This year we performed pilot studies that will be used as a basis for all other investigations. In these pilot experiments we studied mainly laboratory population to develop the most suitable techniques, to assess their reproducibility and to determine major compounds present in fruit fly samples. We also studied several wild population to determine what differences can be seen using our methods and what type of multiple analysis are relevant to data sets obtained.

METHODS AND MATERIAL

INSECTS

Laboratory population of C. capitata and A. fraterculus originated from entomological laboratory FAO/IAEA. Wild populations of C. capitata originated from Greece and wild population of A. fraterculus originated from North East and South Brazil. The wild flies of C. capitata were collected from different host plants as last instar larvae during the season (August-September) near Athens and Thessaloniki. They were allowed to pupate in Greece and fresh pupae were sent to our laboratory. There, the flies were allowed to emerge, sexes were kept separated. Sexually mature flies were used for experimentation.

The CHC and pheromone samples from wild flies of A. fraterculus were prepared at the Institute of Chemistry and Biochemistry, Federal University of Alagoas, Brazil, using standard aeration and extraction protocol (see below). Samples were taken to our laboratory and analyzed.

Flies of all populations were fed by an artificial diet composed of sugarcane and mineral water. The temperature of the insectarium was 25°C, relative humidity 60%, and a photoperiod of 14 hours.

EXTRACTION PROTOCOL OF CHCs

The flies were immobilized (-18°C, 5 males and 5 females each) and placed for 15 minutes into a desiccator to remove the surface moisture. The CHCs were extracted with dichloromethane (0.5 ml, 5 min) in small glass vials. The volume of the solvent was then reduced under a stream of argon. The total extract was fractionated using thin layer chromatography (TLC) plates using hexane as the mobile phase. The CHC fractions were visualized by spraying Rhodamine 6G
solution onto the plates, scrapped off the plates, and extracted with diethyl ether. This extracts were analysed.

MALDI/TOF-MS ANALYSIS
The CHCs extracts were analyzed using Reflex IV (Bruker Daltonik, Ltd., Bremen, Germany) operated in reflectron mode with the acceleration voltage of 20 kV and 200 ns ion extraction pulse. Desorption and ionisation was be achieved using a nitrogen UV laser. Matrix ions were suppressed below m/z 300 (Vrkoslav et al. 2009 article in press). Data were collected and analyzed using FlexAnalysis 3.0 software (Bruker Daltonics).

CHEMICAL IONIZATION
To determine the positions of double bonds in unsaturated CHC, samples were analyses using GC-MS either in EI or chemical ionisation mode. Acetonitrile was used as ionisation reagens.

DYNAMIC HEAD SPACE PROTOCOL
A group of 20 calling virgin males were placed in a glass chamber (500 ml) (10 replicates). The chambers were aerated by clean air (1 mL/min). Volatiles produced by calling males were carried away by the air stream and were captured by a filter placed at the chamber outlet. Male volatiles were trapped 24 hours – starting at 9.00 am and finishing at 9.00 am on the following day. The filter was made from glass tube (0.7 mm I.D., 5 cm length) with 100 mg SuperQ (Chrompack). Entrapped volatiles were subsequently eluted with 500 µl of hexane. The eluent was analyzed using GC-MS, GCxGC/TOF-MS and GC-EAD techniques.

GC-EAD ANALYSIS
To identify which components of the volatile bouquet are perceived, fly antennae were used as biological detector coupled to GC (GC-EAD). In GC-EAD experiment, the GC effluent is divided into two parts: one flows onto the insect antenna (biological detector, EAD) and the other one into flame ionization detector (FID). Data are fed into PC and simultaneously analysed.

RESULTS:

Analysis of male pheromone

FIG. 1
Example of GCxGC-MS analysis of A. fraterculus male effluvia (laboratory population). Each dot represents one compound. Its concentration is colour coded: scale ranging from blue to red represents increasing concentrations.
Several EAD active compounds were identified (Tab. 1). Identification was based on comparison of antennal activity, retention indexes and mass spectra with synthetic standards. Identified compounds probably play a role in mediating female attraction to mating spots.

**Tab 1:**
1. Z3-NONENOL
2. Z3,Z6-NONADIENOL
3. GERANYLACETONE
4. 3E,6E-α-FARNESENE
5. SUSPENSOIDE
6. EPIANASTREPHIN

GC-EAD experiments performed on both male and female *A. fraterculus* antennae suggest that in this species there exist sex specific differences in perception of individual pheromone components. Specifically, the females are likely more sensitive to Z3-nonenol and Z3,Z6-nonadienol then are the males.

**Ontogeny of male pheromone production**
Samples have been already collected and GCxGC-MS analysed. Data are currently evaluated.
Fig. 3
GCxGC-MS analysis of *Ceratitis capitata* male effluvia. Each dot represents one compound. Its concentration is colour coded: scale ranging from blue to red represents increasing concentrations.

Fig. 4
GC-EAD Analysis of *Ceratitis capitata* male pheromone. Figure shows that there are four prominent EAD areas, but also several small ones, identity of which has not yet been unambiguously identified.

Tab. 2:
Compounds identified in male effluvia of *Ceratitis capitata* with area above the selected threshold (compounds identified based on agreement of retention behavior, MS, KI and with synthetic standards whenever possible). Red colour depict four most active compounds.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>RT 1ST; 2RD (SEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-PINENE</td>
<td>845; 2,520</td>
</tr>
<tr>
<td>Dihydro-3-methyl-2(3H)-furanone</td>
<td>870; 4,330</td>
</tr>
<tr>
<td>CAMPHENE</td>
<td>880; 2,650</td>
</tr>
<tr>
<td>SABINENE</td>
<td>925; 2,610</td>
</tr>
<tr>
<td>β-PINENE</td>
<td>935; 2,650</td>
</tr>
<tr>
<td>6-METHYLHEPT-5-EN-2-ONE</td>
<td>940; 2,980</td>
</tr>
<tr>
<td>OCTAN-3-ONE</td>
<td>940; 2,810</td>
</tr>
<tr>
<td>MYRCENE</td>
<td>950; 2,570</td>
</tr>
</tbody>
</table>
Our data show that there are many compounds in male sex pheromone that had not been previously reported. Further analysis is necessary to show whether there exist differences in composition between populations originating from different hosts. I)

**ANALYSIS OF CUTICULAR CHCs**

In majority of CHC studies including those on Tephritids, GC-MS had been the primary tool. However, GC-MS identification of high-mass hydrocarbons is problematic because the molecular ion is virtually absent. To obtain a more complete picture, we employed Laser desorption/ionization (LDI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometries. In our pilot experiments, we compared CHC profiles of laboratory _A. fraterculus_ flies using these available methods to i) see the scope and limitations of each of them. Pilot experiments showed that CHC of _A. fraterculus_ contain c14-c37 saturated, unsaturated and branched CHC. To determine the position of double bonds in unsaturated CHC chemical ionization was performed. Though many CHC already have been identified, there are still areas where identity of CHC is still obscure. Our experiments showed, that all methods has to be combined together to get the whole picture.

**Fig.5**
CHC profile of females of _A. fraterculus_ obtained using GC-MS.
FIG.6.
CHC profile of males of A. fraterculus using GC-MS

CONCLUSIONS:

In conclusion, we standartized our analytical methods and determined the scope and limitation of each of particular one. Using an unique combination of method, we identified number of so far unidentified compounds in male pheromones of studied fruit fly species. Our pilot experiments provided solid basis for further studies of population CHC and pheromone variability. The data were already presented on numerous conferences and currently we are preparing a manuscript about differences in pheromone composition in different population of Ceratitis capitata (see attachment).

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RNDr. Blanka Kalinova, CSc.,