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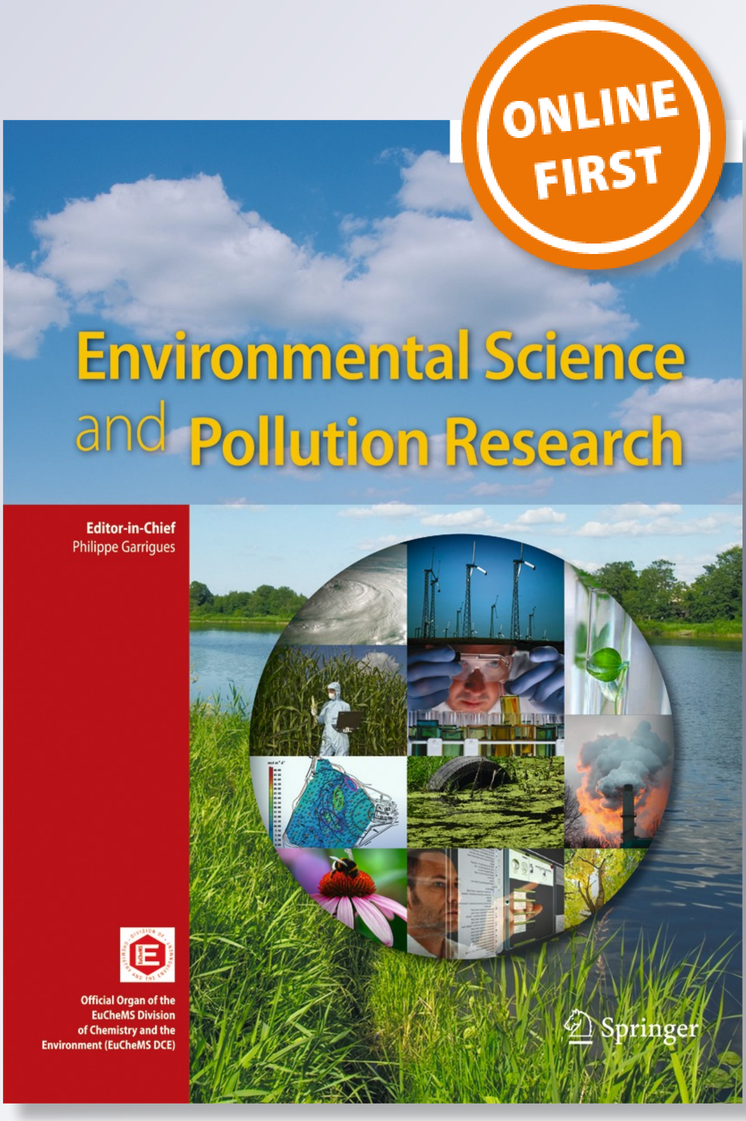

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ADVANCES & PROSPECTS IN THE FIELD OF WASTE MANAGEMENT

Assessing the volatile profile of carob tree (*Ceratonia siliqua* L.)

Andriana Krokou[1] · Marinos Stylianou[1] · Agapios Agapiou[1]

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Abstract

Biogenic volatile organic compounds (VOCs) contribute to the communication, growth, breeding, and defense of plant; their role in plant kingdom is vital. Carob tree is cultivated mainly in Middle East and eastern European countries (e.g., Spain, Italy, Greece, Cyprus) and lately in Australia, the USA, and South Africa. Therefore, it is examined as a case study for its volatile emissions in the environment. Apart from the VOCs emitted from carob flowers and fruit, carob is considered of great interest for the food industry (carob powder), not only for its health benefits but also due to its characteristic strong aroma, which can be maintained even after processing (roasting). Solid-phase microextraction/gas chromatography-mass spectrometry (SPME/GC-MS) analyses of carob flowers, fruit, and powder (commercial samples) were performed and the detected VOCs are presented and discussed. The most prominent chemical classes emitted from carob fruit and powder appeared to be acids followed by esters and aldehydes/ketones, whereas from carob flowers the terpenoids. The strongest VOCs both in carob fruits and powder were propanoic acid, 2-methyl (isobutyric acid) and in flowers ethanol. The uniqueness of carob benefits is well known in the agriculture, pharmaceutical, cosmetic, and food sector and is closely related to the agro-economy and long history of eastern Mediterranean countries.

Keywords Biogenic · VOCs · SPME/GC-MS · Emissions · Odor · Plantomics

Introduction

Ceratonia siliqua L., also known as carob tree, is a drought-tolerant tree of limited soil requirements as it thrives on various types of soil such as rocky, dry, and sloping, as long as it is lightly fertile, and can be penetrated by the root system. This evergreen long-lived tree belongs to the family of *Leguminosae*, originates from the Middle East, and is cultivated mostly in the Mediterranean coast. Nowadays, due to crop migration, carob tree is widespread almost all around the world (e.g., Spain, Italy, Morocco, Greece, Cyprus, Australia, South Africa, and the states of California and Arizona in the USA). Carob tree has a long ancient history; according to archeological evidence, the first charcoal from carob wood, dated to 8000–6000 BC, was found in Jericho (Israel) (Ramón-Laca and Mabberley 2004). A variety of carob cultivars exist worldwide; only in Spain, there are five cultivars, whereas in Greece two and in Cyprus three (Ministry of Agriculture Cyprus 2016). The main cultivars grown in Cyprus are *Kountourka*, *Koumpota*, and *Tilliria* (Batlle and Tous 1997). Carob cultivars usually differ in their morphological (e.g., shape, size), nutritional (e.g., flavor, nutrition content), and agronomical characteristics. Despite the cultivars' wide variation, Fourier transform infrared spectroscopy technique (FTIR) combined with chemometrics enabled to distinguish the carob origins and type (Christou et al. 2018). According to the Food and Agriculture Organization of the United Nations (FAO), the countries with the largest production of carob in 2016 were Portugal (40,385 t), Italy (28,925 t), Spain (26,185 t), Morocco (22,032 t), and Turkey (13,405 t), followed by Greece (12,150 t), Cyprus (8280 t), and Algeria (3257 t) (FAO 2016).

Biogenic volatile organic compounds (BVOCs) contribute to the growth, breeding, and defense of the plant. They help in the interconnection with various parts and the communication between other plants and between insects. Floral scents may function as long- and/or short-distance attractants, not only to pollinators but also to key insect pests. Since floral scent can be crucial in pollination, and therefore, in reserving seed or fruit set, the presence or absence of an attractive scent to the locally available insect pollinators may conflict the yield of

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agronomically essential crops, such as that of carobs (Custódio et al. 2006).

The flowering season of carob tree begins in the mid-September and is completed in November. Carob flowers are small and during the flowering season have been observed to attract a great number of insect visitors, which help in the pollination (Custódio et al. 2004, 2006). The scent of carob flowers is considered distinct and is mostly produced by the male flowers.

Carob has been used in the past as human food in famine and lately as animal fodder. In the last years, there has been an increasing interest in the use of plants rich in tannins (such as carobs) and their extracts in the diet of ruminants for improving the quality of their edible products. Literature studies on the effects of tannins on animal performance and quality of their products are controversial. Some results show that tannin-rich diet is effective in improving the fatty acid profile of meat and milk, increasing the level of health-beneficial fatty acids, as well as improving the oxidative stability of the products. On the other hand, the use of tannin-rich feed in animal diets demands great care, because of its possible harmful effects on animal performance and induction of metabolic disorders (Jerónimo et al. 2016). It is mostly cultivated for its edible pods, as well as a decorative tree in fields and streets, gardens, parking areas, and the sidewalks. Carob is a multipurpose food crop tree that produces large fruit pods, which consist of pulp (90%) and seeds (10%). Carob pulp is widely used in a variety of sectors, such as in food industry, where carobs are usually roasted between 120 and 180 °C and typically used in food and drinks (Boublenza et al. 2017). Due to its high sugar content (48–56%), the carob pulp is used in the production of traditional carob food and beverages and as a substitute for cocoa in cakes and sweets (Papaefstathiou et al. 2018). In addition, the endosperm of carob seeds can be extracted to yield the locust bean gum (LBG), also known as E410. LBG is mainly used as a thickening and stabilizing agent in food industry, as well as in cosmetics and pharmaceuticals. The production of bioethanol from carob pod by solid-state fermentation (SSF) and solid submerged fermentation using *Zymomonas mobilis* and *Saccharomyces cerevisiae* was also reported (Mazaheri et al. 2012; Saharkhiz et al. 2013). The extraction of sugars from the carob pods is a costly process; solid-state and solid submerged fermentations do not require sugar extraction; thus, these techniques are economical processes for bioethanol production. Carob pods are also well known for their health benefits (Goulas et al. 2016), their high pinitol content, and their characteristic strong persistent aroma.

In modern life, VOCs beyond their environmental applications are widely applicable in the areas of food, drinks, disease diagnosis, cosmetics, aromatherapy, personal hygiene, and fragrances. VOCs emitted from food are vital for the perception of odor and flavor; thus, their non-invasive detection and understanding are of high importance in the food industry. Various parameters may affect the emitted VOCs such as the microclimate, soil content, drought moisture, cultivars, and even the process of harvest. Although there have been a plethora of studies on non-volatile metabolites of carob (Loullis and Pinakoulaki 2018; Stavrou et al. 2018), very few studies exist on the sensory perception of carob aroma.

For the study of carob aroma, it was reported that solid-phase microextraction (SPME) technique is quite effective for the extraction of volatile compounds. SPME is a quick and powerful extraction green technique (solvents are not used), which enables the enrichment of VOCs from the headspace of the sample to the stationary coating phase of a fused silica fiber (Farag and El-kersh 2017). In this way, the sample is pre-concentrated. Then, by applying high temperature at the inlet of the gas chromatography (GC), the analytes are thermally desorbed and inserted to the GC for separation and subsequently to the mass spectrometer (MS) for chemical detection and identification (SPME/GC-MS). A number of factors affect the SPME sampling process and performance such as the selection of the fiber coating phase, the sample volume, and the extraction and desorption time, sample modifications (e.g., temperature, ionic strength). The use of SPME technique has been previously reported for the analysis of cocoa products' odor profile (Counet et al. 2004; Ducki et al. 2008), coffee (Masayuki et al. 2003; Akiyama et al. 2007), pecans (Gong et al. 2018), and wine (Perestrelo et al. 2012; Arcari et al. 2017). In the case of carobs, a limited number of studies were performed in order to decode the aroma profile of carob powder and flower. These studies are presented in detail in Table 1; it should be noted that in the literature, there are only qualitative data.

In the present study, the VOCs emitted from the different parts of the carob tree such as flowers, pods, and commercial samples of carob powder were analyzed using the SPME/GC-MS method. So far, a small number of studies, focused especially on powder VOCs, were reported (Table 1), whereas a more limited number exists on carob fruit and flower. Therefore, this is the first study focused on the carob cultivars originated from Cyprus. According to our knowledge, the aroma characterization of Cyprus carob cultivars is missing from the literature and this is the first study for its characterization.

Experimental part

SPME fibers 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) and 75 μm carboxen/polydimethylsiloxane (CAR/PDMS) (Supelco) were examined for the carob aroma analysis. The latter was eventually selected for the respective VOCs analysis. Before each analysis, the PDMS/DVB SPME fiber was conditioned for 30 min at 270 °C and CAR/PDMS for 30 min at 300 °C in the GC injection port.

Table 1 Analytical methods for the analysis of carob powder and flowers

Analytical method	Sampling	Results	Literature
SPME/GC-MS	SPME: 50 μm/30 μm divinyl benzene/carboxen/polydimethylsiloxane (DVB-CAR-PDMS)	31 VOCs (carob powder)	Farag and El-kersh 2017
GC-MS	Solvent extraction (pentane-dichloromethane 2:1) followed by liquid injection	137 VOCs (carob powder)	Cantalejo 1997
ITEX/GC-MS	ITEX (in-tube extraction)	12 VOCs (carob powder)	Racolta et al. 2014
GC-MS	Solvent extraction (2-methylbutane) followed by liquid injection	169 VOCs (carob powder)	Macleod and Forcen 1992
GC-MS and Aroma Scanner e-nose	SPME with a Hamilton 7000 series syringe	Acids, alcohols, aldehydes, furans, esters, ketones, pyrroles, pyrans, thiazoles, and sulfur compounds (carob powder)	Cantalejo 1999
SPME/GC-MS	SPME: polydimethylsiloxane (PDMS) 100 μm	25 VOCs (carob flower)	Custódio et al. 2006

The extraction time of VOCs from carob powder was examined (15, 30, 45, and 60 min) and the 30-min time was found as the optimum. Also, the sample volume of carob powder was tested (1 g, 3 g, and 5 g); the amount of 5 g was selected since the major compounds were detected in more abundance.

Initially, the commercial carob powder samples were sieved through a 125-μm molecular sieve. Five grams of the grounded carob pod was then placed in a 20-ml glass vial (Agilent) and sealed. The analysis of the sample was carried out the next day; the fiber was exposed to the headspace above the sample for 30 min at room temperature. Double sampling was performed along with a blank sample serving as a control.

Carob pods (294 g) were placed into 1-l in-house made glass jars. The analysis of the samples was carried out at different time intervals: days 1, 4, 8, 12, 18, 22, 26, 32, and 64. The SPME sampling procedure is described in detail above.

Carob flowers from three different trees were collected during mid-day (November 2017). Five grams of the flowers was placed in a 20-ml glass vial (Agilent) and sealed. The analysis was carried out the next day; SPME sampling is as described above.

SPME fiber CAR/PDMS was thermally desorbed at 280 °C for 1 min in the inlet of an Agilent GC 7890B/MS 5977B in split mode 1:10 for carob powder samples and splitless mode for the carob pods and flowers. VOCs were separated using an SPB-624 capillary column (60 m × 0.25 mm × 1.4 film thickness, Supelco). The GC conditions that were applied are the following: inlet 280 °C, column oven 35 °C for 5 min then at a rate of 4 °C/min to 180 °C and kept for 20 min. The carrier gas flow rate was set at 1.7 ml/min. The MS source, transfer line, and quadrupole temperature were 230 °C, 250 °C, and 150 °C, respectively. The MS was operated in the electron ionization (EI) mode at 70 eV and the scan range was set at 35–350 m/z. Volatile components were identified by retention time (R_t) relative to analytical standards of EPA 524 VOC Mix A (Supelco), isobutyric acid $\geq 99\%$ (Sigma), butyric acid $\geq 99\%$ (Aldrich), valeric acid $\geq 99\%$ (Aldrich), acetic acid $\geq 99.7\%$ (Sigma-Aldrich), and volatile free acid mix (Supelco), using the mass spectrum matching of library database. The library used for chemical identification was NIST11.

Results and discussion

Sampling optimization

The selection of SPME fiber coating, sample volume, and extraction time are important parameters for the SPME sampling process. So, these factors were examined in order to find the best experimental conditions. SPME fiber coatings CAR/PDMS and PDMS/DVB were examined and it was found that with CAR/PDMS, a greater amount of VOCs were extracted from the headspace of the carob samples; PDMS/DVB resulted in less extracted VOCs (Fig. 1a). Extraction time affects the mass transfer of the analytes on the fiber and finding the optimum time for the fiber to reach its equilibrium is necessary. The extraction was studied at four different time intervals (15, 30, 45, 60 min) and three different sample volumes (1 g, 3 g, 5 g). The key odorant compounds were extracted more at 5 g and 30 min (Fig. 1b, c), allowing these to be the optimum extraction values. All the analyses were conducted at room temperature, in order to resemble the natural emissions of the samples.

VOC analysis

The aroma profile of carob fruits, carob powder, and flowers was decoded using SPME/GC-MS analysis. Each part of the carob tree was found to emit a complex mixture of VOCs contributing to carob sustainability in the ecosystem and

Fig. 1 Comparison of the **a** SPME fiber coating, **b** carob sample volume, and **c** SPME extraction time that were studied

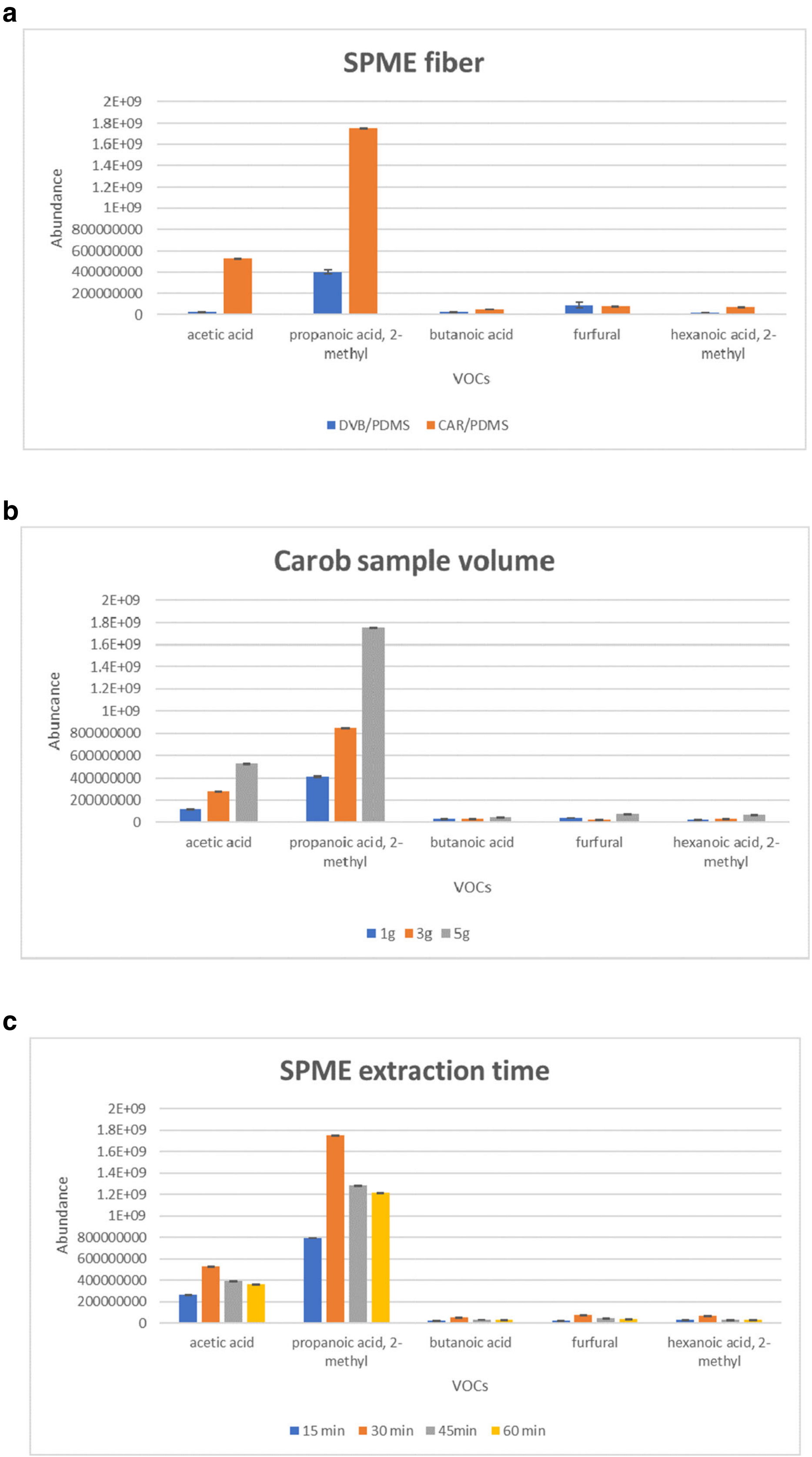

Table 2 VOCs found in carob fruit ($n = 7$) of Cyprus cultivars, commercial carob powder ($n = 6$), and carob flowers ($n = 4$)

Volatile organic compounds (VOCs)	Literature
Acetaldehyde* (C, CP)	
Cyclopropane ethyl (CP)	
Pentane (CP, CF)	
Ethanol (C, CP, CF)	
Furan (CP)	
Propanal (CP)	
Acetone (C, CP)	
Isopropyl alcohol (CP)	
Acetic acid, methyl ester (C, CP, CF)	
Methylene chloride (CP)	
Propanal, 2-methyl (C, CP)	
Methacrolein (CP)	
Furan, 2-methyl (C, CP, CF)	
Butanal (CP)	
2,3-Butanedione (C, CP)	CP (Cantalejo 1997)
2-Butanone (C, CP)	
Ethyl acetate (C, CP, CF)	
Methyl propionate (C, CP)	
Butanal, 3-methyl (C, CP, CF)	
Acetic acid (C, CP)	
Butanal, 2-methyl (C, CP)	
Propanoic acid, 2-methyl-, methyl ester (C, CP)	
Furan, 2-ethyl (C)	
2-Pentanone (C, CP)	CP (Cantalejo 1997)
Butanoic acid, methyl ester (C, CP)	CP (Cantalejo 1997)
Disulfide dimethyl (C, CP)	CP (Cantalejo 1997)
Acetoin (C, CP)	
Propanoic acid, 2-methyl-, ethyl ester (C, CP)	
Propanoic acid (C, CP)	CP (Farag and El-kersh 2017)
Toluene (C)	
Octane (C, CP, CF)	
Isobutyl acetate (CP)	
Butanoic acid, 2-methyl-, methyl ester (C, CP)	
Butanoic acid, ethyl ester (C, CP)	
2-Hexanone (C, CP)	
Propanoic acid, 2-methyl (C, CP)	CP (Cantalejo 1997; Racolta et al. 2014; Farag and El-kersh 2017)
Butanoic acid (C, CP)	CP (Cantalejo 1997; Racolta et al. 2014; Farag and El-kersh 2017)
Butanoic acid, 2-methyl, -ethyl ester (C, CP)	
Furfural (CP)	CP (Cantalejo 1997; Racolta et al. 2014; Farag and El-kersh 2017)
1-Butanol, 3-methyl-, acetate (C)	
Butanoic acid, 3-methyl (C, CP)	
Butanoic acid, 2-methyl (C)	
2-Heptanone (C, CP)	CP (Cantalejo 1997; Racolta et al. 2014)
Propanoic acid, 2-methyl-, 2-methylpropyl ester (CP)	
Hexanoic acid, methyl ester (C, CP)	
3-Hepten-2-one (C, CP)	CP (Cantalejo 1997)
Hexanoic acid, ethyl ester (C, CP)	

Table 2 (continued)

Volatile organic compounds (VOCs)	Literature
Benzaldehyde (C, CP)	CP (Cantalejo 1997; Racolta et al. 2014; Farag and El-kersh 2017)
Butane, 1,1′-oxybis[3-methyl] (C)	
2-Octanone (C)	
Propanoic acid, 2-methyl-, 2-methylbutyl ester (C, CP)	
b-Pinene (CF)	
a-Phellandrene (CF)	
a-Pinene (CF)	
d-Limonene (C, CP, CF)	CF (Custódio et al. 2006)
o-Cymene (C, CP, CF)	
γ-Terpinene (CF)	
Eucalyptol (CF)	
Hexanoic acid (C, CP)	CP (Cantalejo 1997; Farag and El-kersh 2017)
2(3H)-Furanone, dihydro-3-methyl (CP)	CP (Racolta et al. 2014)
2-Nonanone (C, CP)	CP (Cantalejo 1997)
Linalool (CF)	CF (Custódio et al. 2006)
Nonanal (C, CP, CF)	
Allo-ocimene (CF)	
Ethanone, 1-(1H-pyrrol-2-yl) (CP)	
2H-Pyran-2-one, tetrahydro-6-methyl (CF)	
Ethyl benzoate (CF)	
Linalool oxide (CF)	CF (Custódio et al. 2006)

**C*, carob fruit; *CP*, carob powder; *CF*, carob flower

agriculture ecology. The VOCs presented in Table 2 were identified in all examined samples (100% identification). Some of them, such as toluene and methylene chloride, are probably of exogenous origin. Representative chromatograms of carob fruit, powder, and flower are shown in Fig. 2a, b, c, respectively, whereas their pie charts are shown in Fig. 3a, b.

Carob fruit VOCs

The aroma of carob fruit is due to the emission of more than 45 VOCs. These VOCs were monitored in time (day 1–day 64) to verify the strong and perceptive aroma of carobs. The most abundant VOCs emitted from carob fruit are shown in Table 2, where in Fig. 2 a, a representative SPME/GC-MS chromatogram is presented. According to our knowledge, the volatile emissions from carob fruit were not reported in previous studies. The few studies existed are focused only on the carob powder VOCs (the "Carob powder VOCs" section). Following the carob fruit pie chart (Fig. 3a), the aroma profile of the fruit is mainly due to the presence of acids and esters; these classes are responsible for the 96% of the emitted VOCs. The most dominant VOCs emitted from carob fruit belong to the chemical group of acids and include the acetic acid, propanoic acid, 2-methyl (isobutyric acid), butanoic acid, and hexanoic acid, whereas from the class of esters are the propanoic acid 2-methyl-, methyl ester, butanoic acid methyl ester, hexanoic acid methyl ester, and propanoic acid, 2-methyl-, 2-methylbutyl ester. Among them, isobutyric acid was the most abundant peak; its concentrations ranged between 11 and 36 $\mathrm{ppb_v}$ (external calibration curve, $R^2 = 0.999$). Carob fruit is richer in acids (81.6%) compared with carob powder (72.6%) and poorer in esters (14.2%) and aldehydes/ketones (3.7%). This can be explained on the basis that the whole fruit is analyzed, compared with the powder. The main difference with carob powder VOCs is the addition of the thermal process stage (roasting) of the fruit particles in order to produce the carob powder.

Carob powder VOCs

The carob powder appears to be the richest source of VOCs compare with fruit and flower. Figure 2b shows a representative chromatogram of carob powder. The most abundant VOCs emitted from the carob powder are presented in Table 2; more than 50 VOCs were identified. In carob powder, propanoic acid, 2-methyl, butanoic acid, pentanoic acid, hexanoic acid, furfural, and heptanoic acid were previously detected with SPME/GC-MS analysis (Farag and El-kersh 2017) and with in-tube extraction (ITEX) coupled with GC-MS (Racolta et al. 2014). In general, the present results are in

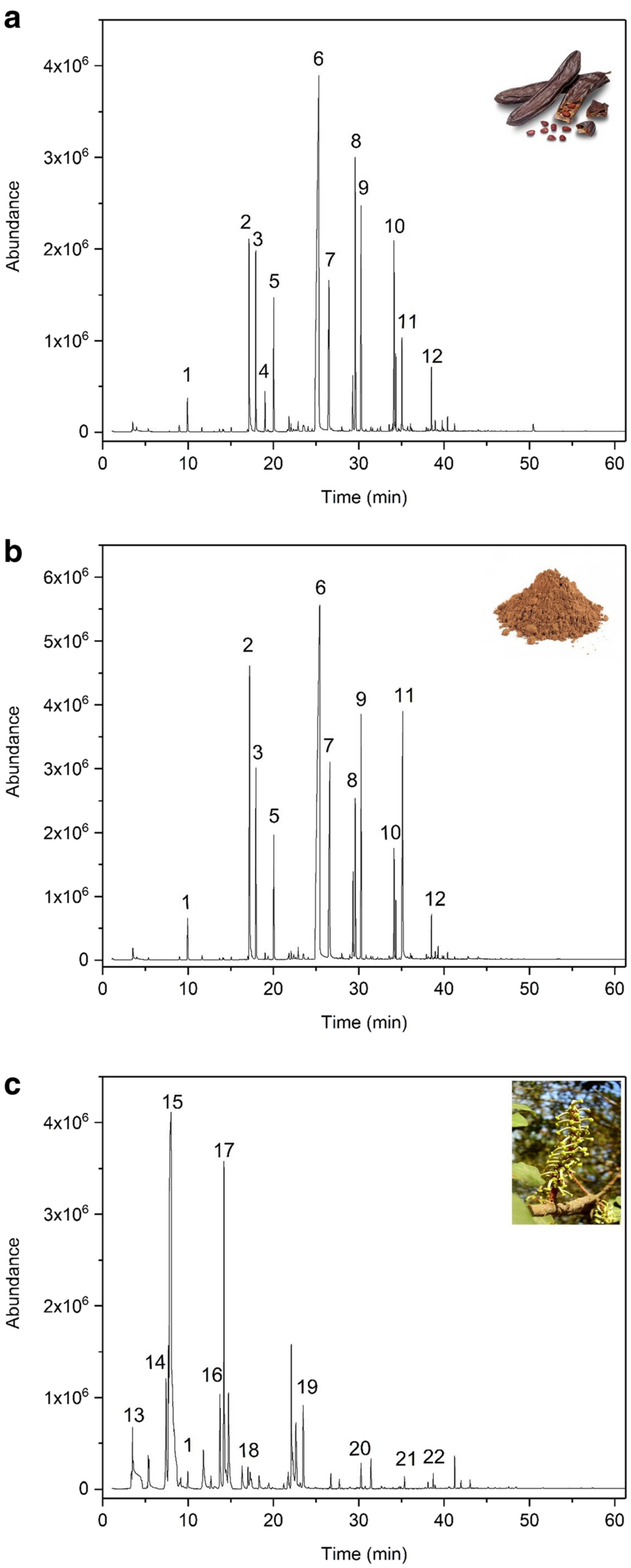

Fig. 2 SPME/GC-MS chromatogram of **a** carob fruit, **b** powder, and **c** flower

agreement with the literature studies presented in Table 1. The most abundant volatile chemical groups that distribute to the aroma of carob powder are acids followed by esters and aldehydes/ketones (Fig. 3b). Isobutyric acid, which is the most abundant compound in the aroma profile of carob powder, is responsible for the sweet and buttery flavor; its concentration in the commercial carob powder samples was ranged between 22 and 28 ppb_v. The roasting process appears to affect negatively the concentration of isobutyric acid in the carob kibbles; however, this depends on the time and the heating temperature (Berna et al. 1997). The fruity, floral, woody, and almond flavor is a result of the presence of furfural (Racolta et al. 2014).

Furan and its derivatives (e.g., 2- and 3-methyl furans, furfural) probably originated from the thermal processing of carob powder. Generally, these chemical contaminants naturally form during heated food processing, including cooking (present in cooked or heated foods). According to the European Food Safety Authority (EFSA), furans are formed from a variety of substances naturally present in food including vitamin C, carbohydrates, amino acids, unsaturated fatty acids, and carotenoids. The cooking or processing conditions help to determine the quantity of furan that is formed and as well lost (due to evaporation), along with how much is present when food is consumed.

Moreover, EFSA reported that consuming regularly furan or its derivatives through dietary could lead to long-term liver damage (EFSA 2017).

Carob flower VOCs

An indicative SPME/GC-MS chromatogram of carob flower is presented in Fig. 2c. The most common VOCs emitted from carob flower are summarized in Table 2. The majority of the detected VOCs are terpenoids. More than 20 VOCs were identified (Table 2) in the headspace of carob flowers; some of them such as linalool, linalool oxide, d-limonene, a-pinene, and b-pinene were previously detected with SPME/GC-MS analysis (Custódio et al. 2006). The study of carob flower VOCs presents ecological interest, as this way, plants are communicating with the outside and inside world.

According to Knudsen et al., a dozen of VOCs are considered the most common compounds emitted in floral scents. The majority of them are monoterpenes such as limonene, (E)-ocimene, myrcene, linalool, a-pinene, and b-pinene, followed by benzaldehyde, methyl 2-hydroxybenzoate, benzyl alcohol, 2-phenyl ethanol, caryophyllene, and finally the terpene 6-methyl-5-hepten-2-one. Some of these volatile moieties were also detected in the present study (e.g., linalool, a-pinene, b-pinene, and limonene). The widespread distribution of these substances suggests alternative and synergistic roles, in addition to pollinator attraction. The primary function of floral scent in flowering plants is to attract and guide pollinators. However, additional functions may be ascribed, including plant defense and protection against abiotic stresses (Knudsen et al. 2006).

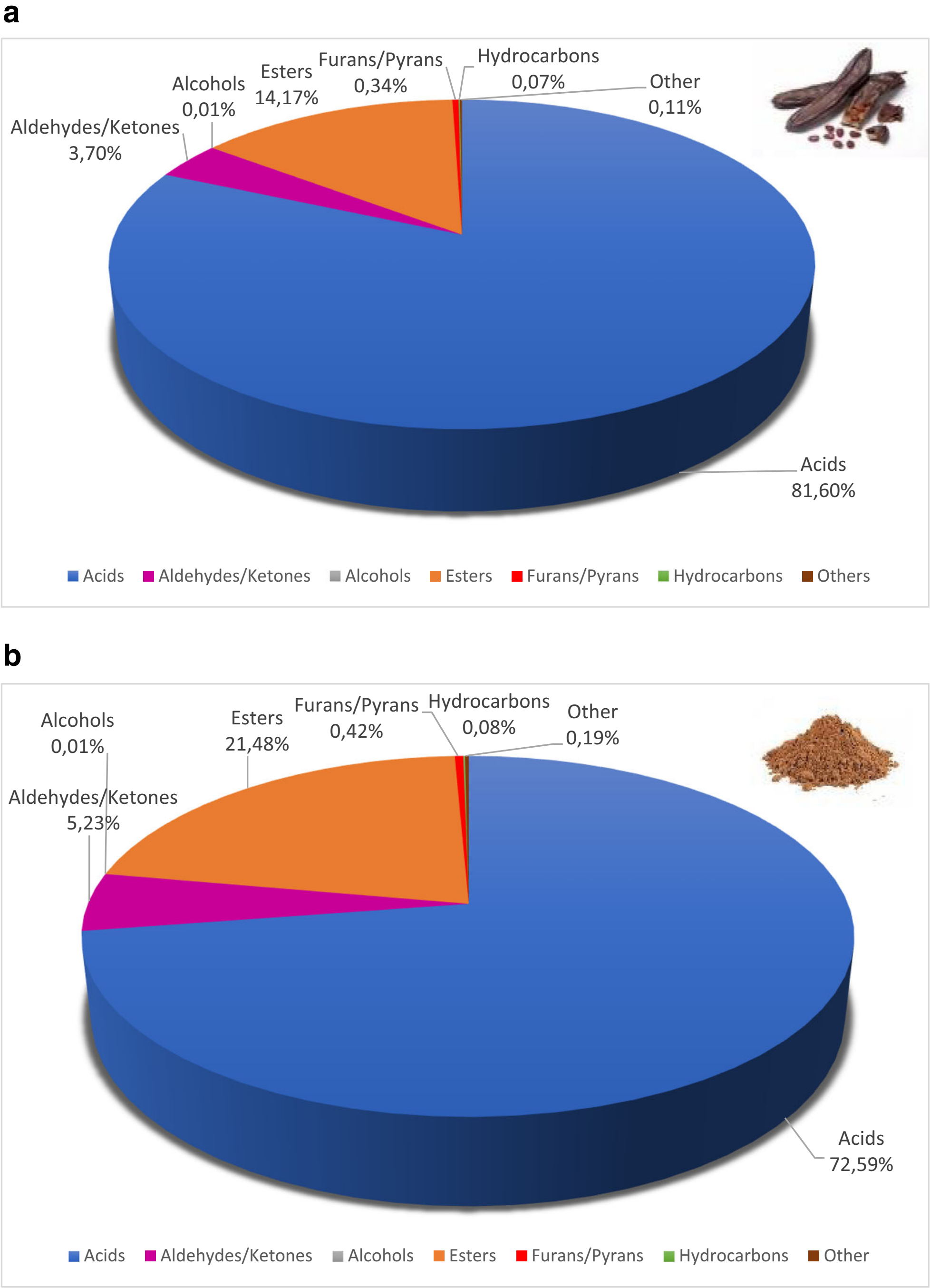

Fig. 3 Pie chart of **a** carob fruit and **b** carob powder VOCs

Carob leaves

Carob leaves are not known for their aroma profile, but for their associated beneficial health effects. Recently, a phytochemical isolation study on the leaves of carob tree in Australia was performed. Carob leaf extracts have shown antibacterial, antioxidant, and antiproliferative activity towards hepatocellular carcinoma cells, colon cancer cells, and the gastrointestinal tract (Deans et al. 2018). Another study revealed that the methanolic extract of carob leaves inhibited the growth of *Listeria monocytogenes* (Aissani et al. 2012). Thus, carob leaves can be a promising natural replacement of antimicrobial chemicals for food safety and preservation.

Agronomic and environmental importance

Although carob is a great decorative plant, it is even more important as a forest tree. It prevents the spread of fire, as opposed to pine and is suitable for afforestation. As long as the commercial role of carob bean is currently degraded, its environmental role is enlightened, because it can survive in barren and dry limestone soils (Morton 1987). Many areas owe their green color to the locust beans, while at the same time, their rich root system retains and protects the soil from erosion. Carob can cover abandoned or barren and bushy areas, even rocky soils. Reforestations in these areas can stop erosion, change the physiognomy of areas, provide new opportunities, and make places more attractive. As climate change is ahead, these advantages make carobs ideal candidate trees. Carob processing shows limited zero waste compared with other common Mediterranean species, as for example, the olive tree (olive mill wastewater). According to Geraldo et al. (1990), carob trees can absorb 15.56 t CO_{2eq}/ha (Geraldo et al. 1990). However, since they are particularly deep rooted and a considerable proportion of their biomass may exist below ground (especially in mature trees), their potential carbon dioxide storage is probably underestimated.

Conclusions

VOCs evolved from plants play a very important role, serving a wide array of processes in nature. *Ceratonia siliqua* L. is being examined as a case study, because of its direct relation with the history, agriculture, medicine, and food culture of eastern Mediterranean countries. The VOCs emitted from the carob tree (i.e., pods, flower, powder) were determined to serve as a link between the socioeconomic and cultural backgrounds (tradition), as well as the modern food and agriculture trends. In both carob fruit and powder, acids were the most dominant volatile class followed by esters, whereas in carob flower the terpenoids. Similarly, the most abundant VOC in both fruit and powder was isobutyric acid, whereas in flower ethanol.

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