# Journal of Entomology

ansinet
Asian Network for Scientific Information

OPEN ACCESS Journal of Entomology

ISSN 1812-5670
DOI: 10.3923/je.2019.98.107

# Research Article
# Identification and Discrimination of the Developmental Stages of Two Mosquito Vectors, *Aedes caspius* and *Culex pipiens* by Using Cuticular Hydrocarbons Analysis

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## Abstract

**Background and Objective:** Although the usage of cuticular hydrocarbons profiles in differentiating between mosquitos' species and subspecies, their role in identification and differentiation of the developmental stages is scarce. The objective of this study was to determine differences in cuticular hydrocarbons ($CHC_S$) among developmental stages of *Aedes caspius* and *Culex pipiens* mosquito vectors in order to examine their reliability in mosquito classification. **Materials and Methods:** Mosquito immatures were collected from breeding habitats found around Al-Hfouf Oasis, eastern Saudi Arabia by the aid of special long aquatic net and chemical analysis of cuticular compounds was performed by gas chromatography. **Results:** Eleven hydrocarbons, n-alkanes, with carbon numbers from C9-C19 were identified from the developmental stages of both mosquito species and their levels varied significantly among these stages. The variation was quantitative rather than qualitative and the major n-alkane in all developmental stages was C12 except for *C. pipiens* females (C11) whilst the lowest one was differed according to the stage. **Conclusion:** The present findings reporting qualitative differences in $CHC_S$ among developmental stages of *A. caspius* and *C. pipiens* mosquitoes and supporting their application and reliability as a marker to assist in identifying the immature stages of mosquitoes.

Key words: Mosquitoes, developmental stages, *Aedes caspius*, *Culex pipiens*, cuticular hydrocarbons analysis, Saudi Arabia

Citation: Essam Abdel-Salam Shaalan, Mohamed A. El-Kersh and Zeinab Abdelmoaty, 2019. Identification and discrimination of the developmental stages of two mosquito vectors, *Aedes caspius* and *Culex pipiens* by using cuticular hydrocarbons analysis. J. Entomol., 16: 98-107.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Mosquitoes transmit serious infectious diseases such as malaria, dengue and yellow fever that kill and weaken millions of people yearly[1]. Identification of these vectors relied for long time on morphological features of both immature stages, including larvae and pupae and adult stage. Due to much variation in such morphological characters and sometimes damaging of preserved samples of the mosquito species particularly mosquito vectors, problems in proper identification have been aroused. Additionally, mosquito species complex such as *Anopheles gambiae*, *Culex pipiens* and *C. univittatus* is another morphological identification challenge resulting from morphological similarities among their species. The only reliable method of mosquito identification and other insects at the present time is depending entirely on chromosome analysis. Recently, the cuticular hydrocarbon analysis is used in chemical taxonomy[2].

Insect cuticle is a hard outer body cover providing support and protection from desiccation, ultraviolet radiation and pathogenic microorganisms[3]. It consists of a number of layers that differ in their physico-chemical characters. The outermost waxy layer is composed of hydrocarbons, free fatty acids, free alcohols, wax esters, glycerides, sterol esters and aldehydes[4]. Cuticular hydrocarbons (CHCs) generally represent the most abundant lipids on insect cuticle and play major role in semiochemical functions[5]. They play a major role in species and mate recognition in various insect groups[6] such as *Drosophila*[7], termites[8], *A. gambiae* complex[9], blow flies[10] and other medically important Diptera[11] as well as female sexual receptivity in *A. gambiae* and *A. aegypti*[12]. Furthermore, it has been used to determine age and sex among mosquitoes such as *A. gambiae*[4,13], *A. aegypti*[14,15] and age and survivorship among three Australian mosquito vectors *A. aegypti*, *A. farauti* and *Ochlerotatus vigilax*[16].

In addition to the afore mentioned usage of cuticular hydrocarbons profile in mosquito chemo-ecology research, they were also useful for the identification of mosquito species, siblings allopatric populations[13,14,17-23]. In contrast, literatures revealed scarcity of the role of cuticular hydrocarbons analyses in the identification and differentiation of the immature stages, larvae and pupae, of mosquitos. Only, a few investigations were conducted on mosquito larvae but no studies are available on mosquito pupae. Three investigations were conducted on *Anopheles* larvae to differentiate between larval stages of *A. gambiae*, *A. arabiensis* and *A. melas*[24], *A. gambiae* strains[9] and between larval stages of *A. gambiae* and *A. arabiensis*[20]. Contrarily, only one was carried out on larvae of *C. pipiens*[25]. Compared to other insects, there are several recent studies on other insects in particular forensically important insects such as blow flies[26-29], flesh flies[30,31] and social insects including ants[30,32-34], bees[35] and termites[30,36].

Accordingly and for the first time, the present work was conducted to differentiate between immature stages within and between two mosquito vectors *A. caspius*[37] and *C. pipiens*[38] as well as sexes of the latter species collected from Al-Hfouf oasis, eastern Saudi Arabia utilizing cuticle hydrocarbon analysis. Furthermore, to ascertain the reliability of cuticular hydrocarbon technique for the identification of the immature stages of mosquitoes.

## MATERIALS AND METHODS

**Mosquitos' collection and identification:** Immature stages of mosquitoes "larvae and pupae" were collected from breeding sites found around Al-Hfouf oasis by the aid of special aquatic nets. Information on collecting locations and specimens are shown in Table 1. These stages were divided into two halves, the first one was preserved in 70% ethanol for species identification whilst the second half was kept in the same breeding field water inside plastic pans at 25°C, 75-80% RH and 12L: 12D photoperiod cycle and fed on aquarium fish food. Such pans were placed in plastic rearing cages for adult emergence. Morphological identification was done with the aid of light microscope using keys of Harbach[39] and Al Ahmad *et al.*[40] whereas, both emerged adult mosquitoes and immatures were identified as *A. caspius* and *C. pipiens*. Fresh and alive batches of newly emerged one day old 4th larval instar, pupae and adults were selected for cuticular hydrocarbons analysis.

Table 1: Collecting locations of immature stages of mosquitoes in Al-Hfouf oasis, Eastern Saudi Arabia

| | Mosquitoes | | | | | |
|---|---|---|---|---|---|---|
| | *Aedes caspius* | | *Culex pipiens* | | | |
| Collection information | Larvae | Pupae | Larvae | Pupae | Males | Females |
| Location | Ash-Shu'bah 28°59'N/44°49'E | | Al-Battaliyah 25°26'N/49°38'E | | | |
| Method of collection | Larval collection by the aid of special aquatic nets | | Emerged from collected larvae | | | |
| Number of specimens | 105 | 70 | 194 | 86 | 53 | 33 |
| Date of collection | March 2015 | | | | | |

Table 2: MRM method setup using SRM

| n-alkanes | RT (min) | Width (min) | Start (min) | Stop (min) | Precursor mass (m/z) | Product mass (m/z) | Collision energy (eV) |
|---|---|---|---|---|---|---|---|
| C9 | 3.88 | 1.50 | 3.13 | 4.63 | 128.17 | 57.12 | 10.00 |
| C10 | 5.07 | 2.00 | 4.07 | 6.07 | 142.18 | 57.12 | 10.00 |
| C11 | 6.25 | 2.00 | 5.25 | 7.25 | 156.20 | 57.12 | 10.00 |
| C12 | 7.32 | 2.00 | 6.32 | 8.32 | 170.23 | 57.12 | 10.00 |
| C13 | 8.32 | 2.00 | 7.32 | 9.32 | 184.23 | 57.12 | 15.00 |
| C14 | 9.24 | 2.00 | 8.24 | 10.24 | 198.25 | 57.11 | 15.00 |
| C15 | 10.12 | 2.00 | 9.12 | 11.12 | 212.23 | 57.12 | 15.00 |
| C16 | 10.94 | 2.00 | 9.94 | 11.94 | 226.29 | 57.11 | 15.00 |
| C17 | 11.71 | 2.00 | 10.71 | 12.71 | 240.31 | 57.13 | 15.00 |
| C18 | 12.44 | 2.00 | 11.44 | 13.44 | 254.34 | 57.12 | 15.00 |
| C19 | 13.14 | 2.00 | 12.14 | 14.14 | 268.34 | 57.12 | 15.00 |
| C20 | 13.81 | 2.00 | 12.81 | 14.81 | 282.32 | 57.12 | 15.00 |

**Extraction of cuticular hydrocarbons:** Cuticular hydrocarbons were extracted according to the method described by Suarez *et al.*[4], Anyanwu *et al.*[9], Phillips *et al.*[11] and Caputo *et al.*[21]. Ten individuals from 1 day old larvae, pupae and adults were immersed in 10 mL n-hexane to extract the CHCS by gently swirl for 5 min at room temperature. Each sample was transferred through a silica gel column (2 cm, 70e 230 mesh, 60 A°) to obtain only hydrocarbon compounds and was washed with 1 mL of n-hexane. Each sample was collected in a 1 mL glass vial. The n-hexane was removed and the samples were re-suspended in 2 µL n-hexane and the entire extract was injected into the capillary column of TSQ 8000 GC-MS/MS.

**GC-MS/MS Analysis of cuticular hydrocarbons:** Analysis was performed on Thermo Fischer Scientific, Triple quadrupole MS instrument, TSQ 8000 gas chromatograph-mass spectra (GC-MS/MS) with Programmable Temperature Vaporizing (PTV) split less injector at 300°C. We used a HP-5MS, 30 m fused silica capillary GC column with 0.25 mm internal diameter and 0.25 µm film thickness. Electron ionization (EI) method was used for MS at 70 eV with 300°C source temperature and electron multiplier voltage at ~2000 eV, detector gain $3.1 \times 10^6$. The GC injection port temperature was maintained at 300°C and the samples were injected in the split less mode, followed by a purge of 1min after injection. The GC was paired to a computer and data were processed with Xcalibur software. A microliter was injected into the column and elution was performed with helium as a carrier gas at 1 mL $min^{-1}$. The initial temperature of the oven was about 65°C for 2 min, followed by a ramp at 15°C $min^{-1}$ to 250°C and sec ramp at 8°C $min^{-1}$ to 300°C and held in that temperature for 2 min.

**Hydrocarbons identification:** Alkanes (from C9-C20) were purchased from Sigma Aldrich. The super position of chromatograms of all other peaks were used with fingerprint method. The proximity of each peak was undertaken using a combination of visual pattern recognition and retention time data. Qualitative analysis was performed with a selected reaction monitoring (SRM). Three different concentrations including 100, 200 and 300 ppm of n-alkane standards (C9-C20) were injected with every set of samples. Qualification of n-alkanes probability was made by comparing them with those of standard retention time and mass to charge ratio (m/z) of hydrocarbons analytes. Quantification was made by comparing the peak area of the sample with that of the standard. As a result of the SRM program, the generated SRM transition is shown in Table 2. The table represents at the same time the system method of TSQ 8000 GC-MS/MS MRM acquisition using timed modes-SRM with a short 60s acquisition window around the compound retention time. No other setting of scan segments is necessary or required in case additional compounds should be added to acquisition, other than the compound retention time.

**Statistical analysis:** Differences in cuticular hydrocarbons quantities were analyzed using a one-way ANOVA and the Tukey HSD *post hoc* test. Hierarchical cluster analysis was applied using Euclidean distance to prepare dendrogram assessing the relationship of the hydrocarbon profiles among the developmental stages. Statistical analysis were performed utilizing SPSS statistical package ver. 16.

## RESULTS

Eleven aliphatic cuticular hydrocarbons (CHCS) with chain lengths $C_9$-$C_{19}$ were totally recognized at retention time (RT) 3.85-14.64 in the chromatogram profiles of the immature stages, larvae and pupae, of the two mosquito species *A. caspius* and *C. pipiens* as well as both sexes of *C. pipiens* species (Table 3). Peaks in Fig. 1 showed the characteristic retention time (RT) for the developmental stages of both mosquito species.

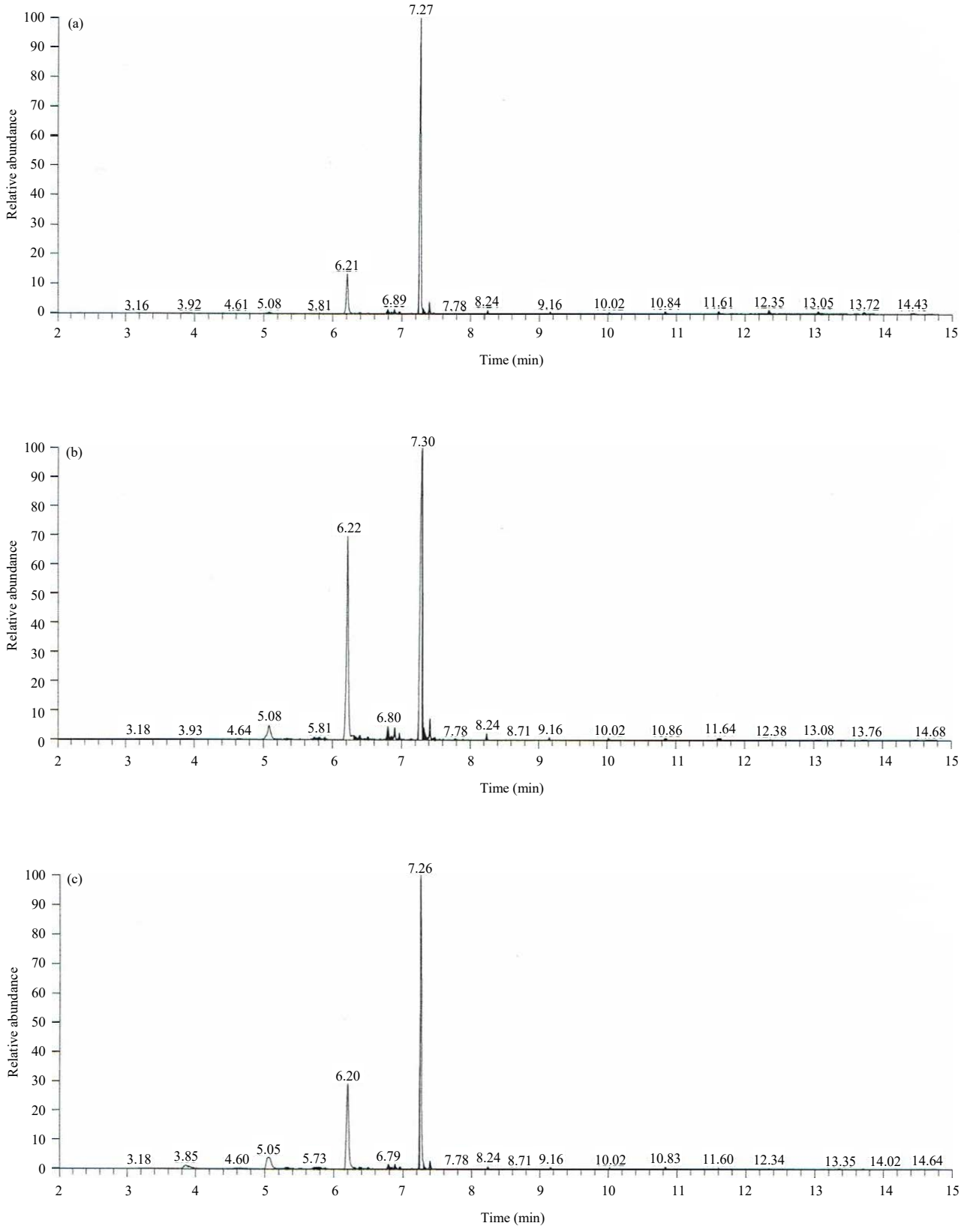


Fig. 1(a-f): Continue

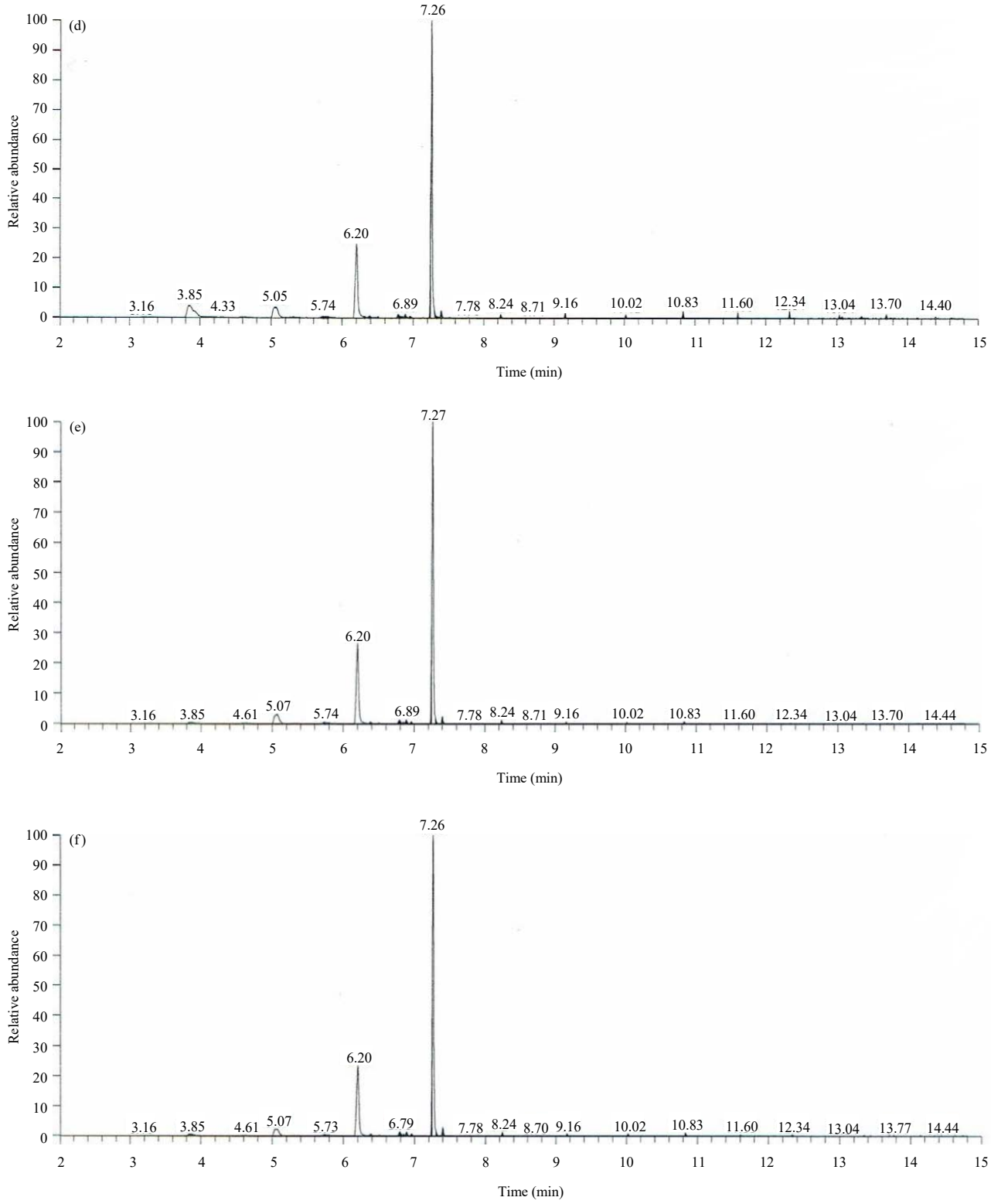


Fig. 1(a-f): GC profile, indicating hydrocarbons relative abundance against retention time (min) for the mosquito species of Al-Hfouf Oasis, King Saudi Arabia. (a) *Aedes caspius*-larvae, (b) *Aedes caspius*-pupae, (c) *Culex pipiens*-larvae, (d) *Culex pipiens*-pupae, (e) *Culex pipiens*-adult females and (f) *Culex pipiens*-adult males

The precise retention time of each hydrocarbon is shown on the relevant peak

Table 3: Hydrocarbon masses based on integratable peaks of *Aedes caspius* and *Culex pipiens* mosquitoes collected from Al-Hfouf Oasis, Eastern Saudi Arabia

| | Mean/individual (ppm±SE) | | | | | |
|---|---|---|---|---|---|---|
| | *Aedes caspius* | | *Culex pipiens* | | | |
| n-alkanes | Larvae | Pupae | Larvae | Pupae | Female | Male |
| C9[a] | 4.90±0.51[b] | 47.07±2.34 | 2.74±0.046[bc] | 3.84±0.25[bc] | 85.19±0.68 | 19.46±1.8 |
| C10[a] | 13.05±0.58[b] | 740.81±1.76 | 15.77±1.31[bc] | 6.96±0.48[bc] | 890.98±3.1 | 144.04±0.60 |
| C11[a] | 242.98±1.4 | 7405.75±3.03 | 65.26±2.97 | 24.97±0.64 | 4253.27±1.81 | 818.43±3.1 |
| C12[a] | 1536.64±3.08 | 16215.28±1.81 | 162.39±2.17 | 69.98±1.09 | 2069.77±1.45 | 2496.00±2.33 |
| C13[a] | 27.80±0.63 | 277.76±4.5 | 2.02±0.02[b] | 0.16±0.006[b] | 200.67±2.48 | 47.37±1.29 |
| C14[a] | 14.11±0.69 | 117.09±0.61 | 0.53±0.017[b] | 0.26±0.011[b] | 87.31±1.27 | 27.52±0.68 |
| C15[a] | 11.46±0.55 | 86.62±1.33 | 0.12±0.005[b] | 0.51±0.017[b] | 70.15±1.64 | 25.77±1.5 |
| C16[a] | 30.86±1.3[c] | 158.74±4.81 | 1.42±0.017[b] | 1.80±0.057[b] | 120.15±1.22 | 45.23±2.37[c] |
| C17[a] | 54.92±1.43 | 399.39±2.41 | 0.85±0.017[b] | 1.59±0.046[b] | 25.77±0.8 | 11.22±0.3 |
| C18[a] | 90.58±1.34 | 169.09±1.2 | 0.79±0.005[b] | 3.08±0.04[b] | 64.75±2.07 | 20.55±1.32 |
| C19[a] | 62.21±2.95 | 173.01±0.58 | 0.13±0.005[b] | 1.94±0.03[bc] | 34.02±0.58 | 8.87±0.41[c] |

[a]Hydrocarbons found in all developmental stages, [b,c]Insignificant difference in the hydrocarbons level (p>0.005)

The hydrocarbon of C12 had the highest concentrations detected in all stages except for the females of the *C. pipiens* whilst the lowest one varied according to stage (C9 in larval and pupal stages of *A. caspius* and C15, C13, C17 and C19 in larvae, pupae, females and males of *C. pipiens*, respectively).

Qualitatively, results indicated the presence of high degree of similarity between the immature stages of the two mosquito species, where along chain of hydrocarbons (CHCS) lengths C9-C19 have been extracted. Quantitatively in contrast, results showed different trend. Quantity of CHCs of chain lengths C10-C14 are larger in larval than pupal stage of *C. pipiens* mosquitoes whilst quantity of CHCS lengths C9 and C16-C19 are larger in pupal than larval stage (Table 3). The contrary completely happened in *A. caspius* whereas, quantity of all hydrocarbons, C9-C19, are larger in pupal than larval stage (Table 3). According to sex, results (Table 3) indicated that quantities of all hydrocarbons are larger in female than males with the exception of C12.

Significant difference in the hydrocarbons quantities were found between immature stages of both mosquito species (all hydrocarbons of *A. caspius* larvae are larger than *C. pipiens* larvae except for C10 as well as all hydrocarbons of pupae in the first species that are the larger).

The statistical analysis (Table 3) showed significant difference in the levels of CHCs between immature stages of *A. caspius* ($p = 0.05$, $df = 5$) whilst insignificant difference ($p>0.05$, $df = 5$) were found between immature stages of *C. pipiens* in the levels of all CHCs except for C11-12. Unlike immature stages, all hydrocarbons levels in both sexes of *C. pipiens* differed significantly ($p = 0.05$). With the exception of three hydrocarbons (C9-10,16) from larval stage, immature stages of *A. caspius* mosquitoes significantly differ in the hydrocarbons levels from both immature and adult stages of *C. pipiens* (*post hoc* test, Tukey HSD).

Cluster analysis in Fig. 2 showed the relationships between chemical profiles of the developmental stages of the same mosquito species as well as between the two mosquito species.

## DISCUSSION

The present study revealed that 11 aliphatic cuticular hydrocarbons with chain lengths C9-C19 were identified in larval and pupal stages of both mosquitoes as well as both sexes of *C. pipiens* species. Furthermore, quantitative rather than qualitative differences have been also detected in these developmental stages of both mosquito species and both sexes of *C. pipiens* species. The statistical analysis showed significant differences in CHCS quantities among all stages except for larval and pupal stages of *C. pipiens* (C9-10, 13-19), a couple of hydrocarbons (C9-10) between *A. caspius* larvae and immature stages of *C. pipiens*, C16 between *A. caspius* larvae and males of *C. pipiens* and C19 between pupae and males of *C. pipiens*.

Results indicated quantitative differences in the compounds found in both larval and pupal stages of the same mosquito species and between both mosquito species (Table 3). The difference in the quantity of CHCS between larval and pupal stages of *A. caspius* is statistically significant with larger tendency towards pupal stage. In contrast, insignificant statistical difference in CHCS quantities was found between larval and pupal stages of *C. pipiens* for all hydrocarbons with the exception of C11 and C12. Although this the first study for the immature stages of *A. caspius* mosquito, the CHCS of *C. pipiens* larvae has been evaluated through only one study by Amira *et al*.[25] but no studies are available for the pupal stage. Even though both studies showed that the CHCs classes of *C. pipiens* larvae are consisted of n-alkanes that differ quantitatively, they showed differences in both retention time (RT) and the hydrocarbons

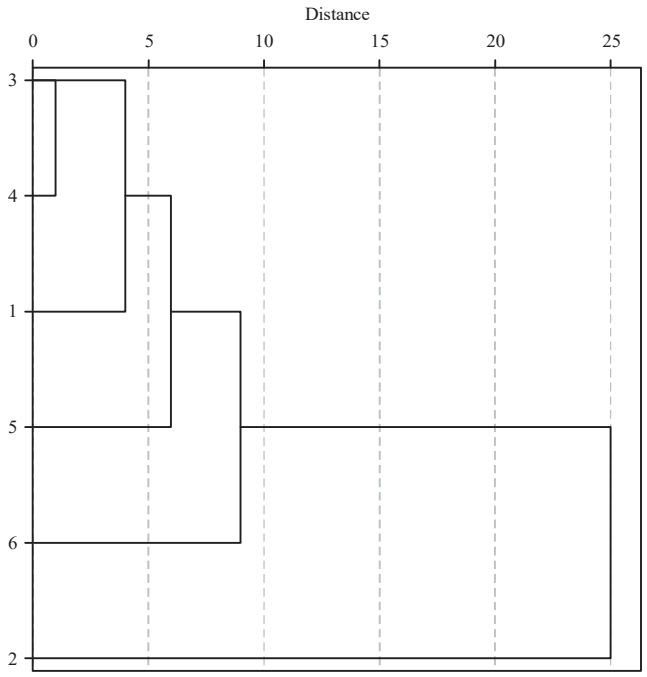


Fig. 2: Cluster analysis based on Euclidean distance showing the relationships among the hydrocarbon profiles of *Aedes caspius* and *Culex pipiens* mosquitoes. (1) *Aedes caspius*-larvae, (2) *Aedes caspius*-pupae, (3) *Culex pipiens*-larvae, (4) *Culex pipiens*-pupae, (5) *Culex pipiens*-adult males and (6) *Culex pipiens*-adult females

chain length. Present hydrocarbons chain length is $C_{9-19}$ at retention time (RT) range 3.88-13.81 min, compared to hydrocarbons chain length C23-29 at retention time (RT) range 21.04-24 min recorded by Amira *et al.*[25]. Such difference could be attributed to differences in studied hydrocarbon chains length that directly proportioned with retention time. The reason behind the huge and statistically difference in $CHC_S$ levels between the immature stages of both *A. caspius* and *C. pipiens* mosquitoes is unknown but it could be due to environmental conditions. Toolson[41] has stated that environmental conditions, particularly extreme variations in temperature and humidity, could influence the cuticular hydrocarbons phenotype of the developmental stages of dipterous insects by either increasing or decreasing their relative abundance in the profile (quantitative change). Accordingly and owing to immature stages, larvae and pupae, of *A. caspius* are living in highly brackish aquatic habitat[42,43], they may need harder cuticle to avoid the stress derived by salts.

Furthermore, the hydrocarbon compositions of immature stages differ markedly from those of the adults, both sexes, of *C. pipiens* mosquitoes. The quantity of $CHC_s$ in the adult stage, both sexes, of *C. pipiens* was generally higher for all the alkanes compared to both larval and the pupal stages (Table 3). Similarly, Paula *et al.*[29] and Zhu *et al.*[44] have mentioned that levels of hydrocarbons increased with progression of the developmental stages of the blow flies. Such differences in amounts of $CHC_S$ among developmental stages of mosquitoes is generally unknown but it could be due to dietary differences, since adults fed on either blood (females) or sugary materials (male) but larvae fed on algae, plankton and other microorganisms in the water whilst pupae are non-feeders. Furthermore, adult mosquitoes generally inhabiting drier habitats requiring harder and impermeable cuticle compared to both larvae and pupae that are sharing the same aquatic habitat. Likely, Paula *et al.*[29] and Zhu *et al.*[44] have identical conclusions but for blow flies.

The present findings (Table 3) indicated that significant quantitative differences in $CHC_S$ profiles was found between sexes of *C. pipiens* but no qualitative differences was detected. Chen *et al.*[45] found significant quantitative difference in hydrocarbon profiles in the young rather than old aged male and female *C. quinquefasciatus* mosquito and both sexes undergo strong $CHC_S$ profile changes with age whereas, individuals aged 0-2 days differ remarkably from the older ones. Similar results for the Afrotropical malaria vector *A. gambiae* were recorded by Caputo *et al.*[13] and such differences were more observed in 0-2 days aged individuals than in older ones. Like mosquitoes, differences in $CHC_S$ were observed between the sexes of other dipterous insects such as the small hairy maggot blowfly[46] and the black dump fly, *Hydrotaea aenescens*[47].

Cuticular hydrocarbons profile of female mosquitoes in particular *A. gambiae* received much attention. Polerstock *et al*.[12] analyzed $CHC_S$ of female *A. gambiae* Giles sensu stricto and *A. aegypti* (L.) mosquitoes before and after they mated. Significant reduction in the percentage of some $CHC_S$ has been observed in female mosquitoes after the matting whilst no changes in the hydrocarbon composition of males occurred after matting. Recently, Al Ahmed *et al*.[23] identified 9 wild collected mosquito vectors from Saudi Arabia by using cuticular hydrocarbons profile analysis of females. These findings for *C. pipiens* females have 2 differences compared to findings of Al Ahmed *et al*.[23] for the same species. The first is the chain length $CHC_S$ ($C_8$-$C_{18}$ in RT range 2.78-50.85 min compared to $C_9$-$C_{19}$ in RT range 2-15 min) and the second is 7 of the $CHC_S$ extracted by Al Ahmed *et al*.[23] differ qualitatively compared to quantitative difference in all $CHC_S$ extracted in the present work. Unlike females, cuticular hydrocarbons of mosquito males received little attentions because they neither blood feeder nor vectors.

For reliability of cuticular hydrocarbon analysis in mosquito species identification, current data were reliable not only in differentiating between the immature stages of the same mosquito species but also between other species. Previous studies have reported the usefulness of hydrocarbons quantitative differences in distinguishing immature stages of the same insect species. Kranz *et al*.[28] recently reported that analysis of chemical profiles of immature stages of blow flies have been suggested as an alternative for species identification rather than morphological identification and DNA-extracting technique as they are easily conducted on instruments and would be more objective. Braga *et al*.[48] stated that cuticular hydrocarbon profile may be used as an additional tool for the taxonomic differentiation of insect species and also for the determination of the age and sex of adult and immature forms. Furthermore, hydrocarbon composition in social insects is assumed to be species specific and may be used for chemotaxonomy[8]. For mosquitos' identification, Rasoolian *et al*.[22] indicated its reliability as a marker for identification of mosquitos' species and its developmental stages and considered as an efficient tool to identify the old museum specimens whose morphological characters are lost over time. Additionally, Al Ahmed *et al*.[23] stated that it is fast and reliable diagnostic tool for field collected mosquitoes.

## CONCLUSION

In conclusion, the cuticular hydrocarbons (CHCs) extracted from the developmental stages, larvae and pupae, of *A. caspius* and *C. pipiens* mosquitoes are only quantitatively and statistically differed and could be used for the discrimination between the two mosquito species as well as sexes. Furthermore, present findings indicates reliability of this method as a marker for identification of mosquitos' species and its developmental stages.

## SIGNIFICANCE STATEMENT

The present study is the second work to use such technique in mosquitoes' identification in Saudi Arabia in general but it is the first one to use it for the developmental stages identification and differentiation. This study will assist researcher to reliably identify mosquito immatures by using of cuticular hydrocarbons analysis.

## ACKNOWLEDGMENTS

Authors are gratitude to Deanship of scientific research, King Faisal University, Saudi Arabia for supporting the present work (Grant No.: 160042). We are also appreciate the project technician, Mr. Youssif Al-Gassim, Biological Sciences Department, College of Science, King Faisal University, Saudi Arabia for his assistance in collecting developmental stages of mosquitoes.

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