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Identification of Iranian Vectors of Malaria by Analysis of Cuticular Hydrocarbons

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Abstract: Twenty-eight *Anopheles* species has been so-far identified in Iran, while only 8 species was proved as malaria vector. In this study, we principally examined the cuticular hydrocarbon (CHC) potency in identification of Iranian vectors of malaria and then differentiation of vector and non-vector species of *Anopheles*. Seven species of malaria vectors and the non-vector species, *Anopheles claviger* were collected throughout Iran. Female extracts were made out of every five conspecific specimens by surface immersion in pure n-hexane. Each sample was injected into a FID-GC instrument along with the known concentrations of standards. CHC profiles of the eight *Anopheles* species indicated no qualitative difference. The average mass of each eluted CHC were compared using Repeated ANOVA and Mann-Whitney tests. Results confirmed a significant difference in mass of each single CHC at a specific retention time (RT). Statistical comparison of CHC mass in *An. sacharovi, An. stephensi, An. culicifacies* and *An. fluviatilis* at RT 39.6 indicated significant differences ($P<0.05$) among these species. Analysis of CHC mass of *An. dthali, An. superpictus* & *An. sacharovi* at RT 28.5, *An. stephensi* & *An. sacharovi* at RT 30.7 and *An. sacharovi* & *An. claviger* at RT 30.6 similarly indicated significant differences ($P<0.05$). *An. sacharovi* could be distinguished from other species, which showed only trace, by integratable peaks at retention times of 29.7, 31 and 32.6. Similarly, *An. claviger* could be distinguished from the other species with a trace peak at RT 30.6. In order to separate *An. stephensi* from the five other species, the integratable peak at RT 30.7 was used. *An. dthali* could be identified at RT 26.2 by an integratable peak v.s. the trace peaks of other species. *An. superpictus* had indicator peaks at RTs 27.4 & 28.5 v.s. trace peaks of other species. *An. maculipennis* with its trace peak at RT 39.6 could be easily differentiated from *An. fluviatilis* & *An. culicifacies*. This study proved that all of the examined species of *Anopheles* could be well identified based on their quantitative differences in CHCs, except for *An. fluviatilis* & *An. culicifacies* for which no CHC indicator peak was detected.

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Key words: *Anopheles*, vector, cuticular hydrocarbon, malaria, Iran

INTRODUCTION

Malaria, transmitted by dipterans of genus *Anopheles* (Diptera: Culicidae), is one of the most important parasitic diseases in Iran and an important health concern in the tropics (Edrissian, 2002). Number of patients in Iran was 13821, 18966 and 15869 cases in 2004, 2005 and 2006 respectively (WHO, 2006). Twenty-two to twenty-eight *Anopheles* species has by-far reported from Iran (Dow, 1953; Sedaghat et al., 2003; Doosti et al., 2006), while only 8 species proved as malaria vectors (Zaim et al., 1993; Sedaghat et al., 2003; Doosti et al., 2006). Control measures are first based on the identification of vector & non-vector species, followed by other detailed studies on the biology, ecology and behaviour of vectors and their distribution (Manoucheri et al., 1976). *Anopheles* mosquitoes are normally identified by morphologic keys (Deane, 1946), which is a reliable method. Mosquitoes, identified by this method should be preferably intact and fresh, since morphological characters may be damaged or faded by time. Meanwhile subspecies and siblings cannot be identified by morphological characters (White, 1977 & 1978). To cope with more advanced needs, complementary techniques such as surface patterns of eggs (Ramsdale and Leport, 1967), hybridization and species mating (Davidson, 1964), cytogenetic & chromosomal investigations (Coluzzi and Sabatini, 1967), isoenzyme evaluation by electrophoresis (Miles, 1978), specific DNA features (Gale and Crampton, 1987) and analysis of cuticular hydrocarbons (CHCs) (Carlson and Service, 1979) were developed to differentiate species, sub species, siblings and sympatric & allopatric populations.

Most of studies on the cuticular lipids of insects indicated that CHCs were present in all insect orders (Howard, 1993). Epicuticular wax layer of the integument contains long chain hydrocarbons, including methyl-alkanes, n-alkenes and n-alkanes (Gibbs, 1998; Howard & Blomquist, 2005). Analysis of insect CHCs using gas chromatography has shown that there are qualitative or quantitative differences among species and between male and female of the same species (Howard, 1993; Rasoolian et al., 2008). CHC analysis of mosquitoes was first performed on *Anopheles gambiae* sensu stricto (Carlson and Service, 1979) and soon followed by other works on *An. culicifacies* (Milligan et al. 1986), *An. arabiensis* (Phillips et al., 1987), *An. maculipennis* siblings (Phillips et al., 1990), *An. stephensi* siblings (Anyanwu et al., 2000) and allopatric and sympatric populations of *Anopheles gambiae* s.s. (Caputo et al., 2007).

MATERIALS AND METHODS

Collection and identification of mosquito specimens

Indoor and outdoor collection of adult *Anopheles* was done by hand catch if there was a high abundance. In case of low frequency, larvae were collected and then reared in the insectary at 25°C, 75-80% RH, 12L:12D on aquarium fish food. Collecting sites, dates and number of collected mosquitoes are indicated in Table 1. Female mosquitoes were identified right after the field collection using the pictorial key to the Iranian species of *Anopheles* (Shahgudian, 1960) and immediately stored at –20°C until the time of extracting.

Chemical extraction

Twenty specimens of each species were randomly chosen and groups of five specimens were extracted according to Phillips et al. (1987) in 800 μL n-hexane for five minutes to get enough cuticular wax without extra contamination from the internal lipids. Extracts were 100 fold condensed using a Nitrogen flow.

Quantitative gas chromatography

One μL of identical extract of each species was four time (n=4) injected into a GC Varian 3800 gas chromatograph in split mode (split ratio 50%), equipped with a CP-Sil 8cb (5% Phenyl and 95% dimethyl polysiloxane, non-polar) bounded phase fused silica capillary column (Varian, FT 0.12 μm, ID 0.25 mm, length: 50 m) connected to a flame ionization detector (FID) and their CHC profiles were monitored. Helium was used as the carrier gas at 1 mL/min velocity. Injector and detector temperature were set at 300°C. The temperature programme was started at 32°C and ramped at 2°C/min to 52°C, then maintained for 3 min. Thereafter, the temperature was raised at 5°C/min to 72°C, maintained for 2 min, followed by a ramp at 5°C/min to 230°C and another one at 2°C/min to 285°C, ultimately cooled to the starting temperature. CHC peak areas were integrated by Saturn® Workstation package, Saturn view™ version 5.2.1, 1989-1998, Varian Associates, Inc.

Mass calculation and carbon chain estimation

Using mixture of a given value of the three external standards of n-pentadecane ($C_{15}H_{32}$), n-pentacosane ($C_{25}H_{52}$) and n-dotriacontane ($C_{32}H_{66}$) and their relevant integrated peak areas, mass of each CHC was calculated. The chain length of eluted CHCs was estimated by the elution time of the external standards. The solvent (n-hexane) was eluted at RT 6.3.

Statistical analysis

CHC mass comparisons of the 8 *Anopheles* species was achieved by Repeated ANOVA and Tukey HSD Post Hoc Test. Kolmogorov-Smirnov test was applied in order to verify the normal distribution of the data. If an integratable

Table 1. Collecting locations of *Anopheles* mosquitoes in Iran, 2006 & 2007

Anopheles Species	*An. dthali*	*An. fluviatilis*	*An. superpictus*	*An. sacharovi*	*An. stephensi*	*An. culicifacies*	*An. maculipennis*	*An. claviger*
Geographical location & Reference grid	Kazeroon County (*Islam-Abad*)* 29°47'N 51°33'E	Kazeroon County (*Pirsabz*) 29°48'N 51°38'E	Darab County (*Arab chegini*) 28°47'N 54°22'E	Marvdasht County (*Garm-Abad*) 30°3'N 52°47'E	Iranshar Research Station	Iranshar County (*Sarbaz district*) 27°12'N 60°41'E	Noor County (*Abasa*) 36°32'N 51°59'E	Shiraz County (*Arjan district*) 29°48'N 51°54'E
Collecting date	September 2006 June 2007	June 2007	May & June 2006	September 2006	December 2006	December 2006	September 2007	December 2006
Method of collection & type of habitat	Hand Catch Indoor	Larval collection	Larval collection	Hand catch Outdoor	Lab Colony	Hand Catch Indoor	Hand Catch Indoor	Hand Catch Outdoor
Number of specimens	360	75	110	94	45	42	40	62
Number of females	175	40	48	45	45	32	28	48

*Names in parentheses refer to a village except those followed by the term, district.

peak was appeared in only two species at a specific RT, the non-parametric test of Mann-Whitney test was used. All statistical analyses were fulfilled with SPSS statistical package ver.11.5.0 (GS-35F-5899H, USA).

RESULTS

Statistical analyses on the CHC mass quantity (Table 2) of the 8 *Anopheles* species indicated significant differences among species. Seventeen hydrocarbons were totally recognized at RTs 6.6-39.6 in the total ion chromatograms of the 8 studied species, while no peak was appeared between RTs 40-54 (Fig. 1).

An. sacharovi was distinguished from *An. fluviatilis*, *An. culicifacies* ($P<0.001$) and *An. stephensi* ($P<0.05$) at RT 39.6 (Fig. 2). *An. sacharovi* v.s. *An. dthali* and *An. superpictus* v.s. *An. sacharovi* ($P<0.05$) could be identified at RT 28.5 (Fig. 3). CHC mass of *An. sacharovi* and the non-vector, *An. claviger*, indicated significant difference ($P<0.05$) between the two species at RT 30.6 (Fig. 4). The

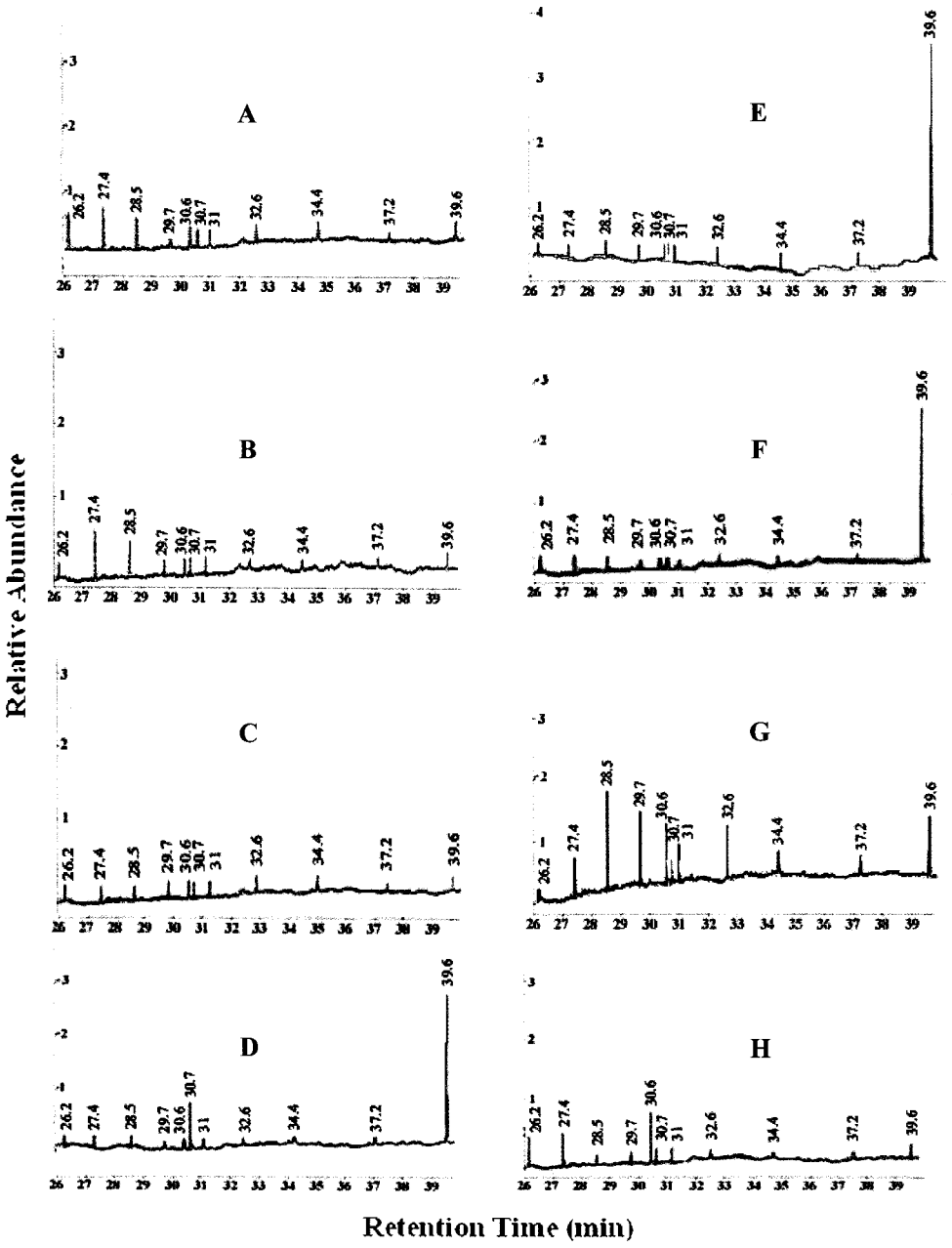

Fig. 1. FID-GC profile, indicating CHC relative abundance against Retention Time (minute) for the *Anopheles* species of Iran (2008). (A): *Anopheles dthali*, (B): *Anopheles superpictus*, (C): *Anopheles maculipennis*, (D): *Anopheles stephensi*, (E): *Anopheles fluviatilis*, (F): *Anopheles culicifacies*, (G): *Anopheles sacharovi*, (H): *Anopheles claviger*. The precise retention time of each CHC is shown on the relevant peak. RTs 6.3-26.2 & 40- 54 have not been indicated because of no qualitative or quantitative difference. RT solvent (hexane): 6.3.

Table 2. Mean±SEM of CHC masses based on integratable peaks (RTs 6.6-39.6) of the seven vectors & a non-vector (*An. claviger*) species of *Anopheles*

RT (min)	dthali	fluviatilis	superpictus	sacharovi	stephensi	culicifacies	maculipennis	claviger
6.6	0.532769± 0.111569	0.476821± 0.041207	0.452945± 0.02759	0.473932± 0.039788	0.475052± 0.018885	0.478± 0.023341	0.461434± 0.027873	0.483955± 0.047792
6.7	117.689± 27.10233	106.9748± 6.292474	104.6112± 9.2705	104.256± 4.555323	103.5037± 3.413888	104.6167± 4.219187	104.3792± 4.112728	109.864± 11.84868
7.3	5.609779± 1.145782	5.056554± 0.302906	4.988285± 0.515373	5.028963± 0.154837	4.956803± 0.212616	5.00432± 0.282541	4.874503± 0.416858	5.209775± 0.558479
7.4	0.498516± 0.111942	0.52593± 0.049299	0.434256± 0.125741	0.491363± 0.027454	0.423586± 0.121838	0.441684± 0.136395	0.41315± 0.154064	0.427535± 0.111488
7.5	0.513136± 0.130154	0.478236± 0.033066	0.460255± 0.022462	0.507065± 0.054564	0.471102± 0.016768	0.478531± 0.031207	0.458506± 0.017913	0.489496± 0.058129
7.8	3.579812± 0.811787	3.370996± 0.232274	3.217421± 0.28693	3.360915± 0.263919	3.328019± 0.128243	3.300782± 0.147032	3.04781± 0.085447	3.35502± 0.356273
8.1	3.743174± 0.790008	2.681351± 1.318438	3.301961± 0.301144	3.382315± 0.192317	3.361446± 0.137025	3.360326± 0.119221	3.227089± 0.1272	3.443097± 0.355154
8.7	0.6956± 0.146436	0.512076± 0.118555	0.584235± 0.025135	0.617544± 0.062876	0.619569± 0.035264	0.589188± 0.040378	0.572327± 0.020586	0.639357± 0.063603
26.2	0.249848± 0.019866	Trace	Trace	Trace	Trace	Trace	Trace	0.262918± 0.027227
27.4	0.319649± 0.049581	Trace	0.313223± 0.054279	0.454242± 0.232649	Trace	Trace	Trace	0.304027± 0.029359
28.5	0.265571± 0.006818	Trace	0.280872± 0.010705	1.187962± 0.141204	Trace	Trace	Trace	Trace
29.7	Trace	Trace	Trace	1.033937± 0.152574	Trace	Trace	Trace	Trace
30.6	Trace	Trace	Trace	0.989014± 0.245982	Trace	Trace	Trace	0.431966± 0.080615
30.7	Trace	Trace	Trace	0.414389± 0.052293	0.297074± 0.034028	Trace	Trace	Trace
31	Trace	Trace	Trace	0.668894± 0.127561	Trace	Trace	Trace	Trace
32.6	Trace	Trace	Trace	0.965669± 0.262363	Trace	Trace	Trace	Trace
39.6	Trace	2.029908± 0.209234	Trace	1.026332± 0.40069	1.71981± 0.097058	1.872018± 0.05016	Trace	Trace

two species, *An. stephensi* and *An. sacharovi* were differentiated by significant difference ($P<0.05$) at RT 30.7 (Fig. 5).

Similarly, *An. claviger* could be distinguished from other species by a trace peak at RT 30.6. In order to separate *An. stephensi* from the five other species, the integratable peak at RT 30.7 was used (Fig. 6). *An. dthali* could be differentiated from the other *Anopheles* species which had only a trace peak at RT 26.2 by an integratable peak at the same RT. *An. superpictus* had some integratable peaks at RTs 27.4 & 28.5 v.s. the trace peaks of other species. *An. maculipennis* with its trace peak at RT 39.6 could be easily differentiated from *An. fluviatilis* & *An. culicifacies* (Fig. 6).

DISCUSSION

Our study proved the efficiency of CHC quantitative analysis by FID-GC to differentiate malaria vectors and also vector & non-vector *Anopheles* of Iran; however no qualitative difference was observed. Quantitative difference at a given RT in many cases includes integratable peak (IP) for one species, while others showed only trace amounts. *An. sacharovi* for instance was distinguished from other species, which showed only trace, by integratable peaks at retention times of 29.7, 31 and 32.6 (Fig. 6). Trace is a peak area whose related mass is lower than the detector sensitivity and measurement level and cannot be calculated. *An. sacharovi* and *An. maculipennis* showed the highest and the lowest number of integratable peaks respectively (Table 3). All quantitative differences of the 8 *Anopheles* species were observed at RTs 26.2 to 39.6 (representing long chain hydrocarbons) with no observed difference at RTs 6.6- 26.2 (Fig. 1).

The approximate length of carbon chain in each CHC could be estimated comparing RT of the eluted standards and

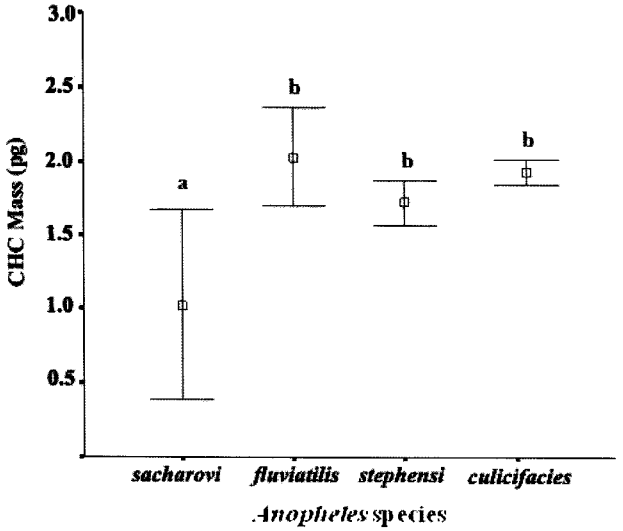

Fig. 2. CHC mass quantity of *An. sacharovi*, *An. stephensi*, *An. culicifacies* and *An. fluviatilis* at RT 39.6. Rectangles and bars respectively indicate mean and standard deviation. ANOVA (Tukey *HSD* post hoc test), n=4; Confidence levels: (*An. sacharovi* v.s. *An. fluviatilis* and *An. sacharovi* v.s. *An. culicifacies*; $P<0.001$), (*An. stephensi* v.s. *An. sacharovi*; $P<0.05$). Bars with different letters indicate statistically significant differences. Pg: Picogram.

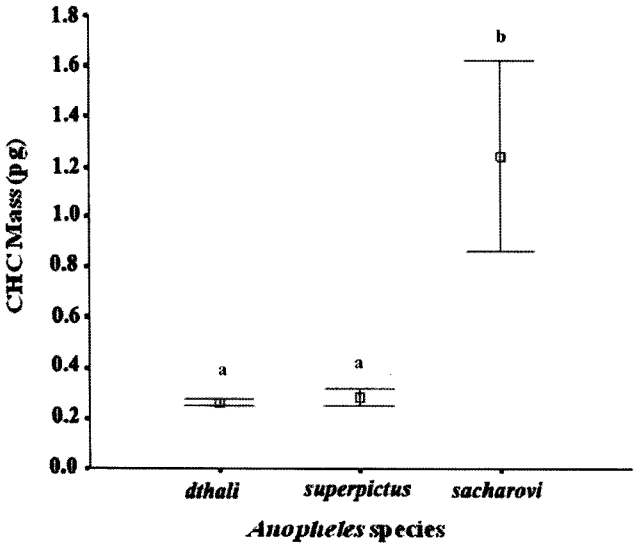

Fig. 3. CHC mass quantity of *An. sacharovi*, *An. dthali* and *An. superpictus* at RT 28.5. Rectangles and bars respectively indicate mean and standard deviation. ANOVA (Tukey *HSD* post hoc test), n= 4; Confidence levels: (*An. superpictus* v.s. *An. sacharovi* and *An. dthali* v.s. *An. sacharovi*; $P<0.05$). Bars with different letters indicate statistically significant differences. Pg: Picogram.

CHCs. Peaks, eluted earlier than 27.51 min (RT of $C_{15}H_{32}$) have shorter chain than pentadecane (10 compounds); those eluted between 27.51 and 38.64 min (RT of $C_{15}H_{32}$ and $C_{25}H_{52}$ respectively) have a carbon chain of 15-25 (6 compounds) and just one hydrocarbon appeared between 38.64 and 39.64 min (RT of $C_{25}H_{52}$ and $C_{32}H_{66}$ respectively) and thus a C chain between 25-32.

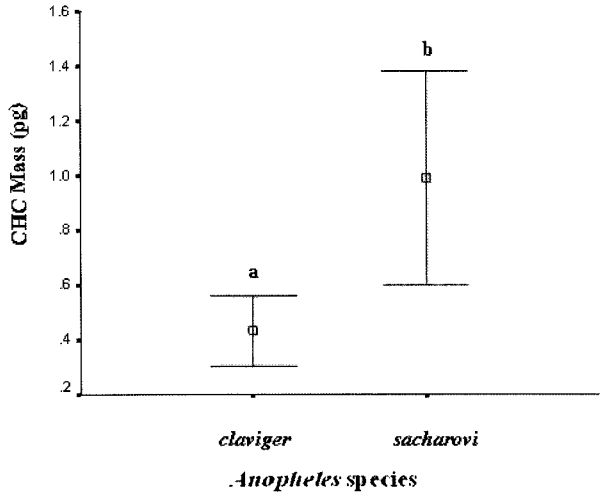

Fig. 4. CHC mass quantity of *An. sacharovi* and the non-vector, *An. claviger*, at RT 30.6. Rectangles and bars respectively indicate mean and standard deviation. Mann-Whitney test, n=4, $P<0.05$. Bars with different letters indicate statistically significant differences. Pg: Picogram.

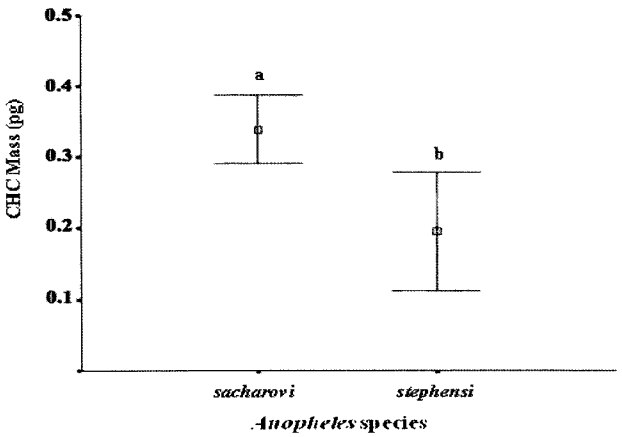

Fig. 5. CHC mass quantity of *An. sacharovi* and *An. stephensi* at RT 30.7. Rectangles and the bars respectively indicate mean and standard deviation. Mann-Whitney test, n=4, $P<0.05$. Bars with different letters indicate statistically significant differences. Pg: Picogram.

This study proved that all of the examined species of *Anopheles* could be well identified based on their quantitative differences in CHCs, except for *An. fluviatilis* & *An. culicifacies* for which no CHC indicator peak was detected. Differentiation of *An. fluviatilis* & *An. culicifacies* may require more precise analyses. Similar results were obtained on *An. gambiae* s.s. (Carlson and Service, 1979; Caputo et al., 2007), *An. culicifacies* (Milligan et al., 1986) and *An. arabiensis* (Phillips et al. 1987; Caputo et al., 2007). All other workers similarly reported CHC quantitative differences without any qualitative diversity in mosquitoes (Hamilton and Service, 1983; Kittayapong et al., 1993).

We also detected some statistically significant differences in the CHC mass of vector and non-vector *Anopheles*

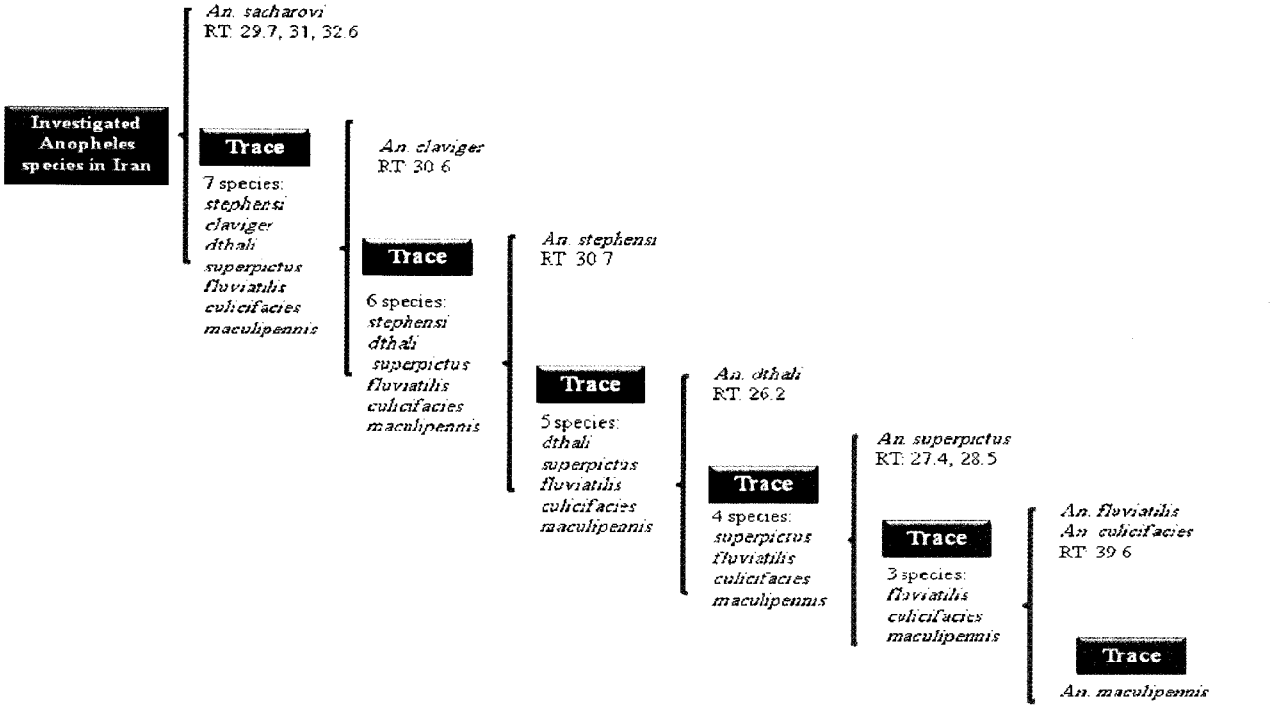

Fig. 6. Differentiation of Iranian vectors of malaria (seven species) and the non-vector, *An. claviger* by analysis of cuticular hydrocarbons. RT of integratable peaks is indicated.

Table 3. Number of integratable peaks in the seven vectors and a non-vector (*Anopheles claviger*). Total peaks (integratables and non-integratables): 17

Anopheles Species	IP
Anopheles sacharovi	16
Anopheles dthali *Anopheles claviger*	11
Anopheles superpictus *Anopheles stephensi*	10
Anopheles fluviatilis *Anopheles culicifacies*	9
Anopheles maculipennis	8

species (Fig. 4 and 6). In a similar study, vectors and non-vector siblings of *Anopheles maculates* could be successfully differentiated by their CHC quantitative differences (Kittayapong et al. 1993). We believe that such differences can be similarly marked among other vector and non-vector species of Iranian *Anopheles*. On the other hand geographic, physiologic, genetic and ecologic parameters have some impacts on the CHC profile of a species; however the extent of such impacts is not yet clear (Anyanwu et al. 2000; Caputo et al., 2007). CHC analysis which does not harm the specimen and its taxonomic characters is in particular a useful technique when we study the old museum specimens or damaged ones for which all morphologic characters may not be simultaneously present.

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