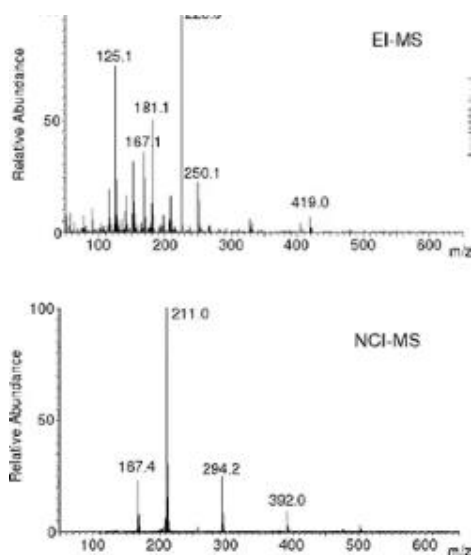
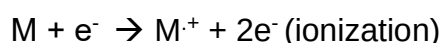


Figure 8: Fragmentation caused by EI and CI [62]

- EI [61]: In EI, electrons are emitted from a heated strand of Wolfram or Rhenium and they are accelerated by a voltage of 70eV. In this source, molecules are bombarded with a high-energy pack of electrons. Negative ions, positive ions, and neutral species are produced through this process. The positive ions are directed toward the analyzer by electrostatic repulsion. Electrical impact is a hard source which results in an extended fragmentation helping in creating libraries of mass spectrum which demonstrate the chemical imprint of an analyte



EE⁺: ions with even number of electrons and

OE⁺: ions with single number of electrons

4.3 Low-Resolution Mass Spectrometry (LRMS) and High-Resolution Mass Spectrometry (HRMS)

4.3.1 Low Resolution Mass Spectrometry (LRMS)

LRMS measurements provide information about nominal mass of the analyte i.e., the m/z for each ion is measured to single-digit mass units. The most common LRMS instruments are Quadrupole, Ion trap and Triple Quadrupole.

- Triple Quadrupole (QqQ)

A triple quadrupole is presented in Figure 9. There are three quadrupoles, from which the first and third are used as mass filters, while the second through interaction with a collision gas, such as argon or nitrogen, factions for fragmentation of the precursor ion. Multiple scan modes can be applied via QqQ, for example modes of precursor ion, product ion, neutral loss, single reaction monitoring (SRM), multiple reaction monitoring (MRM), and MS_n scans. MS/MS provides us with numerous advantages, like an increased selectivity, lower limits of quantitation (LOQ), improved S/N, an

improved accuracy and wider linear range. In more cutting-edge QqQ instruments, the basic linear quadrupole structure is modified with the curved quadrupoles, making it ideal for a higher resolution mass spectrometry and selections of more accurate m/z ratios. The unit mass resolution achieved typical quadrupole instruments corresponds to 0.7Da. Nevertheless, an ultra-selective resolution of 0.1Da can be achieved via advanced quadrupole instruments.

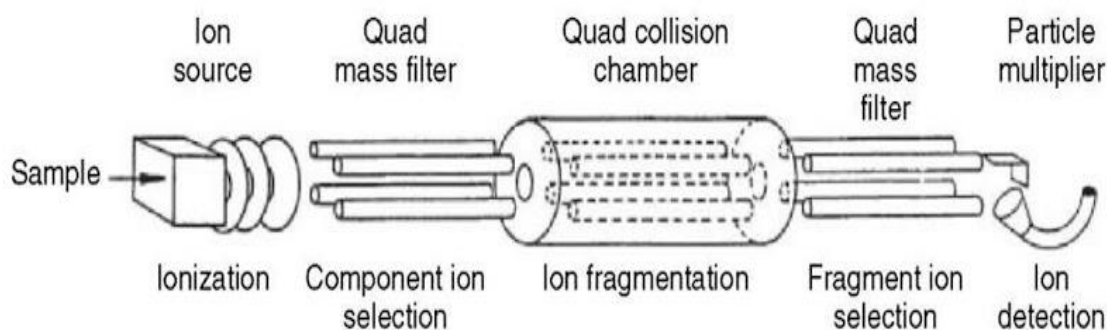


Figure 9: Linear QqQ instrument parts

4.3.2 High Resolution Mass Spectrometry (HRMS)

HRMS provides information about the exact mass of each analyte, the m/z of their ions is measured from four to six decimal points. This means that co-eluting molecules with the same nominal mass can be identified. The most widely used HRMS mass analyzers are Time of Flight (TOF) and Orbitrap.

4.4 Data analysis workflows

Having analyzed the samples, obtained data can be processed with three different approaches. These are target, suspect and non-target screening. A flow chart of them is attached in Figure 10 and more extensive details are mentioned in the following lines.

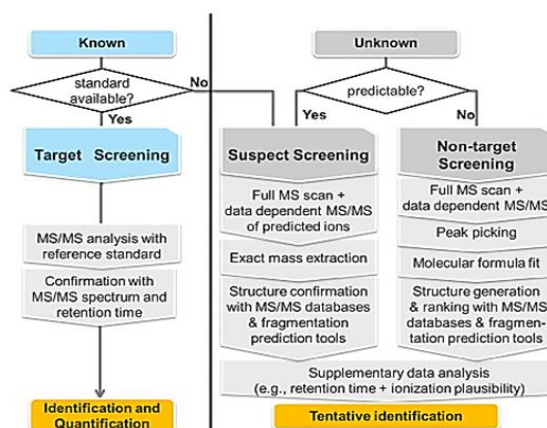


Figure 10: Flow chart of screening procedures. “Known” compounds have been confirmed or confidently identified before, other compounds are considered ‘Unknown’ [63]

4.4.1 Target screening

In target screening, reference standards are used in order to compare retention time, MS spectrum profile (precursor ion, adducts, in-source fragments) and MS/MS spectrum (fragment ions and ion ratios) with those respective parameters of analytes in each sample. In other words, by target screening known compounds can be detected and quantified. The triple quadrupole (QqQ) allows us to conduct several applications of MS/MS modes, such as production scan, precursor-ion scan, neutral-loss scan and selected reaction monitoring (SRM).

4.4.2 Suspect screening

In this approach, a list of suspect compounds, found in literature that are possible to be found in honey or olive oil is built. The screening is based on the exact m/z of the expected ions. The molecular formula allows the calculation of the exact m/z of the expected ion. A positive finding must be verified and several confirmatory steps have to be followed in order to reach structure-derived information[64]. The exact mass of each analyte is extracted from the chromatogram and checked by comparing it with control samples. An intensity-threshold value is applied to cut off unclear spectra. The plausibility of the chromatographic retention time, isotopic pattern, and ionization efficiency are used as further filters to narrow down the number of candidate peaks.

Furthermore, using MS/MS or MSⁿ, structures of suspected volatile compounds are suggested based on the observed fragmentation pattern and diagnostic fragment ions. Depending on the above criteria, there are different confidence levels of identification in HR-MS analysis

4.4.3 Non target screening

In non-target screening, no previous information is available. This infers that corresponding a non-target extracted m/z is a difficult task. There are five levels related to identification of an analyte and are proposed by a system, described in Figure 11

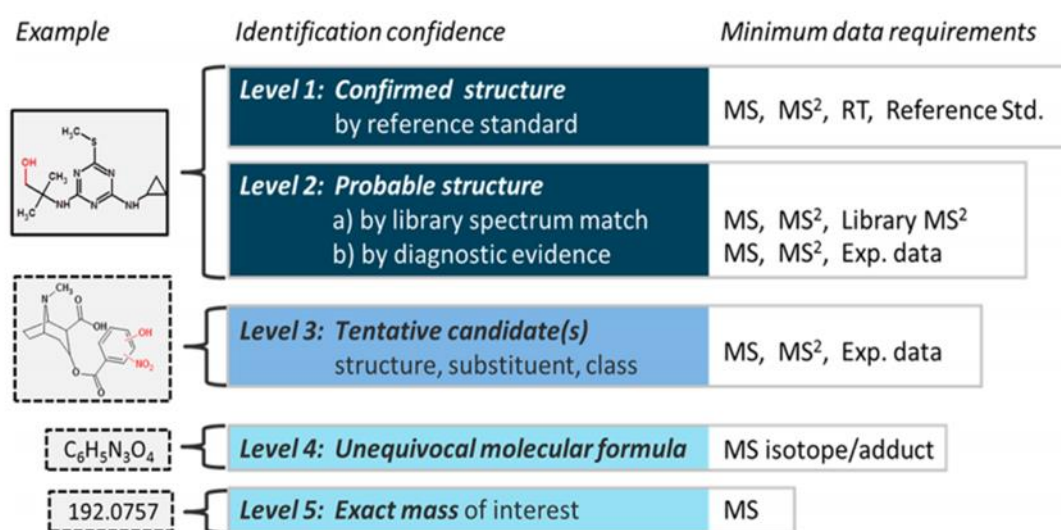


Figure 11: Proposed identification confidence levels in HR-MS analysis [65]

- Level 1: Confirmed structure is the perfect situation where the candidate structure is confirmed by the measurement of a reference standard with MS, MS/MS and retention time matching.
- Level 2: Probable structure refers to a proposal for an exact structure based on different evidence.
 - Level 2a: Library which includes indisputable matching between literature or library spectrum data and experimental.

- Level 2b: Diagnostic which refers in the case of no other structure fits in experimental data, but no standard or literature information is available.
- Level 3: Tentative candidate(s) is the situation where there is evidence for possible structure(s) but the experimental information is insufficient to the exact proposal.
- Level 4: Unequivocal molecular formula describes the case of an unambiguous formula which is assigned by the spectral information but there is no sufficient evidence to propose possible structures.
- Level 5: Exact mass (m/z) is detected in the sample but no experimental information exists in order to propose even a formula.

Chapter 5: Materials and methods

5.1 Honey Samples

A total of 36 Greek honey samples from 12 different botanical origins produced in various geographical regions of Greece during the harvesting period of 2019 were collected directly from Greek producers. More specifically, the honey samples collected were:

Botanical Origin	Geographical Origin		
Pine (N=3)	Nothorn Evoia	Crete	Thasos
Thyme (N=3)	Crete	Lefkas	Attica
Fir (N=3)	Argolis	Korinthus	Aitolia and Akarnania
Oak (N=3)	Elis	Grevena	Evros
Heather (N=3)	Andros	Arta	Antiparos
Chestnut (N=3)	Olympus	Kozani	Agion Oros
Orange (N=3)	Argolis	Argolis	Arta
Arbutus (N=3)	Arta	Nothorn Evoia	Aitolia and Skarnania
Sage (N=3)	Kozani	Kefalonia	Athos
Cotton (N=3)	Larissa	Kilkis	Xanthi
Carob (N=3)	Crete	Crete	Samos
Vanilla (N=3)	Vitina	Vitina	Vitina

All honey samples were stored in glass containers and maintained at 4 ± 1 °C before analysis



Figure 12: An Arbutus honey sample which was analyzed

5.2 Chemicals and Reagents

All reagents which were used in this study were of analytical grade and used as received. More specifically, a mix of carboxylic acids, including Butyric acid, Valeric acid, Hexanoic acid, Heptanoic acid, Octanoic acid, Nonanoic acid, Decanoic acid, Undecanoic acid, Dodecanoic acid, Tridecanoic acid, Tetradecanoic acid and Pentadecanoic acid, was purchased from CPA Chem. The same Vendor provided us with a mix of Alkanes (Pentane, Hexane, Heptane, Octane, Nonane, Decane, Undecane, Dodecane, Tetradecane, Pentadecane and Hexadecane) and other chemical substances, Isophorone, 1-Hexanol, 1-Octanol, 1-Nonanol, Ethyl Octanoate, Ethyl Nonanoate, β -Damascenone and Ethyl Ether. In addition, Undecanol, Ethyl Hexanoate, and a series of Aldehydes (Butanal, Pentanal, Hexanal, Heptanal, Octanal, Nonanal, Decanal, Benzaldehyde) were purchased from Sigma Aldrich.

5.3 Database construction

In order to construct our database, we applied single Ion Monitoring (SIM) and Multiple Reaction Monitoring (MRM) scan modes to identify the analytes. At least two MRMs or three SIMs were set as a requirement to claim the determination of the analyte. The retention time, the precursor ions, and ion ratios have to be given in the SIM scan mode. The necessary information in the MRM scan mode is the retention time, precursor and product ions, and the optimized collision energy. Each mix or single analyte was injected in different concentrations during the first step, and a Full scan mode was applied. Their molecular ions (M^+) were used to create the extracted ion chromatogram of each mass. Mass spectra are generated from each EIC, and with this procedure, the retention time and the three most abundant ions from each mass

spectra were recorded. Product scan mode was adopted during the following step, in which the three most abundant ions for each analyte were used as precursor ions (Q1), and three different collision energies were applied (Q2) in order to record all product ions. The most abundant transition is used as a quantifier, while the second and/or the third transitions are used as qualifiers.

5.4 Optimization of methods

In static Headspace analysis, the volatile molecules from the gas phase are injected into the gas-chromatography system. Similarly, during the Headspace Solid-Phase microextraction, the absorbed in the fiber analytes are desorbed into the injector. This means that the migration of compounds from the liquid phase to the gas phase is critical in those techniques. There is a thermodynamic energy associated with its presence in the headspace phase and in the liquid phase for every chemical substance. Partition coefficient (K) represents the equilibration between the two phases and its value is connected with the concentration of the compound in both the headspace and liquid phase. This ratio is presented below in equation 1

$$K=C_s/C_g \text{ (Equation 1)}$$

C_s : The concentration of a specific compound in the sample (liquid) phase

C_g : The concentration of a specific compound in the sample (gas) phase

Many factors influence partition coefficient, making the optimization of those headspace methods necessary to determine volatile compounds in honey samples. Therefore, several parameters were tested and optimized to lead to a final method. These were the weighted mass of a honey sample, the volume of water (Honey-Water ratio), the mass of NaCl added into the vial, and incubation time and incubation temperature. These parameters are crucial for a wide variety of reasons which are explained below. A pine sample was chosen randomly for the optimization experiments.

5.4.1 Optimization of static headspace

All optimization experiments were performed in 20mL vials. The experimental work was performed in five steps which are presented in the following pages. A brief presentation of the parameters which were optimized is found in Table 16.

Table 16: The under -optimization parameters of static HS

Parameter	Static HS		
Phase Ratio	12:8	9:11	-
Honey (g): Water (mL) ratio	4:8	6:6	8:4
Quantity of NaCl (g)	0.25	0.50	-
Incubation Time (min)	15	20	30
Incubation Temperature (°C)	45	60	-

5.4.1.1 Honey-Water ratio

Concerning this parameter, three different levels were tested in triplicates.

- Honey-water: 1:2 (4g of honey, dissolved with 8mL distilled water)
- Honey-water: 1:1 (6g of honey dissolved with 6mL distilled water)
- Honey-water: 2:1 (8g of honey dissolved with 4mL distilled water).

These quantities of honey and water were added in a 20mL vial, while the remaining parameters remained constant (0.25g NaCl were added into the vial and incubation was performed at 60° C for 15 minutes)

5.4.1.2 Addition of NaCl

Generally speaking, the equilibration between the liquid and gas phase is affected by adding inorganic salts. This is because their presence changes the nature of the molecular interactions and increases the ionic activity, leading to a higher concentration of volatile compounds in the gas phase. During our experiments, Sodium Chloride (NaCl) was used for this purpose in two different quantities, 0.25 and 0.50 g. So, as a second step, the optimum amount of NaCl was investigated.

Based on the first step results, 8g of honey and 4mL distilled water were placed in a 20mL vial. The selected quantity of NaCl was then added and each sample into the vial was vortexed for 1 minute to be well-homogenized. Next, a mix of 100 mg/L of analytes, including every chemical compound of our constructed database, was prepared. From this mix, a spiked injection was performed into the sample. The injected spiked volume was selected, keeping in mind that the final concentration of each analyte should have been 100 µg/L. For the extraction of analytes, incubation at 60° C for 15 minutes was applied.



Figure 13: Sodium Chloride (NaCl)

5.4.1.3 Phase Ratio (β)

The ratio of the volume of the two phases is called phase ratio and is expressed with equation 2

$$\beta = V_G/V_S \text{ (Equation 2)}$$

V_G : the volume of the gas (headspace) phase

V_S : the volume of the liquid phase

Equation 3 demonstrates the link between C_G and β .

$$C_G = C_O / (K + \beta) \text{ (Equation 3)}$$

A conclusion of this equation is the fact that an increase in the concentration of analytes can be achieved with a decrease of β . In other words, an increase in sample volume will boost the concentration of analytes in the gas phase.

As a third step in this optimization procedure, two different values of β were examined

$\beta_1=1.5$, derived from a dilution of 8g honey in 4 mL of water

$\beta_2=0.82$, derived from a dilution of 6g honey in 3 mL of water

More specifically, based on the results of the previous steps, in a 20mL vial, 8g of honey and 4mL of distilled water or 6g of honey with 3mL of water were added. In addition, 0.25 g NaCl was also placed in the vial. The vial was vortexed for 1 minute to be well-homogenized. From the mix of all analytes, a spiked injection was performed into the sample. The injected spiked volume was selected, keeping in mind that the final concentration of each analyte should have been 100 $\mu\text{g/L}$, similar to the previous step. For the extraction of analytes, incubation at 60° C for 15 minutes was applied.

5.4.1.4 Incubation Temperature

Extraction temperature is another crucial parameter [51] . It is associated with partition coefficient, and by increasing extraction temperature, the concentration of analytes in the headspace phase is also increased. Furthermore, high temperatures increase the transfer of semi-volatile compounds into the gas phase, while the opposite is noticed for compounds with high volatility whose retention will be increased by using a lower incubation temperature. If a column or detector is particularly susceptible to water, then caution must be exercised before increasing the temperature significantly.

Following the conclusions from the previous three tests, 8g of honey, 4mL of distilled water and 0.25g NaCl were placed in a 20mL vial. The vial was vortexed for 1 minute to be well-homogenized. From the mix of all analytes, a spiked injection was performed into the sample. The injected spiked volume was selected, keeping in mind that the final concentration of each analyte should have been 100 $\mu\text{g/L}$, similar to the previous

steps. A fifteen-minute incubation time was applied for the extraction of analytes, while two different incubation temperatures, 45° C and 60° C, were evaluated.

5.4.1.5 Incubation Time

Incubation time is another critical aspect that should be evaluated during a Headspace analysis as all analytes have to reach an appropriate equilibration between the sample and the gas phase before being injected into the system [51]. An increase in the extraction time facilitates and boosts this process. Nevertheless, it should be pointed out that too long extraction times can lead to undesirable results, such as sample deterioration due to physical, chemical or enzymatic reactions. Generally, short incubation times are more favorable for volatile analytes than for semi volatiles.

Using the results produced from the previous optimization steps, 8g of honey, 4mL of distilled water and 0.25g NaCl were placed in a 20mL vial. The vial was vortexed for 1 minute to be well-homogenized. From the mix of all analytes, a spiked injection was performed into the sample. The injected spiked volume was selected, keeping in mind that the final concentration of each analyte should have been 200 µg/L, similar to the previous steps. For the extraction of analytes, the incubation temperature was at 60° C, while three different incubation times, 15, 20 and 30 minutes, were evaluated.

5.4.2 HS-SPME Optimization

Similarly, with the previous static Headspace method, the optimization of this SPME method was also critical. A brief presentation of the parameters which were optimized is found in Table 17.

Table 17: The optimization parameters of HS-SPME

Parameter	Static HS		
Phase ratio	12:8	9:11	6:14
Incubation Time (min)	15	20	30
Incubation Temperature (°C)	45	60	-
Sampling time (min)	10	20	30

5.4.2.1 Parameters of pre-treatment

Similar to the previous static Headspace method, the optimization of this SPME method was also critical. The honey-water ratio and NaCl concentration values were kept the same as with the previous optimized static headspace method.

First of all, the parameter of phase ratio was tested. Since HS-SPME seemed to be more sensitive than static Headspace, less honey sample was weighted than the optimized phase ratio of static headspace (8g honey with 4 mL water). Thus, 6g honey with 3 mL water and 4g honey with 2 mL water were tested for peak areas and %RSD. The experiments were conducted in triplicates.

5.4.2.2 Parameters of extraction

Incubation time and temperature are important aspects of every SPME method, and their importance has been described in paragraphs 5.4.1.4 and 5.4.1.5. Sampling time is another factor that is crucial to be optimized. It determines the duration of the interaction between the fiber and the analytes which have reached the gas phase. Since the compounds of honey present a broad range of polarity, the ideal sampling time in order to reach the equilibration needs to be investigated.

Having optimized the pretreatment parameters, in 20mL vials, 4g of honey along with 2mL water containing 6,25% v/v NaCl were added. Vials were vortexed for 1 min and from a prepared mix in which all analytes of the target database were included, a spiked injection was performed into the sample. The injected spiked volume was selected, keeping in mind that the final concentration of each analyte should have been 30 µg/L. We selected 30 µg/L rather than 100 µg/L, which was the concentration

of the analytes during the optimization of static headspace due to the high analyte signals observed during preliminary tests. Some analytes had enormous peak areas and intensities when their concentration was 100 µg/L, so to limit the risk of destroying the detector due to too high signals, we adopted the value of 30 µg/L, which proved to provide us satisfactory results. The effect of 15min, 20min and 30 min incubation time, 45°C and 60 °C incubation temperature, along with the impact of 10min, 20min and 30 min sampling time, were evaluated. Every combination, 18 in total, of these parameters were tested in triplicates.

The using fiber was a 2cm long DVB/CAR/PDMS. It was selected due to the triple solid phase, which can absorb analytes of different polarities. Before the beginning of the experiments, it was preconditioned at 240° C for 1h, following the manufacturer's instructions.



Figure 14: HS-SPME-GC/MS instrumentation

5.5 Selection of the Internal Standards

The proper internal standard for each analyte was selected among 4 compounds, Chlorobenzene, Benzophenone, 2-Pentanol and 4methyl-2-pentanone in terms of r^2 of standard addition curve, RSDr %, Recovery %, LOD's and LOQ's.

5.6 Validation of methods

Our optimized analytical methods were validated to confirm that they are suitable for the intended application. The analytical validation focused on the aspects of linearity by constructing the calibration curves for every included compound in our database, the working ranges, the limits of quantification (LOQ) and quantification (LOD), as well as accuracy, which is comprised of trueness and precision, sensitivity and selectivity.

5.6.1 Linearity

Linearity is the analytical procedure to obtain test results directly proportional to the analyte concentration in the sample. Linearity was evaluated by the determination of the least square regression coefficients (R^2) of the calibration curves, which consisted of 10 different concentration levels (2 µg/L, 5 µg/L, 10 µg/L, 25 µg/L, 50 µg/L, 100 µg/L, 200 µg/L, 500 µg/L, 1 mg/L and 2 g/L) for each of the target analytes. More specifically, the honey matrix was spiked with each analyte in these 10 different levels. Each calibration curve was created from the peak area ratio of the selected analyte/peak area ratio of the internal standard, if used, versus the corresponding concentration.

5.6.2 Precision

The repeatability of our methods was determined by analyzing three replicates in 3 different levels (5 µg/mL, 50 µg/mL, 200 µg/mL) of standard solutions under the same conditions on the same day (intraday precision) and over a period of three days (inter-day precision). The precision was expressed as the relative standard deviation (%RSD).

5.6.3 Limits of Detection (LOD) and Quantification (LOQ)

The LOD defines the lowest concentration of a chemical component which can be reliably detected. Based on the LOD, an analyte is not necessarily quantifiable but is distinguishable from zero (signal /noise ratio ≥ 3). On the other hand, LOQ is the lowest concentration that can be determined, achieving an acceptable level of precision and accuracy (signal /noise ratio ≥ 10). Equations 4 and 5 demonstrate the calculation type of LOD and LOQ.

$$\text{LOD} = (\text{SD}_{\text{intercept}} / \text{slope}) \times 3.3 \text{ (Equation 4)}$$

$$\text{LOQ} = (\text{SD}_{\text{intercept}} / \text{slope}) \times 10 \text{ (Equation 5)}$$

These two limits evaluate methods' sensitivity.

5.6.4 Working ranges

It determines the range over which the method can be applied. The values of LOD and LOQ restrict the lower end of the working range, while the upper end can face limitations due to various reasons which can abrogate the linearity. For example, when the concentration of an analyte is high, a curved line may be noticed.

5.6.5 Trueness

It is determined by the term of recovery. In this study, it is expressed by the ratio of the calculated from the calibration curves concentration versus the reference concentration, which is presented in Equation 6.

$$R = (\text{C}_m \times 100) / \text{C}_{\text{ref}} \text{ (Equation 6)}$$

5.7 Final optimized and validated Methods

5.7.1 Isolation of volatile compounds, using HS-SPME-GC-MS

For the extraction of volatile compounds, a 50/30 μm thick divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco) was used. The fiber was preconditioned and thermally cleaned before use, following the manufacturer's recommendations. This was achieved by exposing the fiber in the injection port for 1h at 240°C.

4g of honey and 2mL distilled water and 0.25g NaCl were introduced into a 20mL screw-cap vial. Next, 10 μL of IS (mix of Banzophenone, Chlorobenzene, 4methyl-2-Pentanone and 2-Pentanol 100 $\mu\text{g/mL}$) was then spiked into the vial. Afterward, the sample was vortexed for 1.5 minutes to be well-homogenized and then was incubated for 15min at 60°C under continuous stirring. After that, the fiber was introduced into the vial and exposed for 10 min to absorb the volatile compounds. After sampling time, the fiber was transferred to the injector port and desorbed for 8 min.

5.7.2 Isolation of volatile compounds, using static HS-GC-MS

8g of honey along with 4mL distilled water and 0.25g NaCl were introduced into a 20mL screw-cap vial. 10 μ L of IS (mix of Banzophenone, Chlorobenzene, 4methyl-2-Pentanone and 2-Pentanol 100 μ g/mL) was then spiked into the vial. Afterward, the sample was vortexed for 1.5 minutes to be well-homogenized and then was incubated for 15min at 60°C under continuous stirring. Then 1000 μ L of the Headspace was injected into the chromatographic system.

5.7.3 Chromatographic analysis by GC-MS

The chromatographic analysis was the same for both static HS and HS-SPME methods. A SCION 456-EVOQ TQ system (Bruker Daltonics) was used. The injection was made in spitless mode. A Varian CP-Select 624 column (30m $\times$ 0.32mm i.d $\times$ 1.80 μ m film thickness) was used to separate the analytes. The temperature of the oven was initially maintained at 35°C for 2 min, then was increased to 140°C at a rate of 10°C/min, in which it was held for 5 min and as a last step, it was increased to 240°C at 10°C/min, in which it was held for another 5 min, meaning that the total time of the chromatographic separation was 32.5minutes. Helium was used as the carrier gas (purity 99.999%) at a 1.5 mL/min flow rate. The mass spectra were recorded at 35-500 mass range, while the spectrum was obtained using 70eV ionization energy. The injector temperature was maintained at 240°C during the analysis, while the transferline temperature was 250 °C. The ionization source was held at 240°C.

Chapter 6: Results and discussion

6.1. Database Construction

Having followed the workflow described in Unit 5.3, the final database was developed, including chemical compounds belonging to different classes, such as alkanes, organic acids, esters, aldehydes, ketones, alcohols, and an ether. This database has been constructed after the conduction of both static headspace and Headspace SPME experiments, using the same separation column, CP-Select 624. These experiments included SIM and MRM trials. The majority of analytes are detected using SIM as a scan type. Nevertheless, for some analytes with higher molecular weight, MRM mode is preferable. The final database is presented in Tables 18,19 and 20. In Figure 15, an example of the identification of Decanal, an analyte that is included in the database, is shown.

Table 18: The final developed database

Compound	Retention time (RT)	Scan type	Quantifier ion	Qualifier ion (I)	Qualifier ion (II)
Ethyl ether	3.02	SIM	59	74	45
Pentane	3.23	SIM	43	59	72
Acetone	3.37	SIM	43	58	42
Hexane	4.41	SIM	41	43	57
Butanal	5.08	SIM	44	72	57
Heptane	6.47	SIM	43	71	57
Pentanal	7.29	SIM	44	55	58
3- Pentanone	7.34	SIM	57	86	56
2-Pentanol	7.50	SIM	45	55	73
2-Pentanone,4methyl	8.27	SIM	43	58	100
Octane	8.58	SIM	43	57	85

Table 19: The final developed database (Continued)

Compound	Retention time (RT)	Scan type	Quantifier ion	Qualifier ion (I)	Qualifier ion (II)
Hexanal	9.46	SIM	56	44	72
Chlorobenzene	10.28	MRM	112→77(10eV)	112→51(20eV)	112→62(30eV)
Nonane	10.58	SIM	43	57	85
Hexanol	10.96	SIM	56	43	69
Heptanal	11.46	SIM	70	44	55
Pentanoic acid	11.76	SIM	60	73	39
Decane	12.44	SIM	43	57	71
Ethyl Hexanoate	12.93	SIM	88	93	43
Benzaldehyde	12.99	SIM	77	106	55
Octanal	13.39	SIM	43	57	84
Hexanoic acid	13.54	SIM	60	73	41
Undecane	14.54	SIM	43	57	71
1-Octanol	15.09	SIM	56	69	83
Benzyl Alcohol	15.17	MRM	79→77(10eV)	100→77(10eV)	100→79(10eV)
Nonanal	15.98	SIM	67	98	70
Heptanoic acid	16.02	SIM	60	73	41
Dodecane	17.65	SIM	57	71	85
Isophorone	18.14	SIM	82	54	138

Table 20: The final developed database (Continued)

Compound	Retention time (RT)	Scan type	Quantifier ion	Qualifier ion (I)	Qualifier ion (II)
Nonanol	18.40	SIM	56	70	98
Ethyl Octanoate	18.51	SIM	88	101	127
Octanoic acid	19.28	SIM	60	73	101
Decanal	19.36	SIM	43	57	70
Ethyl Nonanoate	21.13	SIM	88	101	141
Nonanoic acid	21.67	SIM	60	55	73
Tetradecane	22.66	MRM	85→43(10eV)	85→41(10eV)	198→57(10eV)
Undecanol	23.11	SIM	55	69	97
Decanoic acid	23.54	SIM	60	73	43
β-Damascenone	23.73	MRM	121→105(10eV)	121→77(20eV)	105→77(10eV)
Pentadecane	24.40	MRM	85→43(10eV)	85→41(10eV)	99→41(10eV)
Hexadecane	25.88	MRM	85→43(10eV)	85→41(10eV)	226→57(10eV)
Dodecanoic acid	26.53	SIM	73	60	129
Heptadecane	27.21	MRM	85→43(10eV)	85→41(10eV)	99→43(10eV)
Tridecanoic acid	27.77	SIM	73	60	43
Benzophenone	27.84	MRM	105→77(10eV)	77→51(10eV)	182→105(10eV)
Octadecane	28.51	MRM	85→43(10eV)	85→41(10eV)	254→57(10eV)
Tetradecanoic acid	29.20	SIM	73	60	43
Pentadecanoic acid	30.95	SIM	73	60	43

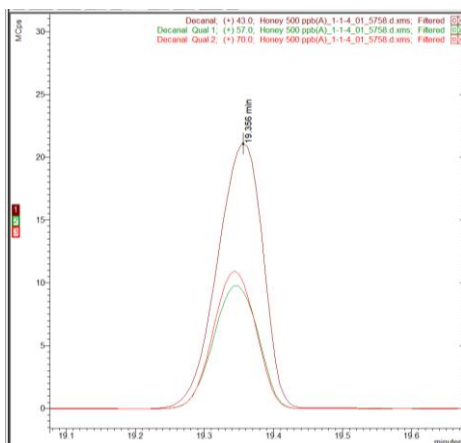


Figure 15: Identification of Decanal based on the developed database

6.2. Optimization of methods

6.2.1. Headspace optimization

6.2.1.1 *Honey-water ratio*

Evaluating the results, we concluded that the best honey-water ratio is 2:1 (8g honey with 4mL water). A suspect list containing volatile compounds found in pine samples during other scientific research was created for the evaluation. After the suspect list was screened in the samples, we detected a wider portion of analytes, simultaneously achieving higher sensitivities and lower %RSD in the 2:1 ratio. In Table 21, 10 analytes that were identified during the optimization of the honey-water ratio are presented.

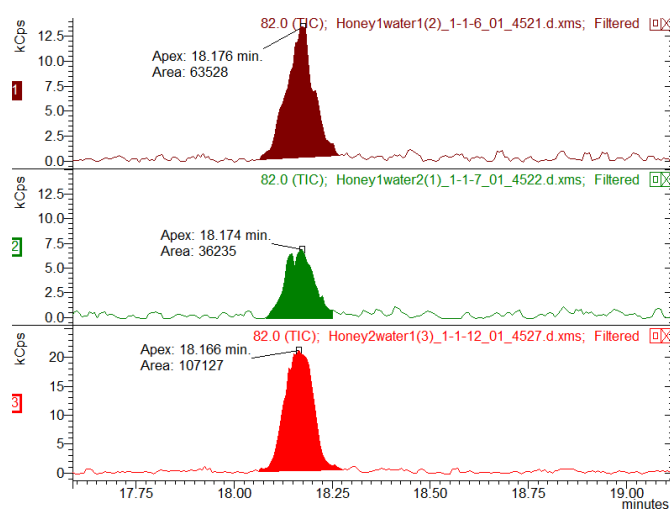


Figure 16: Sensitivity of Isophorone in different honey-water ratios [63]

Table 21: The results from the optimization of honey-water ratio through 10 analytes

Analyte	RT	Average area honey- water 1:2	%RSD honey- water 1:2	Average area honey- water 1:1	%RSD honey- water 1:1	Average area honey- water 2:1	%RSD honey- water 2:1
Hexane	4.41	1935422	6.9	3125116	4.0	4943358	4.0
Dimethyl Disulfide	8.18	339556	4.5	672591	5.5	946511	5.8
Octane	8.58	1268272	4.3	1672012	9.2	2680305	3.3
Hexanal	9.47	244020	15	472524	8.1	785512	1.9
Nonane	10.58	1167815	4.9	1456782	10	2437561	3.3
Furfural	10.68	205227	9.8	680584	16	1512333	6.0
Benzaldehy de	12.99	158709	11	326529	5.3	817649	6.3
Benzeneac etaldehyde	15.09	ND	-	232367	4.9	366041	4.3
Nonanal	15.98	694934	5.6	1050237	4.0	1317056	2.7
Isophorone	18.14	36235	7.8	63528	6.8	176893	6.2

6.2.1.2 Addition of NaCl

Having compared analytes' response under the addition of 0.25g NaCl and 0.50g NaCl we concluded that these two quantities of salt give similar results. More specifically, Alkanes show a bit higher peak area and lower %RSD with the addition of 0.50g NaCl. Aldehydes present similar peak areas in both cases, but when 0.25 g of salt are used,

their %RSD decreases. Alcohols and Ketones also have similar responses in both two levels of NaCl. Regarding these observations, we concluded that 0.25g was the most suitable quantity. If 0.50 g of NaCl had been selected without improving the response of analytes, it would have been a waste of reagent.

Moreover, in the case of analytes that appear in honey in minor concentrations, a lack of NaCl could pinpoint their existence. This scenario made us decide to use 0.25 g NaCl instead of omitting it.

In Tables 22 and 23, the results from the optimization of NaCl are presented.

Table 22: The results of the optimization of NaCl

	0.25g NaCl		0.50 g NaCl	
Analyte	AVERAGE	%RSD	AVERAGE	%RSD
Dodecane	2397861	12	3061805	0.62
Pentadecane	662337	22	883830	17
Benzophenone	90481	22	78971	7.0
Hexadecane	935691	25	1205394	17
Octanal	51789	8.9	59284	24
Diethyl ether	961227	5.2	1063894	6.9
1-Octanol	36340	22	42155	14
Heptadecane	699480	28	873004	20
Ethyl Octanoate	9906	65	58811	78
Decanal	81650	25	97248	34
Butyraldehyde	115246	8.6	132792	24
β-Damascenone	46838	11	45138	6.8

Table 23: The results of the optimization of NaCl

	0.25g NaCl		0.50 g NaCl	
Analyte	AVERAGE	%RSD	AVERAGE	%RSD
Pentanone-3-	71474	6.6	72778	12
Undecane	4633112	14	5625104	3.0
4Methyl-2-Pentanone	843984	9.4	835769	5.1
Chlorobenzene	7663831	9.3	8358454	4.5
Heptanal	70590	16	94334	26
Octane	4664250	11	5075426	2.4
Nonane	3706834	14	4208813	4.1
Hexane	24833015	24	26142405	0.59
Tetradecane	684204	14	850917	9.9
Hexanol	70866	11	69345	10
Heptane	3746155	15	4305742	2.9
Octadecane	338970	28	433519	23
Hexanal	93606	10	126541	34
Decane	4786925	16	5537176	2.7
Isophorone	15269	10	13118	10
Ethyl Nonanoate	8924	92	25992	87
Nonanol	42389	22	44733	10
2-pentanol	101130	7.5	99280	9.7

6.2.1.3 Phase Ratio

The third parameter which was optimized was phase ratio. It can be clearly understood that a higher phase ratio increases peak areas for the majority of analytes. Furthermore, %RSD are also lower when $\beta=1.5$. Considering this, the value of 1.5 was selected. The experimental results about phase ratio are appended in Tables 24 and 25.

Table 24: The results of the optimization of phase ratio

	$\beta=1.5$		$\beta=0.82$	
Analyte	AVERAGE	%RSD	AVERAGE	%RSD
Dodecane	6632005	3.6	2429587	10
Pentadecane	1870535	9.0	425618	11
Benzophenone	171406	14	59710	17
Hexadecane	1384347	9.5	483065	17
Octanal	371919	10	124375	9.2
Diethyl ether	3000882	0.27	1549197	3.2
1-Octanol	117176	7.5	40383	13
Heptadecane	633845	6.2	313758	26
Ethyl Octanoate	845381	26	43946	39
Decanal	590044	6.4	130535	10
Butyraldehyde	572615	8.0	211216	6.6
β -Damascenone	108402	5.3	39751	6.5
Pentanone-3-	253725	12	120605	12
Undecane	12382314	3.0	5156177	7.8

Table 25: The results of the optimization of phase ratio (Continued)

	$\beta=1.5$		$\beta=0.82$	
Analyte	AVERAGE	%RSD	AVERAGE	%RSD
4Methyl-2-Pentanone	3006240	4.3	1292540	14
Chlorobenzene	18866208	0.28	8091049	6.3
Heptanal	664030	13	194335	8.0
Octane	8549175	0.31	4536035	3.9
Nonane	9155713	2.4	4234719	4.3
Hexane	38570854	4.2	18542412	2.6
Tetradecane	1896734	6.7	537011	12
Hexanol	211262	3.5	105103	10
Heptane	8409376	3.2	4938381	6.0
Octadecane	263723	4.4	138749	29
Hexanal	858535	12	310864	5.7
Decane	12975752	1.2	5503102	4.8
Isophorone	41168	11	30636	11
Ethyl Nonanoate	524761	33	20026	43
Nonanol	96984	2.7	37661	8.4
2-pentanol	386026	10	178864	8.2

6.2.1.4 Incubation Temperature

Comparing the results of the two temperatures, at 60°C, analytes show a higher peak area. Most of them also show a lower %RSD, which verifies that a sixty-degree incubation temperature is the optimum for this headspace method. The results about incubation Temperature are mentioned in Tables 26 and 27

Table 26: The results of the optimization of incubation temperature

	T=60°C		T=45°C	
Analyte	AVERAGE	%RSD	AVERAGE	%RSD
Dodecane	6444114	3.7	4064757	10
pentadecane	2431628	10	1473829	16
Benzophenone	119329	4.8	142632	4.5
Hexadecane	1744012	6.3	1084416	21
Octanal	368976	3.7	174248	11
diethylether	2108010	8.0	2149066	8.7
1-Octanol	107223	4.5	63074	10
Heptadecane	839898	4.2	489202	18
Ethyl Octanoate	604758	12	541096	12
Decanal	620170	3.4	231304	10
Butyraldehyde	408387	5.6	378808	6.8
b-Damascenone	103052	5.6	60117	6.9
Pentanone-3-	162977	4.3	169657	5.6
Undecane	12085571	6.2	8866133	10

Table 27: The results of the optimization of incubation temperature

	T=60°C		T=45°C	
Analyte	AVERAGE	%RSD	AVERAGE	%RSD
4Methyl-2-Pentanone	2371948	8.7	2169441	2.4
Chlorobenzene	17130055	8.3	15146960	6.9
Heptanal	658368	5.8	375295	23
Octane	8241965	8.4	7059628	4.3
Nonane	8488195	4.2	7496147	5.1
hexane	31282922	4.7	32300605	4.5
Tetradecane	2385541	9.2	1127137	24
Hexanol	187274	6.4	125059	3.2
pentane	864972251	9.4	887421269	16
Heptane	6694634	7.0	7018647	1.5
Octadecane	338962	5.2	147754	21
Hexanal	670261	4.6	484978	18
Decane	11777232	4.9	9558367	9.8
Isophorone	51161	5.0	20479	2.3
Ethyl Nonanoate	371003	16	293507	37
Nonanol	84384	8.7	61628	0.90
2-pentanol	256929	13	231973	1.7

6.2.1.5 Incubation time

Increasing incubation time does not provide us with a significant improvement of either peak area or %RSD of most analytes. For this reason, as analysis time is an important aspect, we've selected an incubation time of 15 minutes, enabling us to analyze more samples during the same period. In Tables 28 and 29, the results of the optimization of incubation time are presented.

Table 28: The results of the optimization of incubation time

	Incubation time 15min		Incubation time 20min		Incubation time 30 min	
Analyte	MEAN	%RSD	MEAN	%RSD	MEAN	%RSD
Dodecane	36752350	2.6	40369861	2.4	42692447	3.3
Pentadecane	23830927	5.0	19998077	16	19511153	13
Benzophenone	3291324	14	3445907	5.3	2974458	9.3
Hexadecane	13504846	4.3	12712689	13	13423130	16
Octanal	2911435	2.1	4033332	2.4	4440012	10
Diethyl ether	15124627	6.1	21994139	2.2	22993780	5.3
1-Octanol	2619217	4.3	3248434	17	2720869	27
Heptadecane	6461036	4.4	5795492	9.1	6616805	16
Ethyl Octanoate	20823983	2.2	12785257	18	4445686	29
Decanal	6976824	3.4	7395375	4.7	6063923	19
Butyraldehyde	3091067	7.4	4357178	10	4239079	10
β-Damascenone	9341122	6.38	5313597	18.4	4340093	27.2

Table 29: The results of the optimization of incubation time

	Incubation time 15min		Incubation time 20min		Incubation time 30 min	
Analyte	MEAN	%RSD	MEAN	%RSD	MEAN	%RSD
Pentanone-3-	3141686	3.4	4778704	12	4775611	15
Undecane	68942226	4.2	82535520	3.0	93550560	3.9
4Methyl-2-Pentanone	25466180	5.4	37081902	12	38023210	21
Chlorobenzene	171293283	2.7	229268812	5.2	239729867	9.9
Heptanal	4210726	2.7	4880100	8.8	3871675	6.9
Octane	48831691	4.9	67596085	4.4	79892288	3.8
Nonane	51847020	5.0	68942754	4.8	81974240	2.8
Hexane	160548580	2.4	207352789	7.4	251929696	6.0
Tetradecane	31432535	7.3	21032249	16	19425799	9.9
Hexanol	2782402	6.5	4157712	18	4098977	24
Heptane	52881973	6.6	73922429	5.9	91897792	4.2
Octadecane	3297137	7.3	2386120	5.8	2780278	12
Hexanal	4847703	2.1	6027156	6.6	5156524	2.6
Decane	67639321	5.6	87573866	4.4	100977960	2.2
Isophorone	484913	15	708933	22	685174	29
Ethyl Nonanoate	30019038	2.0	10243732	24	2493034	29
Nonanol	4612599	4.9	3408905	14	2918333	23
2-pentanol	3321202	4.6	5106368	16	5083405	23

6.2.2. HS-SPME optimization

As referred to in unit 5.4.2, the values of the honey-water ratio and NaCl concentration were kept the same as those of the optimized static Headspace Method. The optimization results of phase ratio, incubation time, incubation temperature, and sampling time are presented in units 6.2.2.1 and 6.2.2.2 below.

6.2.2.1 Phase ratio

Evaluating the results, the phase ratio at the value of 0.43 which derives from the addition of 4g honey and 2mL water, was selected as the best. Having made a suspect list with volatile compounds identified in pine samples during other scientific research, we compared the average peak areas along with the %RSD of some identified analytes. Although the average peak area of all analytes was increased when the value of phase ratio was higher, the phase ratio at the value of 0.43 also provided high peak areas, as shown in Table 30. Furthermore, the %RSD of this value was also satisfactory. So, due to the great response at this value and in order to avoid wasting sample and reagent, reaching a plateau in case of extremely high peak areas, saturating the fiber and simultaneously protecting the detector from potential destruction, we selected this value for our final optimized method.

Table 30: The results from the optimization of phase ratio through 10 analytes

Analyte	RT	Average area $\beta=0.43$	%RSD $\beta=0.43$	Average area $\beta=0.81$	%RSD $\beta=0.81$	Average area $\beta=1.5$	%RSD $\beta=1.5$
Dimethyl Disulfide	8.18	4019069	3.9	5092896	6.5	7513678	4.9
Octane	8.58	133706968	4.1	1970203672	7.3	233569812	3.9
Nonane	10.58	33726571	3.3	52218885	6.8	67889004	2.2
Furfural	10.68	112136640	7.2	135588792	8.8	156218337	8.0
Benzaldehyde	12.99	68934591	8.0	78092384	7.8	86783463	8.3
Benzeneacetaldehyde	15.09	38654991	6.2	49921979	5.9	60692494	7.7
Nonanal	15.98	163889012	4.3	237991018	5.8	257990191	5.7
Isophorone	18.14	59483992	9.7	72189951	8.9	89978887	9.8
Methyl Nonanoate	19.34	363318907	11	567819982	9.8	899817885	12
Decanoic acid	23.54	2409578	9.8	401677	12	5567810	10

6.2.2.2 Parameters of extraction

Based on the results, the final optimized combination is:

- 60°C incubation temperature
- 15 minutes incubation time
- 10 minutes sampling time

In experiments at 60 °C incubation temperature, all analytes had significantly higher peak areas than experiments at 45 °C. Thus, three factors, peak area, %RSD and total analysis time, made us characterizing which combination was the best.

Justifying our selection, when 60°C as incubation temperature, 15 minutes as incubation time and 10 minutes as sampling time were applied, all alkanes had enormous peak areas and satisfactory %RSD, less than 8.5% for all of them under these conditions. In other cases, alkanes showed either lower peak areas and worse RSDs% or a minor improvement in these two factors. For instance, Dodecane, whose peak area was only 4% higher under the combination of 60°C incubation temperature, 20 minutes incubation time and 20 minutes sampling time, or nonane whose %RSD was 2.51% lower under 60°C incubation temperature, 20 minutes incubation time and 20 minutes sampling time. However, we should still point out that in the case of 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time, their peak areas along with their %RSD were still excellent. These examples of minor differences clearly stated that do not worth the extension of analysis time.

Similarly, Alcohols had huge areas and low RSDs%, less than 7.5%, under 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time. Considerable improvement for the class of Alcohols was not observed under other conditions. For instance, although Undecanol showed a 3.93% higher peak area under 60°C incubation temperature, 30 minutes incubation time and 10 minutes sampling time, the corresponding %RSD was seriously worse. Aldehydes also had similar behavior. Their low values of %RSD and their high peak areas under 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time show that these conditions are the ideal choice for this group. Esters appeared to have grand peak areas but a higher %RSD than the other chemical compounds, under 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time. Nevertheless, a better response under an alternative combination is not noticeable, meaning that the adoption of these parameters should not be rejected.

Organic acids were not excluded from the previous observations. Even if their peak areas were lower than those of the other chemical substances, a phenomenon which can be explained due to their hydrophilic behavior, they were still at the same or a better level under 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time in comparison with the other combinations. %RSD areas were acceptable, lower than 14%. Ketones followed the same tendency, with excellent %RSD, less than 5.6%, under 60°C incubation temperature.

Except for some cases, such as the higher peak area of β -Damascenone under 60°C incubation temperature, 20 minutes incubation time and 20 minutes sampling time, all the other compounds seem to stay stable under any different combination of the parameters. Thus this combination, which keeps the analysis shorter than anything else, a priceless aspect in every analytical technique, is optimum. Last but not least, all the potential Internal Standards at 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time had efficient RSDs%, less than 7.1%, and their areas were either higher in these conditions in comparison with any other circumstances or the same level with the respective values of the different combinations. Therefore, all things considered under 60°C incubation temperature, 15 minutes incubation time and 10 minutes sampling time lead to a conclusion that this set of parameters is the best choice providing us both good peak areas and %RSD as well as reduced analysis time.

The results for some selected analytes are appended in Table 31.

Table 31: The results of the optimization of the incubation time, temperature and sampling time for five targeted analytes

	Decanoic acid		β -Damascenone		Nonane		Benzaldehyde		Nonanol	
	AVERAGE	%RSD	AVERAGE	%RSD	AVERAGE	%RSD	AVERAGE	%RSD	AVERAGE	%RSD
451510*	387429	14	13499280	21	59433489	9.1	2379426	6.1	10300649	16
453020	375313	8.0	15883266	19	81984909	5.7	3418024.667	11	12206780	6.9
601510	1768132	14	26807471	5.6	209335424	5.2	4696502.667	5.6	17876818	7.2
601520	1884314	16	25302270	3.2	131892104	7.2	4005937.333	3.9	15360094	4.4
601530	2328378	13	30399815	5.6	107853072	15	4672965.333	2.8	17188514	0.8
602010	597849	13	17679161	8.8	137633125	7.2	3127153	2.4	13100992	9.0
602030	2509293	11	33096023	15	86711848	0.79	4903691.667	7.9	18413376	16
603010	1049400	24	28718447	2.8	240764565	2.5	5831795.333	4.2	18578610	40

***451510: Incubation temperature= 45°C, Incubation time=15min and sampling time= 10min**

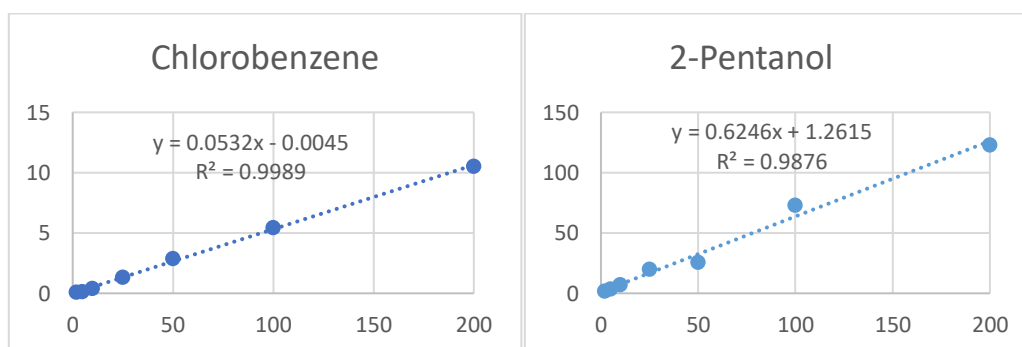
6.2.3. Comparison of the two optimized methods

The same incubation time and incubation temperature values seemed to be the most suitable for both of these two methods. However, a significant difference in sensitivity was noticed. HS-SPME was a more sensitive method. The peak area and intensity of the extracted analytes were significantly higher when HS-SPME was applied. Moreover, hydrophilic compounds, such as organic acids, were not detected when static HS was used, while these compounds were detected with the HS-SPME.

6.3. Selection of Internal Standards

6.3.1. Selection of Internal Standards for the static Headspace

Selecting the appropriate IS for every analyte was an important aspect. We constructed five different calibration curves for every chemical component, one without using any internal standard for the calibration and the other four for the different internal standards. By comparing the linearities between these five curves and their working ranges, their %RSD in terms of repeatability and levels of LOD and LOQ we selected the most suitable internal standard for each analyte, presented in Table 32. For example, in the case of Nonane, a more linear curve was obtained when Chlorobenzene was used as IS, as can be noticed in Figure 17.



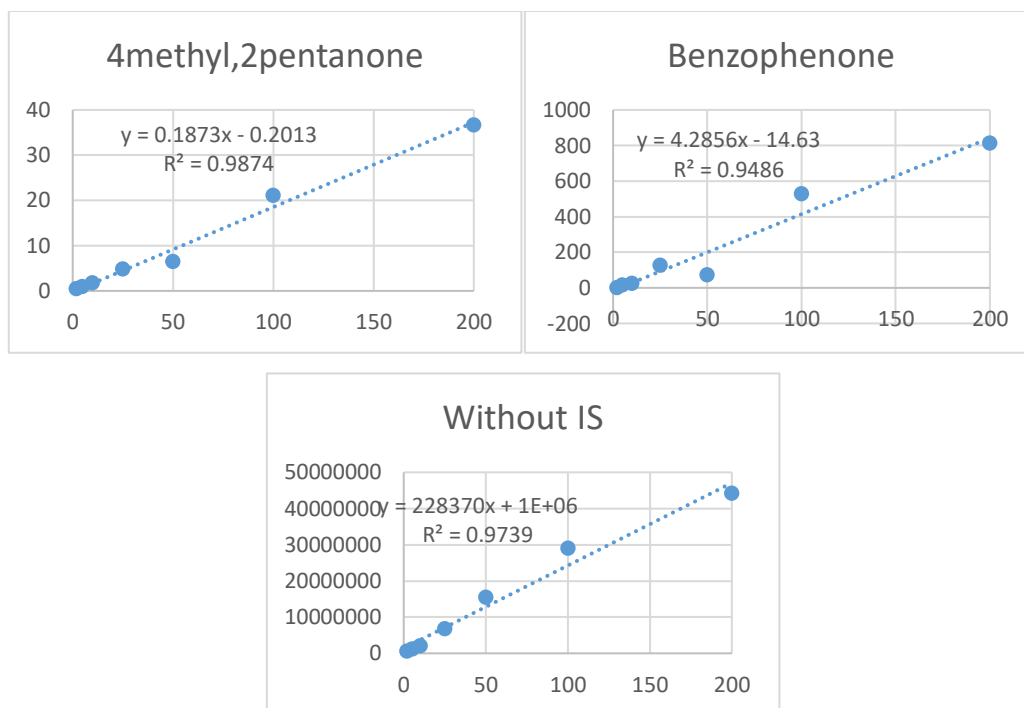


Figure 17: Calibration curves for Nonane with each of the IS and without IS

In another example, for the normalization of Octanal 2-Pentanone, 4-Methyl was selected as the proper IS.

First of all, as shown in Figure 18, the normalization of Octanal with 2-Pentanone, 4-Methyl provided a more satisfactory value of R^2 than the corresponding one when Chlorobenzene or Benzophenone was used as Internal standards. The absence of internal standard also provided unsatisfactory linearity.

However, normalization with 2-Pentanol provided an almost equal value of R^2 . In this case, the repeatability of the values regarding three different levels of concentration along with the levels of LOD and LOQ were compared. With 2-Pentanone, 4-Methyl, both improved repeatability and lower values of LOD and LOQ were achieved. Thus, 2- Pentanone, 4-Methyl was selected as the most suitable for the normalization of Octanal.

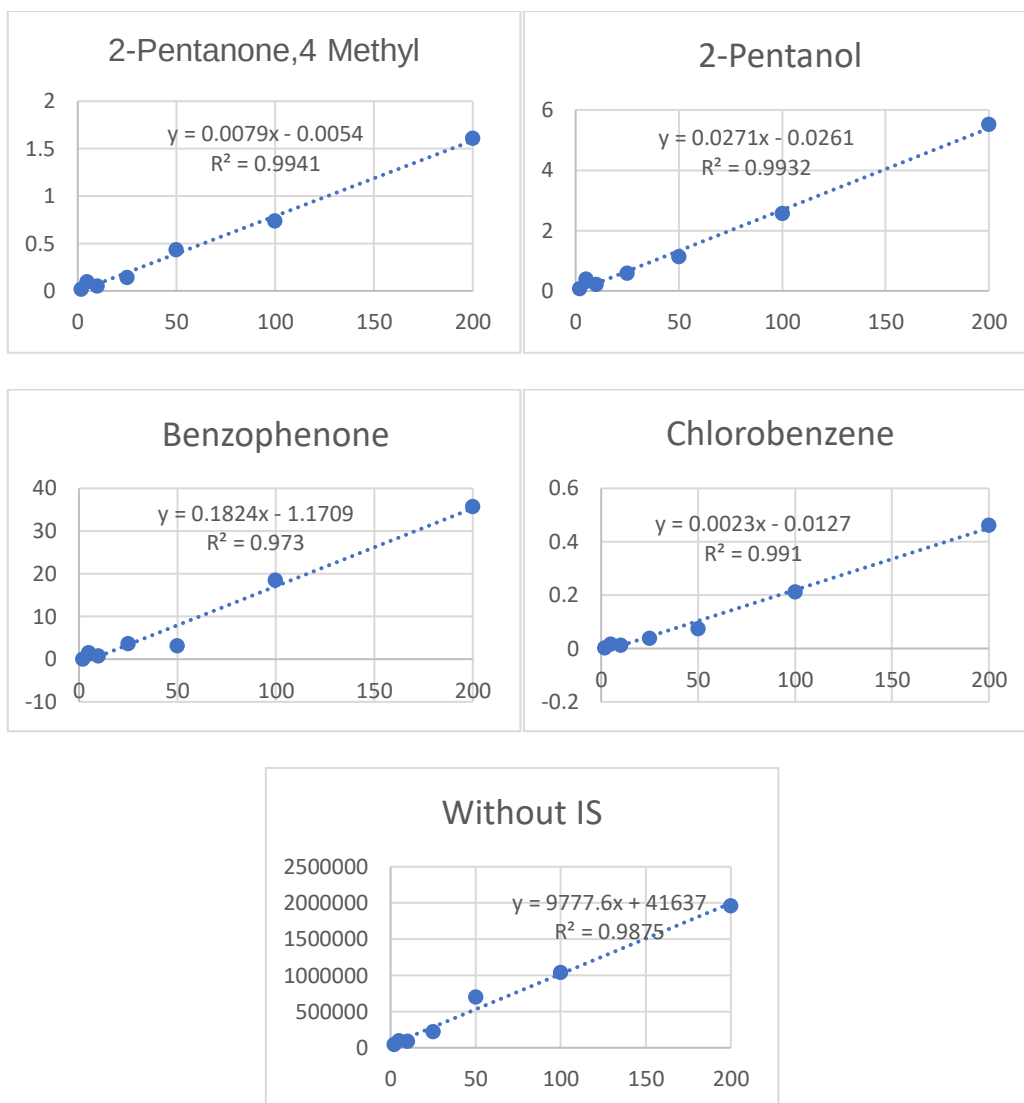


Figure 18: Calibration curves for Octanal with each of the IS and without IS

Table 32: RSD %, LOD's and LOQ's of Octanal after the normalization with 2-Pentanol and 2-Pentanone,4-Methyl

	Normalization with 2-Pentanone,4-Methyl	Normalization with 2-Pentanol
%RSD (5µg/Kg) Repeatability	15	19
%RSD (25µg/Kg) Repeatability	9.6	12
%RSD (100µg/Kg) Repeatability	8.7	10
LOD (µg/Kg)	8.96	22.2
LOQ(µg/Kg)	26.9	66.7

6.3.2. Selection of Internal standards for the HS-SPME method

The workflow was the same as that of static headspace. For example, Undecane was selected to be normalized with Chlorobenzene.

By evaluating the least square regression coefficient presented in Figure 19, we concluded that Benzophenone and the absence of internal standard should be rejected. Chlorobenzene provided the best improved least square regression coefficient. However, both 2-Pentanol and 2-Pentanone,4-Methyl provided a satisfactory value of R^2 , meaning that a further investigation between these three internal standards should be made.

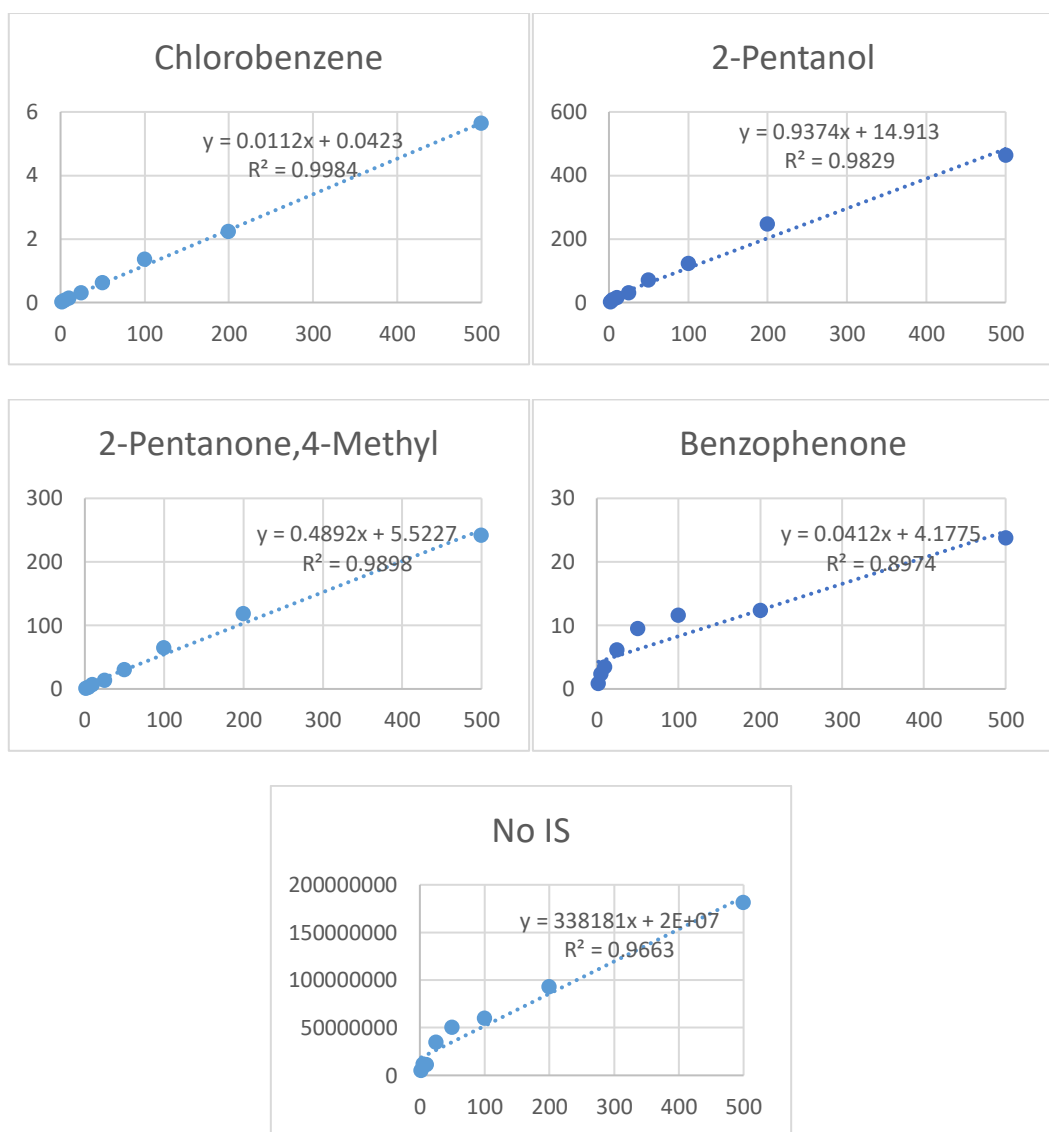


Figure 19: Calibration curves for Undecane with each of the IS and without IS

As a second step, the repeatability in three levels, 5, 50 and 200 µg/L, was evaluated. The results are presented in Table 33. Similarly, with the comparison of linearity, Chlorobenzene seems to be the ideal option.

For the final step, the LOD and LOQ were determined and presented in Table 33. These do also demonstrate that Chlorobenzene should be selected for the normalization of Undecane

Table 33: RSD %, LOD's and LOQ's of Undecane after the normalization with 2-Pentanol and 2-Pentanone,4-Methyl

	Normalization with Chlorobenzene	Normalization with 2-Pentanol	Normalization with 2-Pentanone,4-Methyl
%RSD (5µg/Kg) Repeatability	4.9	10	9.6
%RSD (50 µg/Kg) Repeatability	7.3	10	10
%RSD (200 µg/Kg) Repeatability	3.0	8.5	9.2
LOD(µg/Kg)	3.72	7.90	9.11
LOD (µg/Kg)	11.2	23.7	27.3

6.3.3. Selection of Internal Standard for both of the two methods

The selected Internal standards for both methods are presented in Tables 34,35, and 36. Summarizing the results, in this static HS method, the most suitable Internal Standard for the normalization of Alkanes is Chlorobenzene, while 2-Pentanone,4-Methyl is the most appropriate choice for both Aldehydes and Ketones, apart from Benzaldehyde and β -Damascenone. For these two chemical compounds, Benzophenone should be used. This may be explained by the fact that the structure of those two components, comprised of a benzene ring as depicted in Figure 19, is more relative to Benzophenone's structure than that of 2-Pentanone 4-Methyl. Benzophenone also corrects more efficiently the two esters, Ethyl Octanoate and Ethyl

Nonanoate, along with 1-Undecanol, the highest Alcohol of this database. The Other Alcohols, whose chain is smaller, are normalized with 2-Pentanol.

On the other hand, in the HS-SPME-GC/MS method, the ideal internal standard for each compound seems to be the same as the selected one of the static-HS-GC/MS method, as seen in Table 34. The only exception was β -Damascenone. For this ketone, Benzophenone was the ideal internal standard for the static HS method, while 2-Pentanone, 4-Methyl proved to be the best for the HS-SPME method

Table 34: The selected Internal Standards for the static Headspace method and HS-SPME methods

Analyte	Chemical class	Selected IS (Static HS)	Selected IS (HS-SPME)
β -Damascenone	Ketones	Benzophenone	2-Pentanone,4-Methyl
Isophorone	Ketones	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
3-Pentanone	Ketones	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
1-Hexanol	Alcohols	2-Pentanol	2-Pentanol
1-Octanol	Alcohols	2-Pentanol	2-Pentanol
1-Nonanol	Alcohols	2-Pentanol	2-Pentanol
1-Undecanol	Alcohols	Benzophenone	Benzophenone
Ethyl Hexanoate	Esters	Not Tested	Chlorobenzene
Ethyl Octanoate	Esters	Chlorobenzene	Chlorobenzene
Ethyl Nonanoate	Esters	Chlorobenzene	Chlorobenzene

Table 35: The selected Internal Standards for the static Headspace method and HS-SPME methods (Continued)

Analyte	Chemical class	Selected IS (Static HS)	Selected IS (HS-SPME)
Butanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Pentanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Hexanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Heptanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Octanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Nonanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Decanal	Aldehydes	2-Pentanone,4-Methyl	2-Pentanone,4-Methyl
Benzaldehyde	Aldehydes	Benzophenone	Benzophenone
Hexane	Alkanes	Chlorobenzene	Chlorobenzene
Heptane	Alkanes	Chlorobenzene	Chlorobenzene
Octane	Alkanes	Chlorobenzene	Chlorobenzene
Nonane	Alkanes	Chlorobenzene	Chlorobenzene
Decane	Alkanes	Chlorobenzene	Chlorobenzene
Undecane	Alkanes	Chlorobenzene	Chlorobenzene
Dodecane	Alkanes	Chlorobenzene	Chlorobenzene
Tetradecane	Alkanes	Chlorobenzene	Chlorobenzene

Table 36: The selected Internal Standards for the static Headspace method and HS-SPME methods (Continued)

Analyte	Chemical class	Selected IS (Static HS)	Selected IS (HS-SPME)
Pentadecane	Alkanes	Chlorobenzene	Chlorobenzene
Hexadecane	Alkanes	Chlorobenzene	Chlorobenzene
Heptadecane	Alkanes	Chlorobenzene	Chlorobenzene
Octadecane	Alkanes	Chlorobenzene	Chlorobenzene
Valeric acid	Organic acids	ND	Benzophenone
Hexanoic acid	Organic acids	ND	Benzophenone
Heptanoic acid	Organic acids	ND	Benzophenone
Octanoic acid	Organic acids	ND	Benzophenone
Nonanoic acid	Organic acids	ND	Benzophenone
Decanoic acid	Organic acids	ND	Benzophenone
Dodecanoic acid	Organic acids	ND	Benzophenone
Tridecanoic acid	Organic acids	ND	Benzophenone

To conclude, the majority of analytes are corrected more efficiently with an Internal Standard which belongs to the same or a relative chemical class, while the size and the volatility of the internal standard was also an aspect that affected the final selection. Thus, for example, Benzophenone, the least volatile internal standard, was the most suitable one for the normalization of the hydrophilic organic acids.

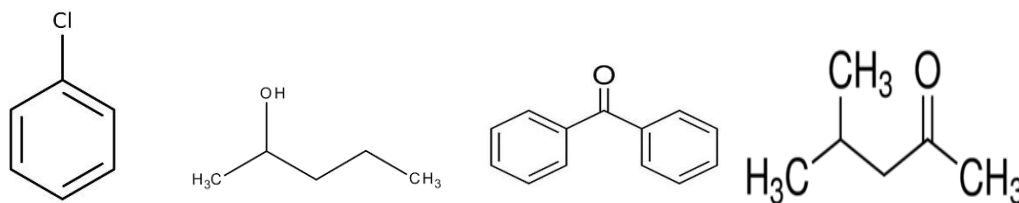


Figure 20: Chemical Structures, from left to right, of Chlorobenzene, 2-Pentanol, Benzophenone and 2-Pentanone,4methyl

6.4. Validation of methods

6.4.1. Linearity

6.4.1.1. Linearity of Static Headspace GC/MS

For the evaluation of the linear response of the analytes, the squared adjusted regression coefficient (R^2_{adj}) was determined. Nevertheless, the linearity of the calibration curve is not always guaranteed by these coefficients. Therefore, the relative standard deviation of the regression (RSD_r) and the distribution of regression residuals have to reach a suitable conclusion. R^2_{adj} , RSD_r , and the other validation data of the static Headspace method are presented in Table 37.

It can be noticed that R^2_{adj} ranged between 0.9645 and 0.9996. There were analytes whose great $R^2_{adj}=0.9996$ is accompanied by satisfactory $RSD_b=0.0311<0.05$, which guarantees the linear response. However, although other analytes had a pretty fine R^2_{adj} , their RSD_r was not the ideal one. In such cases, the regression residual plots have to be constructed to be used as a tool for detecting homoscedasticity. To achieve linearity, residuals should be uniformly distributed (random distribution), no trend has to be noticed, and the numbers of positive and negative residual values must be approximately equal. For example, both Benzaldehyde and 3-Pentanone show $R^2_{adj}>0.99$ but an $RSD_b>0.05$. The importance of residuals can be understood with the comparison of their residual plot. While the residual plot of Benzaldehyde (Figure 23) shows a linear behavior in full range, the corresponding one of 3-Pentanone (Figure 24) shows a clear trend. In cases like this, outlier values were excluded in order to achieve linear working ranges.

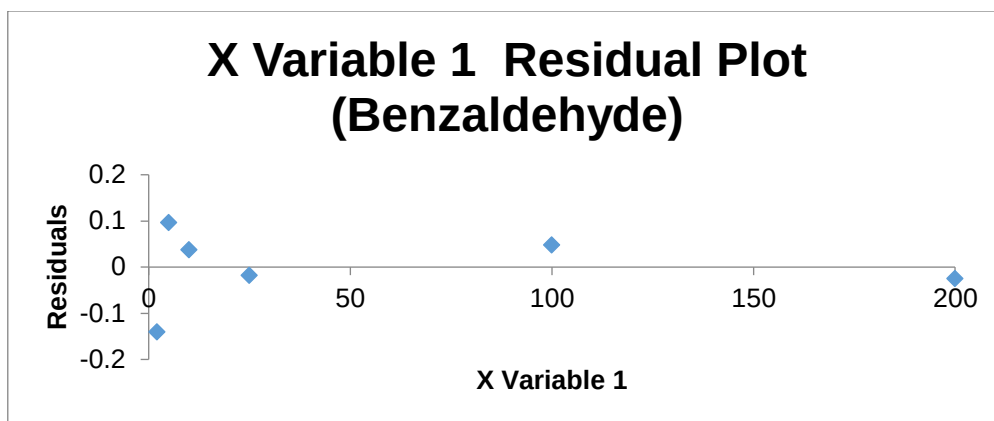


Figure 21: The residual plot of Benzaldehyde

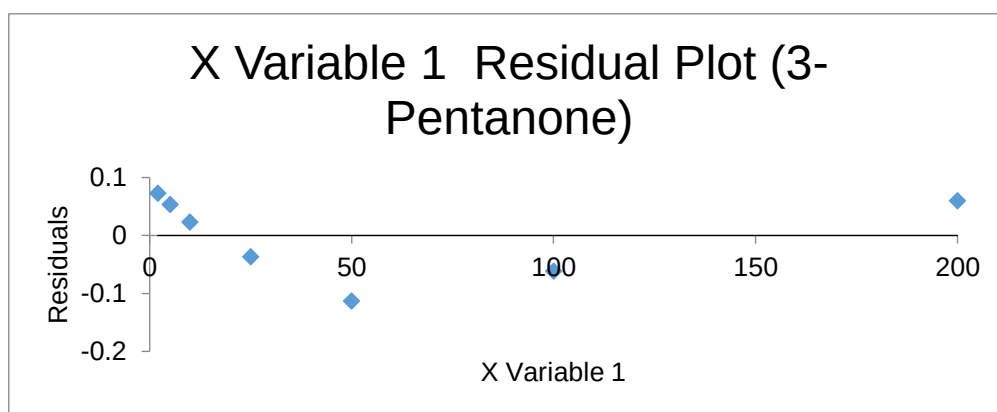


Figure 22: The residual plot of 3-Pentanone

6.4.1.2. Linearity of HS-SPME-GC/MS

Similarly, with static HS, the linear response of the analytes in this HS-SPME method was evaluated. Again, most of the analytes had satisfactory ranges of R^2_{adj} . More specifically, all Ketones, Alcohols, Aldehydes (apart from Benzaldehyde), Alkanes except Heptadecane and Octadecane, the heaviest of this class, and organic acids C₅-C₉ had an $R^2_{adj} > 0.99$. On the negative side, the lowest value was those of Dodecanoic acid, $R^2_{adj} = 0.9677$.

The results about the linearity of both of these two methods are presented in Tables 37 and 38.

Table 37: The data for the linearity of the validation of static HS-GC/MS and HS-SPME-GC/MS methods

Analyte	R²_{adj} (Static HS)	RSD_b (Static HS)	R²_{adj} (HS-SPME)	RSD_b (HS-SPME)
β-Damascenone	0.9996	0.0311	0.9991	0.0458
Isophorone	0.9674	0.0041	0.9985	0.1328
3-Pentanone	0.9885	0.0437	0.9994	0.0076
1-Hexanol	0.9955	0.0495	0.9959	0.2530
1-Octanol	0.9995	0.0500	0.9974	0.1837
1-Nonanol	0.9938	0.0194	0.9985	0.1965
1-Undecanol	0.9768	0.1812	0.9949	0.2614
Ethyl Hexanoate	NOT TESTED	NOT TESTED	0.9879	0.1078
Ethyl Octanoate	0.9730	0.0972	0.9981	0.0480
Ethyl Nonanoate	0.9645	0.0499	0.9899	0.0567
Butanal	0.9959	0.0428	0.9984	0.006
Pentanal	0.9971	0.0725	0.9941	0.08
Hexanal	0.9934	0.0458	0.9935	0.09
Heptanal	0.9891	0.0497	0.9946	0.199
Octanal	0.9929	0.0484	0.9993	0.1326
Nonanal	0.9896	0.0102	0.9992	0.125
Decanal	0.9880	0.0499	0.9971	0.1789
Benzaldehyde	0.9996	0.0915	0.9832	0.1403

Table 38: The data for the linearity of the validation of static HS-GC/MS and HS-SPME-GC/MS methods (Continued)

Analyte	R²_{adj} (Static HS)	RSD_b (Static HS)	R²_{adj} (HS-SPME)	RSD_b (HS-SPME)
Hexane	0.9914	0.0946	0.9959	0.0045
Heptane	0.9943	0.1608	0.9988	0.00073
Octane	0.9981	0.1412	0.9992	0.0003
Nonane	0.9986	0.1396	0.9991	0.0085
Decane	0.9969	0.1849	0.9887	0.065
Undecane	0.9978	0.1225	0.9854	0.1422
Dodecane	0.9948	0.1309	0.9926	0.169
Tetradecane	0.9669	0.0117	0.9838	0.194
Pentadecane	0.9915	0.0123	0.9881	0.176
Hexadecane	0.9845	0.0055	0.9980	0.0426
Heptadecane	0.9968	0.0103	0.9789	0.20
Octadecane	0.9812	0.0622	0.9832	0.1052
Valeric acid	-	-	0.9937	0.0069
Hexanoic acid	-	-	0.9843	0.017
Heptanoic acid	-	-	0.9945	0.016
Octanoic acid	-	-	0.9931	0.123
Nonanoic acid	-	-	0.990	0.1012
Decanoic acid	-	-	0.9739	0.5501
Dodecanoic acid	-	-	0.9677	0.347
Tridecanoic acid	-	-	0.9704	0.0312

6.4.2. Precision

6.4.2.1. Precision of static HS-GC/MS

Two types of precision, repeatability and intermediate precision, were evaluated and the results are presented in Table 39 and 40. The repeatability evaluation showed that 6 analytes had an %RSD < 10 for every level, while 6 analytes showed an %RSD < 15 for every level. An %RSD < 20 for every of the three tested concentration levels showed 10 analytes, while 7 compounds had a value of %RSD > 20 for at least one level. However, in most cases, by increasing the concentration of the tested level, the %RSD was decreased. For example, if we had excluded the lowest level, 5 µg/L, 14 analytes would have shown an %RSD < 10, 5 analytes an %RSD < 15, 5 an %RSD < 20 and 5 an %RSD > 20, which means that in higher concentration the precision was improved.

The results were a little worse for the intermediate precision: 5 analytes had an %RSD < 10, 6 an %RSD < 15, 10 an %RSD < 20, while 8 showed an %RSD > 20. If the lowest level is excluded, 8 analytes show an %RSD < 10, 11 analytes an %RSD < 15, 5 chemical compounds an %RSD < 20 and other 5 an %RSD > 20.

Table 39: The data for the precision of the validation of static HS-GC/MS method

Analyte	%RSD (5µg/L) Repeat	%RSD (25µg/L) Repeat	%RSD (100µg/L) Repeat	%RSD (5µg/L) Inter Precision	%RSD (25µg/L) Inter Precision	%RSD (100µg/L) Inter Precision
β-Damascenone	19	9.6	9.6	18	10	8.0
Isophorone	18	9.2	2.5	12	6.8	7.3
3-Pentanone	14	8.5	2.5	8.5	9.8	7.5
1-Hexanol	10	12	6.8	12	11	9.6
1-Octanol	15	11	6.4	18	13	7.2
1-Nonanol	9.8	9.9	8.6	9.1	10	8.4
1-Undecanol	-	28	22	-	26	23
Ethyl Octanoate	27	25	22	29	24	21
Ethyl Nonanoate	28	24	21	29	23	26
Butanal	13	5.5	2.8	14	9.1	3.0
Pentanal	6.1	7.0	2.4	9.9	8.2	4.6
Hexanal	5.6	9.9	7.0	7.4	11	13
Heptanal	16	11	8.0	27	10	8.3
Octanal	15	9.6	8.7	16	9.2	10
Nonanal	8.0	4.2	1.9	6.7	9.3	5.0

Table 40: The data for the precision of the validation of static HS-GC/MS method (Continued)

Analyte	%RSD (5µg/L) Repeat	%RSD (25µg/L) Repeat	%RSD (100µg/L) Repeat	%RSD (5µg/L) Inter Precision	%RSD (25µg/L) Inter Precision	%RSD (100µg/L) Inter Precision
Decanal	9.7	7.7	4.4	9.0	8.8	6.0
Benzaldehyde	8.8	9.9	7.2	7.9	9.5	8.0
Hexane	13	9.8	6.0	12	9.9	8.8
Heptane	12	3.8	7.8	17	14	9.0
Octane	18	17	6.1	17	19	10
Nonane	16	10	5.1	16	15	11
Decane	18	7.7	5.3	23	11	8.9
Undecane	18	19	11	15	19	14
Dodecane	10	12	7.2	18	14	11
Tetradecane	27	17	8.0	16	22	5.1
Pentadecane	19	18	8.6	15	19	10
Hexadecane	17	21	12	18	18	15
Heptadecane	27	23	15	29	21	17
Octadecane	24	19	12	25	17	13

6.4.2.2. Precision of HS-SPME-GC-MS

Similarly, with static HS, two types of precision, repeatability and intermediate precision, were evaluated. The results are presented in Tables 41, 42 and 43.

The repeatability evaluation showed that 14 analytes had an %RSD < 10 for every level, while other 10 analytes showed an %RSD < 15 for every level. The remaining compounds had an %RSD < 20. In most cases, by increasing the concentration of the tested level, the %RSD was decreased. For example, if we had excluded the lowest level, 5 µg/L, 27 analytes would have shown an %RSD < 10, while the remaining analytes would have had an RSD % < 15, apart from Heptadecane and Tridecanoic acid, whose %RSD would have still been lower than 20.

For the intermediate precision, the results were a little worse. 19 analytes had an %RSD < 10 and other 10 had an %RSD < 15. No analyte had an %RSD of more than 20. Excluding the lowest level, 5 µg/L, all analytes have an %RSD < 10, apart from all the organic acids, ethyl octanoate and 1-undecanol

All in all, HS-SPME improves the results of repeatability and intermediate precision in comparison with static Headspace. More analytes had an %RSD < 10, and no analytes had an %RSD > 20 under these three tested concentration levels.

Table 41: The data for the precision of the validation of HS-SPME-GC/MS method

Analyte	%RSD (5µg/L) Repeatability	%RSD (50µg/L) Repeatability	%RSD (200µg/L) Repeatability	%RSD (5µg/L) Inter Precision	%RSD (50µg/L) Inter Precision	%RSD (200µg/L) Inter Precision
β-Damascenone	5.4	0.54	3.9	4.5	9.7	7.9
Isophorone	8.1	6.5	2.3	6.3	7.8	4.7
3-Pentanone	4.3	6.2	5.8	9.0	9.1	6.9
1-Hexanol	14	4.5	7.7	13	8.2	6.5
1-Octanol	11	8.2	1.2	10	9.2	7.6
1-Nonanol	11	7.4	5.3	11	8.7	9.8
1-Undecanol	10	10	8.2	13	12	9.3
Ethyl Hexanoate	15	5.7	2.0	8.2	8.7	4.9
Ethyl Octanoate	19	13	3.5	19	12.0	8.4
Ethyl Nonanoate	18	11	3.8	19	9.4	8.2
Butanal	6.2	5.6	2.7	6.0	7.1	3.9
Pentanal	8.1	6.3	1.4	7.9	7.4	4.6
Hexanal	8.8	5.7	4.7	9.9	7.9	6.6
Heptanal	11	10	4.1	7.2	8.2	2.9
Octanal	12	6.8	2.5	3.2	9.0	7.7

Table 42: The data for the precision of the validation of HS-SPME-GC/MS method (Continued)

Analyte	%RSD (5µg/L) Repeatability	%RSD (50µg/L) Repeatability	%RSD (200µg/L) Repeatability	%RSD (5µg/L) Inter Precision	%RSD (50µg/L) Inter Precision	%RSD (200µg/L) Inter Precision
Nonanal	7.8	6.3	4.5	9.9	8.0	7.9
Decanal	8.7	9.7	6.0	8.0	7.8	4.3
Benzaldehyde	12	7.1	2.7	11	9.9	7.9
Hexane	8.8	6.9	5.5	9.7	9.0	6.4
Heptane	7.9	7.1	5.2	8.1	6.3	8.3
Octane	4.7	6.2	4.7	8.8	6.7	5.5
Nonane	9.2	7.6	5.1	9.8	8.3	8.2
Decane	6.7	4.4	4.7	10	7.5	8.3
Undecane	12	7.7	4.0	13	9.0	8.2
Dodecane	6.7	5.1	2.4	8.6	7.3	6.4
Tetradecane	15	7.9	7.0	7.7	9.2	7.4
Pentadecane	11	8.1	4.5	13	13	12
Hexadecane	13	11	7.2	13	11	9.8
Heptadecane	18	15	8.3	19	18	13
Octadecane	17	11	4.3	12	12	8.9
Valeric acid	-	12	10	-	13	15

Table 43: The data for the precision of the validation of HS-SPME-GC/MS method (Continued)

Analyte	%RSD (5µg/L) Repeatability	%RSD (50µg/L) Repeatability	%RSD (200µg/L) Repeatability	%RSD (5µg/L) Inter Precision	%RSD (50µg/L) Inter Precision	%RSD (200µg/L) Inter Precision
Hexanoic acid	-	7.7	8.8	-	14	12
Heptanoic acid	-	7.4	4.9	-	13	12
Octanoic acid	-	5.1	7.2	-	11	11
Nonanoic acid	-	13	6.3	-	14	9.9
Decanoic acid	-	8.0	6.4	-	10	8.3
Dodecanoic acid	-	12	6.0	-	12	7.7
Tridecanoic acid	-	17	12	-	19	16

6.4.3. Limits of Detection (LOD) and Quantification (LOQ)

The determined LOD's and LOQ's for these two methods are presented in Tables 43 and 44. With the application of HS-SPME, we managed to identify a wider variety of analytes, such as the hydrophilic organic acids, which were undetectable when static HS was used. Moreover, all analytes had lower LOD's and LOQ's with HS-SPME in comparison with Static HS. The LOD's for the static HS ranged between 6.25 and 14.1 µg/Kg, while the corresponding values for the HS-SPME method were between 2.19 and 9.74 µg/Kg, apart from organic acids whose LOD's ranged between 7.12 and 13.1 µg/Kg. Similarly, the LOQ's for the static HS fluctuated between 18.7 and 42.3 µg/Kg while the corresponding values for the HS-SPME method were between 6.57 and 29.2 µg/Kg.

Table 44: LOD's and LOQ's for the two methods

Analyte	LOD static HS (µg/Kg)	LOQ static HS (µg/Kg)	LOD HS-SPME (µg/Kg)	LOQ HS-SPME (µg/Kg)
β-Damascenone	7.92	23.7	3.01	9.03
Isophorone	8.20	24.6	3.21	9.71
3-Pentanone	14.1	42.3	9.74	29.2
1-Hexanol	10.1	30.5	4.79	14.3
1-Octanol	11.1	33.4	3.82	11.4
1-Nonanol	11.7	35.1	3.20	9.60
Ethyl Hexanoate	NOT TESTED	NOT TESTED	7.84	23.5
Ethyl Octanoate	6.2	18.7	5.29	15.8
Ethyl Nonanoate	9.4	28.4	5.24	15.7
Butanal	11.0	33.3	6.78	20.3
Pentanal	9.21	27.6	5.80	17.4
Hexanal	13.0	39.0	3.07	9.21
Heptanal	8.47	25.4	3.09	9.27
Octanal	8.79	26.3	3.48	10.4
Nonanal	6.78	20.3	2.81	8.43
Decanal	8.67	26.0	2.52	7.58
Benzaldehyde	5.97	17.9	2.19	6.57

Table 45: LOD's and LOQ's for the two methods (Continued)

Analyte	LOD static HS (µg/g)	LOQ static HS (µg/g)	LOD HS-SPME (µg/g)	LOQ HS-SPME (µg/g)
Hexane	12.4	37.2	3.67	11.0
Heptane	7.04	21.1	3.22	9.66
Octane	8.08	24.2	3.15	9.45
Nonane	8.12	24.3	3.21	9.63
Decane	6.71	20.1	2.12	6.16
Undecane	9.04	27.1	3.72	11.1
Dodecane	10.1	30.3	6.89	20.6
Tetradecane	9.42	28.2	7.57	22.7
Pentadecane	9.08	27.2	7.73	23.1
Hexadecane	12.0	36.1	5.38	16.1
Heptadecane	11.8	35.4	7.09	21.2
Octadecane	11.3	33.9	8.14	24.4
Valeric acid	ND	ND	12.0	36.0
Hexanoic acid	ND	ND	13.1	39.5
Heptanoic acid	ND	ND	11.3	33.9
Octanoic acid	ND	ND	7.51	22.5
Nonanoic acid	ND	ND	8.02	24.0
Decanoic acid	ND	ND	7.12	21.3

6.4.4. Accuracy

6.4.4.1. Accuracy of static HS

For three levels, a lower, a medium and a higher, the apparent accuracy was determined. The results are presented in Tables 46 and 47. 20 volatile compounds showed $80 < \% \text{Recovery} < 120$ in every one of these three levels. Concerning the medium and higher level, all compounds were inside the above optimum range. However, regarding the lower level, 9 compounds had a recovery, which was out of the desirable range.

Table 46: The results for the accuracy of the static HS-GC/MS method

Analyte	Accuracy Recovery% (Low Concentration, 5µg/Kg)	Accuracy Recovery% (Medium Concentration) (25µg/Kg)	Accuracy Recovery% (High Concentration) (100µg/Kg)
β-Damascenone	138	119	117
Isophorone	84	107	116
3-Pentanone	73	85	94
1-Hexanol	129	96	102
1-Octanol	93	96	98
1-Nonanol	83	107	119
1-Undecanol	-	103	119
Ethyl Octanoate	147	102	82
Ethyl Nonanoate	79	99	91
Butanal	73	88	88
Pentanal	86	100	103

Table 47: The results for the accuracy of the static HS-GC/MS method

Analyte	Accuracy Recovery% (Low Concentration, 5µg/Kg)	Accuracy Recovery% (Medium Concentration) (25µg/Kg)	Accuracy Recovery% (High Concentration) (100µg/Kg)
Hexanal	101	89	92
Heptanal	87	89	86
Octanal	76	87	93
Nonanal	85	82	94
Decanal	124	106	93
Benzaldehyde	127	114	105
Hexane	84	91	103
Heptane	102	118	111
Octane	81	107	117
Nonane	82	100	102
Decane	80	95	107
Undecane	78	95	106
Dodecane	74	114	109
Tetradecane	79	89	87
Pentadecane	113	93	87
Hexadecane	145	82	96
Heptadecane	140	96	97
Octadecane	136	112	104

6.4.4.2. Accuracy of HS-SPME

For three levels, a lower, a medium and a higher, the apparent accuracy was determined. The results are presented in Tables 48 and 49. All analytes apart from Heptadecane showed $80 < \% \text{Recovery} < 120$ in all of these three levels.

Table 48: The results for the accuracy of the HS-SPME-GC/MS method

Analyte	Recovery%, (5µg/Kg)	Recovery% (50µg/Kg)	Recovery% (200µg/Kg)
β-Damascenone	109	94	98
Isophorone	95	94	95
3-Pentanone	107	114	100
1-Hexanol	114	95	98
1-Octanol	113	95	100
1-Nonanol	119	98	99
1-Undecanol	-	103	119
Ethyl Hexanoate	96	95	102
Ethyl Octanoate	95	101	99
Ethyl Nonanoate	88	102	100
Butanal	73	88	88
Pentanal	113	108	104
Hexanal	83	107	103
Heptanal	92	95	96
Octanal	91	94	97
Nonanal	115	97	102
Decanal	117	103	102

Table 49: The results for the accuracy of the HS-SPME-GC/MS method (Continued)

Analyte	Accuracy Recovery% (5µg/Kg)	Accuracy Recovery% (50µg/Kg)	Accuracy Recovery% (200µg/Kg)
Benzaldehyde	107	97	104
Hexane	84	91	103
Heptane	109	102	104
Octane	106	109	108
Nonane	109	100	94
Decane	104	97	100
Undecane	108	92	99
Dodecane	91	97	93
Tetradecane	92	93	96
Pentadecane	82	87	92
Hexadecane	119	107	104
Heptadecane	125	83	103
Octadecane	86	88	108
Valeric acid	84	89	81
Hexanoic acid	89	95	101
Heptanoic acid	91	92	98
Octanoic acid	84	108	92
Nonanoic acid	90	93	98
Decanoic acid	95	100	98

6.5. Results from the analysis of 36 honey samples of 12 different botanical origin

6.5.1. Target screening

Following the target screening process, several volatiles were identified in these honey samples. Therefore, the results of the HS-SPME method are presented in Tables 50-53, while those of static HS in Tables 54-56.

Table 50: Target results from HS-SPME

C (µg/Kg)								
Analyte	Octanoic acid ethyl ester		Hexanoic acid		Octanoic acid		Nonanoic acid	
	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE
Pine	ND-24.9	<LOQ	ND-<LOQ	<LOQ	ND-67.0	29.5	ND-64.7	27.9
Fir	ND-42.3	<LOQ	ND-59.8	<LOQ	ND-53.2	28.1	ND-41.5	<LOQ
Oak	ND-39.0	<LOQ	ND-53.1	<LOQ	ND-60.1	36.2	ND-51.3	<LOQ
Thyme	ND-15.9	<LOQ	ND-<LOQ	<LOQ	ND	-	ND-25.9	<LOQ
Heather	ND-31.3	<LOQ	ND-48.3	<LOQ	ND	-	ND	-
Orange	ND	-	ND	-	ND	-	ND	-
Sage	ND	-	ND	-	ND-29.4	<LOQ	<LOQ-42.8	<LOQ
Chestnut	ND-65.9	14.7	ND-47.4	32.6	ND-42.6	28.9	ND-71.4	29.6
Arbutus	156.7-238.3	198.9	ND	-	ND	-	ND	-
Cotton	ND-12.4	<LOQ	ND-37.9	<LOQ	ND-37.9	23.1	ND	-
Vanilla	ND	-	ND	-	ND	-	ND-24.0	<LOQ
Carob	ND-51.7	23.3	ND-38.1	<LOQ	<LOQ-41.4	29.1	ND	-

Table 51: Target results from HS-SPME (Continued)

C (µg/Kg)										
Analyte	Decanoic acid		Isophorone		B-Damascenone		1-Hexanol		1-Nonanol	
	RANGE	AVER-AGE	RANGE	AVER-AGE	RANGE	AVER-AGE	RANGE	AVER-AGE	RANGE	AVER-AGE
Pine	ND	-	ND-34.9	11.1	ND	-	ND-34.4	13.7	18.9-57.8	32.6
Fir	ND	-	13.6-59.9	29.2	ND-11.7	<LOQ	ND-18.6	13.8	12.2-21.9	16.3
Oak	ND-26.3	<LOQ	11.2-76.7	19.6	ND-10.2	<LOQ	13.9-76.9	33.2	14.9-32.1	22.6
Thyme	ND	-	ND	-	ND-11.1	<LOQ	ND	-	11.7-15.4	13.9
Heather	ND	-	87.2-189.8	123.3	ND-18.7	11.2	ND	-	ND	-
Orange	ND	-	ND-29.9	12.1	ND	-	ND	-	ND-14.2	<LOQ
Sage	ND	-	ND	-	ND-11.9	<LOQ	ND	-	ND	-
Chestnut	ND-44.8	<LOQ	22.1-69.8	39.7	<LOQ-11.6	<LOQ	ND-24.9	13.7	17.8-39.3	27.7
Arbutus	ND	-	2580-7870	5292	ND-13.9	<LOQ	ND-17.1	<LOQ	ND	-
Cotton	ND	-	ND	-	ND	-	ND	-	ND	-
Vanilla	ND	-	21.7-75.4	44.2	ND	-	ND	-	12.9-20.2	17.6
Carob	ND	-	24.6-149	82.8	27.2-75.8	43.9	ND	-	ND	-

Table 52: Target results from HS-SPME (Continued)

C (µg/Kg)										
Analyte	Heptane		Octane		Nonane		Decane		Heptanal	
	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE
Pine	29.7-72.6	49.8	182.7-469.8	289.9	<LOQ-	27.5	ND-	28.7	ND-14.9	9.4
Fir	42.9-98.2	74.6	129.6-630.8	389.4	14.7-42.9	30.3	ND-16.2	8.9	9.3-16.9	10.8
Oak	ND-41.8	15.8	102.9-202.1	174.4	12.1-	60.2	9.0-18.9	15.9	ND	-
Thyme	18.9-62.7	31.3	82.9-429.8	226.1	17.6	51.7	ND-32.7	19.8	ND-21.7	12.9
Heather	ND-49.0	23.2	49.9-120.4	88.9	ND-49.8	29.6	ND-13.5	7.8	12.3-32.7	19.9
Orange	11.8-72.7	38.4	32.9-102.7	81.9	ND-39.8	24.6	6.5-12.2	9.9	ND-14.2	9.8
Sage	14.7-42.8	25.9	66.7-202.4	145.2	12.9-	39.7	ND-21.0	14.7	ND-16.3	9.4
Chestnut	12.9-42.6	26.3	78.9-292.6	189.8	16.9-59.7	32.6	ND-10.8	7.3	ND-9.9	<LOQ
Arbutus	ND	-	ND-78.7	32.7	ND-22.1	11.7	ND- 11.1	7.2	12.4-43.9	25.1
Cotton	ND-32.7	21.9	123.7-414.2	239.9	38.1-89.2	56.6	9.7-16.1	12.3	ND	-
Vanilla	35.9-82.8	57.3	159.8-247.9	221.1	37.4-92.6	58.8	ND-24.3	16.9	ND	-
Carob	ND	-	149.4-322.8	241.7	27.4-61.1	43.9	7.4-9.2	8.1	ND	-

Table 53: Target results from HS-SPME (Continued)

C (µg/Kg)								
Analyte	Octanal		Nonanal		Decanal		Benzaldehyde	
	RANGE	AVER- AGE	RANGE	AVERAGE	RANGE	AVER- AGE	RANGE	AVERAGE
Pine	10.7-19.8	13.6	59.9-182.6	93.9	8.4-52.4	32.1	29.5-76.3	45.7
Fir	ND-33.1	19.9	78.9-183.9	142.8	ND-50.2	25.6	21.2-49.8	33.5
Oak	<LOQ-52.8	22.4	13.7-42.1	28.2	10.1-40.7	27.6	13.1-29.9	22.6
Thyme	11.7-29.9	18.2	12.5-51.8	30.4	9.8-38.9	25.2	179.8- 301.2	246.3
Heather	15.7-25.9	19.3	9.7-67.2	30.1	15.6-42.8	29.4	92.9-249.4	189.3
Orange	ND-16.2	11.1	ND-50.7	27.2	13.4-37.2	25.3	12.7-61.6	33.9
Sage	10.5-20.1	14.8	10.1-58.4	32.3	ND-24.7	14.1	72.3-115.8	83.9
Chestnut	18.9-59.8	36.3	13.4-62.6	33.2	8.7-30.2	18.1	53.9-102.6	83.7
Arbutus	12.9-29.9	22.7	30.6-72.9	51.1	ND-25.5	14.1	19.0-41.4	28.6
Cotton	10.7-19.2	14.5	15.6-74.9	51.9	17.2-59.8	26.9	67.8-112.9	80.6
Vanilla	13.6-18.7	15.9	79.9-160.3	138.7	12.1-47.6)	32.7	15.1-28.4	23.7
Carob	ND-14.2	11.1	20.1-37.2	26.9	13.9-32.8	21.5	15.3-37.2	25.1

Table 54: Target results from static HS

C (µg/Kg)										
Analyte	Octanoic acid ethyl ester		Isophorone		B-Damascenone		1-Hexanol		1-Nonanol	
	AVERAGE	MEAN	AVERAGE	MEAN	AVERAGE	MEAN	AVERAGE	MEAN	AVERAGE	MEAN
Pine	ND-21.3	<LOQ	ND-26.3	<LOQ	ND	-	ND-<LOQ	<LOQ	<LOQ-48.9	<LOQ
Fir	ND-33.1	<LOQ	<LOQ-44.2	25.9	ND	-	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ
Oak	ND-31.7	<LOQ	<LOQ-59.6	<LOQ	ND	-	<LOQ-65.2	27.9	ND-<LOQ	<LOQ
Thyme	ND-<LOQ	<LOQ	ND	-	ND	-	ND	-	ND-LOQ	<LOQ
Heather	ND-31.3	<LOQ	59.8-151.7	98.9	ND-<LOQ	<LOQ	ND	-	ND	-
Orange	ND	-	ND-<LOQ	<LOQ	ND	-	ND	-	ND-<LOQ	<LOQ
Sage	ND	-	ND	-	ND-<LOQ	<LOQ	ND	-	ND	-
Chestnut	ND-47.8	<LOQ	<LOQ-49.8	27.8	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	<LOQ-37.9	<LOQ
Arbutus	117.9-189.6	167.8	2480-7680	5279	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	ND	-
Cotton	ND	-	ND	-	ND	-	ND	-	ND	-
Vanilla	ND	-	<LOQ-68.6	37.9	ND	-	ND	-	ND-<LOQ	<LOQ
Carob	ND-38.9	19.7	<LOQ-129	71.9	<LOQ-59.8	35.7	ND	-	ND	-

Table 55: Target results from static HS (Continued)

C (µg/Kg)										
Aanalyte	Heptane		Octane		Nonane		Decane		Hexanal	
	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVERAGE
Pine	22.9-71.8	47.4	175.4- 189.8	296.7	ND-<LOQ	<LOQ	ND-23.1	<LOQ	ND-<LOQ	<LOQ
Fir	38.7-100.3	71.2	117.9- 679.8	362.9	ND-36.9	<LOQ	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ
Oak	ND-33.8	<LOQ	91.4-214.9	185.9	ND-51.7	31.2	ND-<LOQ	<LOQ	ND	-
Thyme	<LOQ- 57.6	27.9	75.9-412.9	208.7	<LOQ- 43.1	32.8	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ
Heather	ND-42.9	<LOQ	39.8-112.9	79.9	ND-38.4	<LOQ	ND-<LOQ	<LOQ	ND-40.1	<LOQ
Orange	<LOQ- 66.9	33.9	28.5-108.9	78.9	ND-37.9	<LOQ	ND-<LOQ	<LOQ	ND	-
Sage	<LOQ- 38.8	23.7	58.9-211.4	139.8	ND-35.2	<LOQ	ND-<LOQ	<LOQ)	ND-<LOQ	<LOQ
Chestnut	<LOQ- 41.9	22.4	76.4-298.9	192.6	ND-54.7	-28.4	ND-<LOQ	<LOQ	ND	-
Arbutus	ND	-	ND-55.9	<LOQ	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	ND	-
Cotton	ND-27.9	<LOQ	136.8- 429.9	252.1	29.3-66.9	43.8	ND-<LOQ	<LOQ	ND	-
Vanilla	31.4-74.9	49.8	139.3- 222.9	200.7	30.5-79.1	-50.3	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ
Carob	ND	-	137.9- 308.6	224.6	<LOQ- 52.0	-37.2	ND-<LOQ	<LOQ	ND	-

Table 56: Target results from static HS (Continued)

C (µg/Kg)										
Analyte	Heptanal		Octanal		Nonanal		Decanal		Benzaldehyde	
	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVER- AGE	RANGE	AVERAGE
Pine	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	43.9- 161.6	80.7	ND-41.2	28.7	22.6-69.7	39.8
Fir	ND-<LOQ	<LOQ	ND-29.7	<LOQ	68.9- 159.8	112.9	ND-39.7	<LOQ	<LOQ- 47.1	28
Oak	ND	-	<LOQ- 39.8	<LOQ	ND-33.2	24.7	ND-33.8	<LOQ	<LOQ- 24.2	20.1
Thyme	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	ND-47.3	26.2	ND-22.7	22.3	154.8- 289.1	222.2
Heather	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	ND-58.9	27.9	<LOQ- 37.1	23.4	83.1- 232.9	178.2
Orange	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	ND-42.8	22.1	<LOQ- 30.2	21.5	<LOQ- 54.5	27.9
Sage	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	ND-48.9	29.8	ND-21.4	<LOQ	60.3- 104.9	74.9
Chestnut	ND-<LOQ	<LOQ	ND-51.7	31.3	ND-52.9	28.5	ND-23.8	<LOQ	42.8-90.7	83.7
Arbutus	ND-<LOQ	<LOQ	ND-<LOQ	<LOQ	23.5-64.7	44.2	ND-22.1	<LOQ	<LOQ- 35.5	23.8
Cotton	ND	-	ND-<LOQ	<LOQ	<LOQ- 59.8	40.1	<LOQ- 52.4	22.2	64.4- 106.9	75.9
Vanilla	ND	-	ND-<LOQ	<LOQ	70.1- 150.2	128.5	<LOQ- 41.9	28.9	<LOQ- 22.9	20.8
Carob	ND	-	ND	-	<LOQ- 31.5	22.5	<LOQ- 28.5	<LOQ	<LOQ- 31.1	22.4

Several important conclusions are extracted from these results. To begin with, it is verified that organic acids can only be identified with HS-SPME, as they are undetectable with static HS. Moreover, Isophorone is detected in significantly higher concentrations in Arbutus honeys, with both static HS and HS-SPME, which makes it a potential marker of this botanical origin. Similarly, β -Damascenone is a potential marker of Carob honey. Octane is present in most samples, and the higher mean concentration of this analyte is detected in Fir samples. Nonanal concentration is higher in Vanilla and Fir samples, while Benzaldehyde's mean concentration is higher in Thyme and Heather samples compared to the other varieties.

Moreover, other analytes were identified in all honey samples of a specific variety but simultaneously were undetectable in all samples of a different botanical origin. For example, Heptanal was Detected in all Arbutus, Fir and Heather samples with HS-SPME, while it was not present in all Oak, Cotton, Vanilla and Carob samples. Another example is 1-Nonanol. While this compound was present in every Pine, Oak, Fir, Thyme, Chestnut and Vanilla sample, it was not detected in Heather, Sage, Arbutus, Carob and Cotton samples.

In conclusion, the HS-SPME method is more efficient in determining volatile compounds in honey. However, apart from hydrophilic compounds, such as organic acids, which cannot be detected with static HS, the sensitivity and LOD's and LOQ's are not satisfactory enough in some cases in static HS technique. For instance, applying HS-SPME enabled us to quantify the mean concentration of Octanal in every variety. However, when static HS was used, we both did not manage to qualify Octanal in some varieties such as in Pine Samples and we were not able to quantify its mean concentration in all varieties, apart from Chestnut.

6.5.2. Suspect Screening

A screening of analytes that have previously been reported in other similar scientific researches was conducted. In Table 57, 10 analytes that were identified through suspect screening are presented.

Table 57: The analytes which were identified through suspect screening

Analyte	RT (min)	Quantifier Ion	Qualifier Ion (I)	Qualifier Ion (II)
Furan,2,5-dimethyl	6.99	96	81	43
2,3 Butanediol	10.09	45	43	57
Furfural	10.63	96	95	39
α -Pinene	11.54	93	91	77
2-Furancarboxaldehyde, 5methyl	13.19	110	109	53
Benzeneacetaldehyde	15.03	91	92	120
Octanoic acid methyl ester	16.02	74	87	127
2-Hydroxy,4-Oxoisophorone	18.29	84	56	126
Ketoisophorone	18.55	68	96	152
Nonanoic acid methyl ester	19.34	74	87	141

The mean absolute intensities for each analyte identified through suspect screening in the HS-SPME method, in every different botanical origin, are presented in Tables 58 and 59. The corresponding results for static HS are those in Tables 60 and 61.

Table 58: Suspect results from HS-SPME

Analyte	α -Pinene	2,3 Butanediol	Ketoisophorone	2 Furancarboxal- dehyde ,5 Methyl	2-Hydroxy Isopho- rone
Pine	4.59×10^6	1.16×10^7	9.87×10^5	1.52×10^6	ND
Fir	$\times 10^5$	1.31×10^6	7.54×10^5	1.07×10^6	ND
Oak	1.17×10^6	1.11×10^8	ND	5.87×10^5	ND
Thyme	5.02×10^5	3.16×10^5	3.67×10^5	1.48×10^6	ND
Heather	1.01×10^6	3.36×10^5	5.31×10^6	9.99×10^5	3.89×10^6
Orange	1.12×10^5	8.11×10^6	ND	8.97×10^6	ND
Sage	2.66×10^5	8.82×10^6	1.02×10^6	1.27×10^6	ND
Chestnut	1.12×10^6	2.82×10^7	8.34×10^5	1.62×10^6	ND
Arbutus	1.64×10^5	2.01×10^6	6.98×10^7	2.99×10^5	2.19×10^7
Cotton	1.97×10^5	6.82×10^6	ND	1.87×10^6	ND
Vanilla	5.29×10^5	1.01×10^6	7.59×10^5	6.21×10^5	ND
Carob	6.98×10^5	1.42×10^6	ND	5.88×10^5	ND

Table 59: Suspect results from HS-SPME (Continued)

Analyte	Benzeneacetalde- hyde	Furan,2,5 Dimethyl	Furfural	Methyl Nonanoate	Methyl Octanoate
Pine	6.31×10 ⁶	6.23×10 ⁵	1.29×10 ⁷	2.42×10 ⁷	2.47×10 ⁷
Fir	2.89×10 ⁶	7.17×10 ⁵	2.01×10 ⁷	1.89×10 ⁷	1.23×10 ⁷
Oak	5.48×10 ⁶	2.89×10 ⁶	3.51×10 ⁶	1.57×10 ⁶	2.21×10 ⁷
Thyme	4.89×10⁷	ND	1.84×10 ⁷	1.41×10 ⁷	7.42×10 ⁶
Heather	1.77×10 ⁷	ND	5.89×10⁷	8.89×10 ⁶	6.41×10 ⁶
Orange	1.37×10 ⁷	ND	1.98×10 ⁷	7.64×10 ⁶	5.61×10 ⁶
Sage	3.93×10⁷	ND	1.30×10 ⁷	4.68×10 ⁶	9.87×10 ⁶
Chestnut	6.98×10 ⁶	2.17×10 ⁶	9.92×10 ⁶	1.67×10 ⁷	1.49×10 ⁷
Arbutus	4.89×10 ⁶	1.19×10⁷	9.01×10 ⁶	9.76×10 ⁶	5.89×10 ⁶
Cotton	1.82×10 ⁷	ND	2.84×10 ⁷	1.01×10 ⁷	8.63×10 ⁶
Vanilla	7.29×10 ⁶	1,89×10 ⁶	1.76×10 ⁷	2.34×10 ⁷	1.87×10 ⁷
Carob	6.02×10⁶	5.28×10⁵	1.09×10⁷	2.37×10⁷	1.16×10⁷

Table 60: Suspect results from Static HS

Analyte	α -Pinene	2,3 Butanediol	Ketoisophorone	2 Furancarboxal- dehyde ,5 Methyl	2-Hydroxy Iso- phorone
Pine	5.52×10^5	ND	ND	ND	ND
Fir	1.01×10^5	ND	ND	ND	ND
Oak	ND	1.04×10^7	ND	ND	ND
Thyme	ND	ND	ND	ND	ND
Heather	2.11×10^5	ND	ND	ND	ND
Orange	1.21×10^5	ND	ND	ND	ND
Sage	ND	ND	ND	ND	ND
Chestnut	ND	3.76×10^6	ND	ND	ND
Arbutus	ND	ND	1.09×10^6	ND	ND
Cotton	ND	ND	ND	ND	ND
Vanilla	ND	ND	ND	ND	ND
Carob	ND	ND	ND	ND	ND

Table 61: Suspect results from Static HS (Continued)

Analyte	Benzeneacetaldehyde	Furan,2,5 Dimethyl	Furfural	Methyl Nonanoate	Methyl Octanoate
Pine	ND	2.88 ×10 ⁵	0.89×10 ⁵	4.58×10 ⁵	2.84×10 ⁵
Fir	1.19×10 ⁵	7.49×10 ⁴	7.83×10 ⁵	2.01×10 ⁵	1.89×10 ⁵
Oak	ND	5.57×10 ⁵	ND	2.60×10 ⁵	3.01×10 ⁵
Thyme	9.87×10⁵	ND	1.12×10 ⁵	1.50×10 ⁵	1.15×10 ⁵
Heather	2.28×10 ⁵	ND	8.85×10 ⁵	1.67×10 ⁵	1.51×10 ⁵
Orange	2.12×10 ⁵	ND	2.76×10 ⁵	2.41×10 ⁵	1.02×10 ⁵
Sage	1.90×10⁶	ND	4.17×10 ⁵	1.95×10 ⁵	0.78×10 ⁵
Chestnut	4.49×10 ⁵	2.89×10 ⁵	6.23×10 ⁴	2.30×10 ⁵	2.77×10 ⁵
Arbutus	ND	9.89×10⁶	ND	1.30×10 ⁵	1.13×10 ⁵
Cotton	9.87×10 ⁴	ND	5.83×10 ⁵	1.99×10 ⁵	3.51×10 ⁵
Vanilla	ND	6.78×10 ⁶	4.23×10 ⁵	4.93×10 ⁵	4.13×10 ⁵
Carob	ND	ND	8.83×10 ⁴	2.28×10 ⁵	7.91×10 ⁴

Based on the results of suspect screening, with both static HS and HS-SPME, α -Pinene is more abundant in Pine samples, making it a potential marker of this variety. The absolute intensity of 2,3 Butanediol is significantly higher in Oak samples with both of these two methods. Moreover, while this analyte is detected in all botanical origins with HS-SPME, it is identified only in Oak and Chestnut samples with static HS. Similarly, Ketoisophorone is a potential marker of Arbutus honey with both of these two methods. In the case of 2 Furancarboxaldehyde,5 Methyl, this compound is identified in a higher absolute intensity in orange samples when HS-SPME is applying. On the contrary, when static HS is used, it is not identified in any sample. Furthermore, with HS-SPME, 2-Hydroxy Isophorone is identified in Arbutus and Heather samples. However, this analyte is undetectable with static HS. Moreover, the absolute intensity of Benzenacetaldehyde is higher in Sage and Thyme samples, while Furan,2,5

dimethyl seems to be more abundant in Arbutus honey. Finally, the absolute intensity of Furfural is higher in Heather samples while the esters Methyl Octanoate and Nonanoate were identified in all varieties.

Chapter 7: Conclusions

Both the static HS and HS-SPME methods seemed to be efficient for determining volatile compounds in honey. HS-SPME enabled us to identify hydrophilic compounds, such as organic and simultaneously provided us with improved LOD'S and LOQ's compared to static HS. Finally, several potential markers were identified through target and suspect screening, which may indicate the botanical origin of honey. More specifically, Isophorone is a potential marker of Arbutus honey, while β -Damascenone is a potential marker of Carob honey. Nonanal concentration is higher in Vanilla and Fir samples, while Benzaldehyde's concentration is higher in Thyme and Heather samples. Through suspect screening, Ketoisophorone and 2 Hydroxy Isophorone seemed to be a potential marker of Arbutus honey, 2,3 Butanediol of Oak honey, 2 Furancarboxaldehyde, 5 Methyl of Orange variety, while the concentration of Benzeneacetaldehyde was higher in Sage and Thyme samples.

ABBREVIATION AND ACRONYMS

Table 62: Abbreviations and acronyms

GC-MS	Gas Chromatography coupled to Mass Spectrometry
LR-MS	Low Resolution Mass Spectrometry
HS	Headspace
SPME	Solid Phase Microextraction
PDO	Protected Designation of Origin
LODs	Limits Of Detection
LOQs	Limits Of Quantification
MS	Mass Spectrometry
QqQ	TripleQuadrupole
QToF	quadrupole time of flight
TOF	Time-Of-Flight
EIC	Extracted Ion Chromatogram
ESI	ElectroSpray Ionization
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High-Performance Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry

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