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ANALÝZA SEXUÁLNÍHO FEROMONU OVOCNÝCH MUŠEK RODU CERATITIS
FASCIVENTRIS, CERATITIS ANONAE A CERATITIS ROSA

Analysis of sex pheromone of fruit flies genus *Ceratitis fasciventris*, *Ceratitis anonae* and
Ceratitis rosa

Diplomová práce

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Declaration

I declare that all the results, which are used and published in this Thesis, have been obtained by my own experimental work and that all the ideas taken from work of others are properly referred to in the text and the literature survey. I also declare that neither this Thesis, nor its significant part, has been submitted in any form for another degree or diploma at any university or other institution of tertiary education. I am conscious that the prospective use of the results, published in this Thesis, outside the Charles University in Prague is possible only with a written agreement of this university.

In Prague, 19.8. 2013

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Signature

KEY WORDS

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KLÍČOVÁ SLOVA

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Plynová chromatografie s hmotnostní detekcí

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Plynová chromatografie s biologickou detekcí

Stopová analýza

Chemotaxonomie

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LIST OF SYMBOLS AND ABBREVIATIONS

1D-GC	One-dimensional gas chromatography
CI	Chemical ionisation
EAD	Electroantennographic detection
EAG	Electroantennogram
ECD	Electron capture detector
EI	Electron impact ionization
FID	Flame ionization detector
FTIR	Fourier transformation infrared spectroscopy
GC×GC	Comprehensive gas chromatography
GC×GC-TOFMS	Two-dimensional gas chromatography with time-of-flight mass spectrometric detection
GC-EAD	Gas chromatography combined with an EAG detector
GC-MS	Gas chromatography combined with mass spectrometry
HPLC	High performance liquid chromatography
MALDI	Matrix-assisted laser desorption/ionisation
<i>m/z</i>	Mass to charge ratio
NPD	Nitrogen phosphorus detector
PTFE	Polytetrafluorethylene
ReTOF	Reflectron time of flight detector
SD %	Standard deviation
SIT	Sterile insect technique
SPME	Solid phase microextraction
TLC	Thin layer chromatography
TOF	Time of flight detector

ABSTRACT

Ceratitis fasciventris, *Ceratitis anonae* and *Ceratitis rosa* are polyphagous agricultural pests originating from African continent. Their behaviour is heavily altered by pheromones. Insect chemical communication channels are species-specific, represents taxonomic and reproduction barriers. Taxonomy of this group (so-called *Ceratitis* FAR complex) is unclear. Therefore new chemical approaches along with genetic tests for identification of entities within the cryptic species FAR complex are being developed.

To study multi-component mixtures of male volatiles originating from the three mentioned fruit fly species, comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometer (GC×GC-TOFMS) was used.

A number of compounds were identified, out of which 23 were found to be distributed and shared among the studied species, 11 out of these were present in all three species. Analyses of male pheromone volatiles, using gas chromatography combined with electroantennographic detector (GC-EAD) revealed 4 common compounds with antennal activity shared among three studied species: methyl (*E*)-hex-3-enoate, 6-methylhept-5-en-2-one, linalool, and methyl (2*E*,6*E*)-farnesoate. The species-specific EAD active compounds in *C. fasciventris* were esters of isomers of hexenoic acid, whereas isomers of α and β -farnesene were special to *C. rosa*. On the contrary, *C. anonae* was found to lack specific compounds. The significance of those common as well as unique (species-specific) compounds was approved by multivariate statistical analyses.

The results represent first study of chemical communication in cryptic species FAR complex and constitute notable support for future taxonomic evaluation.

ABSTRAKT

Vrtulovité mouchy *Ceratitis fasciventris*, *Ceratitis rosa* a *Ceratitis anonae* jsou polyfágní zemědělské škůdci afrického původu. Jejich chování je značně ovlivněno feromony. Chemická komunikace hmyzu je druhově specifická a představuje taxonomickou i reprodukční bariéru. Taxonomie této skupiny vrtulí (tzv. *Ceratitis* FAR komplexu) je dosud nejasněná a pro její objasnění jsou vyvíjeny nové chemické a genetické metody.

Pro studium komplexních směsí těkavých látek emitovaných samci tří výše uvedených druhů vrtulí byla použita metoda dvourozměrné plynové chromatografie s hmotnostně-spektrometrickou detekcí.

Z identifikovaných složek bylo 23 látek společných pro nejméně dva druhy, z toho 11 látek bylo společných pro všechny tři studované druhy, z toho 4 látky vykazovaly antenální aktivitu: šlo o methyl-(Z)-hex-3-enoát, 6-methylhept-5-en-2-on, linalool a methyl-(2E,6E-farnesoát). Druhově specifické antenálně aktivní látky u druhu *C. fasciventris* byly estery izomerů hexenové kyseliny, zatímco izomery α a β -farnesenu byly typické pro *C. rosa*. U *C. anonae* nebyly zjištěny žádné druhově specifické antenálně aktivní látky.

Výsledky této práce jsou první studií chemické komunikace kryptického *Ceratitis* FAR komplexu a představují významný základ pro jeho budoucí taxonomické zhodnocení.

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Macrophotographs of *Ceratitidis anonae* (top), *C. fasciventris* (middle), *C. rosa* (bottom)
Courtesy of Ing. Michal Hoskovec, CSc.

INTRODUCTION

Ceratitis fasciventris, *Ceratitis anonae* and *Ceratitis rosa*, also known under acronym FAR complex, are part of Tephritidae family (fruit flies). These species feed on vast variety of fruits and crops causing economic loss not only in African countries, where they originate from, but also threaten worldwide crop yield due to global trading activities that facilitate their potential migration to new territories.

Studied fruit fly species communicate via pheromones, complex blend of compounds in specific ratios that represent reproduction barriers and function as information channels – modifying sexual behaviour, aggregation or marking of food resources. These signals are conserved and species-specific. Morphological differences between these three species are almost undistinguishable which complicates the taxonomic characterization. Recently the focus has been placed on genetic studies resulting in identification of 5 morphotypes. Nevertheless a development of new methods for differentiation of entities inside the cryptic species FAR complex is necessary. Therefore we opted to use chemical and statistical analyses to investigate chemical composition of released male volatiles.

The research was accomplished within international cooperation funded by IAEA (International Atomic Energy Agency) that focuses on exploitation of atomic energy for peaceful purposes. Our work is part of the efforts to help decrease the economic losses caused by fruit flies. Specifically to clarify the species identification and distribution to allow effective employment of sterile insect technique (SIT) based on release of radiation sterilized males into the wild population.

AIM OF THE THESIS

Due to their high biotic potential, large numbers of host plants, rapid establishment and development, fruit flies have become serious pests of many fruits and vegetables in temperate, sub-tropical and tropical areas. To help understand the taxonomical relationships within the three African species *Ceratitis fasciventris*, *C. rosa* and *C. anonae*, we aimed to analyse the composition of their male sex pheromones. These intraspecific communication signals that control mating behaviour are highly species-specific and play a remarkably important role in reproductive isolation of different species. Detailed study of male sex pheromone composition intends to support current chemical studies of cuticular hydrocarbon composition and molecular genetic studies aiming to clarify the taxonomical classification and investigate evolution relatedness of the *Ceratitis* FAR species. Resolving taxonomy of agricultural pest species that are potentially invasive is particularly important, because inadequate characteristics might lead to deficient or insufficient pest control strategies.

1. *Ceratitis* FAR complex

Kingdom:	Animalia
Class:	Insecta
Order:	Diptera
Family:	Tephritidae
Genus:	<i>Ceratitis</i>

Order Diptera occupies several habitats, from organic matter to decomposing animal tissues. Due to large number of species ($\approx 125\,000$)¹ this order has great impact on the whole mankind. Species belonging to this order are known for being agricultural pests carrying significant economic importance, vectors of diseases², as well as major pollinators of plants. The so-called "true fruit flies", Tephritidae, spread rapidly from their natural habitat and are currently dispersed all over the world³.

The family Tephritidae consists of four major genera, *Ceratitis*, *Bactrocera*, *Anastrepha* and *Rhagoletis*, which are considered the most important insect pest worldwide⁴. The genus *Ceratitis* is most studied, with *Ceratitis capitata* as species most frequently monitored and used as model pest organism. Tephritidae fruit flies are polyphagous. Adults feed on various fruit juices, nectar, pollen and lay eggs in a wide range of wild and cultivated crops (≈ 250 types), the majority of which are economically important⁵. Reproductive maturity is attained within 5 days for most laboratory strains and considerably longer (7–13 days) in wild flies⁶. Males initiate mating by forming small groups, leks, on the vegetation and disperse sexual signal - a volatile pheromone mixture - to attract females to mate. Males are polygynous^{7,8}, while females are monoandrous or oligoandrous⁹. Male sex pheromone is released from mouth and anus during the signalling (calling) behaviour¹⁰. The pre-mating behaviour of fruit flies is complicated and involves the use of visual, auditory, and chemical signals. In the presence of a female, the male follows species-specific pre-mating behavioural pattern consisting of wing vibrations, head rotations, abdomen bending and acoustic signalling. Mating occurs usually during discrete circadian periods, which are species-specific¹. The Afro-tropical group of fruit flies *Ceratitis fasciventris*, *Ceratitis anonae* and *Ceratitis rosa*, often referred to as FAR complex¹¹, are widespread in a number of African countries: *C. fasciventris* and

C. anonae occur sympatrically in both East and West Africa, while *C. rosa* is more restricted to southern and eastern Africa where its distribution partially overlaps with that of *C. fasciventris* but not with *C. anonae*^{12,13}. *C. rosa* is now feared to be a global threat due to tolerance of lower temperatures¹⁴, it may expand not only within Africa, but also across Europe, Asia, Australia and North and South American continent¹⁵. Despite the economic importance, the taxonomy of this group is not clear and taxonomical classification is not easy¹⁶. Resolving invasive agricultural pest species is particularly important, because inadequate morphological/molecular characterization of species might lead to serious economical consequences resulting into inept ecological models and/or pest control strategies¹⁷.

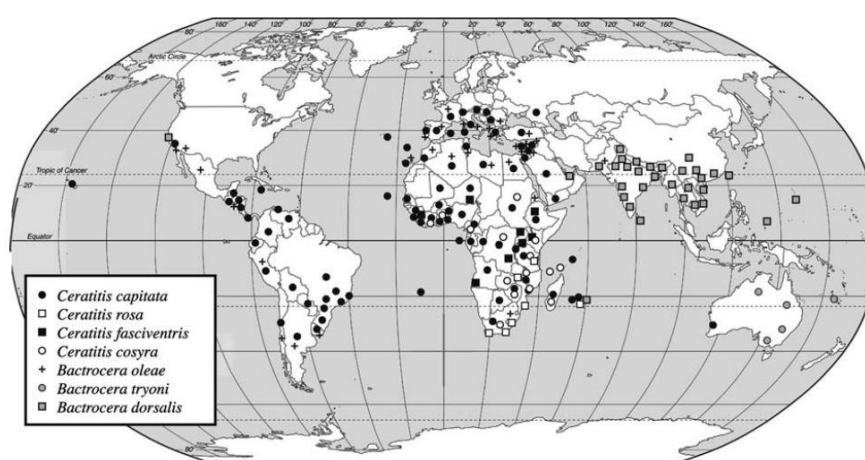


Fig. 1.1: Distribution map of some of the principal fruit fly species (CABI/EPPO 1998)⁴

The needs to develop precise pest detection technique, diagnostic tools and management strategies for these pest species initiated large scale morphological studies, characterization of the genetic variation within and between the species, as well as investigating of their evolutionary relationships⁵. The identification of species of the FAR complex is only possible based on specific small differences of morphological characters of adult male leg patterns (larvae, pupae and females, are even more difficult to distinguish)¹⁸. While the females of *C. anonae* can be identified, females of *C. rosa* and *C. fasciventris* are almost indistinguishable and show only very subtle differences in their scutellar colour patterns. Absence of clear diagnostic morphological characters to identify individual species emphasizes the need for unambiguous identification molecular and/or chemical tools^{13,19}. Molecular approaches for species recognition were developed in the past^{11, 20, 21}. Recently, Virgilio and co-workers provide clear data on specification of 5 different morphotypes using comparison of allelic variations at 16 microsatellite loci¹⁷. Nevertheless the use of microsatellite loci for cryptic species identification is rather laborious and expensive.

Moreover the chemical analyses of cuticular hydrocarbon components extracted from males and females of *C. fasciventris*, *C. anonae*, *C. rosa* showed clear species- and sex-specific differences among the three studied species²². To help to understand the taxonomical relationships within the FAR complex, we aimed to analyse the composition of their male sex pheromones, since these communication signals are highly species specific and are extremely important in reproduction isolation of different species.

1.1 Chemical communication

Insect chemical communication have proved to be some of the richest sources of knowledge for chemical ecology²³. Insects extensively communicate using chemical messages, which are mediated by volatile compounds (semiochemicals). Volatiles are used for *intra*- (within species) and as well as *inter*- (among species) specific communication. Pheromones are semiochemicals used in communication between members of the same species, while allelochemicals mediate communication between different species²⁴. The name pheromone is derived from the Greek *pherein*, to transfer, and *horman*, to excite. Pheromones induce either a specific reaction such as special behaviour or a developmental process. Accordingly, pheromones fall into two distinct classes, releasers and primer. Releasers elicit an immediate, short-lived behavioural response, while primers elicit a delayed, long-lived physiological response²⁵. A myriad of messages are encoded by releaser pheromones: sex pheromones, released by one sex function as powerful attractants of the opposite sex during mating time; aggregation pheromones signal favourable conditions for both sexes; less volatile trail and forage-marking pheromones point to food and other resources. Primer pheromones are at the heart of insect societies delineating the hierarchy and distribution of labour among individuals²⁴. Pheromone components are very diverse in their structure and volatility. They are typically mixtures of several compounds with a species-specific composition and blend ratios. Stereostructure and chirality are essential for eliciting the specific behavioural response²⁶. It has been observed that chiral compounds elicit different responses among sexes of same species²⁷.

Pheromones are perceived by a specialized olfactory system located on insect antennae and palpi. This system consists of numerous and variable olfactory organs, *sensillae*, that cover the antennal surface and house the olfactory receptor neurons responsible for olfactory perception. Olfactory neurons responding to general and pheromone stimuli are housed in distinct sensory hairs. Insect olfactory system is capable of simultaneous detection of specific

and general molecules using large amount of receptors. Pheromone sensillae are usually located only on the antennae, while the sensillae for general odours are located both on the antennae and the mouthparts. Pheromones (and other odours as well) are adsorbed on the waxy surface of sensory hairs and are thought to diffuse to the inner cuticular face through the pores in the cuticular wall of the sensilla²⁴. Then the molecules are desorbed from the pore surface by carrier proteins, the pheromone (general odour) binding proteins (PBPs, GOPBs)²⁸. Binding proteins are believed to transport volatile hydrophobic odour molecules across the aqueous sensillar lymph to the membrane bound olfactory receptor proteins located on the dendritic membrane of olfactory receptor neurons²⁹. Olfactory receptors were recently revealed to be heteromeric ligand-gated ion channels, composing of at least one common subunit (Orco) and one or more subunits that confer odorant specificity. The receptor responds to odorant binding by opening a non-selective cation pore^{30,31}. Interaction of odour with olfactory receptor elicits a process called olfactory transduction, which transforms information coded in the chemical composition of odour into the electrical code of the brain. The electrical message is then carried from the antennal olfactory receptor into the olfactory and integration centres of the brain, where the signals are recognized, integrated with other stimuli and eventually trigger the behaviour³². Due to signal specificity and effectiveness, sex pheromones can be used for detection and monitoring of insect populations.

1.2 Pest control

Pheromones may also represent a basis for development of environmentally safe pest control methods, such as mass trapping^{33,34}, mating disruption³⁵ and sterile insect technique (SIT)^{36, 37} that can help to reduce the traditional use of synthetic insecticides³⁸ such as organochlorines or organophosphates – fenthion (CAS number 55-38-9) and malathion (CAS number 121-75-5), which threaten not only human health³⁹ but also biodiversity. Moreover, since the insecticide overuse has resulted in the development of resistance^{40, 41}, pheromones may represent a possible alternative of control of resistant populations. Synthetic pheromones are utilized not only as a monitoring tool in place of a luring lamp but also for mass trapping and disruption of mating communication in the field⁴². Mating disruption by use of pheromones is a promising and, in many cases, a successful strategy for control (confusion strategy)⁴³.

SIT is the one of such target-specific and environmentally non-disruptive methods that does not introduce new genetic material into existing population. It relies on mating between mass-reared sterilized males and wild females, which as a consequence produce unfertile eggs. SIT however, is only effective when applied on area-wide basis, affecting the whole population and when sterile males are of the same species or population as are the wild females. During the control treatment, large numbers of sterilized males are released over monitored area to reduce the fertilization of wild females. SIT thus enables control, eradication and/or serves as prevention barrier maintaining pest-free areas. SIT is successfully used in many countries to control populations of model pest organism *C. capitata* (Latin America, Southern Australia, U.S.A)³⁶ and currently is attempted for other fruit fly species. SIT helped in eradication process of disease vectors, such as tse-tse fly (genus *Glossina*) and malaria vector *Anopheles gambiae*, in African countries⁴⁴. There are various techniques of sterilization. One of the most frequently used is ionizing radiation, that breaks down the chromosomes of the germ line leading to dominant lethal mutations in the sperm and eggs⁴⁵.

Mass or laboratory reared insect are known to be less competitive than wild ones, due to changed behaviour under artificial conditions⁴⁶. To improve their mating performance and effectiveness, various nutritional (protein rich diet)⁶, hormonal (application of juvenile hormone to accelerate maturation)⁴⁷ and semiochemical supplements (exposure to ginger or citrus oils)⁴⁸ are used. Molecular technologies leading to genetic manipulation, e.g. manipulation of sex-determining genes causing sterility or female-specific lethality, are being developed to

improve strains for mass rearing⁴⁹. Although biological controls like SIT, mass trapping and/or mating disruption technologies are being extensively employed to manage Tephritid fruit flies, use of insecticide chemicals is still the principal control tool⁴⁰.

An already mentioned, essential requirement for successful SIT implementation into the pest management is good mating competitiveness of laboratory mass reared sterilized males with wild populations. This is based on good physical condition of mass released males and precise taxonomical compatibility with the wild local populations.

2. Analytical methods employed in pheromone study

Many pheromones consist of multicomponent mixtures, present in very low amounts – 10^{-9} g or even 10^{-12} g. Therefore, their analysis together with structure elucidation requires separation and identification techniques capable of high resolution and sensitivity^{50,51}. Due to scarce amounts emitted by insect, the acquisition of quantities sufficient to carry out the full range of spectroscopic tests (^1H and ^{13}C NMR^{52,53,54} routinely used in structural organic analysis, is often impossible. Due to chemical character (volatile below 300 °C, low molecular mass), pheromone identifications predominantly rely on GC-MS⁵⁵. Mass spectrum, however, provides insufficient information to determine the geometry of the double bond; for example, the mass spectra of most monounsaturated acetates of a given chain length are quite similar⁵⁶. Therefore complementary supportive methods are always needed. Among them are the comparison with synthetic standards, calculation of retention indices⁵⁷, microderivatization, and their combinations. Microderivatization techniques are used to enhance chromatographic properties, for modification of functional groups, and for determination of absolute configuration of studied semiochemicals⁵⁰. Double bond position is mostly determined using DMDS (dimethyl disulfide) followed by GC-MS analysis⁴² or by chemical ionization with acetonitrile, which occurs directly inside the GC injector⁵⁸. Chiral components of pheromone are subjected to reaction with homochiral agent producing diastereoisomeric products that are separable on achiral stationary phase⁵⁹.

The most widely used method for compounds structure identification is the retention index system developed by Kováts (*I*) for isothermal separation where retention indices are calculated relative to straight-chain alkane standards⁶⁰. The linear-temperature programmed retention index (LPTRI) is used for separations within temperature ramp.

$$I = 100n + 100 \left(\frac{\log t'_{R(i)} - \log t'_{R(n)}}{\log t'_{R(n+1)} - \log t'_{R(n)}} \right) \quad \text{Eq. 2.1}$$

Eq. 2.1: Equation for calculation of Kováts index

$$LPTRI = 100n + 100 \left(\frac{t_{R(i)} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}} \right) \quad \text{Eq. 2.2}$$

Eq. 2.2: Equation for calculation of Linear programmed temperature index.

The difference between Eq. 1 and Eq. 2 is related to logarithmic scale and the use of adjusted retention time [$t'_{R(i)}$] for I and absolute retention times for LPTRI [$t_{R(i)}$]⁶¹.

Comparison of the retention indices of the unknown structure and those suggested with GC-MS library can rapidly eliminate some structures from further consideration, while retention index matches, particularly on more than one type of stationary phase, provide strong evidence for a correct identification⁵⁶. The retention data are robust tool for qualitative GC analysis and have been used extensively in the identification of pheromones, based on correlation between retention time of unknown compound and a standard of known structure providing strong support for tentative identification. Combination of retention indices with mass spectrometry has shown to be effective tool for the pheromone identification.

2.1 Extraction of volatile compounds

Although insect-produced pheromones have most often been isolated by extraction of whole insects or insect glands, there are two main disadvantages to this procedure. First, some substances are synthesized and released immediately by the insect instead of being stored in a glandular structure, and second, glands may contain precursors and inhibitory substances that may also complicate the isolation and purification of the active chemicals^{62,63}. In contrast to this labour-intensive preparation, collection of volatiles by headspace from the air surrounding insects allows information to be gained not only on the qualitative composition but also on the relative concentrations of the components. Ratios of volatiles in a multicomponent semiochemical bouquet are usually critical for biological activity.⁶⁴ Dynamic headspace analysis has been widely used for collecting air-borne pheromones from live organisms and substrates⁶⁵. It is generally based on two main approaches: (a) purge and trap approach, where the volatile fraction is accumulated from the gaseous flow stream stripped *through* the matrix onto a suitable trapping medium (a cold trap, a sorbent, an adsorbent or a specific reagent or solvent for a given class or classes of compound). The second approach, (b) dynamic, is where analytes are sampled from the gaseous flow stream passed *over* the matrix⁶⁶. The sampled volatiles are generally recovered either by solvent elution or (more often) by thermal desorption on-line or off-line to the GC or GC-MS system. The apparatus for pheromone collection consists of an entrainment chamber with an inlet and outlet through which purified air can be drawn using a vacuum pump. The airborne, volatile pheromones are usually trapped on Porapak Q, Tenax, or activated charcoal^{50, 67}. Collecting volatiles from insects sometimes needs to be done over a period of several days, depending on diurnal cycle

also to obtain enough material for chemical analysis⁶². In addition to mentioned collection methods, a technique for solid phase micro-extraction (SPME)⁶⁸ of airborne volatiles is frequently used due to elimination of solvents and short extraction time (min)^{42,69,70}. SPME consists of a fibre coated with an adsorbent that extracts organic compounds from the gas surrounding liquid or solid sample, or from the surface of biological material. The extracted compounds are desorbed in the heated GC injector port operating in splitless mode⁵⁰.

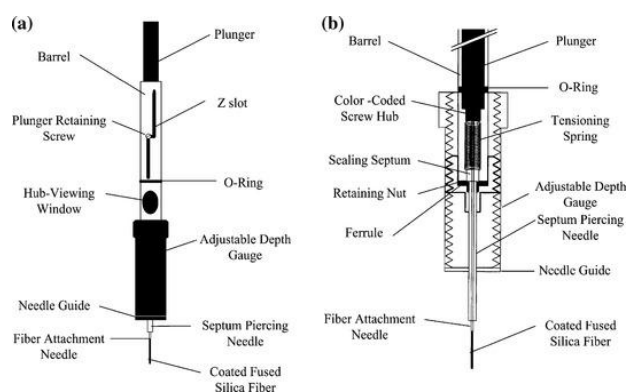


Fig. 2.1: Commercial SPME device: (a) SPME fibre holder; (b) section view of SPME holder and fibre assembly⁷¹.

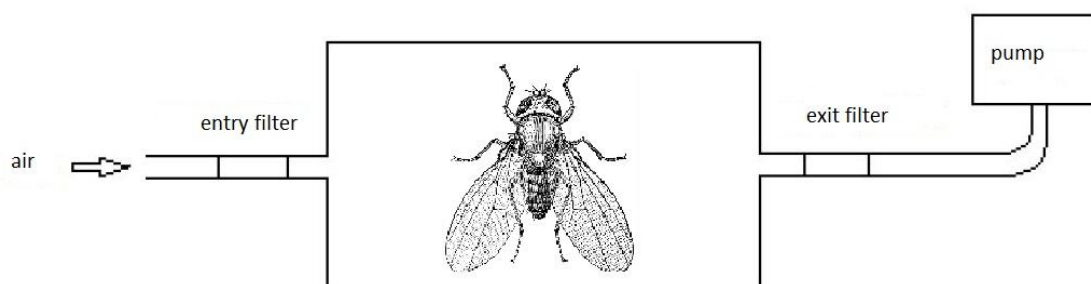


Fig. 2.2: Dynamic headspace trapping of volatiles, fruit fly aeration⁷².

2.2 Separation techniques

Chromatography is one of the most successful techniques to separate and yield quantitative information about components of complex mixtures⁷³. Gas chromatography (GC) is widely used analytical method since it provides high separation efficiency, rapid method development and validation, as well as straightforward coupling to several spectroscopic techniques⁷⁴. One of the basic requirements of GC is that substances under test must be volatile at the operating temperature in order to be eluted and detected, while the stationary phase must be sufficiently non-volatile and thermally stable in order to serve as the substrate on which separation occurs. The molecular mass operating range of GC spans the interval 2–1500 atomic mass units (amu), so that the compounds that can be separated by GC range from permanent gases (i.e., highly volatile substances), volatile compounds (with a mass up to 200 amu), to semi-volatile compounds (>200 amu)⁷⁵.

In the last decade, the implementation of comprehensive two-dimensional gas chromatography (GC×GC) facilitated unravelling of the trace-level composition of complex mixtures⁷⁶; enabling analysis of pheromones⁵⁵, petrochemical analysis⁷⁷, environmental analysis of polychlorinated biphenyls⁷⁸ or even separation of plasma fatty acids⁷⁹ and cigarette smoke⁸⁰. The method, invented in early 90's by Philips⁸¹, has superior chromatographic resolving power that is particularly suitable for separation of low molecular weight analytes in complex samples. The entire sample is subjected to two distinct analytical separations: the whole sample undergoes two fundamentally different separation mechanisms, creating so-called orthogonal conditions⁸², which lead to increased component resolution⁸³. In principle, entire effluent from the first column is introduced to the second column according to a chosen modulation period which determines the sampling duration of the first column effluent⁸⁴. The combination of different column lengths is related to the necessity of slow temperature program (1-5 °C/min)⁸⁵ in the first dimension and a very fast high-resolution secondary separation (high-speed GC). With regards to column dimensions, the most common choice for the primary capillary column is the conventional non-polar 30 m × 0.25 mm i.d. column with film thickness of 0.25–1.0 µm producing relatively broad peaks; the second dimension generally consists of a short polar or shape-selective⁸⁶ 1–2 m × 0.1 mm i.d × 0.1 µm film thickness micro-bore column⁸⁷. The instrumental set-up ensures that the total second dimension separation is completed in the run time of the first-dimension analysis⁸⁸. The modulation time is longer than the retention times of the compounds most strongly retained in

the second dimension⁸⁶. Gas flow accelerates between first and second dimension, which results from the higher phase ratio of the secondary column, and also its smaller internal diameter causing increase of linear carrier gas velocity⁸⁹. The combination of non-polar and polar/shape selective column allows components to be eluted according to distinct separation mechanisms. In the first dimension the compounds are separated according to their boiling points, and in the second dimension according to their interactions with stationary phase, by means of hydrogen bonds, π - π interactions or steric effects^{86, 90}. The separation in second dimension is fast, isothermal – thus the boiling points of analytes with similar volatility will have negligible contribution to division of compounds, furthermore only the specific interactions with the stationary phase will govern the retention. Both dimensions operate statistically independently and the entire 2D plane of the GC $\times$ GC chromatogram, the so-called “separation space” is available for peak separation^{88, 91}. Phenomenon of “wrapped around compounds,” occurs if their elution time exceeds the modulation period, causing co-elution of compounds from sequential modulations. “Wrapped-around compounds” are not problematic as long as the peaks are not overlapping other peaks in the chromatogram, however, it should be avoided whenever possible⁸³.

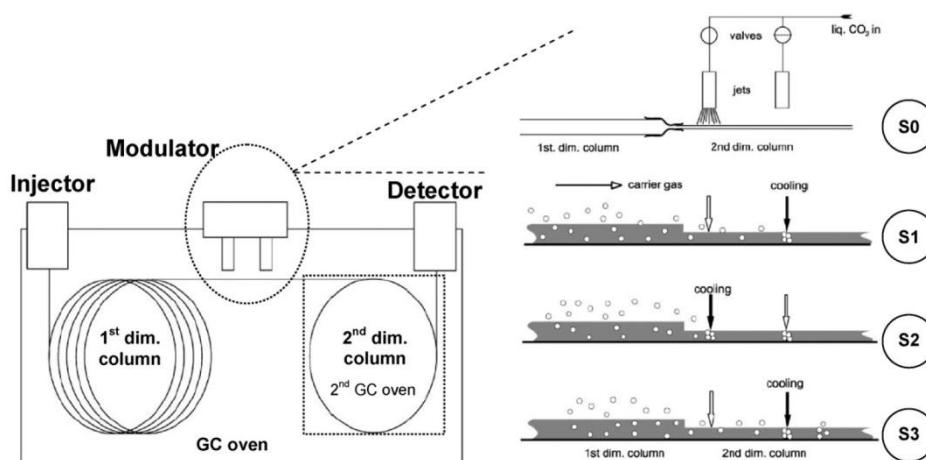


Fig. 2.3: Schematic of two-oven GC $\times$ GC system and explanation of cryo-modulation. Left: typical set-up. Right: (S0) general set-up of dual-jet cryogenic modulator. (S1) Right-hand-side jet traps analytes eluting from first-dimension column; (S2) right-hand-side jet switched off, cold spot heats up rapidly and analyte pulse is released into second-dimension column; simultaneously, left-hand-side jet switched on to prevent leakage of first-dimension column material; (S3) next modulation cycle is started⁸⁸.

Fundamental concept of GC $\times$ GC is based on well-known principles of 1D-GC separation, thus, the columns and injector are not particularly specific to GC $\times$ GC and commercially available hardware is satisfactory. GC $\times$ GC is essentially dependent upon the interface be-

tween the two columns connected by modulator, which is the key component. Its main function is to continuously trap and/or sample the entire primary column effluent during the analysis and periodically inject it into the secondary column⁷³. Following the functional principles modulators are classified into two main categories: thermal and flow modulators. Thermal modulators can be further subdivided into devices called cryogenic and cryogen-free modulators (cooling-based) which retard analytes and cause on-column trapping or focusing of bands – these are most commercially popular; and modulators using elevated temperature to accelerate solute into a narrow band (heater-based)⁹². Flow modulators typically utilize transfer lines and pressure differentials to fill a collection channel (or channels) with primary column effluent that is periodically and rapidly flushed into the secondary column. Location of modulator varies with its working principle: thermal modulators are usually located at the head of the secondary column, whilst flow modulators are placed between the columns⁷³.

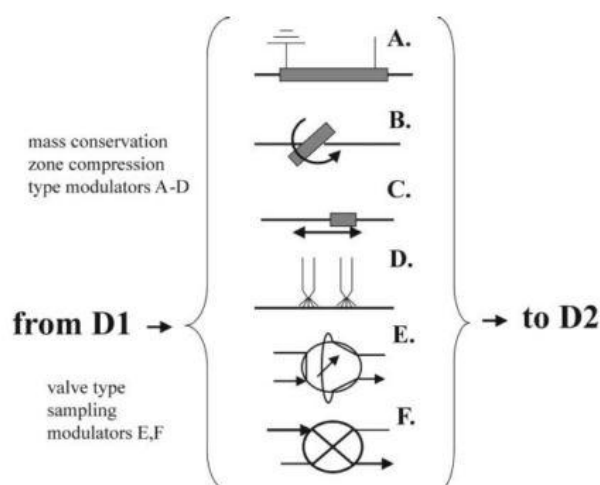


Fig. 2.4: Different modulators may be classified as mass conservation types (A.-D.) or sampling (valve) types (E. and F.) The modulator is located between first and second column, D1 and D2, respectively. A) electrically-heated sleeve, B) rotating heated-sweeper, C) cryogenically-cooled trap, D) dual cryogenic jet system; flow switching modulators E) “large volume” collecting tube, F) small segment sampling⁹².

Compared to conventional 1D-GC, GC×GC results in enhanced peak capacity (improved separation) – due to multiple separation dimensions, and high sensitivity (improved detectability) – due to analyte refocusing process between separations^{76,93}. Furthermore, as the separation followed orthogonal principle – analytes are ordered according to their chemical structures, which facilitates group analysis (homologues, isomers)⁸⁶. Substances belonging to a particular category such as the alkylbenzenes/terpenes etc. fall within clearly recognizable boundaries⁸⁹. The optimization process in comparison with 1D-GC is considerably more complex because the two dimensions are intimately related⁸⁷. For example carrier gas flow

and temperature will affect separations in both columns differently⁸⁶. Optimization involves a combination of factors (modulation frequency and efficiency, T, and/or flow programming, etc.)⁹¹. Optimal injection time into the second-dimension column should be several microseconds (μs)⁹⁴ to minimize band broadening⁹³. GC $\times$ GC-MS analysis produces enormous amount of data, therefore processing is essential. Chromatograms are visualised as contour plots, with x axis corresponding to retention times at first dimension, and y axis depicting retention times at second dimension. Final chromatogram contains spots representing peaks, colour intensity indicates the amount of compound⁸⁵.

2.3 Detectors for GC $\times$ GC

The narrow peaks (50-200 ms) resulting from fast separation in second dimension require detectors with small volume, in order to decrease potential band broadening, and optimal acquisition speed (short rise time of the detector) in order to reconstruct the second dimension chromatograms properly^{86,95}. Universal and robust flame ionization detector (FID) is well suited for GC $\times$ GC analysis as it provides fast ionisation, fast acquisition rate and negligible band broadening; it is often utilized not only for quantification, but also for peak identification based on retention times of structurally similar groups of compounds. Alternatives employing electron-capture (ECD) detectors and nitrogen-phosphorus detectors (NPD) are to be found in analysis of pesticides^{96,97}. A mass spectrometer functioning as GC $\times$ GC detection system enhances the identification capability⁸², enables confirmation of compounds as well as deconvolution of partially overlapping compounds⁸⁵. According to Mondello, nowadays the combination of GC $\times$ GC with mass spectrometric dimension (MS) generates the most powerful analytical tool for volatile and semi-volatile analytes. Almost all GC $\times$ GC-MS applications have been carried out by using either a time-of-flight (TOF) or quadrupole mass analyzer⁸⁷.

Time of flight mass spectrometry

The principle of time of flight detector (TOF) is quite simple: ions with various m/z are dispersed in pulsed ion beam followed by separation by their time of flight in the field-free drift path of known length⁹⁸. Analysis consists of three steps: ionisation, acceleration and flight time measurement. Ionisation is traditionally carried out by either electron impact (EI), or chemical ionisation (CI), however in previous years TOF became more and more popular in analysis of large biomolecules along with development of matrix assisted laser

desorption/ionisation technique (MALDI)⁹⁹. The ions generated in the ion source are accelerated by pulsed electrical field (10-100 ns)¹⁰⁰ as discrete packages leaving the source at the same time and having the same initial velocity. However, the smaller ions travel faster and reach detector sooner than heavier ions. Thus, velocity is proportional to mass and m/z ratio is obtained as the time taken reach detector, which is in order of ms¹⁰¹.

$$v = \sqrt{(2ezU/m_i)} \quad \text{Eq. (2.3)}$$

$$t = s\sqrt{(m_i/2ezU)} \quad \text{Eq. (2.4)}$$

Eq. 2.3: Equation describing velocity v of ion accelerated in electric field; derived from equation describing kinetic energy. e = electron charge, z = integer number of electron charges, U = voltage, m_i = mass of ion.

Eq. 2.4: Time taken for ion to travel in field-free environment of known length can be obtained by substitution of velocity v into $t = s/v$.

Due to its high ion extraction speed, the TOFMS analyzer works at very high repetition rates (5–30 kHz)¹⁰² and is able to generate up to 500 spectra/s, yielding clean spectra thanks to almost simultaneous detection of ions¹⁰³. High acquisition speed, ability to detect very narrow chromatographic peaks enables coupling with (ultra-) fast-GC or GC×GC separation system. TOF instruments are capable of high resolution ($R = 10\,000$ at full width half maxima peak height) what allows acquirement of quantitative and qualitative data¹⁰⁴. Comprehensive GC×GC-TOFMS, was successfully used for separation of groups of natural compounds⁵⁵ or metabolomic analysis¹⁰⁵. TOF can be found either in linear, or reflector (ReTOF) setup. The latter offers increased resolving power due to energy correction by compensating of flight-time differences of ions with different kinetic energy⁹⁹.

2.4 Biologic detection: Electroantennography (EAD)

Insect sensory system relies on specifically adapted hair-like structures “sensillae” on the antenna, which allow insect to sense volatile compounds and respond within milliseconds¹⁰⁶. Almost 60 years ago German biologist Schneider showed that microelectrodes inserted into the base and tip of an insect’s antenna enabled observation and recording of slow depolarization across the antenna in response to stimulation by biologically active volatile compounds. This technique, almost uniquely used in entomological laboratories, is termed electroantennography¹⁰⁷. Electrodes record the signal generated by insect antenna as a negative deflection (depolarization) of several mV and its amplitude is concentration-

dependent. Insect antenna is widely used in pheromone analysis as a biological detector, since it is very sensitive and detects specific chemical signals that affect insect behaviour. Combination of EAG with GC leads to creation of a new analytical technique, GC-EAD (gas chromatography with electroantennographic detection) that is widely used in insect chemical ecology. The GC-EAD is accomplished by splitting effluent into two lines that lead to the two detectors, conventional and insect antenna¹⁰⁶. The antenna works as highly specific biological detector tuned specifically to biologically active compounds. In many cases, antenna is more sensitive than conventional detectors. Thus, EAD helps to focus effort to certain parts of chromatogram where the activity was observed^{108, 109}. Electrical contact between micro-electrodes and antenna surface is usually done using microelectrodes Ag/AgCl with saline solution (Ringer's solution consisting of 7.5 g NaCl, 0.2 g CaCl₂, 0.2 g NaHCO₃ and 0.1g KCl in 1 L of solution); otherwise electro conductive gel can be used. Antennal preparation might differ: animal may remain intact, or its head or antenna can be cut off. Sample preparation affects the signal-to-noise ratio, which is also dependent on insect physiology.

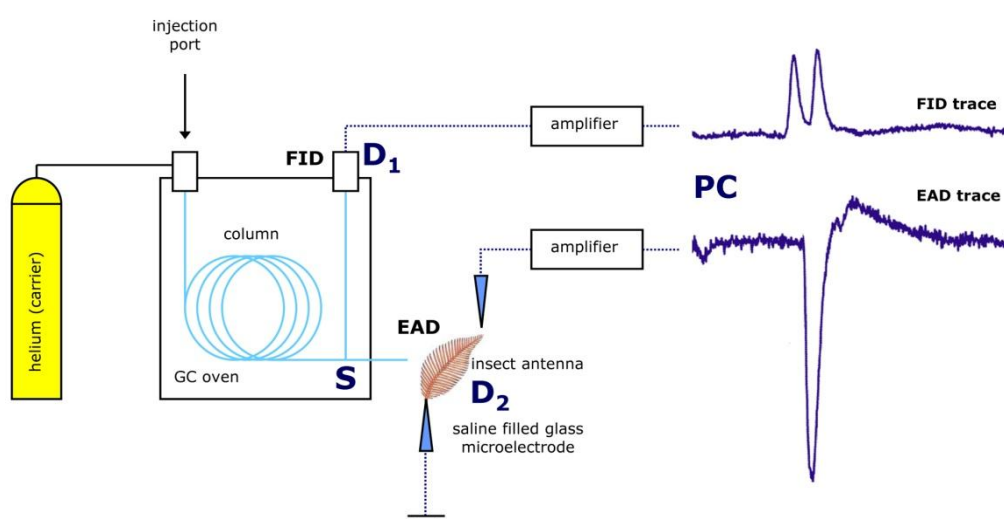


Fig 2.5: GC-FID/EAD scheme: Injected sample undergoes separation in one-dimensional GC. Effluent is split to two detectors – FID and EAD in ratio: 1:1. Pheromone components are detected simultaneously – chromatographic peaks are correlated to biological responses¹¹⁰.

2.5 Additional techniques used in pheromone studies

GC-FTIR

Combination of gas chromatography and Fourier transform infrared spectroscopy has been less employed than GC-MS in pheromone research. GC-FTIR is useful for determination of double bond geometry^{89, 109}.

NMR spectroscopy

Pheromone components and structural analogues in various species have been characterized by ^1H and ^{13}C NMR spectroscopy. Nuclear magnetic resonance is utilized not only for elucidation of molecular structure, but also in explaining intermolecular regulation mechanism for pheromone binding and release¹¹¹.

HPLC

Liquid chromatography is used in very few cases; the technique is suited better for more polar and less volatile compounds. However, species such as various ragworms (*Nereis*) produce peptides as their sex-pheromones¹¹².

3. Methods and technical equipment

Rearing of insect

Ceratitis fasciventris, *Ceratitis rosa* and *Ceratitis anonae* were provided from entomological laboratory of Dr. Sunday Ekesi (International Centre of Insect Physiology and Ecology, African Insect Science for Food and Health, Nairobi, Kenya). Obtained flies were in stadium of 2-3mm long pupae (approximately 100 individuals of each species). Pupae of different species were placed into glass aquariums (30 × 20.5 × 16 cm) separately and were allowed to emerge. Nylon covers were used to prevent hatched flies from escaping. Each aquarium was equipped with water and cane sugar mixed with brewer's yeast (3:1 ratio). Rearing room was tempered to 27.5 °C; humidity was approximately 70% and light: dark regime was 14:10 hours, respectively. Hatched flies were separated according to sex as soon as they emerged (females were distinguished by visible ovipositor organ at the end of abdomen).

Collection of volatiles

Technique of volatile collection was performed as described in by Nation¹¹³. All glassware was scrupulously cleaned with hot water and detergent, then washed with ethanol, distilled water and heated for 1 hour at 150 °C before use to avoid cross contamination. 5 virgin male and female flies were separately placed into round-bottom flasks adapted to volatile collection with volume of 250 mL (Verkon, Praha, Czech Republic) together with lump of sugar and water soaked cotton in vial. Air was sucked by the pump (Pocket Pump 210 Series, SKC Inc., PA, USA) at 100 mL/min from the flask, through a glass pipette-shaped filter with sieve located at thinner end. The filter was filled with layer of silanized cotton (Applied Science Laboratories, Inc. Bedford, Massachusetts, USA), followed by SuperQ[®] (copolymer of ethylvinylbenzene and divinylbenzene, Alltech ARS Inc., Gainesville, Florida, USA) adsorbent layer ($m = 30$ mg), and finished with another layer of glass wool and Teflon ring. Air entering the flask carrying volatiles went through another filter of the same construction as described above that filtered out the potential contaminants. Filters were pre-cleaned with 12.5 mL acetone and heated in oven at 120 °C for 60 minutes. The apparatus for collecting the volatiles was properly isolated from surrounding room air using clamps, Teflon seal tapes, tubes and joints (PTFE, Applied Science Laboratories, Inc. Bedford, Massachusetts, USA). 500 µL of freshly distilled HPLC quality *n*-hexane was used (Lachner, Neratovice, Czech

Republic) to wash volatiles out of each filter capturing the insect pheromone. Eluate was transferred into vial and sealed for analysis. An empty flask, only with sugar and water, acted as control sample and was analysed under same conditions in order to discriminate fruit fly volatiles from background noise. Volatiles in this experiment were collected for 24 hours, age of tested flies was 20 days. Three independent repetitions were made of *C. fasciventris* and *C. rosa*. For *C. anonae*, only two repetitions were possible due to lack of sufficient amount of live flies.

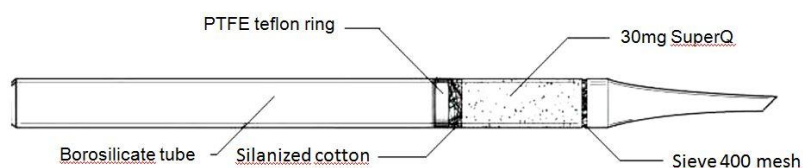


Fig. 3.1: Scheme of filter used for volatile collection: borosilicate tube of 6.4 mm diameter equipped with stainless steel sieve¹¹⁴.

3.1 GC×GC-TOFMS analysis

The separation and analysis of the FAR complex male pheromones were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, Michigan, USA) consisting of Agilent[®] 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) used in splitless mode and followed by TOFMS Pegasus spectrometer (LECO Corp., St. Joseph, Michigan, USA). The key part this instrument is 4-jet cryomodulator, as the modulation frequency and temperature has great impact on separation.

1 μ L of sample was injected into constant flow of helium (1 mL/min), which was used as carrier gas. Injector temperature was 220 °C, temperature in the first dimension was held for 2 min at 40 °C followed by increase 5 °C/min to the target temperature of 270 °C which was held for 10 min. In the second dimension the temperature program was identical, except initial and target temperature, which were 50 °C and 280 °C, respectively. Modulation period was 4 s, hold pulse time was 0.6 s and cold pulse time between stages was set to 1.4 s. Modulation temperature offset relative to the GC oven temperature was 30 °C. Temperature of transfer line connecting secondary column to the TOFMS detector source was operated at 280 °C.

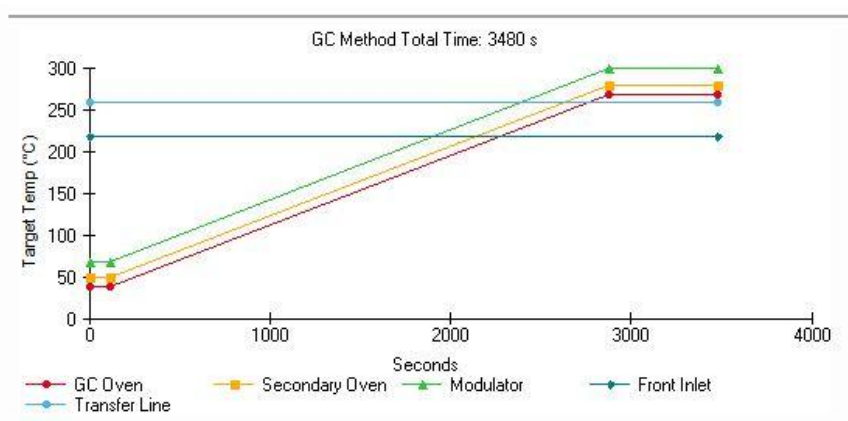


Fig. 3.2: Graphic depiction of temperature program during analysis. (Taken from Pegasus 4D software; LECO Corp., St. Joseph, Michigan, USA).

The first dimension column utilised a weak-polar DB-5 (J & W Scientific; 30 m × 250 μm ID × 0.25 μm film), and the second dimension column was polar BPX-50 (SGE, Austin, TX, USA; 2 m × 100 μm ID × 0.1 μm film). The second column was located in its specific oven with the temperature mode programmable independently of the first oven. The source temperature was 250 °C with a filament bias voltage of −70 V. The data acquisition rate was 100 scans/s, along with a mass range of 29–400 amu and a detector voltage of 1 650 V.

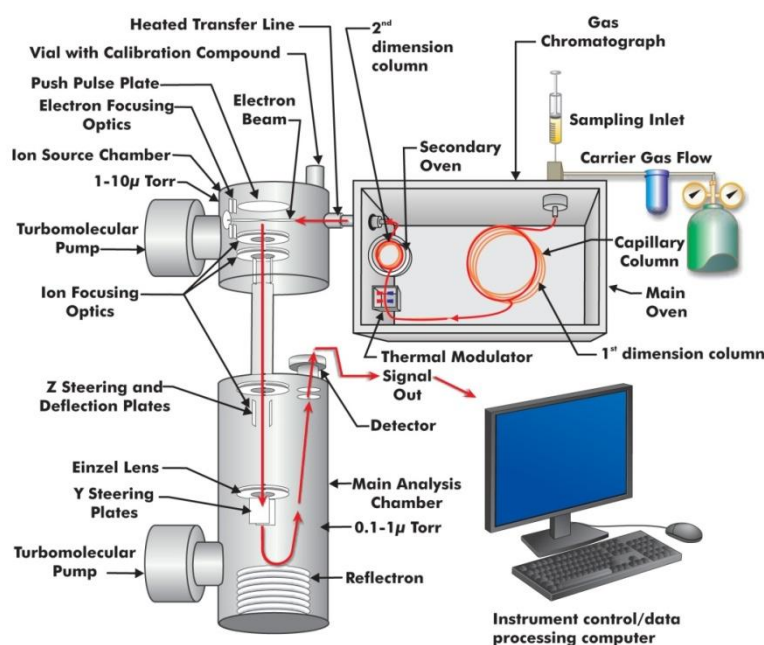


Fig. 3.3: LECO Pegasus IVD scheme. (Taken from Pegasus 4D operation manual; LECO Corp., St. Joseph, Michigan, USA).

As first a mixture of alkanes C₈-C₂₂ (1×10^{-3} μg/μL, Sigma-Aldrich) was analysed at given conditions, followed by the pheromone samples. LECO ChromaTOF™ is equipped with retention index (*RI*) calculation function: manual processing of homologous series of alkanes leads to information on retention times, which are imported to LECO ChromaTOF™ software in order to calculate retention indices of detected compounds in analyzed samples and to incorporate this information into the results of the library search algorithm to match obtained spectra with those provided by mass spectral databases. LECO ChromaTOF™ software allows visualisation of the chromatographic data either in 2D or 3D chromatograms. TIC (Total Ion Chromatogram - sum of intensities of all mass spectral fragments of the same scan). In such chromatograms, each compound is represented by a 3D peak, intensity of which is colour coded. LECO ChromaTOF™ software creates several modulations of each peak and calculates the retention time (*t_R*) from the most abundant modulation, which not always matches the apex of the peak. Software uses mathematical multivariate curve-resolution procedures (deconvolution) to obtain and combine accurately pure-mass spectra of a specific compound in co-eluted peaks in order to identify and quantify compounds correctly¹¹⁵. Analyte identification in the experiment was based on comparison of their mass spectra fragmentation patterns obtained by electron impact ionisation, two-dimensional retention times and retention indices with available standards and/or previously published data. Not all the authentic standards were available and in such cases the identifications were carried out using reference spectra in NIST library, Wiley/NBS registry of mass spectral data and published retention indices¹¹⁶ and available literature. Compounds were identified by GC retention behaviour on two different phases of GC×GC, by MS spectra and biological activity was confirmed by GC-EAD analysis. With regards to quantification, raw area percentages obtained by GC were used for reporting the relative ratios of active compounds in the pheromone blend.

Synthetic standards used in this experiment were purchased from Sigma-Aldrich and tested in concentration 5×10^{-3} μg/μL. (*Z*)-non-3-enal and (*Z*)-non-2-enal were prepared in the laboratory from the corresponding alcohols. Purchased and synthesized chemicals were of analytical grade purity.

Tab. 3.1: List of tested standards with corresponding CAS number.

Name	CAS No.
Methyl (<i>E</i>)-hex-3-enoate	13894-62-7
Methyl (<i>E</i>)-hex-2-enoate	13894-63-8
6-Methylhept-5-en-2-one	110-93-0
Ethyl hexanoate	123-66-0
Ethyl (<i>E</i>)-hex-3-enoate	2396-83-0
Methyl (<i>E</i>)-oct-2-enoate	7367-81-9
Geranyl acetone	3796-70-1
(<i>E,E</i>)- α -Farnesene	21499-64-9
Methyl (2 <i>E</i> ,6 <i>E</i>)-farnesoate	10485-70-8
Linalool	78-70-6
(<i>E</i>)-Non-2-enal	18829-56-6
(<i>Z</i>)-Non-2-enol	31502-14-4
(<i>Z</i>)-Non-2-enal	30551-15-6
(<i>Z</i>)-Non-3-enol	10340-23-5
(<i>Z</i>)-Non-3-enal	31823-43-5

3.2 GC-EAD analysis

Headspace pheromone samples from 5 males were diluted in *n*-hexane and submitted to separation using HP 5890 chromatograph (Hewlett Packard, Palo Alto, CA, USA) with DB-5 colon in splitless mode. The GC column was split into two by Graphpack 3D/2 four-arm splitter (Gertsel Inc., Baltimore, MD, USA) directing the eluate to two detectors working simultaneously – flame ionisation detector (FID) and the antenna (EAD). Volatile compounds were separated in continuous helium stream (1 mL/min). Parameters of GC oven were similar to temperature program applied at GC×GC-MSTOF, and were as follows: injector temperature was set to 200 °C, FID filament temperature was 260 °C. GC colon was operated at temperature program starting at 50 °C for 2 min, followed by 5 °C/min increase reaching temperature of 270 °C, which was held for 10 min. In order to correlate GC×GC-TOFMS and GC-EAD data, the retention indices (*RI*) were calculated using a standard mixture of *n*-

alkanes C₈ – C₂₂ (1×10⁻³ µg/µL) injected and analyzed under the same conditions as the pheromone samples in both GC×GC-MSTOF and GC-FID/EAD systems.

Fruit fly antennal detector, EAD, was prepared by cutting off the head of a narcotized fly, halving the head and fixing it between two Ag/AgCl glass microelectrodes containing Ringer's solution. The reference electrode was inserted into the head capsule, the recording one was positioned to make a contact with the sensory epithelium on the last antennomere surface. The antennal preparation was then placed in a continual air stream (1 L/min) blowing from a glass tube (8 mm in diameter) in which the split GC eluate was directed. The electrical signal generated by the antennal preparation was led to a high impedance pre-amplifier (10¹⁴ Ω; SYNTECH Equipment and Research, Kirchzarten, Germany) and fed to a PC. Data were evaluated using Syntech GC-EAD software, where signals from FID and EAD were displayed and analyzed simultaneously. Not all FID peaks elicited EAD responses. If some FID peaks were associated with EAD activity in at least 3 independent GC-EAD experiments a compound was classified as biologically active. In order to confirm whether an identified compound elicits antennal response, synthetic standards were dissolved in hexane and GC-EAD analyzed under the same conditions as pheromone samples. If synthetic standard eluted at the same *t_R* as the compound from the pheromone and elicited similar GC-EAD reaction, the biological activity of that compound was considered as confirmed. Not all antennal preparation prepared under described conditions was sensitive enough and used in analysis. Their sensitivity was tested by a specific test stimulus injected into the air stream through a small orifice (5 mm diameter) in the air delivering tube wall situated 5 cm downstream from the mixing chamber where GC eluate was directed. These test stimuli were injected from odour cartridges. Odour cartridges were prepared from Pasteur pipettes with filter paper discs (10 mm diameter) to which the respective stimuli were loaded on. Odour cartridges were prepared prior to each experiment in the following way; one male equivalent aliquot of the respective species was loaded onto filter paper discs. Then, the solvent was allowed to evaporate and Pasteur pipettes were sealed with Parafilm M[®] and allowed to equilibrate for about 1 h. Parafilm seal was removed just before stimulation. These odour cartridges were used repeatedly during the day of GC-EAD experiments¹¹⁷.

3.3 Statistics

The data obtained from the chemical analysis ($N = 7$) of male emanations for each species were statistically evaluated. For the statistical analyses, the peak areas of the 22 common compounds identified in the volatile mixtures released by 20 day-old males of all three studied species of FAR complex were used. For the further analysis only the 12 antennally active compounds were used. The differences in the chemical composition of the samples from all of the three species were analyzed by principal component analysis (PCA). Prior to the PCA analysis, the peak areas were subjected to logarithmic transformation, scaling was focused on inter-species correlation, each species score was divided by its standard deviation and the data were centered by species. In the PCA analyses, samples with similar chemical profiles cluster together and segregate from those that are different. PCA was employed for unimodal data whereas correspondence analyses (CA) was used for linear data. The multivariate data analysis software CANOCO 4.5 (Biometris, Plant Research International, Wageningen UR, The Netherlands) was used for both the PCA and CA.

4. Results and discussion

Evaluation of GC×GC-TOFMS analysis

Each gas chromatogram measured was at first compared with control sample to trace artificially introduced contamination.

Fruit fly pheromones are highly complex mixtures (representative GC×GC-TOFMS chromatograms are depicted in Fig.4.1). The pheromone blend contains diverse chemical structures, counting alcohols, aldehydes, terpenes, and esters. GC×GC-TOFMS analysis resulted in identification of 35 compounds found in *C. fasciventris*, 18 compounds in *C. anonae* and 26 compounds found in *C. rosa*. Pheromone components were not found in female volatile emanations and therefore are considered male-specific. Out of all identified male-specific pheromone compounds, 11 compounds were found in all studied species in varying abundance and 23 compounds were common for at least 2 species. Within the 11 shared compounds, biological activity was elicited by methyl (*E*)-hex-3-enoate ($RI_{EAD} = 937$), 6-methylhept-5-en-2-one ($RI_{EAD} = 987$), linalool ($RI_{EAD} = 1104$), and methyl (2*E*,6*E*)-farnesoate ($RI_{EAD} = 1799$).

Tab. 4.1, 4.2, and 4.3 depict list of identified substances in each pheromone emanation. Each substance is characterized by its retention index (RI), retention time in 1st and 2nd dimension (t_R). The amount of each respective compound is represented by its average relative area (Eq. 4.1) determined by ratio of median area ($\tilde{x}_{A_i}$) and sum of all area medians of volatile compounds found in the sample ($\sum \tilde{x}_{A_n}$), where n is number of identified compounds. Average relative area is accompanied with respective standard deviation (SD, %). The amount of collected compounds from studied species was analysed using Grubb's test to detect outliers.

$$\text{Average relative area (\%)} = \frac{\tilde{x}_{A_i}}{\sum \tilde{x}_{A_n}} \quad \text{Eq. (4.1)}$$

Eq. 4.1: Calculation of average relative area, (%)

The components exceeding 8% were considered as major ones. The compounds below the set limit were classified as minor ones. In *C. fasciventris* the most abundant major component was ethyl (*E*)-3-hexenoate ($RI_{EAD} = 1006$) accompanied by methyl (*E*)-hex-3-enoate ($RI_{EAD} =$

932) and minor components (1 - 8%) were methyl (2*E*,6*E*)-farnesoate ($RI_{\text{EAD}} = 1799$) and nonan-2-ol.

In *C. anonae*, the major compounds were (*E,E*)- α -farnesene ($RI_{\text{EAD}} = 1507$), methyl (*E*)-hex-2-enoate ($RI_{\text{EAD}} = 966$). Among minor components (*E*)-non-2-enal ($RI_{\text{EAD}} = 1163$), methyl (2*E*,6*E*)-farnesoate ($RI_{\text{EAD}} = 1799$), linalool ($RI_{\text{EAD}} = 1104$), (*E*)- β -ocimene, heptan-2-ol, (*Z,E*)- α -farnesene, and oct-3-enyl acetate were found.

In *C. rosa*, the major component was represented by linalool ($RI_{\text{EAD}} = 1104$), and 2(*E*)-nonenal ($RI_{\text{EAD}} = 1163$). These were accompanied by minor compounds identified as (*E,E*)- α -farnesene ($RI_{\text{EAD}} = 1507$), (*E*)- β -ocimene, 6-methylhept-5-en-2-one ($RI_{\text{EAD}} = 989$), 2,5-dimethylpyrazine, oct-3-enyl acetate, 2,3,5-trimethylpyrazine, octanal, γ -valerolactone, nonan-2-ol, (*Z*)-non-3-enol, methyl geranate, and one unknown compound (unknown 3).

The remaining compounds listed in Tab. 4.1, 4.2 and 4.3 were present in trace amount and represented less than 1% of the total volatile emanation. In *C. fasciventris* the total area ($\sum \tilde{x}_{A_n}$) of identified volatile compounds was 2.27×10^8 out of which 1.99×10^8 accounts for antennaly active compounds (88%), in *C. anonae* 18 identified compounds altogether comprised area of 6.35×10^7 , antennaly active compounds occupied 5.89×10^7 (92%) and in *C. rosa* the total area of 26 identified compounds was 4.06×10^7 out of which 2.90×10^7 (71%) belongs to antennaly active compounds.

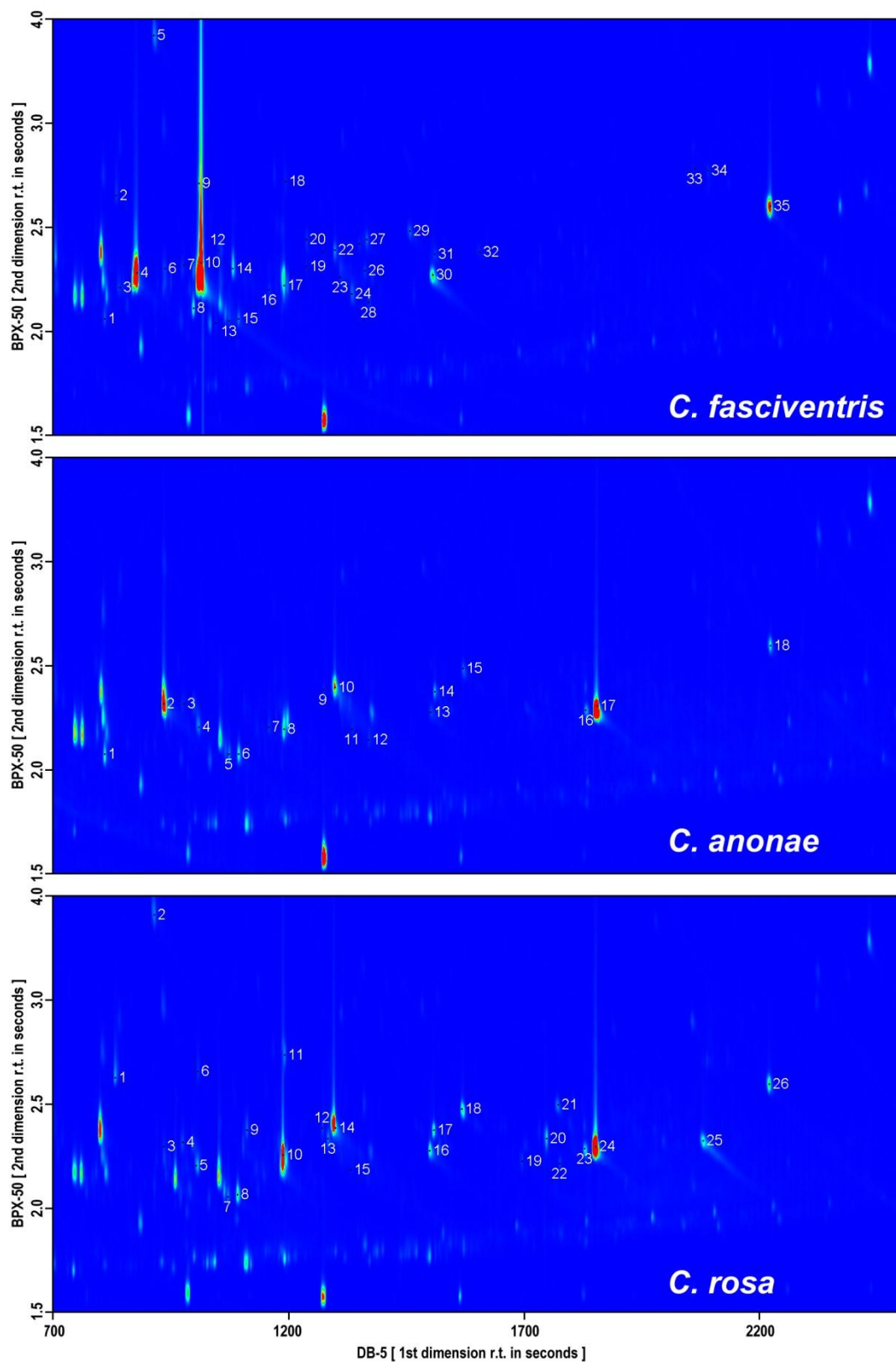


Fig. 4.1: GC×GC-TOFMS chromatograms (TIC mode) of male ($n = 5$) volatiles of *C. fasciventris*, *C. anonae* and *C. rosa*. Each spot represents one compound; identified compounds are numbered in each chromatogram, numbering is valid for each corresponding table of compounds. Intensity of each spot is colour-coded (blue - 0, red - maximum).

Tab. 4.1: Volatile composition of *Ceratitis fasciventris* list of compounds found in calling males using GC×GC-TOFMS

No	Name	RI	RI _{EAD}	t _R [s]	A [%]	SD [%]
1	Heptan-2-ol ^a	901		806, 2.060	0.13	0.01
2	2,5-Dimethylpyrazine ^a	914		830, 2.650	0.23	0.01
3	Ethyl 4-pentenoate	916		834, 2.220	0.27	0.00
4	Methyl (E)-hex-3-enoate * ^s	932	937	870, 2.230	13.59	1.01
5	γ-Valerolactone	956		910, 3.420	1.07	0.33
6	Methyl (E)-hex-2-enoate * ^{a,s}	968	966	934, 2.300	0.16	0.02
7	6-Methylhept-5-en-2-one * ^s	988	989	974, 2.330	0.03	0.01
8	Ethyl hexanoate* ^{a,s}	997	999	990, 2.120	0.87	0.05
9	Ethyl (E)-hex-3-enoate* ^a	1003	1006	1002 2.230	71.49	4.11
10	2,3,5-Trimethylpyrazine	1008		1006, 2.710	0.44	0.03
11	2-Pentylbutyrate	1025		1042, 2.040	0.03	0.01
12	Unknown 1	1027		1046, 2.380	0.07	0.01
13	(Z)- β -ocimene	1040		1070, 2.050	0.32	0.01
14	Ethyl (E)- hex-2-enoate* ^s	1045	1045	1078, 2.300	1.04	0.26
15	(E)- β -ocimene ^a	1051		1090, 2.060	0.24	0.03
16	Unknown 2	1086		1154, 2.210	0.10	0.00
17	Linalool * ^{a,s}	1104	1104	1186, 2.220	0.51	0.04
18	Unknown 3	1106		1190, 2.720	0.08	0.01
19	Methyl (Z)-oct-3-enoate * (?)	1131	1131	1234, 2.310	0.10	0.02
20	Ethyl 3-hydroxyhexanoate	1133		1238, 2.430	0.13	0.05
21	(Z)-Non-2-enal ^s	1151		1270, 2.380	0.01	0.00
22	(E)-Non-2-enal * ^s	1167	1163	1298, 2.390	0.77	0.36
23	(E)-Non-2-enol	1172		1306, 2.310	0.16	0.05
24	Unknown 4	1185		1330, 2.180	0.37	0.04
25	(Z) Non-2-en-4-yn-1-ol	1195		1346, 2.420	0.08	0.01
26	Ethyl (Z)-oct-3-enoate	1201		1358, 2.290	0.12	0.01
27	4-Ethyl-hex-2-ynal	1204		1362, 2.440	0.18	0.03
28	Unknown 5	1206		1366, 2.140	0.11	0.06
29	Unknown 6	1260		1454, 2.480	0.25	0.18
30	Nonan-2-ol	1287		1498, 2.290	1.63	0.64
31	Oct-3-enyl acetate	1292		1506, 2.380	0.19	0.05
32	Unknown 7	1352		1602, 2.380	0.06	0.02
33	Unknown 8	1722		2054, 2.790	0.04	0.01
34	Unknown 9	1735		2082, 2.780	0.11	0.02
35	Methyl (2E,6E)-farnesoate * ^s	1799	1799	2218, 2.600	4.92	0.85

Each component is described by retention index (RI), retention indices of antennally active compounds identified using GC-FID/EAD (RI_{EAD}), retention time (t_R), median of sum of compound abundance ($\bar{x}_{\Sigma A}$) in three repetitions. Compounds listed as “Unknown” have similarity lower than 85% with library, their mass spectra is listed in Annex. Compounds marked with star * are antennally active according to GC-EAD experiments. Compounds marked with “a” are found in aeration extracts of calling males of *C. capitata*^{10, 118, 119}. Compound marked with (?) is biologically active, but of uncertain conformation due to absention standard substance for clear proof. Compounds marked with “s” were tested using commercial or laboratory prepared standards. Compounds marked were identified using published mass spectral data¹¹⁶.

Tab. 4.2: Volatile composition of *Ceratitis anonae*: list of compounds found in calling males using GC×GC-TOFMS

No	Name	<i>RI</i>	<i>RI</i> _{EAD}	<i>t_R</i> [s]	A [%]	SD [%]
1	Heptan-2-ol	901		806, 2.060	1.44	0.49
2	Methyl (<i>E</i>)-hex-2-enoate * ^{a,s}	968	966	930, 2.310	14.46	3.84
3	6-Methylhept-5-en-2-one * ^s	988	988	974, 2.330	0.05	0.07
4	Octanal	1006		1006, 2.200	0.65	0.26
5	(<i>Z</i>)- β -Ocimene	1040		1070, 2.070	0.58	0.26
6	(<i>E</i>)- β -Ocimene ^a	1051		1090, 2.070	1.58	0.59
7	Unknown 2	1086		1154, 2.200	0.17	0.10
8	Linalool * ^{a,s}	1104	1104	1186, 2.180	1.77	0.55
9	(<i>Z</i>)-Non-2-enal ^s	1151		1270, 2.380	0.09	0.03
10	(<i>E</i>)-Non-2-enal * ^s	1167	1163	1298, 2.380	6.90	1.50
11	Unknown 4	1185		1330, 2.190	0.16	0.04
12	Unknown 5	1206		1366, 2.140	0.18	0.06
13	Nonan-2-ol	1287		1498, 2.270	0.37	0.08
14	Oct-3-enyl acetate	1292		1506, 2.370	1.05	0.04
15	Methyl geranate	1329		1566, 2.480	0.43	0.09
16	(<i>Z,E</i>)-α-Farnesene	1491		1826, 2.280	1.12	0.27
17	(<i>E,E</i>)-α-Farnesene * ^a	1507	1507	1850, 2.300	67.01	20.11
18	Methyl (2 <i>E</i> ,6 <i>E</i>)-farnesoate * ^s	1799	1799	2218, 2.590	1.97	0.79

Each component is described by retention index (*RI*), retention indices of antennally active compounds identified using GC-FID/EAD (*RI*_{EAD}), retention time (*t_R*), median of sum of compound abundance ($\tilde{x}_{\Sigma A}$) in three repetitions. Compounds listed as “Unknown” have similarity lower than 85% with library, their mass spectra is listed in Annex. Compounds marked with star * are antennally active according to GC-EAD experiments. Compounds marked with “a” are found in aeration extracts of calling males of *C. capitata*^{10, 118, 119}. Compounds marked with “s” were tested using commercial or laboratory prepared standards. Compounds were identified using published mass spectral data¹¹⁶.

Tab.4.3: Volatile composition of *Ceratitis rosa*: list of compounds found in calling males using GC×GC-TOFMS.

No	Name	<i>RI</i>	<i>RI</i> _{EAD}	<i>t_R</i> [s]	A [%]	SD [%]
1	2,5-Dimethylpyrazine ^a	914		830, 2.620	4.27	0.25
2	γ-Valerolactone ^a	956		910, 3.440	1.97	1.76
3	Methyl (<i>E</i>)-hex-2-enoate * ^{a,s}	968	966	934, 2.290	0.28	0.34
4	6-Methylhept-5-en-2-one * ^s	988	989	978, 2.130	5.04	3.65
5	Octanal	1006		1002, 2.210	2.13	1.85
6	2,3,5-Trimethylpyrazine	1008		1006, 2.650	2.28	1.79
7	(<i>Z</i>)-β-Ocimene	1040		1070, 2.050	0.83	0.63
8	(<i>E</i>)-β-Ocimene ^a	1053		1090, 2.060	5.24	0.78
9	(<i>E</i>)-Oct-2-enal	1062		1106, 2.380	0.79	0.78
10	Linalool * ^{a,s}	1104	1104	1186, 2.150	47.77	9.49
11	Unknown 3	1106		1190, 2.750	2.28	1.79
12	(<i>Z</i>)-Non-2-enal ^s	1151		1270, 2.380	0.17	0.17
13	(<i>Z</i>)-Non-3-enol ^s	1158		1282, 2.330	1.29	1.19
14	(<i>E</i>)-Non-2-enal * ^{a,s}	1167	1163	1302, 2.370	15.92	14.31
15	Unknown 4	1185		1330, 2.180	0.05	0.02
16	2-Nonanol	1287		1498, 2.270	1.34	0.12
17	Oct-3-enyl acetate	1292		1506, 2.370	3.88	0.34
18	Methyl geranate	1329		1574, 2.450	1.06	0.46
19	β-Elemene	1406		1690, 2.230	0.03	0.00
20	β-Caryophyllene	1442		1742, 2.340	0.22	0.04
21	Geranyl acetone * ^{a,s}	1456	1456	1770, 2.480	0.48	0.00
22	(<i>Z</i>)-β-Farnesene	1458		1774, 2.210	0.05	0.03
23	(<i>Z,E</i>)-α-Farnesene	1491		1830, 2.270	0.22	0.21
24	(<i>E,E</i>)-α-Farnesene * ^{a,s}	1507	1507	1850, 2.290	6.36	7.54
25	Unknown 9	1735		2078, 2.320	0.43	0.34
26	Methyl (2 <i>E</i> ,6 <i>E</i>)-farnesoate * ^s	1799	1799	2214, 2.600	0.66	0.20

Each component is described by retention index (*RI*), retention indices of antennally active compounds identified using GC-FID/EAD (*RI*_{EAD}), retention time (*t_R*), median of sum of compound abundance ($\tilde{x}_{\Sigma A}$) in three repetitions. Compounds listed as “Unknown” have similarity lower than 85% with library, their mass spectra is listed in Annex. Compounds marked with star * are antennally active according to GC-EAD experiments. Compounds marked with “a” are found in aeration extracts of calling males of *C. capitata*^{10, 118, 119}. Compounds marked with “s” were tested using commercial or laboratory prepared standards. Compounds were identified using published mass spectral data¹¹⁶.

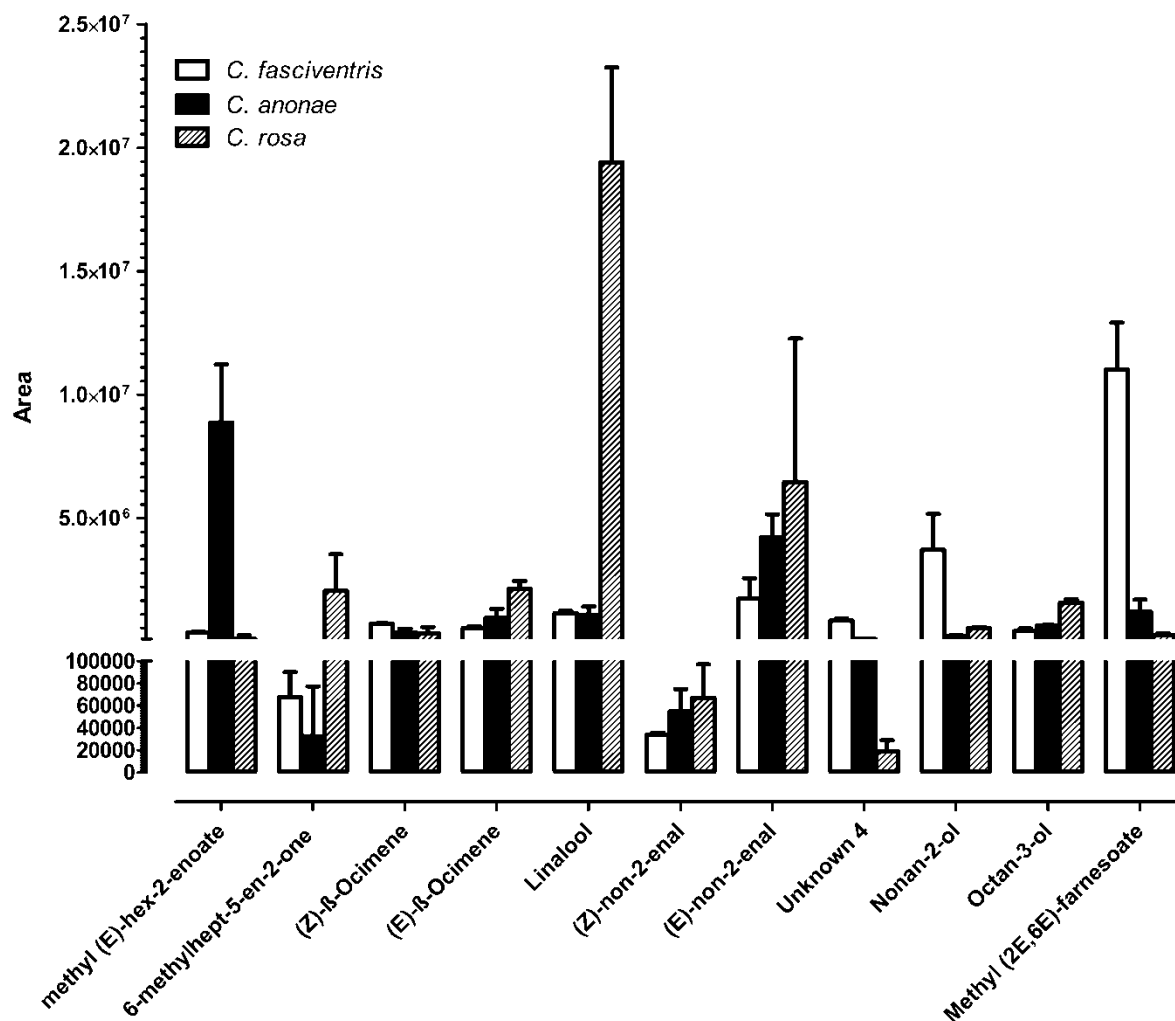


Fig. 4.2: Comparison of absolute area of 11 common compounds found in male emanations of three studied species, depicted is absolute median area of respective components ($\bar{x}_{A_i}$).

As shown in Tab. 4.1, 4.2 and 4.3 the character of the male sex pheromone in the studied species is complex multicomponent blend characterized by different quality and quantitative relationships of the respective compound. The pheromone composition of each species is species specific, though the three species share 11 compounds, out of which 4 are antennally active. Remaining 12 compounds are common between two species.

There are 3 compounds shared among *C. fasciventris* and *C. anonae* and are not present in *C. rosa* (heptan-2-ol, unknown 2, unknown 5), 5 compounds are shared by *C. fasciventris* and *C. rosa* and are not present in *C. anonae* (2,5-dimethylpyrazine, γ -valerolactone, 2,3,5-trimethylpyrazine, unknown 3, unknown 9) and lastly there are 4

compounds shared among *C. anonae* and *C. rosa* (octanal, methyl geranate, (Z,E)- α -farnesene, (E,E)- α -farnesene)

Species specificity is achieved by the presence of specific compounds. *C. fasciventris* was found to have the highest number of specific compounds (17, see Table 4.1) that are not found in other two species. Pheromone specificity in this fruit fly is based on the presence of saturated and unsaturated esters of isomers of hexenoic acid, specifically methyl (E)-hex-3-enoate ($RI_{EAD} = 932$), ethyl hexanoate ($RI_{EAD} = 999$), ethyl (E)-3-hexenoate ($RI_{EAD} = 1006$), ethyl (E)-2-hexenoate ($RI_{EAD} = 1045$). These esters are absent in *C. rosa* and *C. anonae* male pheromone emanations. The only ester of hexenoic acid shared by all three studied species is methyl (E)-hex-2-enoate ($RI_{EAD} = 966$). Furthermore, *C. fasciventris* does not emit isomers of α or β -farnesene. *C. anonae* has no specific compounds. *C. rosa* has 7 species specific compounds (E)-oct-2-enal ($RI_{EAD} = 1062$), (Z)-non-3-enol ($RI_{EAD} = 1158$), β -elemene ($RI_{EAD} = 1406$), β -caryophyllene ($RI_{EAD} = 1442$), geranyl acetone ($RI_{EAD} = 1456$), (Z)- β -farnesene ($RI_{EAD} = 1458$).

The results of principal component analyses are depicted on Fig. 4.3. The PCA shows a clear separation of the three species, indicating that the composition of the male pheromones is specific in each of the species. The two principal components (PC1 and PC2) together accounted for 99% of the total variability. The 23 common pheromone compounds are presented by the italic numbers, which stands for the particular retention indices identified in Tabs. 4.1-4.3. The compounds typical for *C. fasciventris* male emanation were 2,5-dimethylpyrazine ($RI_{EAD} = 914$), γ -valerolactone ($RI = 956$); 2,3,5-trimethylpyrazine ($RI = 1008$), unknown 3 ($RI = 1106$) and (Z)- β -ocimene ($RI = 1040$). *C. anonae* pheromone was characterized by octanal ($RI = 1006$), (E)-non-2-enal ($RI = 1167$), oct-3-enyl acetate ($RI = 1292$), methyl geranate ($RI = 1329$), (Z,E)- α -farnesene ($RI = 1491$), (E,E)- α -farnesene ($RI = 1507$), 6-Methylhept-5-en-2-one ($RI = 988$), (E)- β -ocimene ($RI = 1051$), linalool ($RI = 1104$) and (Z)-non-2-enal ($RI = 1151$) were the compounds typical for *C. rosa* male pheromone.

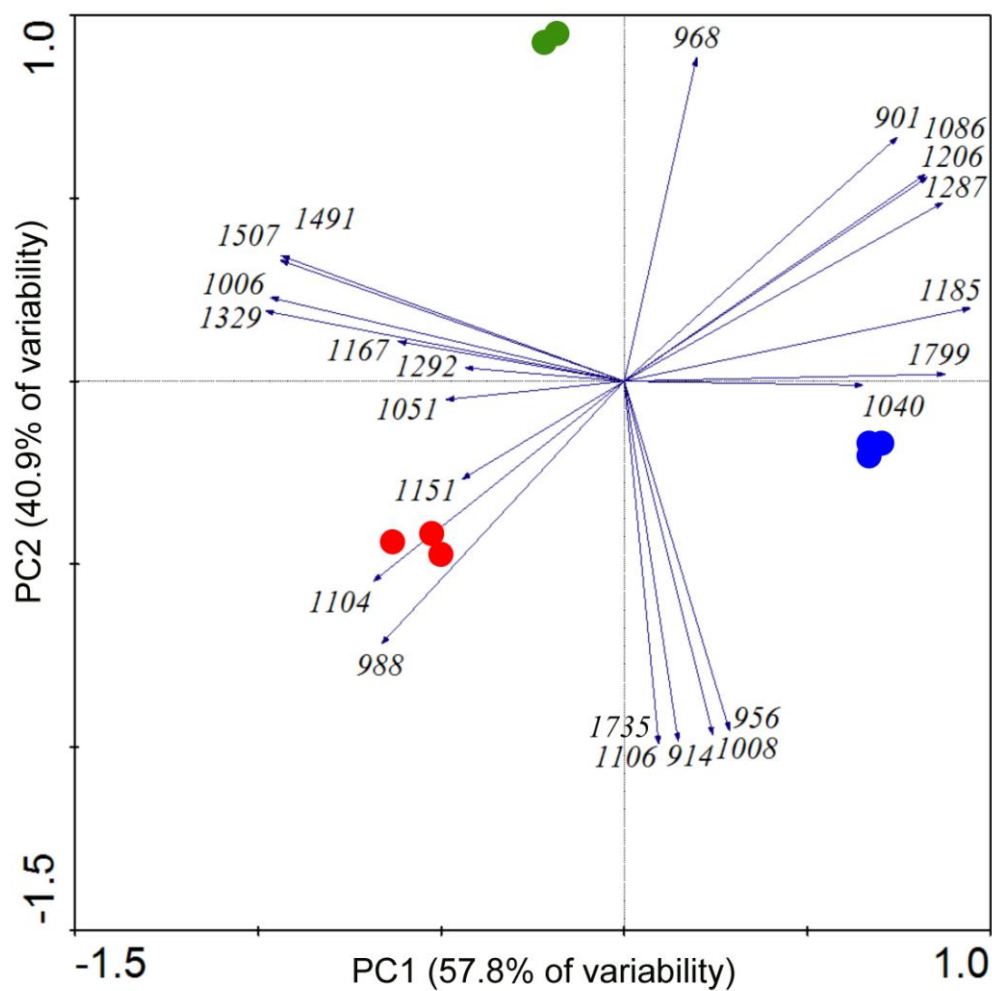


Fig.4.3: The results of the multivariate principal component analysis (PCA) of the 23 common compounds identified in sex pheromone of the males of *C. fasciventris* (blue), *C. anonae* (green) and *C. rosa* (red). The three species are clearly segregated. Each symbol on the plot represents one sample. The italic numbers represent the retention indices (RI) of the species-specific compounds. For the compounds structural identification see the Tab. 4.1-4.3.

Evaluation of GC-EAD-FID analysis

Out of all identified compounds only several elicit biological activity; however the biologically active compounds take up more than 70% of whole volatile emanation. GC-EAD experiments with respective female and male antennae revealed several active components: in *C. anonae* there were 6 antennally active compounds characterized by the following RI_{EAD} : 968, 988, 1104, 1163, 1507, and 1799. In *C. fasciventris* 10 GC-EAD responses were found with RI_{EAD} : 932, 968, 988, 997, 1003, 1045, 1104, 1131, 1163, and 1799. In *C. rosa* 7 antennally active compounds were detected with RI_{EAD} : 968, 988, 1104, 1163, 1459, 1507, and 1799.

The GC×GC-MSTOF analysis resulted in identification of the following compounds, the data are summarized in the Table 4.4 In *C. fasciventris* methyl (*E*)-hex-3-enoate (RI_{EAD} = 932), methyl (*E*)-hex-2-enoate (RI_{EAD} = 968), 6-methylhept-5-en-2-one (RI_{EAD} = 988) ethyl hexanoate (RI_{EAD} = 997), ethyl (*E*)-hex-3-enoate (RI_{EAD} = 1003), ethyl (*E*)-hex-2-enoate (RI_{EAD} = 1045) linalool (RI_{EAD} = 1104), methyl (*Z*)-oct-3-enoate (RI_{EAD} = 1131), (*E*)-non-2-enal (RI_{EAD} = 1163) and methyl-(2*E*,6*E*)-farnesoate (RI_{EAD} = 1799).

In *C. anonae*, the 6 active compounds were identified as methyl (*E*)-hex-2-enoate (RI_{EAD} = 968), 6-methylhept-5-en-2-one (RI_{EAD} = 988), linalool (RI_{EAD} = 1104), (*E*)-non-2-enal (RI_{EAD} = 1163), (*E,E*)- α -farnesene (RI_{EAD} = 1507) and methyl (2*E*,6*E*)-farnesoate (RI_{EAD} = 1799). In *C. rosa* 7 biologically active compounds were identified as methyl (*E*)-hex-2-enoate (RI_{EAD} = 968), 6-methyl-5-hepten-2-one (RI_{EAD} = 988), linalool (RI_{EAD} = 1104), (*E*)-non-2-enal (RI_{EAD} = 1163), geranyl acetone (RI_{EAD} = 1456), (*E,E*)- α -farnesene (RI_{EAD} = 1507) and methyl (2*E*,6*E*)-farnesoate (RI_{EAD} = 1799).

In all the cases synthetic standards possessed comparable antennal activities as the identified compounds in respective authentic pheromone samples. The only exception represents compound presumed to be methyl (*Z*)-oct-3-enoate (RI_{EAD} = 1131), which was not available during the experiment. In this case, the identification was based on GC×GC-TOFMS behaviour, similarity of MS spectra with the spectral library (Fig. 7.23 in Attachment). The antennal activity of this compound has to be tested in the future. Isoprenoid substances originating from plants and eliciting significant biological response were also found in volatile emanations of three species. Namely: linalool, geranyl acetone, myrcene and pinene. The last two mentioned are components of *Ceratitis capitata* pheromone¹¹⁹. However,

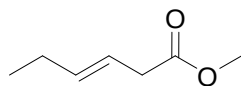
only linalool and geranyl acetone were added to the list of compounds produced by the insect. When comparing area in control samples and insect volatile samples. I decided to add only those, which area in real sample exceeded control sample by at least 2 orders of magnitude. Identified antennaly active structures are depicted in Scheme 4.1.

Tab 4.4: List and relative percentages of antennaly active compounds found in the male pheromone volatiles of studied species (100% is represented by the total area of all antennaly active compounds in each respective species)

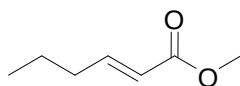
Name	RI	<i>C. fasciventris</i>		<i>C. anonae</i>		<i>C. rosa</i>	
		A %	SD %	A %	SD %	A %	SD %
Methyl (<i>E</i>)-hex-3-enoate	932	14.54	2.17	-	-	-	-
Methyl (<i>E</i>)-hex-2-enoate	968	0.17	0.02	14.08	3.99	0.36	0.45
6-Methylhept-5-en-2-one	988	0.03	0.01	0.07	0.08	6.59	4.77
Ethyl hexanoate	997	0.93	0.06	-	-	-	-
Ethyl (<i>E</i>)-3-hexenoate	1003	76.48	4.38	-	-	-	-
Ethyl (<i>E</i>)-2-hexenoate	1045	1.12	0.28	-	-	-	-
Linalool	1104	0.55	0.04	1.98	0.57	62.44	12.41
Methyl (<i>Z</i>)-3-octenoate (?)	1131	0.10	0.02	-	-	-	-
(<i>E</i>)-non-2-enal	1163	0.83	0.39	6.81	1.56	20.82	18.70
Geranyl acetone	1459	-	-	-	-	0.62	0.01
(<i>E,E</i>)- α -farnesene	1507	-	-	74.81	20.93	8.31	9.86
Methyl (2 <i>E</i> ,6 <i>E</i>)-farnesoate	1799	5.26	0.91	2.25	0.82	0.86	0.26

Each component is described by retention index (*RI*), relative area (*A*, %), standard deviation (*SD*, %). Compound marked with (?) is antennaly active; its conformation has not been tested due to absent standard solution.

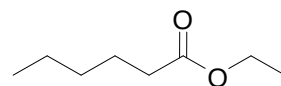
Scheme 4.1.: Structures of identified antennaly active components in *C. fasciventris*, *C. rosa* and *C. anonae* male volatile emanations, RI_{EAD} corresponds to retention index measured by GC-FID/EAD.



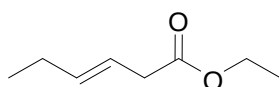
$RI_{EAD} = 937$
Methyl (*E*)-hex-3-enoate



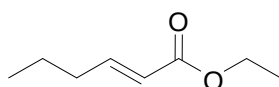
$RI_{EAD} = 966$
Methyl (*E*)-hex-2-enoate



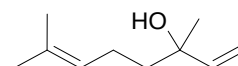
$RI_{EAD} = 999$
Ethyl hexanoate



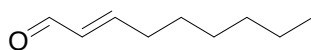
$RI_{EAD} = 1006$
Ethyl (*E*)-hex-3-enoate



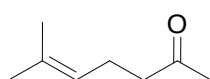
$RI_{EAD} = 1045$
Ethyl (*E*)-hex-2-enoate



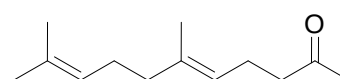
$RI_{EAD} = 1104$
(±)-Linalool



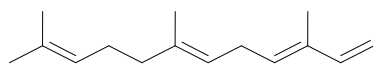
$RI_{EAD} = 1163$
(*E*)-Non-2-enal



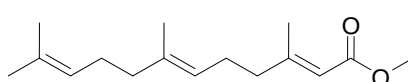
$RI_{EAD} = 989$
6-methylhept-5-en-2-one



$RI_{EAD} = 1456$
Geranyl acetone



$RI_{EAD} = 1507$
(*E,E*)-α-farnesene



$RI_{EAD} = 1799$
Methyl (2*E*,6*E*)-farnesoate

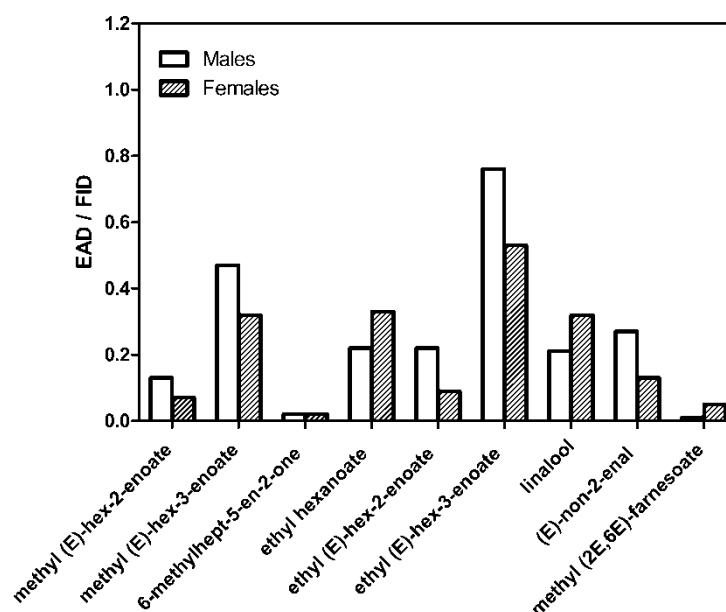


Fig. 4.5: *C. fasciventris* antennal responses to standard solutions, male and female comparison. Ratio between electroantennographic response and conventional detector (EAD/FID). The higher the number, the higher the response. n (males) = 2, n (females) = 2.

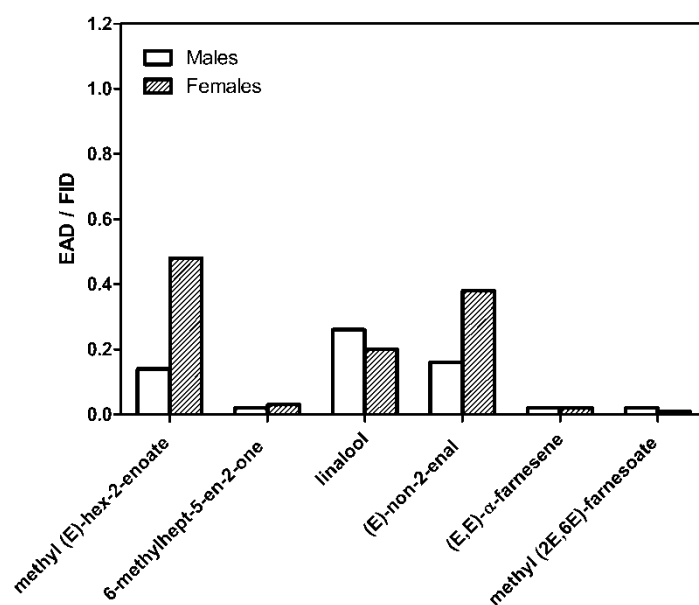


Fig. 4.6: *C. anonae* antennal responses to standard solutions, male and female comparison. Ratio between electroantennographic response and conventional detector (EAD/FID). The higher the number, the higher the response. n (males) = 2, n (females) = 2.

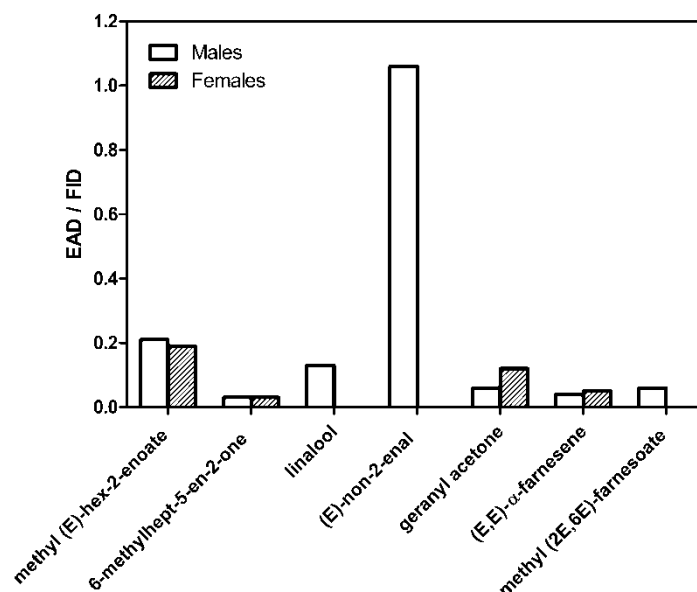


Fig. 4.7: *C. rosa* antennal responses to standard solutions, male and female comparison. Ratio between electroantennographic response and conventional detector (EAD/FID). The higher the number, the higher the response. *n* (males) = 2, *n* (females) = 1.

Graphs depicted in Figs. 4.5, 4.6, and 4.7 represent ratio of peak areas obtained by the conventional FID to peak areas from the biological detector in studied insect. The higher the number of the ratio, the higher is the activity of the compound. Due to lack of live material, only one incomplete measurement was made in case of *C. rosa* females. EAD was successfully recorded only for 4 compounds out of 6 active ones due to the shortage. Thus, information presented for *C. rosa* are indefinite and calls for more experiments.

Differences between the sexes in their responsiveness were found in 12 of the compounds tested (Scheme 1). There was no correlation between the amplitude of the EAD response and the relative abundance of the volatiles identified from the headspace male pheromone analysis. Among the three FAR complex species the relative ranking EAD responses were: methyl and ethyl hexenoates and ethyl hexanoate > linalool > (E)-non-2-enal > (E,E)-α-farnesene, methyl (2E,6E)-farnesoate and 6-methylhept-5-en-2-one.

The antenna of both males and females of *C. fasciventris* responds with the highest sensitivity to ethyl (E)-hex-3-enoate, the most abundant compound in *C. fasciventris* pheromone (Tab. 4.1, Fig. 4.5). Methyl (E)-hex-3-enoate is the second most abundant compound elicits the second highest response (Tab. 4.1, Fig. 4.5). Ethyl (E)-hex-3-enoate was previously reported as a part of *C. capitata* male pheromones with high EAD activity to female^{118, 119}.

However, here we report first evidence of a important role of these two esters in pheromonal communication in *C. fasciventris*. Ethyl hexanoate identified in male *C. fasciventris* volatiles was reported to be a part of *C. capitata*¹¹⁸ pheromone. Among antennally active components of *C. anonae* pheromone, (*E,E*)- α -farnesene is the most abundant one, however it does not yield prominent response in *C. anonae* females (Tab. 4.2, Fig. 4.6). (*E,E*)- α -farnesene was recently identified as a pheromone component with antennal activity in fruit fly species (*A. fraterculus*¹¹⁴, *C. capitata*¹²⁰). (*E*)-Non-2-enal and linalool are present in minor amounts (6.81% and 1.98% of emanation, respectively), but release higher response in females. Studies performed on Mediterranean fruit flies have shown that minor pheromone components may determine pheromone attractiveness^{119, 121}. Linalool was found to be a minor component of *C. capitata* male pheromone and to have an EAD activity.

Incomplete measurement in *C. rosa* females prohibits us to deduce exact conclusions; however, female antennae of *C. rosa* revealed highest activity to methyl (*E*)-hex-2-enoate followed by geranyl acetone. Both compounds were also determined to be EAD active in *C. capitata*^{118, 119}. Interestingly the *C. rosa* males showed the highest antennal activity to (*E*)-non-2-enal, which may lead to the hypothesis, that this aldehyde may serve as a part of male aggregation pheromone. Nevertheless, further GC-EAD analyses and behavioural assays need to be performed. 6-Methylhept-5-en-2-one, and methyl (2*E*,6*E*)-farnesoate revealed the lowest antennal response among all three studied species.

The multivariate statistical analyses (CA) of the 12 EAD active compounds identified by GC $\times$ GC-MS and GC-EAD are depicted on Fig. 4.8. The results reveal species-specific EAD compounds identified by their retention indices. The FAR complex species formed three segregated groups. The CA analyses have revealed exclusive compounds for particular species. methyl (*E*)-hex-3-enoate ($RI_{EAD} = 932$), ethyl hexanoate ($RI_{EAD} = 996$), ethyl (*E*)-hex-2-enoate ($RI_{EAD} = 1045$) and methyl (*Z*)-oct-3-enoate (?) ($RI_{EAD} = 1131$) are characteristic for the aeration extract from *C. fasciventris*. The *C. anonae* emanation has also typical compounds: methyl (*E*)-hex-2-enoate ($RI_{EAD} = 968$), (*E*)-non-2-enal ($RI_{EAD} = 1163$), (*E,E*)- α -farnesene ($RI_{EAD} = 1507$), and methyl (2*E*,6*E*)-farnesoate ($RI_{EAD} = 1799$). *C. rosa* is defined by geranyl acetone ($RI_{EAD} = 1459$) and 6-methylhept-5-en-2-one ($RI_{EAD} = 988$). The terpene linalool ($RI_{EAD} = 1104$) is shared by *C. anonae* and *C. rosa*.

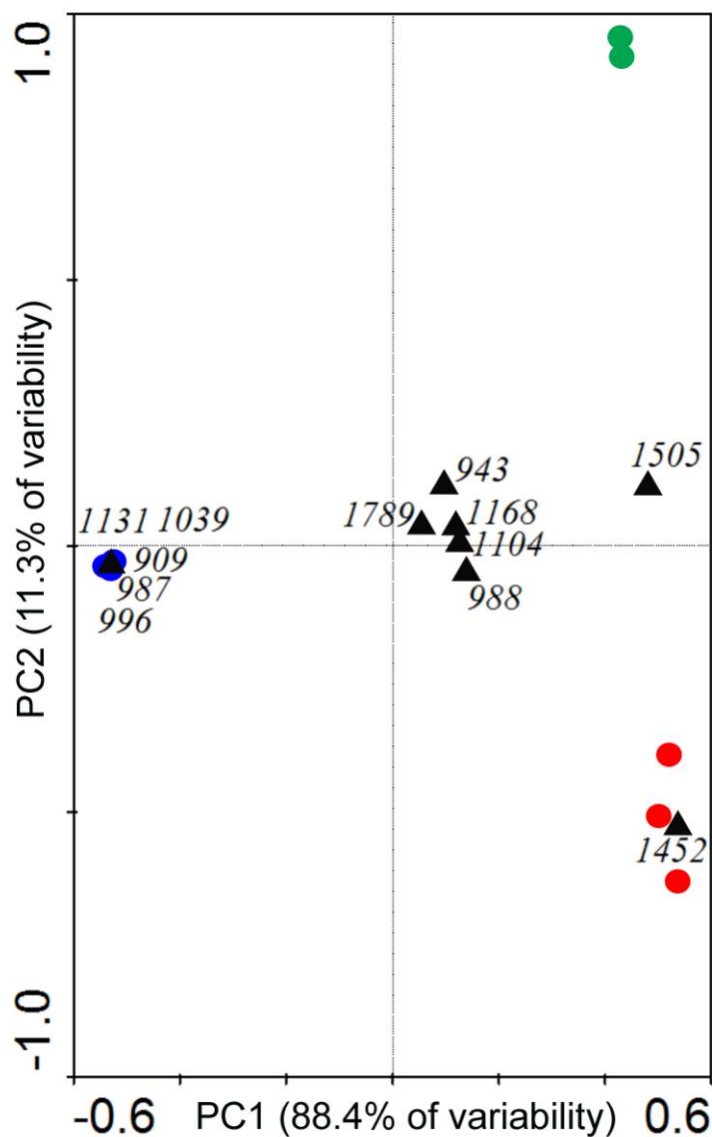


Fig. 4.8: The results of the multivariate correspondence analysis (CA) of the 12 antennal active compounds identified in male pheromone emanations of *C. fasciventris* (blue), *C. anone* (green) and *C. rosa* (red). The three species are clearly segregated. Each circle on the plot represents one sample. The numbers of the analyzed samples (n) for the *C. fasciventris*, *C. anone* and *C. rosa* were 3, 2 and 3, respectively. Black triangles and the italic numbers represent the retention indices (RI_{EAD}) of the species-specific antennal active compounds. For the compounds structural identification see the Scheme 1.

5. Conclusion

In presented thesis volatile composition of three species of agricultural pest insect belonging to fruit fly family were examined for the first time; in particular: *Ceratitis fasciventris*, *Ceratitis anonae* and *Ceratitis rosa*.

Complex mixtures of volatiles with different chemical composition were analysed and identified using GC×GC-TOFMS. Biological activity of present pheromone components was examined using male and female antenna using GC-FID/EAD. Data were evaluated by multivariate statistical analyses. Altogether 35 compounds were identified in *C. fasciventris*, 18 compounds in *C. anonae* and 26 compounds in *C. rosa*. Pheromone represents multicomponent mixture, where only several compounds evoke biological activity. 22 compounds were found between at least two species. It has been observed, that studied species have 23 compound in common (11 among three studied species), out of these 4 compounds elicit biological response in three studied species: methyl (*E*)-hex-3-enoate ($RI_{EAD} = 932$), 6-methylhept-5-en-2-one ($RI_{EAD} = 988$), linalool ($RI_{EAD} = 1104$), methyl (2*E*,6*E*)-farnesoate ($RI_{EAD} = 1799$). These common compounds occur in varying abundance. Species specificity is achieved by production of unique substances, for instance esters of isomers of hexenoic acid found solely in *C. fasciventris*. Interestingly, *C. anonae* was found not to produce any unique compound. Among 8 unique compounds for *C. rosa* we found isomers of farnesene, not observed in other species. Studied species partially share pheromone components with model species *Ceratitis capitata*, in particular ethyl (*E*)-hex-3-enoate, ethyl hexanoate, linalool, 2,5-dimethylpyrazine, γ -valerolactone, 6-methylhept-5-en-2-one and (*E,E*)- α -farnesene, which are known to be antennaly active compound also in other Tephritid species (*A. suspensa*, *A. ludens* and *A. fraterculus*).

The present study shows that the pheromone composition is species- specific and might be used in the chemical diagnosis of the FAR complex. Moreover, our results represent an important first step in the development of a useful chemotaxonomic tool for cryptic species identification. Behavioral assays are needed for subsequent evaluation of the identified EAD compounds. Moreover, it is necessary to perform further analyses that include a large geographic area of sampling in order to fine-tune an effective method, useful for the improvement and application of the SIT to manage this important agricultural pest²⁰.

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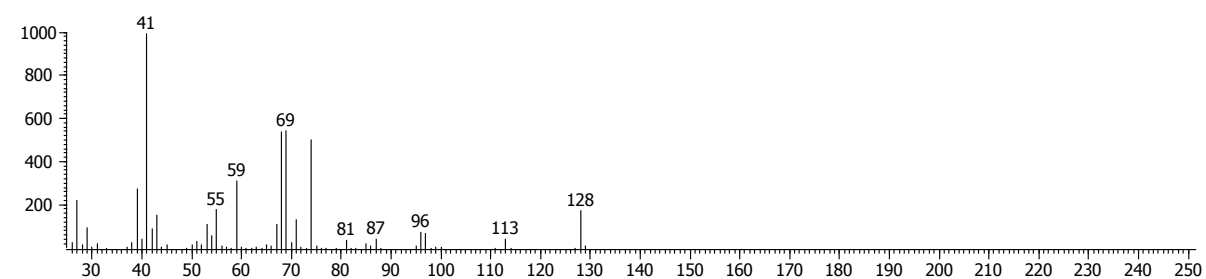
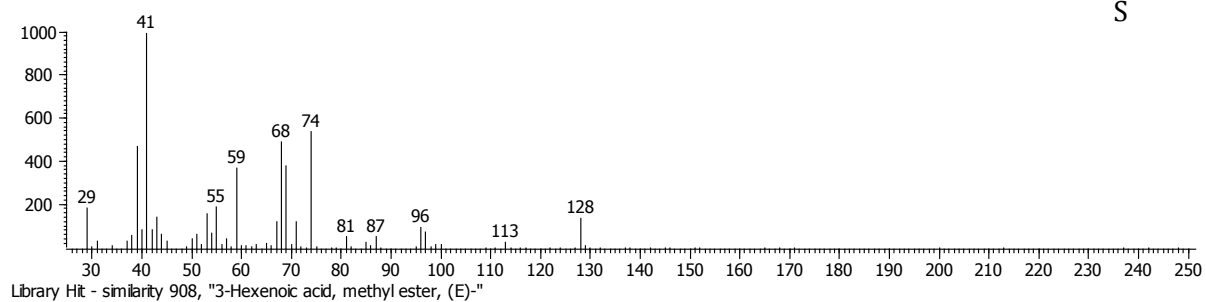
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7. Attachments

Following are fragmentation spectra of mass fragmentation patterns of standard compounds compared to compounds found in volatile emanations of calling males of tree studied species..*Ceratitifasciventris*, *Ceratitisanonae* and *Ceratitisorosa*, $n=5$, t_R is in seconds.

Peak True - sample "methyl-3-hexenoate splitless:1", peak 122, at R.I. 937,5

S



Peak True - sample "C.fasciventris 5 males splitless:1", peak 113, at R.I. 937,5

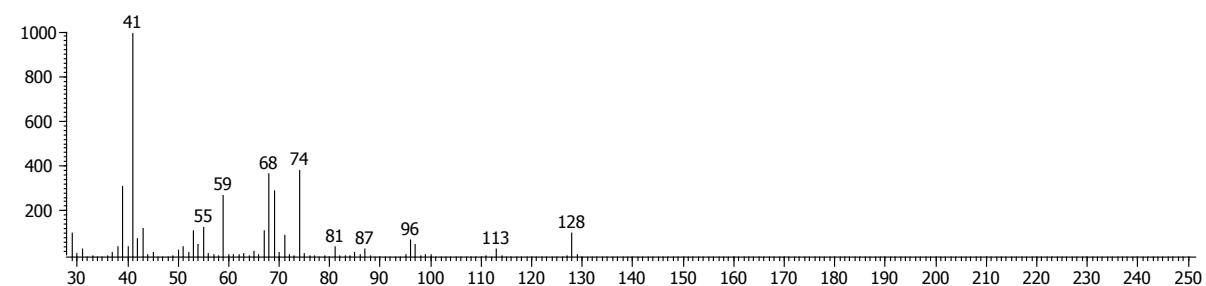
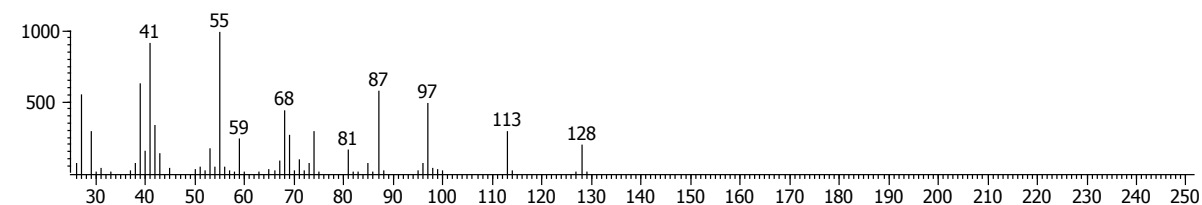
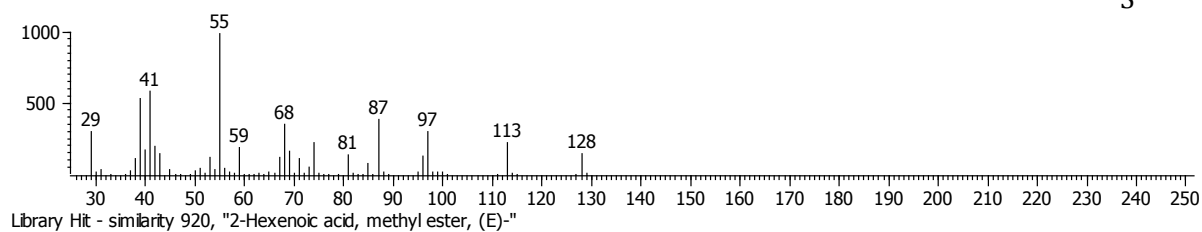


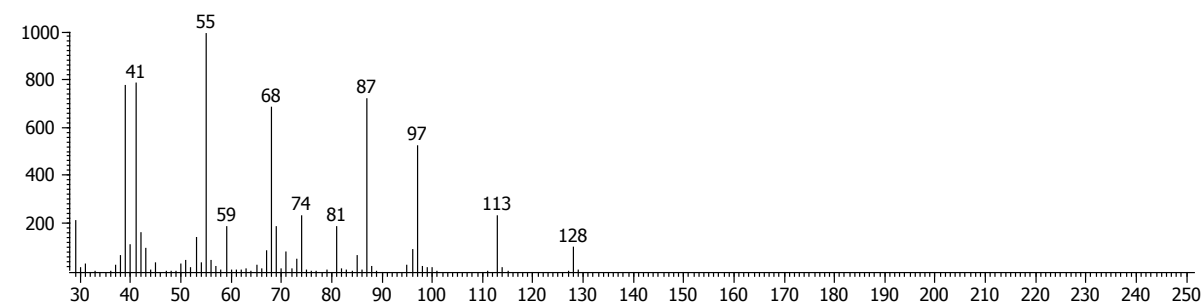
Fig.7.1.: Fragmentation mass spectra of methyl (*E*)-hex-3-enoate ($t_R = 870, 2.230$). Each compound is identified by retention index (*RI*). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes A standard:1", peak 115, at R.I. 966,4

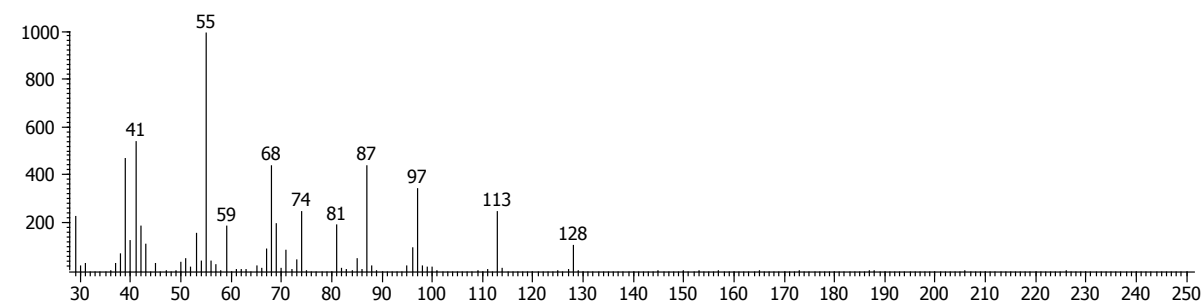
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Peak True - sample "C. Anonae male:1", peak 110, at R.I. 966,4



Peak True - sample "C. Fasciventris male:1", peak 145, at R.I. 968,6



Peak True - sample "C. Rosa male:1", peak 170, at R.I. 968,6

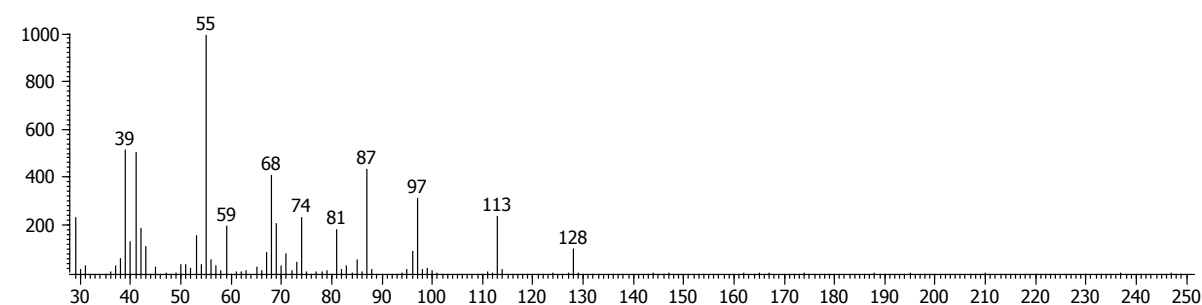
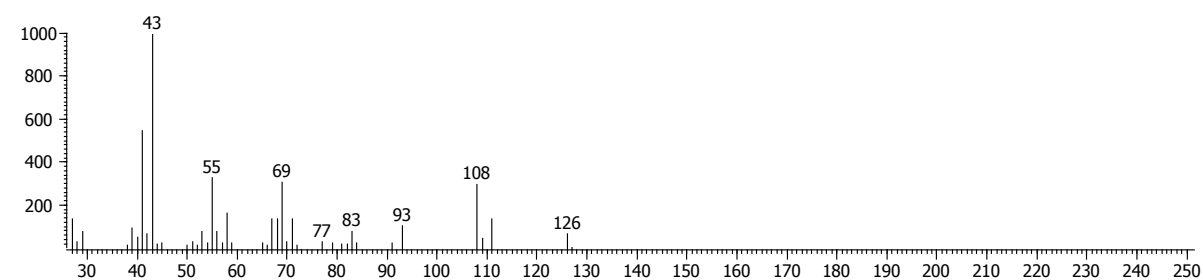
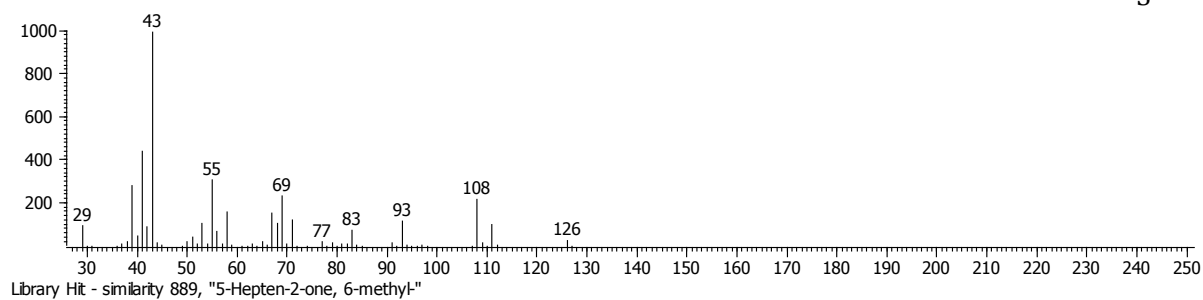


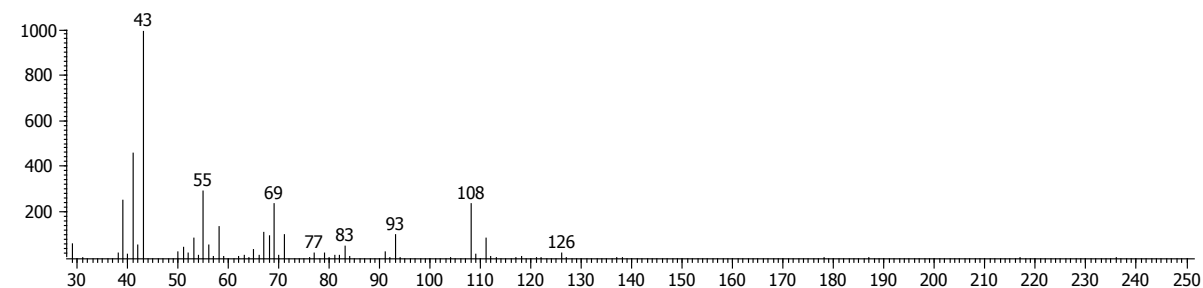
Fig. 7.2: Fragmentation mass spectra of methyl (*E*)-hex-2-enoate ($t_R = 934, 2.300$). Each compound is identified by retention index (*RI*). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes C standard:1", peak 174, at R.I. 986,5

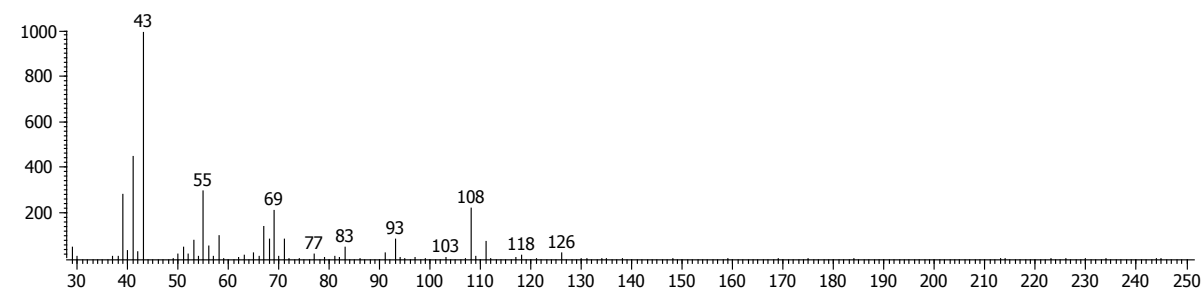
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Peak True - sample "C. Anonae male:1", peak 144, at R.I. 988,7



Peak True - sample "C. Fasciventris male:1", peak 177, at R.I. 988,7



Peak True - sample "C. Rosa male:1", peak 219, at R.I. 988,7

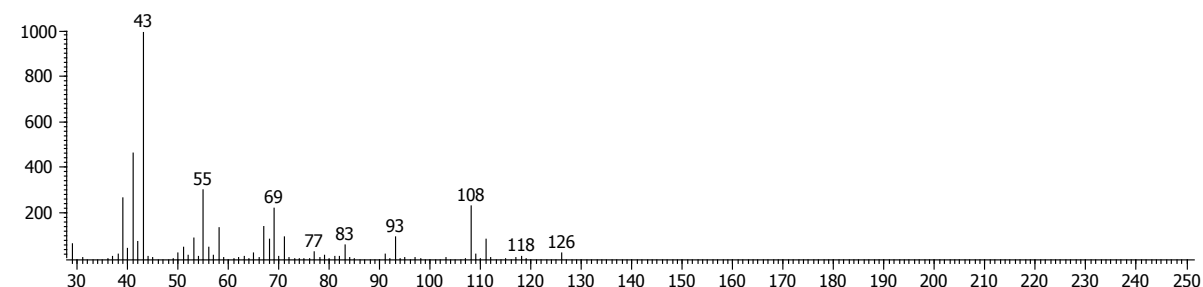
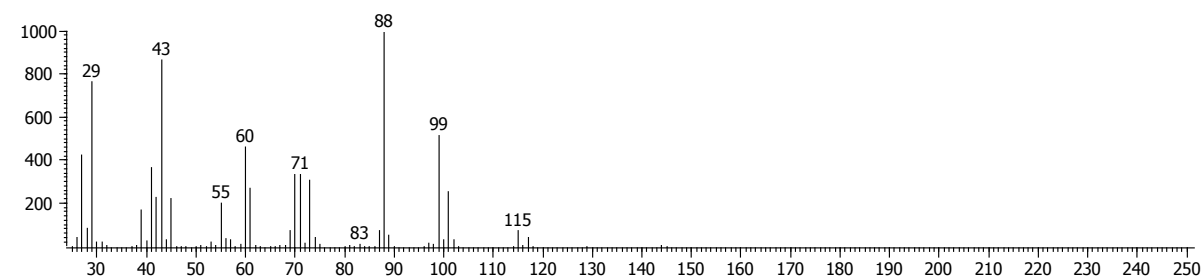
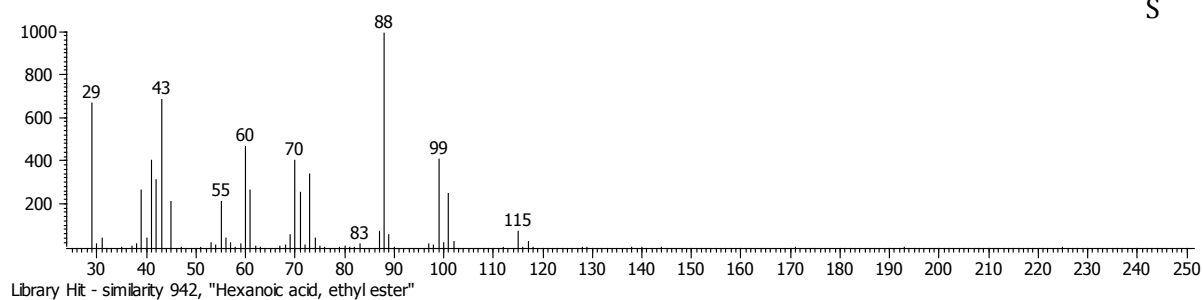


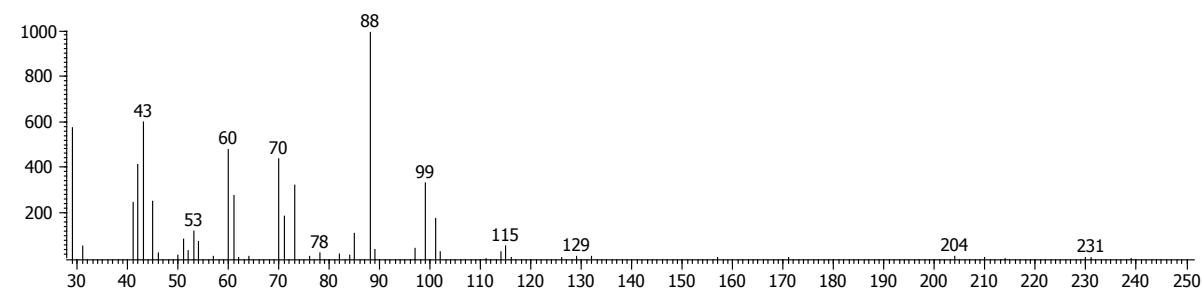
Fig.7.3: Fragmentation mass spectra 6-methylhept-5-en-2-one ($t_R = 978, 2.130$). Each compound is identified by retention index (RI). Mass spectrum marked with (S) is standard solution.

Peak True - sample "ethyl hexanoate splitless:1", peak 197, at R.I. 1001,8

S



Peak True - sample "C.anonae 5 males splitless:1", peak 183, at R.I. 1001,8



Peak True - sample "C.fasciventris 5 males splitless:1", peak 230, at R.I. 1001,8

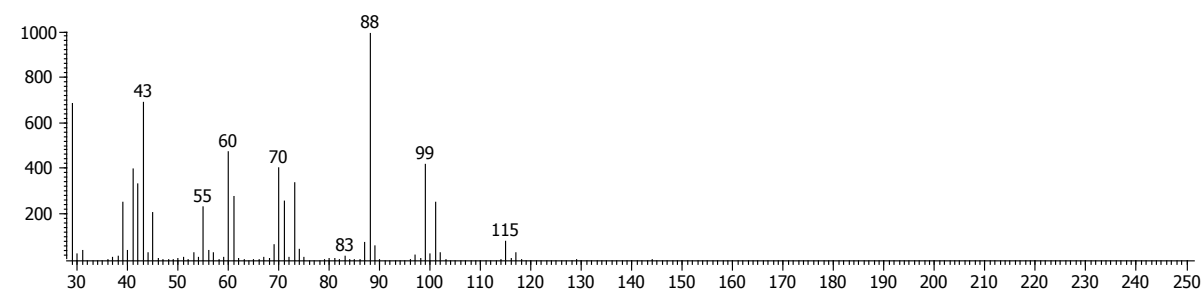
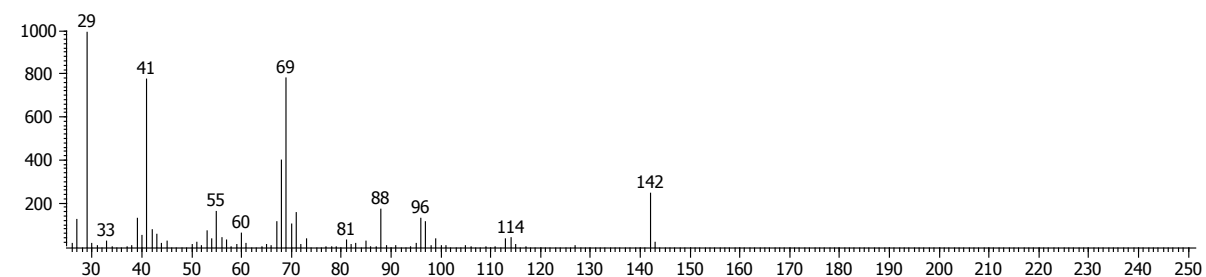
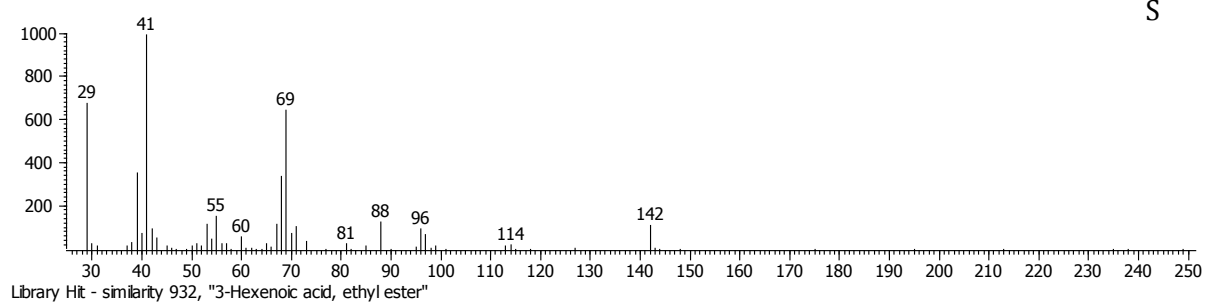


Fig.7.4: Fragmentation mass spectra of ethyl hexanoate($t_R=990$, 2.120 Each compound is identified by retention index (R_I). Mass spectrum marked with (S) is standard solution.

Peak True - sample "ethyl E3-hexenoate splitless:1", peak 195, at R.I. 1010,6

S



Peak True - sample "C.fasciventris 5 males splitless:1", peak 260, at R.I. 1010,6

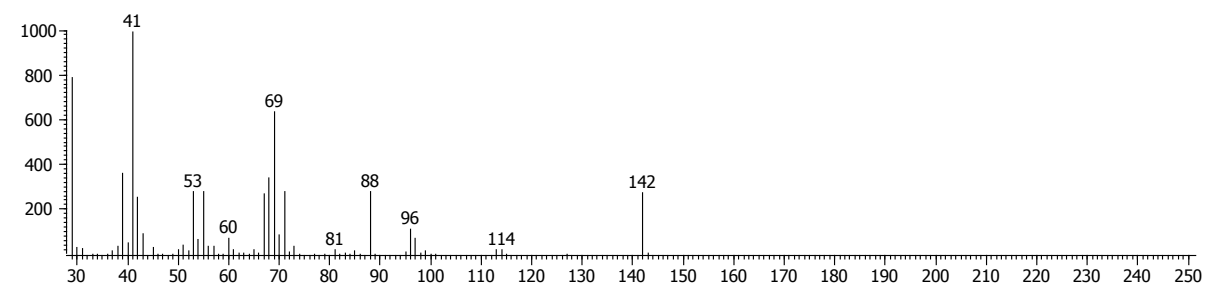
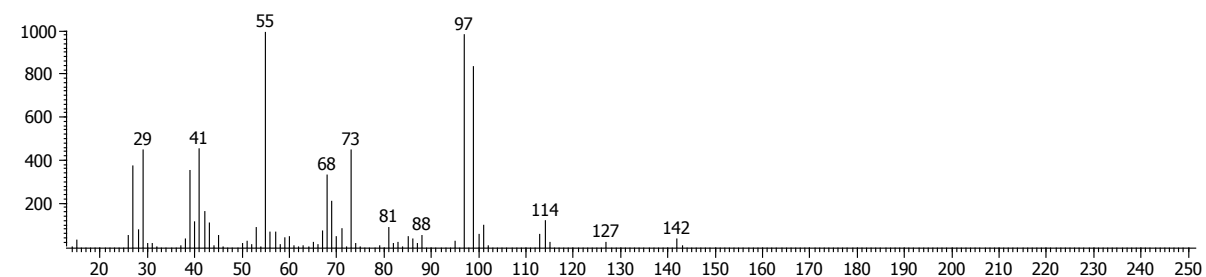
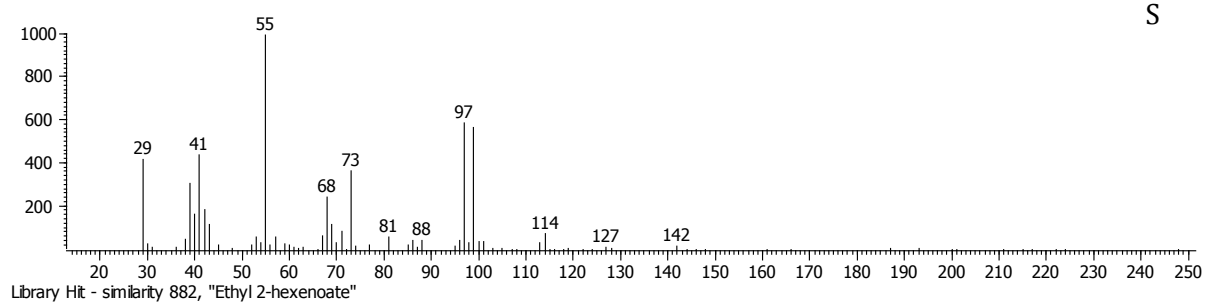


Fig.7.5: Fragmentation mass spectra of ethyl (*E*)-hex-3-enoate ($t_R = 1002, 2.230$). Each compound is identified by retention index (*RI*). Mass spectrum marked with (S) is standard solution.

Peak True - sample "ethyl-E2-hexenoate splitless:1", peak 220, at R.I. 1049,8

S



Peak True - sample "C.fasciventris 5 males splitless:1", peak 342, at R.I. 1049,8

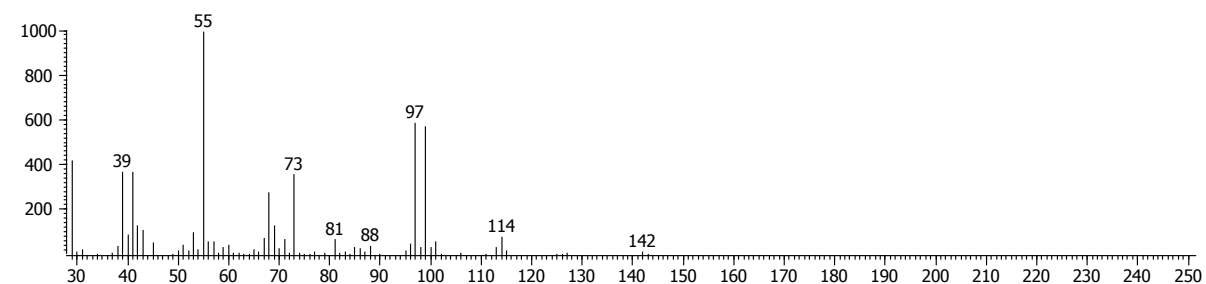
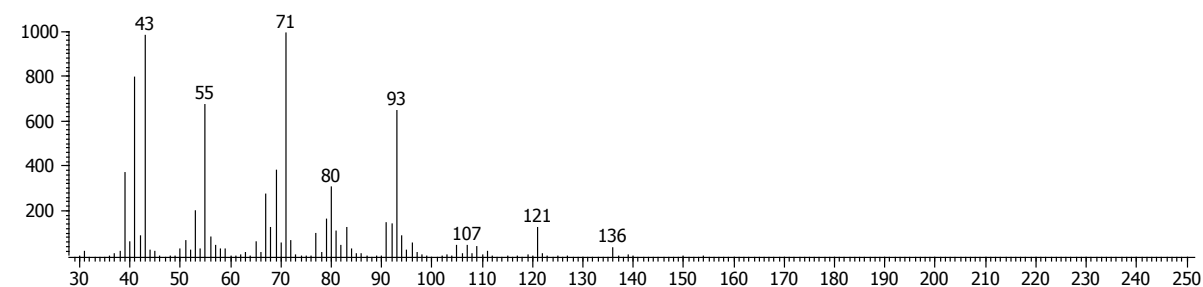
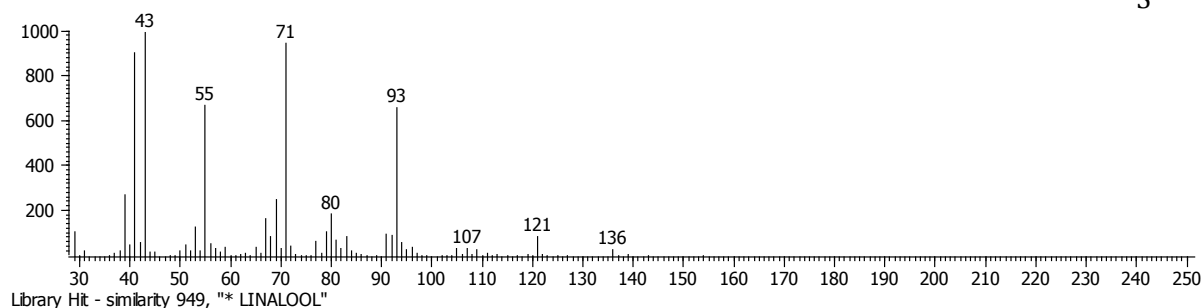


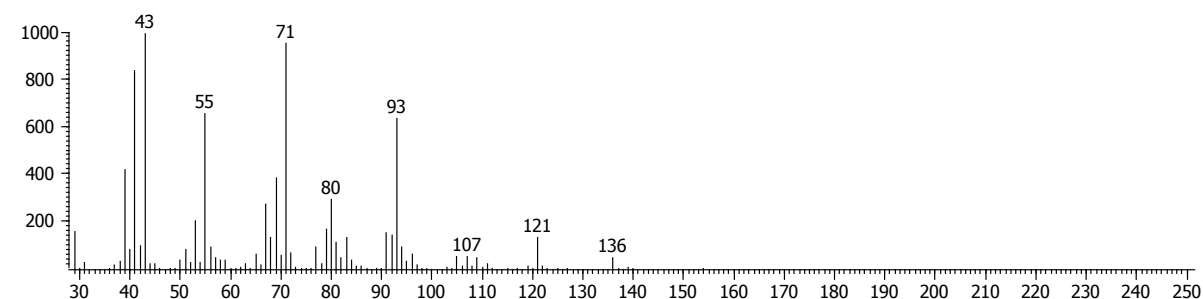
Fig.7.6: Fragmentation mass spectra of ethyl (*E*)-hex-2-enoate ($t_R = 1078, 2.300$). Each compound is identified by retention index (*RI*). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes B standard:1", peak 118, at R.I. 1104,1

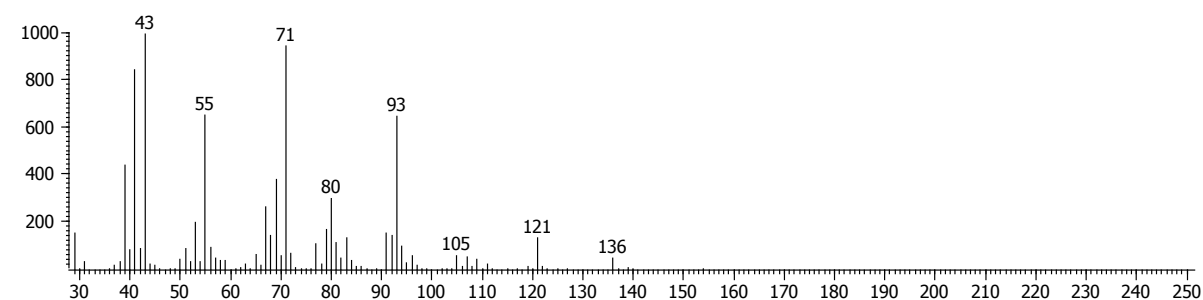
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Peak True - sample "C. Anonae male:1", peak 274, at R.I. 1101,9



Peak True - sample "C. Fasciventris male:1", peak 396, at R.I. 1101,9



Peak True - sample "C. Rosa male:1", peak 490, at R.I. 1101,9

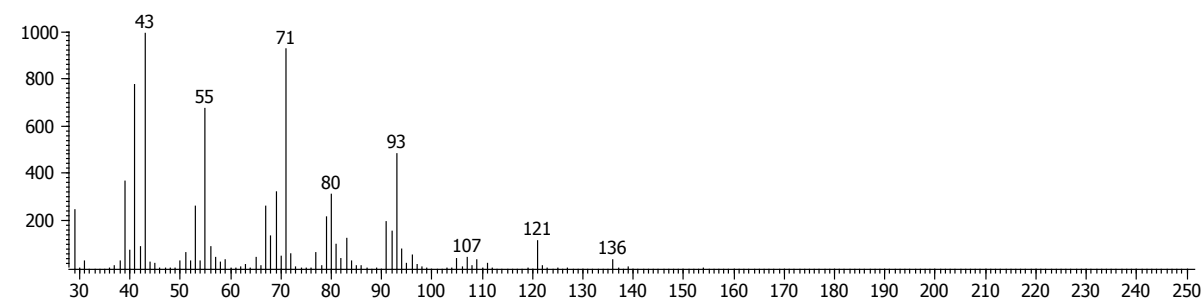
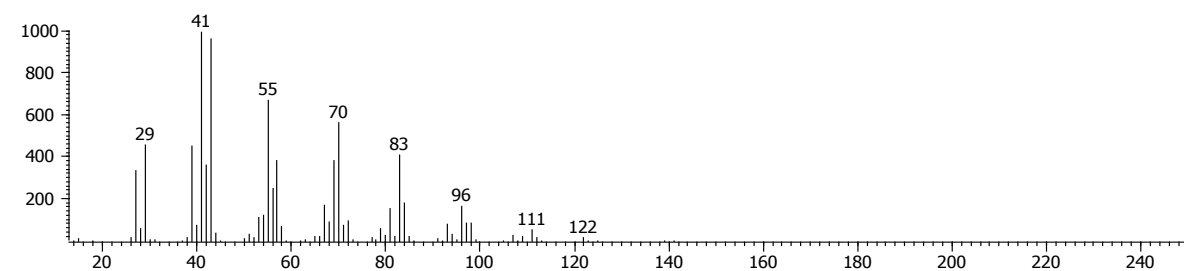
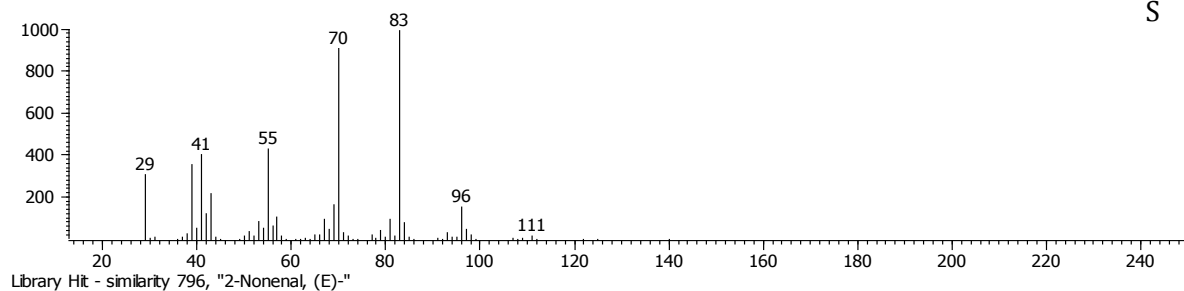


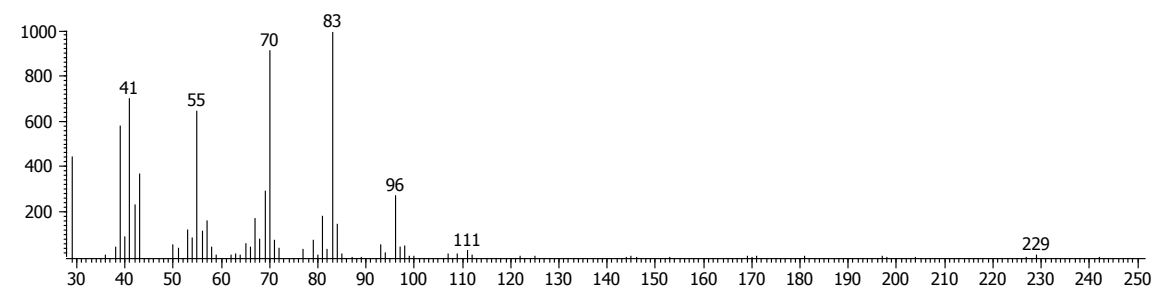
Fig.7.7: Fragmentation mass spectra of linalool ($t_R = 1186, 2.180$). Each compound is identified by retention index (RI). Mass spectrum marked with (S) is standard solution.

Peak True - sample "ZZ-C9-AL:1", peak 261, at R.I. 1149,8

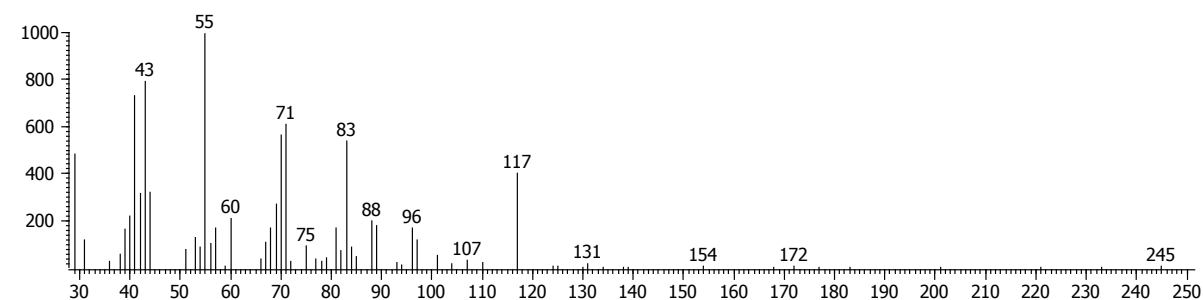
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Peak True - sample "C. Anonae male:1", peak 321, at R.I. 1152,0



Peak True - sample "C. Fasciventris male:1", peak 465, at R.I. 1152,0



Peak True - sample "C. Rosa male:1", peak 569, at R.I. 1149,8

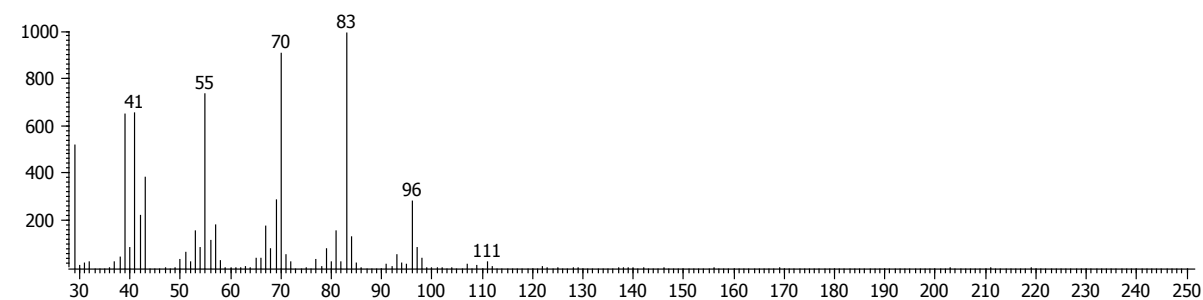
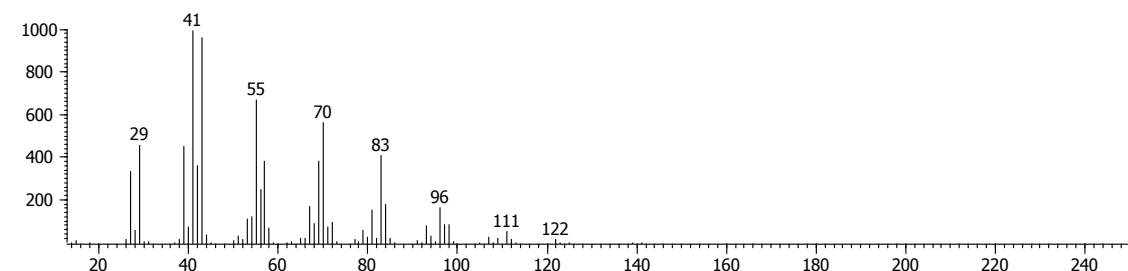
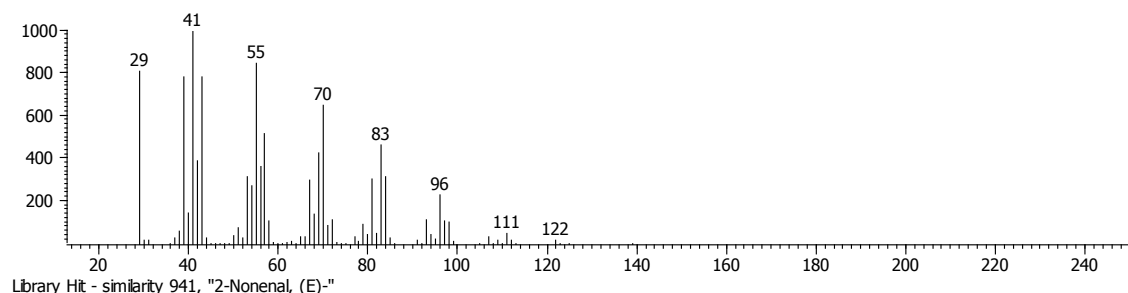


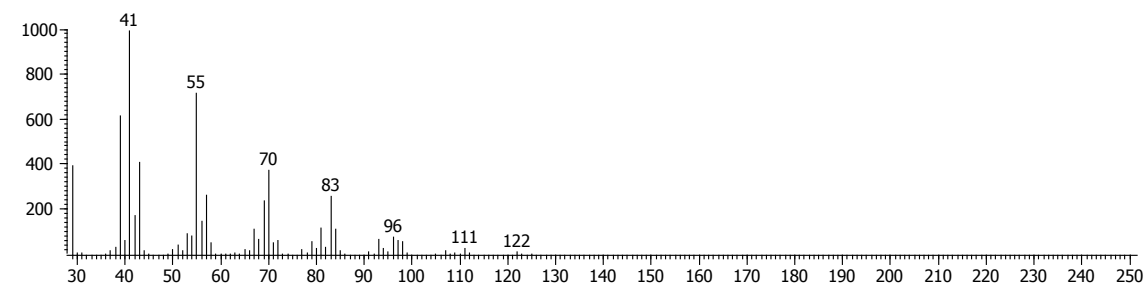
Fig.7.8: Fragmentation mass spectra of (Z)-non-2-enal ($t_R = 1270, 2.380$). Each compound is identified by retention index (RI). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes C standard:1", peak 310, at R.I. 1163,4

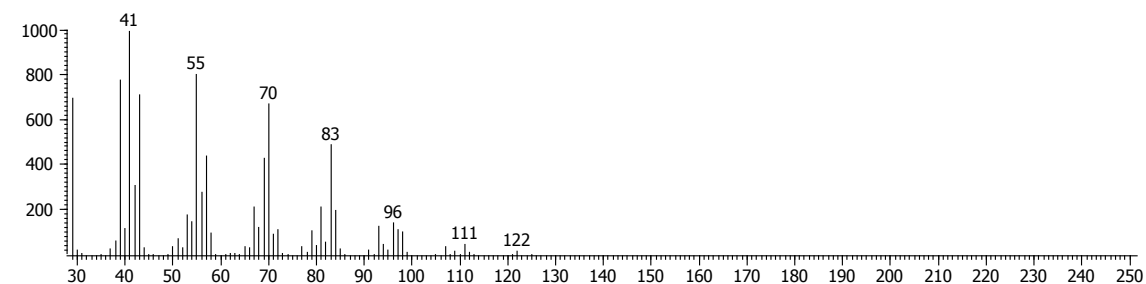
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Peak True - sample "C. Anonae male:1", peak 329, at R.I. 1163,4



Peak True - sample "C. Fasciventris male:1", peak 473, at R.I. 1163,4



Peak True - sample "C. Rosa male:1", peak 590, at R.I. 1163,4

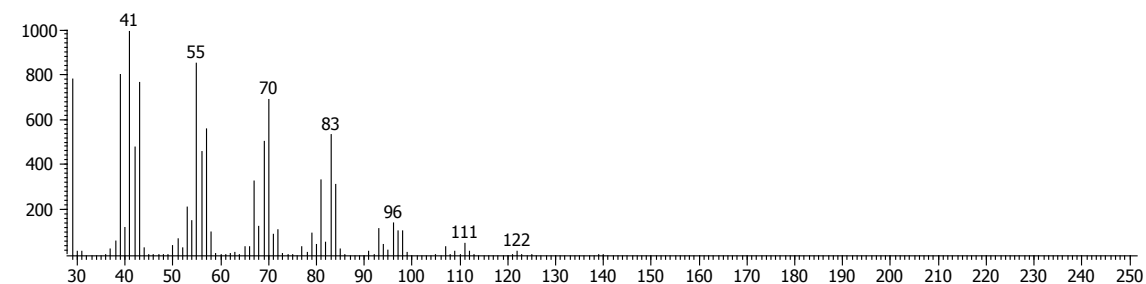
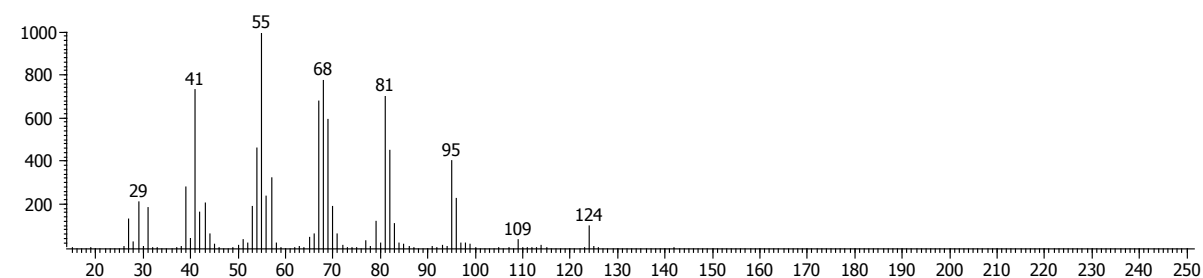
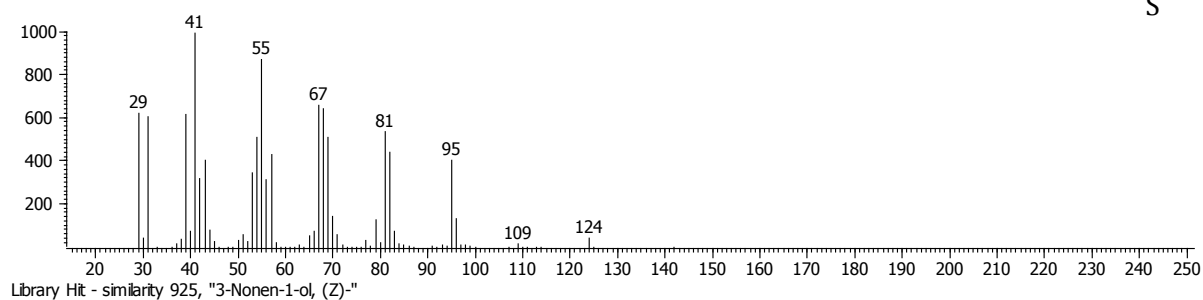


Fig. 7.9: Fragmentation mass spectra of (*E*)-non-2-enal ($t_R = 1298, 2.390$). Each compound is identified by retention index (*RI*). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes C standard:1", peak 301, at R.I. 1156,5

S



Peak True - sample "C. Rosa male:1", peak 631, at R.I. 1190,4

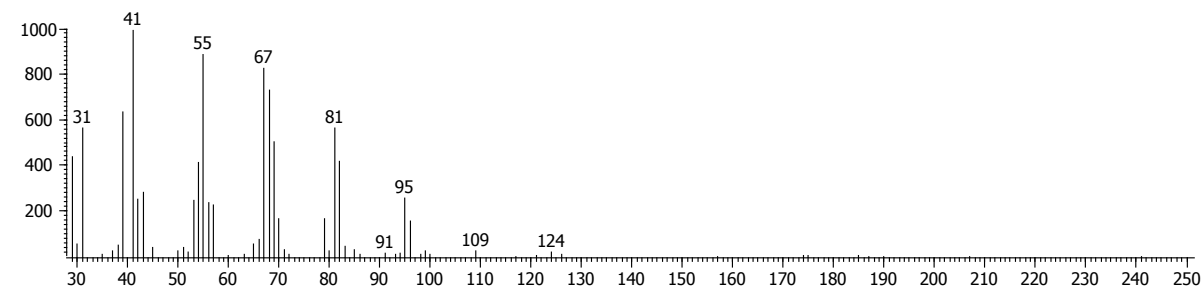
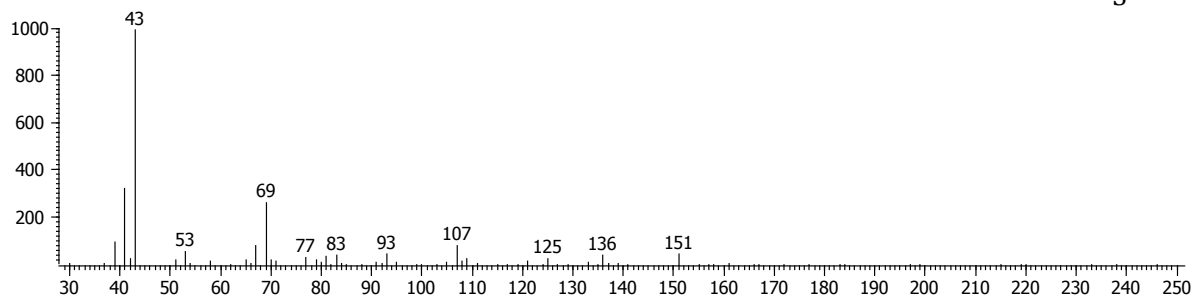


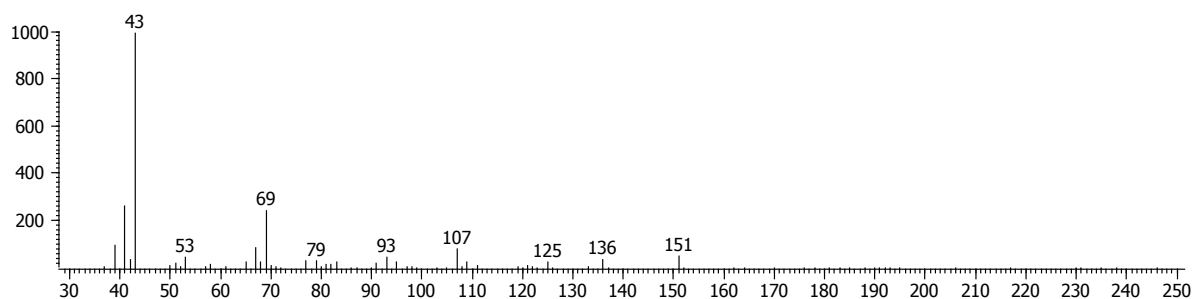
Fig.7.10: Fragmentation mass spectra of (Z)-non-3-en-1-ol ($t_R = 1282, 2.330$). Each compound is identified by retention index (RI). Mass spectrum marked with (S) is standard solution.

Peak True - sample "C. Anonae male:1", peak 450, at R.I. 1449,9

S



Peak True - sample "C. Fasciventris male:1", peak 723, at R.I. 1449,9



Peak True - sample "C. Rosa male:1", peak 988, at R.I. 1449,9

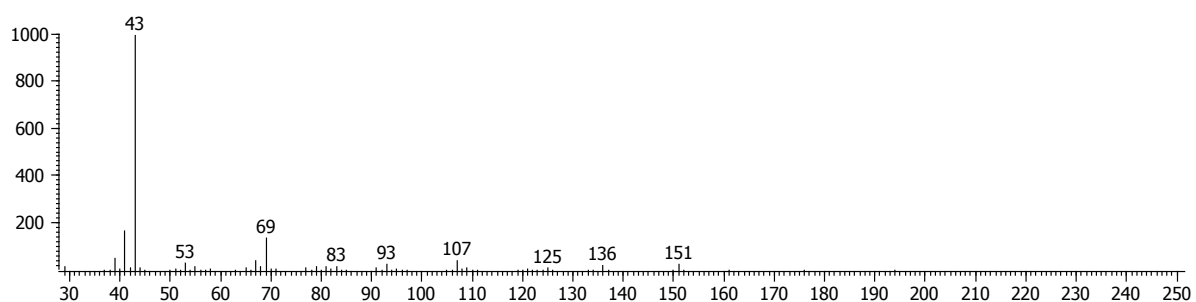
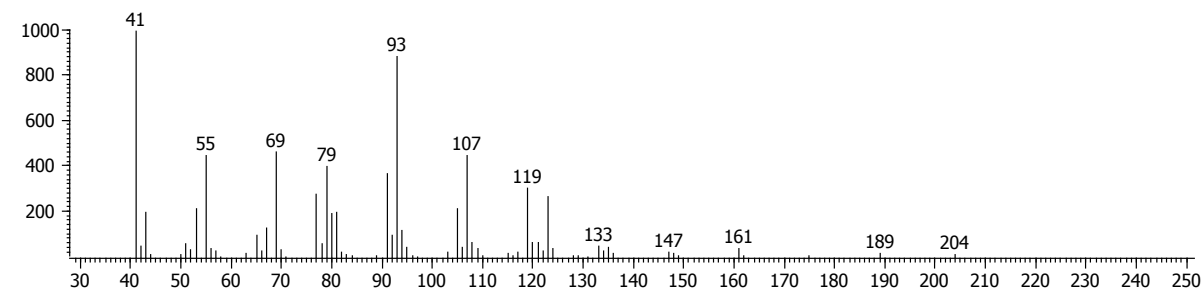
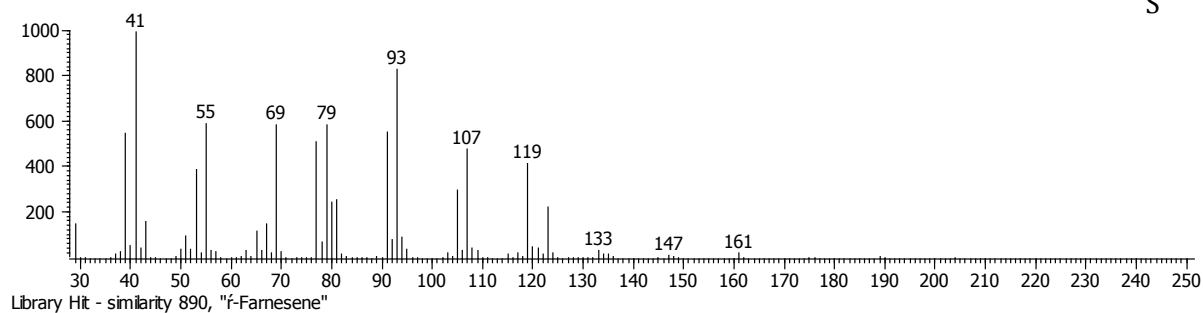


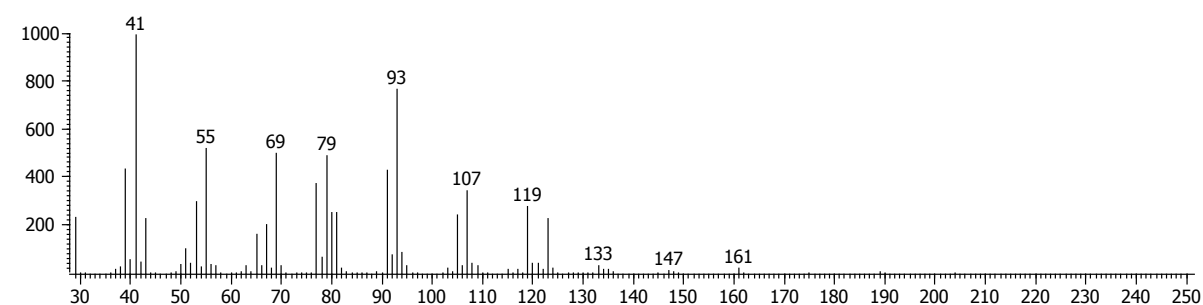
Fig.7.11: Fragmentation mass spectra of geranylacetone ($t_R = 1770, 2.480$). Each compound is identified by retention index (RI). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes C standard:1", peak 484, at R.I. 1507,8

S



Peak True - sample "C. Anonae male:1", peak 469, at R.I. 1505,1



Peak True - sample "C. Rosa male:1", peak 1056, at R.I. 1505,1

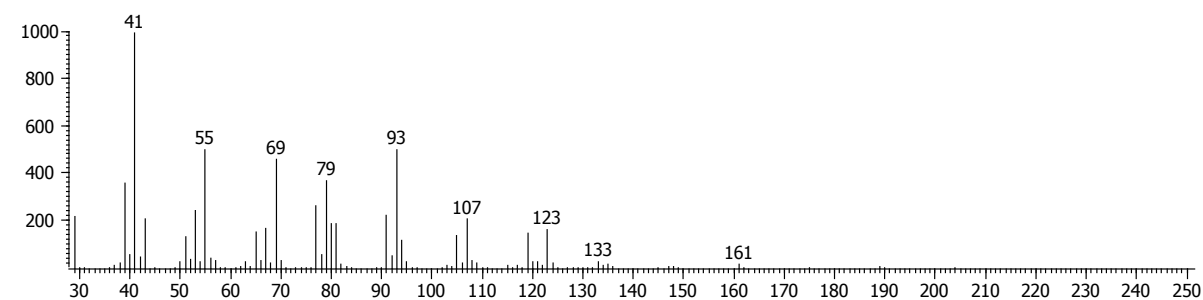
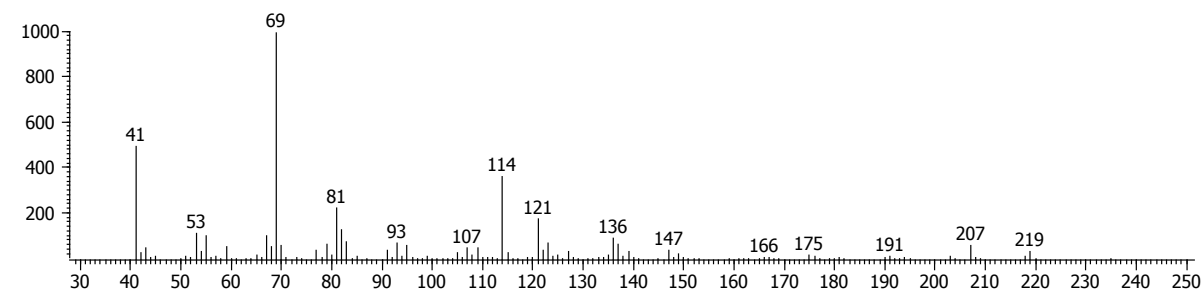
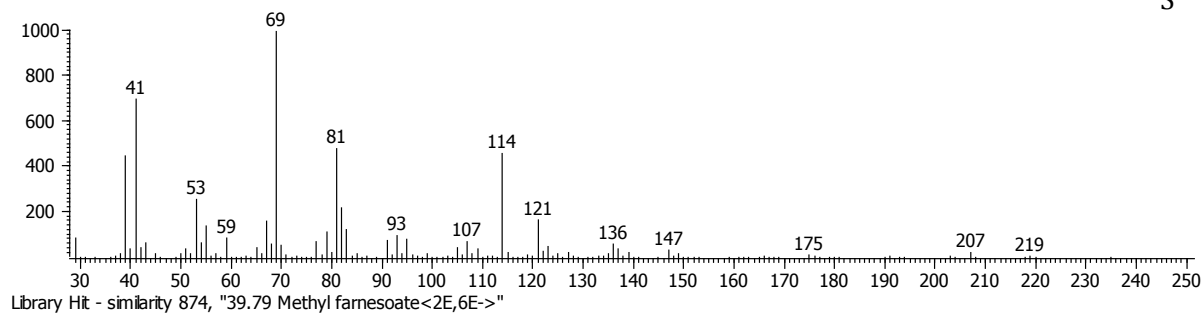


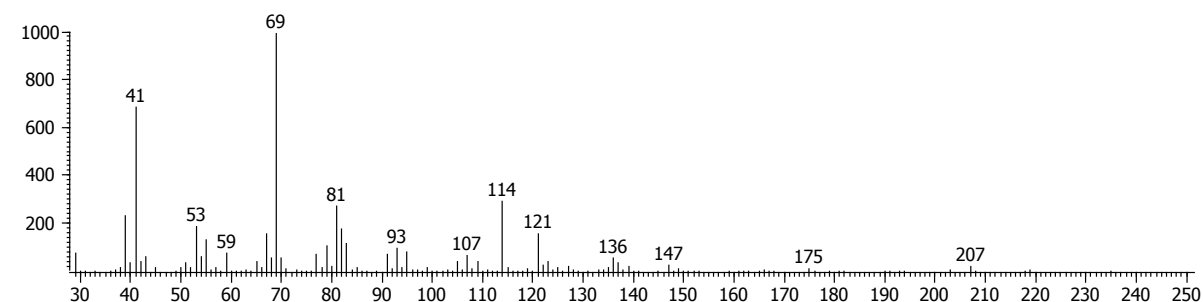
Fig.7.12: Fragmentation mass spectra of (*E,E*)- α -farnesene (t_R = 1850, 2.300). Each compound is identified by retention index (*RI*). Mass spectrum marked with (S) is standard solution.

Peak True - sample "Zmes A standard:1", peak 709, at R.I. 1784,3

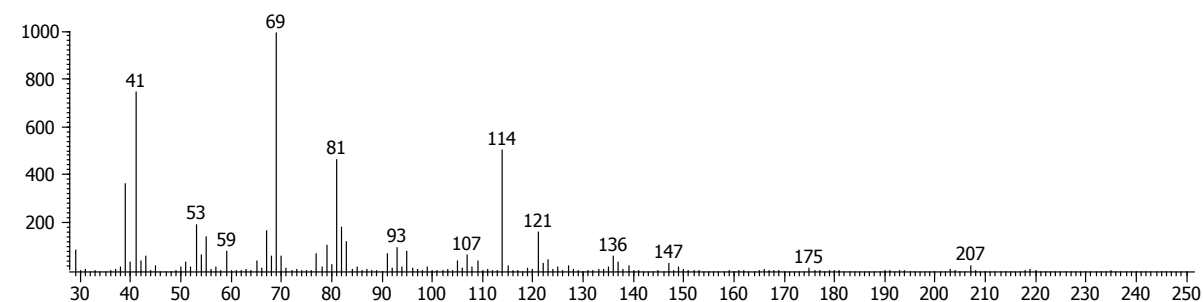
S



Peak True - sample "C. Anonae male:1", peak 561, at R.I. 1781,2



Peak True - sample "C. Fasciventris male:1", peak 838, at R.I. 1781,2



Peak True - sample "C. Rosa male:1", peak 1346, at R.I. 1781,2

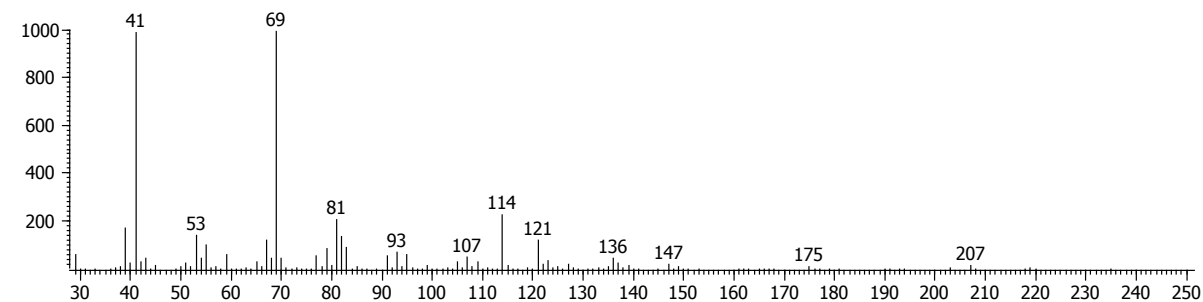


Fig.7.13: Fragmentation mass spectra of methyl (2E,6E)-farnesoate ($t_R = 2218, 2.600$). Each compound is identified by retention index (RI). Mass spectrum marked with (S) is standard solution.

Following are fragmentation spectra of mass fragmentation patterns of compounds listed as “Unknown”, where library hit was < 85%. Each figure is characterized by retention time (t_R), retention index and most probable hit suggested by the library.

Peak True - sample "FAR2012 C. fasciventris 5 males 30-31.III.2012 24h headspace 500 μ L hexan:1", peak 254, at R.I. 1027,4

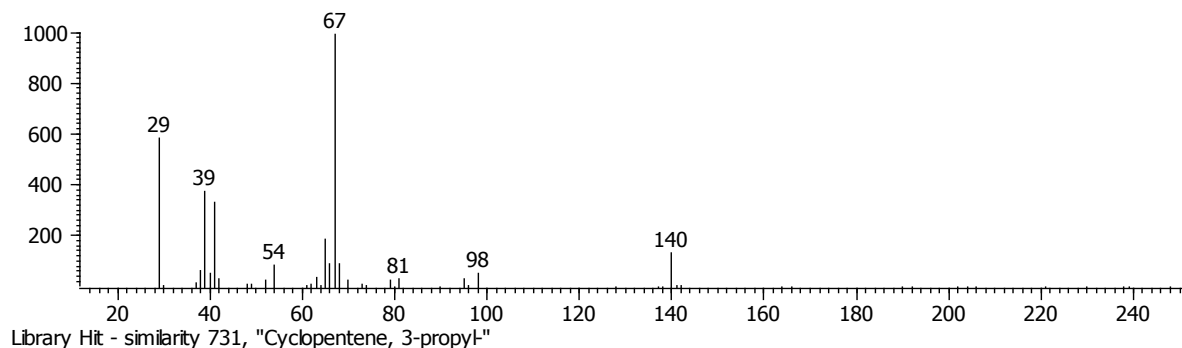


Fig. 7.14: Fragmentation mass spectra of Unknown 1 ($RI= 1027$, $t_R= 1046$, 2.380). Suggested hit: 3-propylcyclopentene, similarity: 73.1%.

Peak True - sample "FAR2012 C. fasciventris 5 males 2. 30-31.III.2012 24h headspace 500 μ L hexan:1", peak 267, at R.I. 1086,0

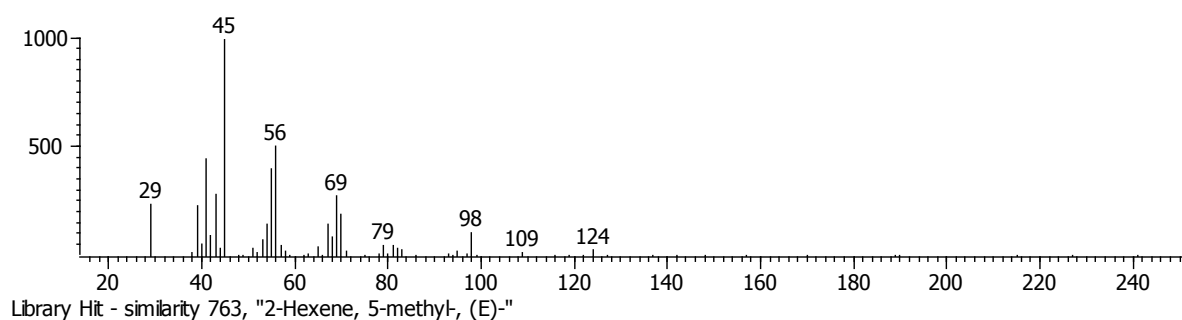


Fig.7.15: Fragmentation mass spectra of Unknown 2 ($RI = 1086$, $t_R= 1046$, 2.380). Suggested hit: 5-Methyl-(E)-hex-2-ene, similarity: 77.5%.

Peak True - sample "FAR2012 C. fasciventris 5 males 2. 30-31.III.2012 24h headspace 500µL hexan:1", peak 288, at R.I. 1106, 1

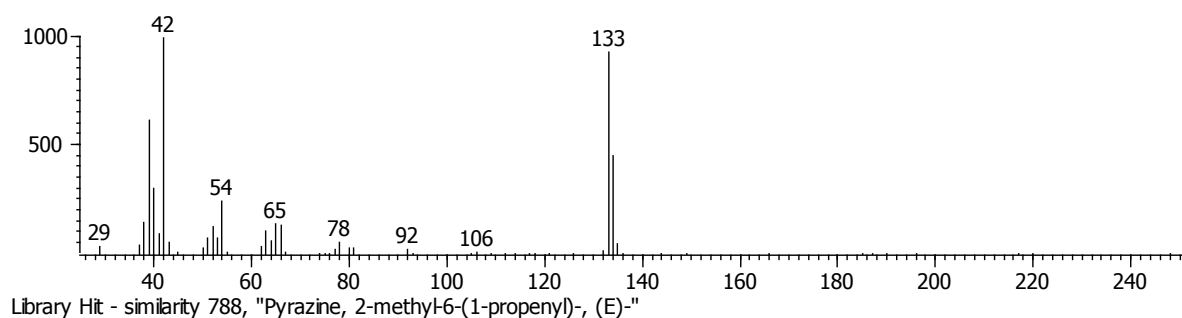


Fig. 7.16: Fragmentation mass spectra of Unknown 3 ($RI = 1106$, $t_R = 1190$, 2.720). Suggested hit: 2-methyl-6-(1-propenyl)-pyrazine, similarity: 78.8%.

Peak True - sample "FAR2012 C. fasciventris 5 males 3. 30-31.III.2012 24h headspace 500µL hexan:1", peak 375, at R.I. 1185, 3

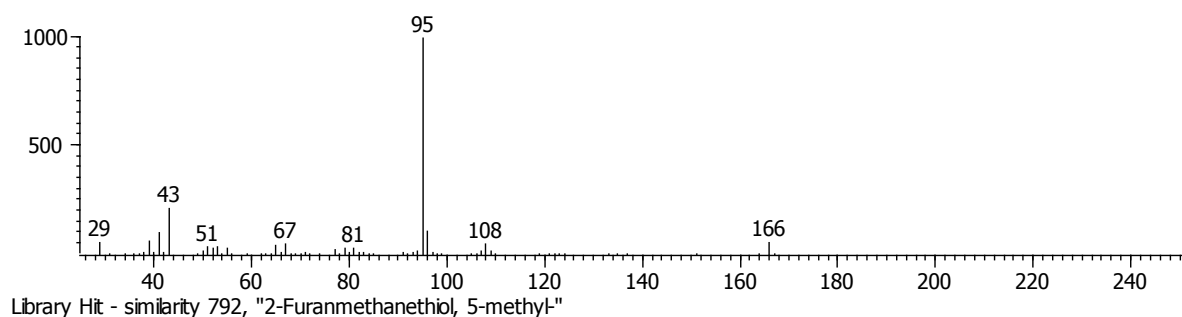


Fig. 7.17: Fragmentation mass spectra of Unknown 4 ($RI = 1185$, $t_R = 1130$, 2.180). Suggested hit: 5-methyl-2-furanmethanethiol, similarity: 79.2%

Peak True - sample "FAR2012 C. fasciventris 5 males 3. 30-31.III.2012 24h headspace 500µL hexan:1", peak 393, at R.I. 1206, 4

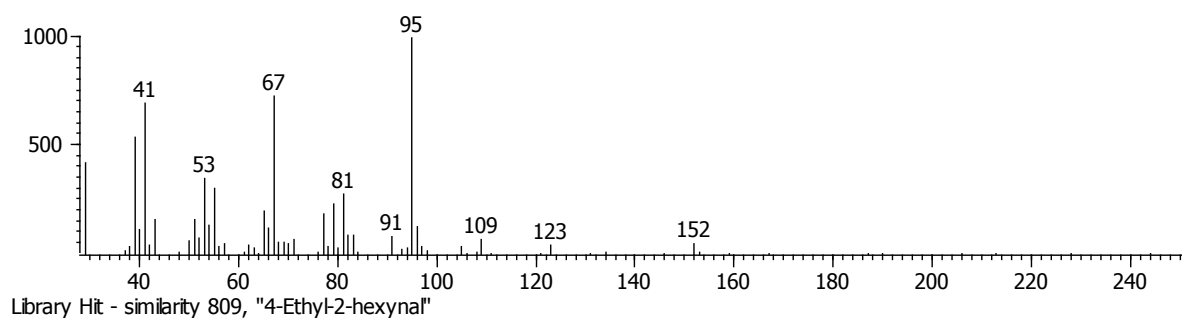


Fig.7.18: Fragmentation mass spectra of Unknown 5 ($RI = 1206$, $t_R = 1366$, 2.140). Suggested hit: 4-ethyl-2-hexynal, similarity: 80.9 %.

Peak True - sample "FAR2012 C. fasciventris 5 males 3. 30-31.III.2012 24h headspace 500µL hexan:1", peak 414, at R.I. 1260, 0

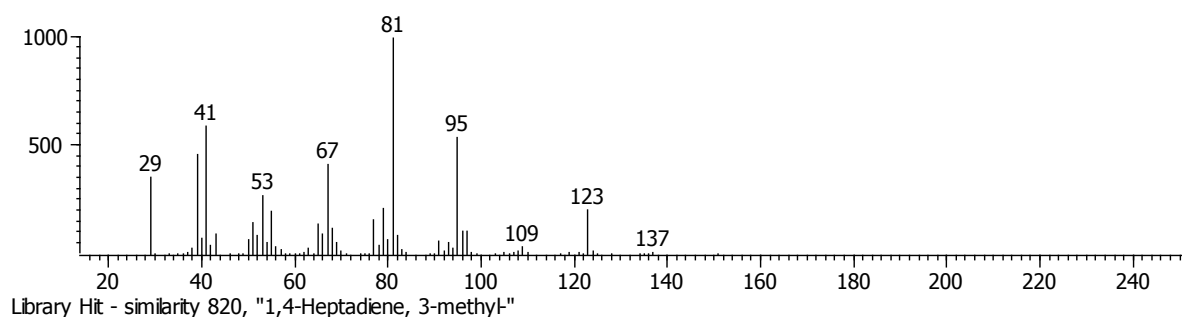


Fig. 7.19: Fragmentation mass spectra of Unknown 6 ($RI = 1260$, $t_R = 1454$, 2.480). Suggested hit: 3-methylhepta-1,4-diene, similarity: 82.0%.

Peak True - sample "FAR2012 C. fasciventris 5 males 3. 30-31.III.2012 24h headspace 500µL hexan:1", peak 463, at R.I. 1351, 5

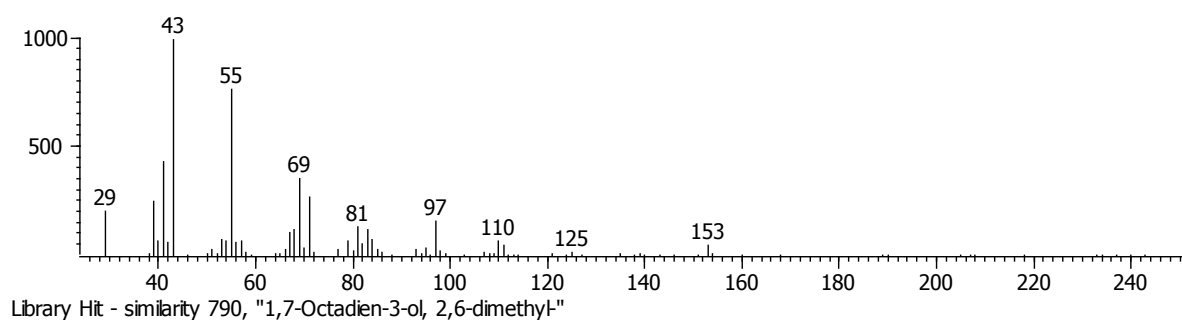


Fig. 7.20: Fragmentation mass spectra of Unknown 7 ($RI = 1352, t_R = 1602, 2.380$) . Suggested hit: 2,6-dimethyl-1,7-octadiene, similarity: 79.0 %.

Peak True - sample "FAR2012 C. fasciventris 5 males 3. 30-31.III.2012 24h headspace 500µL hexan:1", peak 533, at R.I. 1722, 1

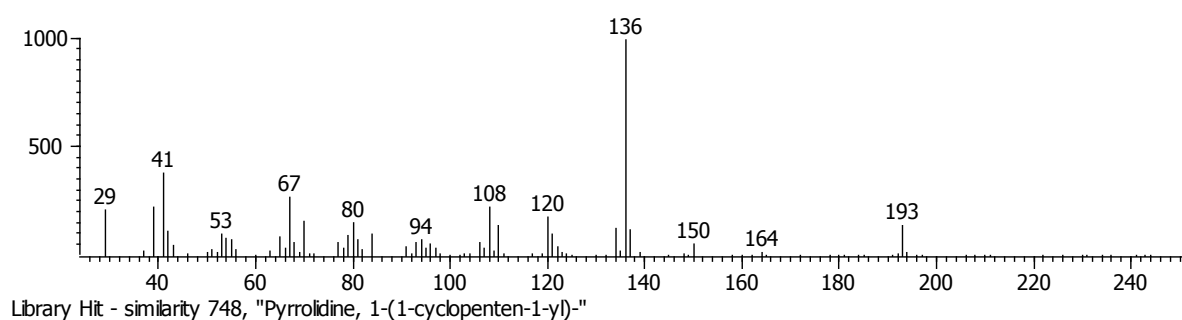


Fig. 7.21: Fragmentation mass spectra of Unknown 8 ($RI = 1722, t_R = 2054, 2.790$) . Suggested hit: 1-(1-cyclopenten-1-yl)-pyrrolidine, similarity: 74.8%.

Peak True - sample "FAR2012 C. fasciventris 5 males 3. 30-31.III.2012 24h headspace 500µL hexan:1", peak 542, at R.I. 1735, 3

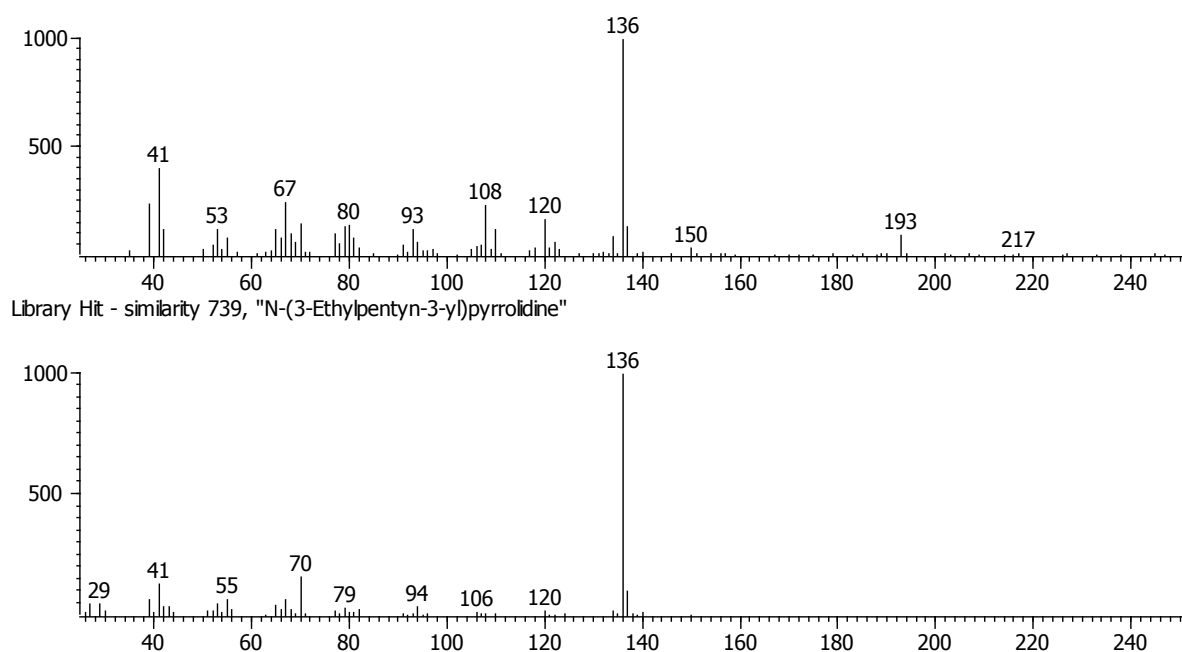


Fig. 7.22: Fragmentation mass spectra of Unknown 9 ($RI = 1735$, $t_R = 2082$, 2.780 . Suggested hit:N-(ethylpentyn-3-yl)-pyrrolidine, similarity:73.9%.

Peak True - sample "FAR2012 C. fasciventris 5 males 2. 30-31.III.2012 24h headspace 500µL hexan:1", peak 297, at R.I. 1130,9

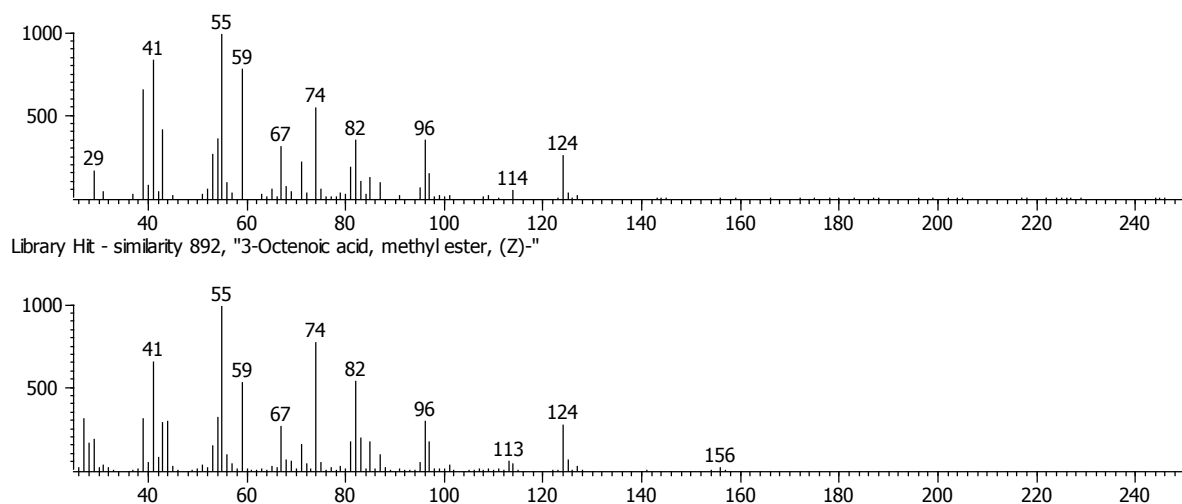


Fig. 7.23: Fragmentation pattern of antennally active compound presumed to be methyl (Z)-oct-3-enoate ($RI = 1131$, $t_R = 1234$, 2.310).