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REVIEW

# Cuticular lipids of insects as potential biofungicides: methods of lipid composition analysis

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**Abstract** The main function of cuticular lipids in insects is the restriction of water transpiration through the surface. Lipids are involved in various types of chemical communication between species and reduce the penetration of insecticides, chemicals, and toxins and they also provide protection from attack by microorganisms, parasitic insects, and predators. Hydrocarbons, which include straight-chain saturated, unsaturated, and methyl-branched hydrocarbons, predominate in the cuticular lipids of most insect species; fatty acids, alcohols, esters, ketones, aldehydes, as well as trace amounts of epoxides, ethers, oxoaldehydes, diols, and triacylglycerols have also been identified. Analyses of cuticular lipids are chemically relatively straightforward, and methods for their extraction should be simple. Classically, extraction has relied mainly on application of apolar solvents to the entire insect body. Recently, several alternative methods have been employed to overcome some of the shortcomings of solvent extraction. These include the use of solid-phase microextraction (SPME) fibers to extract hydrocarbons from the headspace of heated samples, SPME to sample live individuals, and a less expensive method (utilized for social wasps), which consists of the collection of cuticular lipids by means of small pieces of cotton rubbed on the body of the insect. Both classical and recently developed extraction methods are reviewed in this work. The separation and analysis of the insect cuticular lipids were performed by column chromatography, thin-layer chromatography (TLC), high performance liquid chromatography with a laser light scattering detector (HPLC-LLSD), gas chromatography (GC), and GC–mass spectrometry (MS). The strategy of lipid analysis with the use of chromatographic techniques was as follows: extraction of analytes from biological material, lipid class separation by TLC, column chromatography, HPLC-LLSD, derivatization, and final determination by GC, GC-MS, matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS, and liquid chromatography–mass spectrometry (LC-MS).

**Keywords** Cuticular lipids of insects · High-performance liquid chromatography with a laser light scattering detector · Gas chromatography · Gas chromatography–mass spectrometry

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## Introduction

### The composition of the cuticular lipids of insects

The cuticle of insects is covered with complex mixtures of mainly nonpolar and polar compounds which are extractable by organic solvents. The free cuticular lipids of insects vary in composition and quantity, depending on the species and the developmental stage. The major components in the cuticular lipids of insects are hydrocarbons, including straight-chain saturated and unsaturated hydrocarbons and methyl-branched hydrocarbons. In the cuticular lipids, mostly wax esters are present, in addition to aldehydes, alcohols, and free fatty acids.

Mainly long-chain wax esters are present in the cuticular lipids from adult *Bemisia tabaci* [1, 2]. Also, the cuticular lipids of nymphs and exuviae *Bemisia argentifolii* contain wax esters and additionally long-chain aldehydes, hydrocarbons and long-chain alcohols [3]. The wax esters were even-numbered carbon compounds ranging from $C_{38}$ to $C_{64}$. The major acid and alcohol moieties ranged from $C_{14}$ to $C_{28}$ and from $C_{22}$ to $C_{36}$, respectively. The wax esters occurring in the highest percentage in cuticular lipids were the $C_{52}$ ester, dotriacontanyl icosanoate (27.0%) and the $C_{54}$ ester tetratriacontanyl icosanoate (14.2%). The cuticular lipids of nymphs and exuviae contained only four aldehydes and four free alcohols. The major alcohol was dotriacontan-1-ol (36.9%) and the major aldehyde was dotriacontanal (40.1%). The major hydrocarbons were odd-carbon numbered *n*-alkanes ranging from $C_{25}$ to $C_{35}$.

The cuticular lipids of *Pogonomyrmex barbatus* contain wax esters and hydrocarbons [4]. The wax esters ranged from $C_{19}$ to $C_{31}$. The hydrocarbons consisted of *n*-alkanes from $C_{23}$ to $C_{33}$, methyl-branched alkanes from $C_{26}$ to $C_{49}$, and odd-numbered *n*-alkenes from $C_{27}$ to $C_{35}$. The cuticular compounds occurring in the highest concentrations were pentacosane (20.1%) and wax esters $C_{23}$ (8.3%), $C_{21}$ (6.0%), $C_{22}$ (5.4%), and $C_{24}$ (3.3%).

The cuticular lipids of *Calliphora vicina*, *Dendrolimus pini*, and *Galleria mellonella* larvae contain only three group of lipids: hydrocarbons, triacylglycerols, and free fatty acids [5]. The cuticular lipids of *C. vicina* contained fatty acids from $C_5$ to $C_{20}$ and included unsaturated free fatty acids such as $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$. The acids of this insect species occurring in the highest concentration were $C_{16:0}$ (20.1%), $C_{16:1}$ (17.9%), $C_{18:1}$ (18.4%), and $C_{18:2}$ (19.1%). Analysis of the cuticular fatty acids extracted from *D. pini* larvae confirmed the presence of $C_{16:0}$ (5.2%), $C_{18:0}$ (2.6%), $C_{18:1}$ (11.8%), $C_{18:2}$ (5.7%), and $C_{18:3}$ (4.3%). A similar observation was made with the cuticular fatty acids isolated from larvae of *G. mellonella.* Analysis indicated the composition of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$, where $C_{18:1}$ was the major acid, as well as traces of $C_{14:0}$, $C_{16:1}$, $C_{20:0}$, and $C_{20:1}$.

The adults and larvae of *Frankliniella occidentalis* consist of hydrocarbons and free fatty acids [6]. The following hydrocarbons were identified: *n*-alkanes from $C_{25}$ to $C_{29}$ with mainly an odd numbers of carbon atoms, 3-methylalkanes with 26 and 28 carbon atoms, and branched monomethylalkanes containing 26, 28, and 30 carbon atoms. The cuticular lipids of *F. occidentalis* (adults and larvae) contained saturated free fatty acids ($C_{14:0}$, $C_{16:0}$, and $C_{18:0}$) and unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$). The major acid in both larvae and adults was hexadecanoic acid —51.5 and 63.1%, respectively.

The cuticular lipids of adult *Acanthoscelides obtectus* of both sexes contain hydrocarbons, aldehydes, methyl and ethyl esters of fatty acids, triacylglycerols, free fatty acids, alcohols, and sterols [7]. The fatty acids identified in cuticular lipids were $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$. The acid $C_{18:1}$ was found to be dominant (16.0% in males and 10.9% in females). In lipids, methyl and ethyl esters of fatty acids were identified in traces and one of the sesquiterpenes was identified as α-farnesene.

Analysis of the cuticular hydrocarbons from adult *Diaprepes abbreviatus* indicated a composition with four groups of homologous compounds: hydrocarbons from $C_{23}$ to $C_{32}$, monomethyl-branched alkanes from $C_{25}$ to $C_{31}$, dimethyl-branched alkanes from $C_{27}$ to $C_{31}$, and two alkenes—$C_{27}$ and $C_{29}$ [8]. The hydrocarbon group contents were 20.4, 73.1, 3.4, and 3.06%, respectively.

Unusual cuticular lipids were found in *Liposcelis bostrychophila* [9]. The cuticular lipids contained *n*-alkanes, monomethylalkanes and dimethylalkanes, amides, free fatty acids, and aldehydes. *n*-Alkanes ranging from $C_{21}$ to $C_{34}$, monomethylalkanes ranging from $C_{28}$ to $C_{42}$, and dimethylalkanes ranging from $C_{31}$ to $C_{43}$ were identified. The amides were from $C_{16}$ to $C_{22}$ in chain length. The major amide (stearoyl amide $C_{17}H_{35}CONH_2$—76.3%) was identified on the basis of ions at *m*/*z* 59, 72, 240 $[M\text{-}43]^+$, and 283 $[M]^{+\cdot}$. Free fatty acids ($C_{16:1}$, $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, and $C_{18:0}$) and three aldehydes ($C_{15}$, $C_{16}$, and $C_{17:1}$) also occurred as cuticular lipids in *L. bostrychophila*.

In the cuticular extracts, 25 hydrocarbons were identified in *Periplaneta fuliginosa*, 23 in *Periplaneta americana*, 21 in *Periplaneta brunnea*, and 19 in *Periplaneta australasiae* [10]. The hydrocarbons of *P. americana* include compounds with 24 to 43 carbon atoms. The hydrocarbons of *P. fuliginosa*, *P. australasiae*, and *P. brunnea* ranged from 21 to 41 carbon atoms. 6,9-Heptacosadiene (68.5%) was present in the highest concentrations in cuticular lipids of *P. americana*. This compound was absent in the three other insect species analyzed. The major compound found in *P. brunnea* and *P. fuliginosa* was 13-methylpentacosane (59.1 and 56.1%, respectively). The major compounds identified in *P. australasiae* males, females, and nymphs were tricosene (45.8%), 13-methylpentacosane (36.2%), and 13-methylpentacosane (45.4%), respectively.

The larval–larval exuviae (LLE) contained 27 compounds ranging from $C_{8:0}$ to $C_{34:0}$, and larval–pupal exuviae (LPE) contained 26 compounds from $C_{8:0}$ to $C_{34:0}$ [11]. The major free fatty acids present in *D. pini* LLE were $C_{28:0}$ (32.5%), $C_{30:0}$ (24.5%), $C_{22:0}$ (10.7%), $C_{26:0}$ (7.6%), and $C_{32:0}$ (4.3%) in the dichloromethane extract and $C_{28:0}$ (37.7%), $C_{30:0}$ (34.6%), $C_{32:0}$ (7.4%), and $C_{22:0}$ (6.5%) in the petroleum ether extract. The dominant free fatty acids from *D. pini* LPE were $C_{28:0}$ (29.3%), $C_{30:0}$ (19.6%), $C_{22:0}$ (17.8%), $C_{20:0}$ (5.9%), and $C_{26:0}$ (7.9%) in the dichloromethane extract and $C_{28:0}$ (35.0%), $C_{30:0}$ (23.0%), and $C_{22:0}$ (11.5%) in the petroleum ether extract.

The cuticular lipids of male and female *Tenodera sinensis*, *Tenodera angustipennis*, *Mantis religiosa*, *Stamomantis carolina*, and *Bruneria borealis* contained *n*-alkanes, aldehydes, and one alcohol [12]. The alkanes ranged from $C_{29}$ to $C_{33}$, where $C_{31}$ was found to be dominant in *T. sinensis* (21.2% male and 18.0% female), *T. angustipennis* (28.0% male and 33.3% female), *M. religiosa* (34.0% male and 45.0% female), and *S. carolina* (26.0% female). The major compound in *B. borealis* was $C_{33}$ (40.0% female). The cuticular aldehydes (odd-carbon number) ranged from $C_{28}$ to $C_{32}$ in *B. borealis*. *T. sinensis* (3.6% female), *T. angustipennis* (13.0% male and 3.0% female), *M. religiosa* (1.0% male and 2.8% female), and *S. carolina* (7.0% female) contained only one aldehyde—$C_{30}$. The alcohol $C_{30}$ was present only in cuticular lipids of *T. sinensis* females (4.7%), *T. angustipennis* males (5.1%), and *S. carolina* females (2.6%).

Hydrocarbons, which include *n*-alkanes, methylalkanes, and alkenes, and also wax esters were present in the cuticular lipids from adults and nymphs of *Zygogramma exclamationis* [13]. Large amounts of hydrocarbons were identified in males and females (64.4±1.1 and 100.9±10.1 μg/g per insect, respectively). Analysis showed that hydrocarbons were the major lipid class in the cuticle and ranged from $C_{23}$ (tricosene) to $C_{56}$ (trimethyltripentacontane). The most abundant compounds were 13,17,21-trimethylnonatriacontane (19.4%) from females, *n*-nonacosane (17.5%) from larvae, and 11,15-dimethylheptacosane and 9,15-dimethylheptacosane (11.0%) and 13,17,21-trimethylnonatriacontane (11.0%) from males. The wax esters (from 40 to 48 carbon atoms) only detected in larvae were present in the lowest amounts (0.03±0.03 μg/g per insect).

The main cuticular lipids of *Osmia lignaria* Say and *Megachile rotundata* (F.) females were hydrocarbons including alkenes ranging from $C_{25}$ to $C_{31}$ (nearly 63.7%) in *O. lignaria* and alkanes (47.8%) and alkenes (45.1%) both ranging from $C_{23}$ to $C_{33}$ in *M. rotundata* [14]. The cuticular lipids of *O. lignaria* contained 14 alkenes. The alkene constituents occurring in the highest concentrations in the cuticular lipids of *O. lignaria* were 9-heptacosene (48.5±2.2%) and 7-nonacosene (18.4±3.6%). Dominant cuticular alkenes (26 constituents) of *M. rotundata* were 7-pentacosene (30.4±3.2%), 9-pentacosene (13.4±1.4%), and 7-heptacosene (13.2±1.2%). The components occurring in the cuticular lipids of *O. lignaria* and *M. rotundata* in smaller quantities (from traces in *O. lignaria* to 2.3±0.8% in *M. rotundata*) were wax esters. It has been shown that wax esters constitute 4.6% of the lipids in *O. lignaria* and 6.7% of the lipids in *M. rotundata*.

## Diverse biological roles of fatty acids

Fatty acids are needed for the construction of cell membranes, they are an important source of energy, they play a key role in signal transduction as well as in protein acylation, and they are needed for organism growth, differentiation, reproduction, and homeostasis [15]. The fatty acid composition of an organism is determined by the type of biosynthetic pathway of the given species. The abundance of lipids is considered to be a useful biochemical characteristic for taxonomical purposes. Analysis of lipid composition is now routinely used for the identification and differentiation of microorganisms [16–18]. Specific profiles of total cellular fatty acids and phospholipids have also been used to estimate microbial biomass and provide insight into the diversity and nutritional status of microorganisms [19, 20]. Species-specific fatty acid profiles may vary depending on the life cycle, developmental stage, and metabolic activity and are influenced by numerous environmental and stress factors [20–22].

The excellent review by Carballeira [15] deals with the most recent and some background literature on the latest developments with respect to fatty acids as antimalarial agents and the importance of enzyme inhibition, in particular the inhibition of the enoyl–acyl carrier protein reductase (FabI) of *Plasmodium falciparum*, the principal agent responsible for malaria. This review also highlights the mycobactericidal effect of fatty acids and the promising use of specific fatty acids to combat *Mycobacterium tuberculosis* strains resistant to drugs. A possible antibacterial mechanism is postulated to proceed via disruption of the bacterial cell membrane, resulting in a change in membrane permeability. This review also offers information on fatty acids known as inhibitors of topoisomerase I, a key enzyme in the breaking and repair of DNA strands involved in making the necessary topological changes to DNA for key cellular processes such as replication, transcription, and recombination. Topoisomerases have also evolved as key cellular targets for the development of effective anticancer drugs. Carballeira [15] presented data on the cytotoxic effect of fatty acids on cancer cells and their inhibitory effects on topoisomerase I. The last part of the review, the antifungal section, also emphasizes the most recent research with antifungal fatty acids and the importance of enzyme inhibition, in particular *N*-myristoyltransferase inhibition, for antifungal activity against medically important fungal pathogens.

## Role of fatty acids in fungal attack on insects

Numerous insect species transmit highly virulent diseases of humans, animals, and plants, and play a critical role as agricultural pests. Pressure to minimize deleterious effects of synthetic chemical insecticides on environmental and human safety has led to increased interest in the use and study of natural insect enemies. Among various microbial biocontrol agents, entomopathogenic fungi seem to be

most promising. Some 90 genera and 700 species of fungi, representing a large group of *Zygomycetes* and *Hyphomycetes*, are involved with entomopathogenicity of insects [23]. The factors responsible for the initiation and development of mycosis in insects are extremely complex, involving fungal production of biologically active metabolites that could be related to the mechanism of pathogenicity [24]. Asexually produced fungal spores or conidia are generally responsible for infection and are dispersed throughout the environment in which the insect hosts are present [25].

Entomopathogenic fungi invade their hosts by direct penetration of the cuticle, which is composed of two layers: the inner procuticle, comprising chitin fibrils embedded in proteinaceous matrix, and the outer epicuticle, containing phenol-stabilized proteins. The epicuticle is covered by a waxy layer containing variable amounts of fatty acids, lipids, and sterols [26]. Epicuticular lipids play a major role in protecting insects from desiccation and are also often involved in various types of chemical communication between insect species as well as between the instars and sexes of a single species [27, 28]. Epicuticular chemistry is also applied in the chemotaxonomy of insects [29, 30].

The infection process includes (1) prior-to-entry relationships, (2) penetration, and (3) establishment of the pathogen in the host [31]. The first step is the adhesion of fungi to the host cuticle. Surface structure and the chemical composition of the host cuticle are both believed to affect the attachment of fungal propagules to the cuticle [32]. Host recognition mechanisms are keyed to nutrient levels available on the appropriate host cuticle [31]. Penetration of the host cuticle by a parasitic fungus is achieved through a combination of enzymatic and mechanical mechanisms. Entomopathogenic fungi produce a range of cuticle-degrading enzymes, e.g., endoproteases, aminopeptidases, carboxypeptidases, *N*-acetylglucosoaminidases, chitinases, esterases, and lipases, which determine fungal virulence [33]. Invasion of the insect body occurs once the fungus has passed through the cuticle. On the death of the insect host, the fungus emerges from the dead host and sporulation or conidiogenesis usually occurs on the outside of the cadaver [25].

Susceptibility or resistance of various insect species to fungal invasion may result from several factors, including differences in the structure and composition of the exoskeleton, the presence of antifungal compounds in the cuticle, as well as the efficiency of cellular and humoral defense reactions of the invaded insect [34]. The cuticle composition strongly influences conidial germination and hyphal growth of fungi, resulting in the differential susceptibility of various insect species to fungal pathogens [35–37]. The nature of the inductive triggers has not been determined, but whereas the protein and chitin composition of the insect procuticle appears similar in all insects, the epicuticular components are extremely heterogeneous and therefore have the potential to lead to different pathogen responses in particular insects [5, 37–69]. Cuticular fatty acids have a profound effect on fungal spore germination and differentiation: they can be toxic, fungistatic, or occasionally, for some pathogenic species, stimulatory [33, 40]. However the role of host surface lipids and waxes in fungal pathogenesis of insects is still poorly understood. Determination of the cuticular fatty acid profile is therefore of great importance in understanding the background of insect susceptibility or resistance to fungal infection.

## Methods of testing insect susceptibility to fungal pathogens

Experimental surveys of interactions between insects and entomopathogenic fungi aim at establishing simple and reliable quantification of fungal pathogenicity toward insect targets. Different methods are used depending on the fungal and insect species tested. However, submersing the insects in spore suspension, deposition of spores on the cuticle, injection of conidial suspension into the hemocoel, and exposure of tested insects to sporulating fungal colonies are the most popular methods. Injection of conidia into the hemocoel is the most effective way of evoking fungal infection, but the artificial nature of such infections should be pointed out [41]. In the natural environment conidia cannot pass into the hemocoel—the conidium germinates on the surface of a suitable insect cuticle and invasive hyphae penetrate into the hemocoel. The exposure of insects to fungal colonies resembles a natural way of infection: insects wander on a substrate containing sporulating mycelia, the conidia can attach to the insect cuticle and germinate, and consequently invasive hyphae colonize the host hemocoel. This method allows one to distinguish fungal colonies producing conidia with high and low pathogenicity potential. On the other hand, deposition of known amounts of conidia directly on the insect cuticle allows one to perform tests in precisely controlled conditions.

The wax moth, *G. mellonella* (Lepidoptera), an important pest of apiculture, is a frequently used model insect owing to easy and cheap laboratory growth and lack of diapause, making insects available for tests during the whole year.

## Methods of testing the effects of fatty acids on growth and pathogenicity of entomopathogenic fungi

To determine the role of fatty acids identified in the epicuticle of tested insects, one should perform in vitro studies using appropriate liquid or solid culture media (some entomopathogenic fungi do not sporulate in liquid cultures [23, 40]). Each fungal species has its own requirements concerning in vitro maintenance: specific composition of the

artificial diet, temperature, humidity, lighting conditions, etc. [23, 42, 43]. In general, addition of insect host cuticle, extracts, or whole-body homogenates to the culture medium enhances virulence of entomopathogens [23]. Depending on the growing scale, fungal cultures are routinely kept in sterile Petri dishes, culture tubes, flasks, or fermentors [23]. Tested fatty acids are added to culture medium at final concentrations ranging from 0.1 to 0.0001% w/v. Solutions of fatty acids are sterilized by filtration through 0.22-μm filters and added to autoclaved and cooled medium. Medium without fatty acids is used as controls. Culture medium inoculations should always be performed using the same amount of conidia harvested from basic cultures under sterile conditions [40, 44, 45].

After termination of incubation (the incubation time may vary from 1 day to 4 weeks, depending on the fungal species and growing conditions [23]), mycelia are collected from liquid cultures by means of filtration through 0.22-μm filters, lyophilized, and weighed to estimate biomass production [40]. Cell-free filtrates as well as ultrasonicated mycelia can be used to determine the activities of cuticle-degrading enzymes [46–48]. Spectrophotometrical measurements of proteins accumulated [49] in cell-free filtrates followed by electrophoretic analysis (sodium dodecyl sulfate polyacrylamide gel electrophoresis) may give valuable information on proteinaceous metabolites released by the fungus into the incubation medium [50]. Injection of postincubation filtrates into *G. mellonella* larvae or other test insects will provide information on whether accumulated metabolites possess insecticidal potential [50] (M.I. Boguś, unpublished data).

For estimation of sporulation efficiency, cultures propagated on solid mediim are briefly washed with sterile water and the number of harvested conidia is determined manually with the use of a hemocytometer [40] or using automated systems such as a flow cytometer [51]. Identification of germinated conidia in liquid cultures is performed with the use of a microscope [45] (M.I. Boguś, unpublished data). Virulence of fungal colonies, i.e., the ability to evoke infection of the tested insects, can be measured using the methods described above.

## Methods of insect surface lipid composition analysis

### Solvent extraction

The first step in lipid analysis is the selective extraction of lipids from a biological matrix. Several extraction procedures may be found in the literature aiming at the improvement of lipid recovery from an insect's cuticle or body. The most popular lipid extraction procedure is that of Folch et al. [52]. In this method, lipids are extracted with a chloroform/methanol (2:1, v/v) mixture. Usually, external (epicuticular) lipids are extracted with hexane as described previously [53, 54]. For extraction of internal lipids, insects that have already been washed with hexane to remove external hydrocarbons are immersed in 3 ml of chloroform/methanol (1:2 v/v) for 1 h in an ultrasonic bath with ice, before the internal lipids are extracted by the procedure of Bligh and Dyer [55]. Extractions with hexane and chloroform were used to obtain *Semidalis flinti* cuticular lipids. Insects killed by freezing were placed in a glass column fitted with a plug of glass wool and initially rinsed with 5–8 ml of hexane for 1.5 min. This solvent rinse removed hydrocarbons and wax esters but most of the external lipids were found in a subsequent rinse with 5 ml of chloroform for 0.5 min. Extraction with 6–8 ml of chloroform for 1 min was chosen as the usual extraction procedure [56]. The same extraction method was applied by Nelson et al. [57] to analysis of nymphs and pupae of the giant whitefly, *Aleurodicus dugesii*. Adults or exuviae of *Aleyrodes singularis* were also extracted with 5–8 ml of hexane for 1.5 min. However, the hexane rinse did not remove all the hydrocarbons and wax esters from the exuviae. Therefore, both adults and exuviae were subsequently rinsed with 4–6 ml of chloroform for an additional 30 s and the hexane and chloroform rinses were combined [57].

The surface lipids from the insect cuticle can be extracted with petroleum ether for 10 s and then with dichloromethane for 5 min. Use of petroleum ether minimizes the possible extraction of internal lipids that are mostly free fatty acids and glycerides. The method described above has been applied to obtain lipids from *D. pini, C. vicina, G. mellonella*, and *A. obtectus* surfaces [5, 7].

### Solid-phase microextraction and solid injection

Recently, solid-phase microextraction (SPME) has been used as an alternative to solvent extraction for studying the cuticular lipid composition of insects [58–62]. The SPME technique can be used routinely in combination with GC, GC–MS, HPLC, or LC–MS. The SPME technique allows a reduction in preparation time, solvent purchase and disposal costs, and can improve detection limits. In SPME sampling of the headspace, the analyte is distributed among two or three phases: the fiber coating, the headspace, and, in the case of condensed samples, the liquid or solid sample. The kinetics of analyte mass transport within the various intervening media determines the SPME sampling time. The distribution constant depends on temperature but is also affected by ionic strength, pH, stirring, and organic solvent content. Stirring, extraction time, and temperature are interrelated variables, and their effect on the headspace SPME process should be optimized. Seven types of

stationary phase may be used: nonpolar polydimethylsiloxane (PDMS), polar polyacrylate (PA), Carboxen (Car)/divinylbenzene (DVB), and Carbowax/templated resin (polar), and mixed-polarity PDMS/DVB, Car/PDMS, and DVB/Car/PDMS. Compounds of different polarity and volatility can be sampled simultaneously by selecting carefully the polarity and thickness of the fiber coating. A fiber with thin coating is appropriate for semivolatile compounds, whereas thicker coatings permit the retention of highly volatile compounds. Thick coatings offer increased sensitivity but require much longer equilibration times. The PDMS-coated fiber is very sensitive to nonpolar compounds, whereas Car/PDMS is especially sensitive to small molecules. Fibers are selected according to overall performance. There are several aspects to consider: sensitivity, relative lack of affinity for interfering compounds, and the ability to be desorbed easily. The most common SPME fiber used for analyses of cuticular hydrocarbons is 100-μm PDMS [63].

SPME with GC coupled with MS was used to identify *Drosophila* cuticular compounds, to precisely quantify the amount of each compound, and to measure the variation of these substances as a function of aging and mating [64]. In this study 59 compounds, 58 cuticular hydrocarbons ($C_{20}$–$C_{31}$) and *cis*-vaccenyl acetate (cVA), were detected: 19 substances were female-specific, four (including cVA) were male-specific, and 36 were found in both sexes. The effectiveness of SPME was measured as compared with classic solvent extraction. The SPME fiber was gently rubbed on the head, thorax, wings, abdomen, and genitalia of the fly; the volatiles loaded on the fiber were then desorbed in the gas chromatograph injector. With the exception of cVA and $C_{29}$ cuticular hydrocarbons (neither of which were detected with SPME), there were no qualitative differences. However, the two methods revealed quantitative differences: with use of SPME-GC-MS higher levels of unsaturated cuticular hydrocarbons and lower levels of linear and methyl-branched alkanes were generally found. To evaluate the robustness of SPME, the compositions of the same whole-fly extract either after a direct injection or via indirect SPME sampling, by immersing the fiber into the extract, were compared. The authors showed that SPME tended to reveal higher levels of the lighter compounds and lower levels of heavier compounds, but showed no difference in the identification of saturated compounds. Also, in this work, for the first time the effects of fiber polarity on the compounds collected by SPME, using an apolar fiber (Car/DVB) and a polar fiber (PDMS), were compared: both fibers collected all the compounds described here, and significant qualitative differences were observed for only a few compounds.

Compounds occurring on the cuticle of honeybee workers were analyzed by Schmit et al. [65]. Volatile chemicals from the headspace of foraging honeybee workers were collected using 100-μm PDMS fiber. In this study, compounds in low quantity from the cuticle of honeybee workers were identified and, for the first time, the composition of emanated substances in the headspace of foraging honeybees in the field under undisturbed conditions was analyzed. On the cuticle of honeybees besides the known long-chain saturated and unsaturated aliphatic hydrocarbons and long chain esters, there were found traces or minor components, mainly with shorter chain lengths [alkanes, alkenes, one terpene, and (*Z*)-11-eicosen-1-ol]. In the air surrounding bees, major alkanes present on the cuticle up to a chain length of $C_{29}$, minor alkanes as well as alkenes, and in one trial geraniol and farnesol were identified.

The cuticular hydrocarbons of the subterranean termite *Coptotermes formosanus* Shiraki were identified using SPME-GC-MS [66]. Headspace SPME and direct-contact SPME methods were compared with the usually used hexane extraction method. In this work, optimization parameters such as temperature, time, number of termites, condition of the termites, and the type of SPME fiber were considered. Methods were refined to increase the reproducibility as well as the sensitivity. It was found that both SPME methods can be successfully used for the identification of all the major termite cuticular hydrocarbons. Besides cuticular hydrocarbons, fatty acids were also detected using the headspace SPME method. The direct-contact SPME method can be repeatedly applied to monitor chemical changes of the termite cuticular compounds.

Correlation between $C_9$–$C_{31}$ concentration and ovarian activity was studied using SPME with GC on live beta workers which attained alpha status (before the test alpha workers were removed) [58]. In the queenless ant *Dinoponera quadriceps*, it was found that the top worker in the hierarchy ("alpha") produces offspring in each colony, whereas other workers remain virgins. The authors showed that alpha and infertile nest mates consistently differ in their relative proportions of the cuticular hydrocarbon 9-hentriacontene ($C_9$–$C_{31}$). The second-ranking "beta" occasionally lays unfertilized eggs and less $C_9$–$C_{31}$ was detected than in the alpha but more was detected than in infertile workers. The proportion of $C_9$–$C_{31}$ in the replacement alpha increased significantly after 6 weeks, in parallel with her gain in fecundity. In this work it was discussed whether $C_9$–$C_{31}$ provides information about egg-laying ability, enabling ants to recognize the different classes of nest mates involved in reproductive conflicts.

Qualitative and quantitative analysis of male and female *Bagrada hilaris* hydrocarbons were done by headspace SPME, followed by GC-MS [67]. Three types of fiber coatings, several different extraction conditions, and two extraction temperatures (130 and 150 °C) were used to determine the effectiveness of the headspace SPME

method. In this study, PDMS (100 μm), PA (80 μm), and Carbowax/DVB (65 μm) were tested as the stationary phases. Fibers were conditioned in a gas chromatograph injector port as recommended by the manufacturer. Dissected insect body parts were then introduced into vials; sealed vials were then thermostated at 130 and 150 °C for 10 min. Immediately after SPME, a needle was inserted via the septum. The volatiles loaded on the fiber were then desorbed in the gas chomatograph injector port for 2 min. Of the PDMS and PA fibers tested, the PA phase showed the greater extraction efficiency while maintaining reasonably low percentage relative standard deviations. The influences of vial volume and extraction temperature were also investigated. Experiments at two temperatures were performed and 150 °C was selected as the more efficient temperature for maximum recoveries. The extraction method allowed identification of 12 cuticular hydrocarbon peaks that consisted of a homologous series of *n*-alkanes ($C_{17}$–$C_{29}$). Hydrocarbon profiles of males and females were qualitatively similar; however, marked sex-specific quantitative differences were observed (Fig. 1).

The booklouse, *L. bostrychophila*, is an increasingly common pest of stored food products worldwide. The cuticular lipid composition of *L. bostrychophila* was described by Howard and Lord [9]. They used a 7-μm PDMS SPME holder and then analyzed the absorbed lipids by GC-MS. Homologous series of *n*-alkanes ($C_{21}$–$C_{34}$), monomethylalkanes (3-methyl, 4-methyl, 5-methyl, 7-methyl, 9-methyl, 11-methyl, 12-methyl, 13-methyl, and 15-methyl) with a carbon chain range of $C_{28}$–$C_{42}$, and dimethylalkanes (3,7-dimethyl, 9,13-dimethyl, 11,15-dimethyl, 13,17-dimethyl, 9,21-dimethyl, 11,19-dimethyl, and 13,21- dimethyl) with a carbon chain range of $C_{31}$–$C_{43}$ were identified. These hydrocarbons were in small quantities, constituting approximately 0.0125% of the total biomass. Homologous series of amides ($C_{16}$–$C_{22}$ in chain length) were found, with the major amide being stearoyl amide. As the cuticular components, fatty acids ($C_{16:1}$, $C_{16:0}$, $C_{18:2}$, $C_{18:1}$, and $C_{18:0}$ in chain length) and three straight-chain aldehydes ($C_{15}$, $C_{16}$, and $C_{17:1}$ in chain length) were also identified.

The epicuticular composition of different body parts of the cabbage white butterfly, *Pieris rapae* L., was investigated using SPME and then GC and GC-MS [68]. SPME analyses were performed by rubbing 100-μm PDMS fiber, conditioned at 250 °C, on all the respective body parts. It was found that the major group of components, hydrocarbons, occurs in two distinct classes: straight-chain alkanes ranging in chain length from $C_{21}$ to $C_{31}$ (linear group) and dimethyl-, trimethyl-, and tetramethyl-branched alkanes with longer chain length ($C_{35}$–$C_{39}$, branched group). In the first group, small amounts of methyl-branched alkanes and also straight-chain 1-alkenes co-occurred. These groups were in different distributions on the cuticle of the insects. As major compounds on body, head, and wings, unbranched shorter-chain compounds ($C_{21}$–$C_{31}$, linear group) were detected, whereas longer-chain, polymethyl-branched compounds ($C_{35}$–$C_{39}$, branched group) were predominantly found on the antennae. Several other components such as 1,3-pentacosadiene and oxygenated aliphatic compounds occurred in minor amounts on the cuticle.

For the first time the analysis method of reduced-size samples such as pieces of cuticle or single exocrine glands was described by Bagnères and Morgan [69] (the solid injector). This method relies on inserting sealed glass capillaries which contain the insect or part of the insect into the gas chromatograph injector. The main problem with the method is the necessity to modify the injector port of the gas chromatograph. It is also a relatively time-consuming process as the port has to be opened frequently to be cleaned. These complications were overcome by modification of the general idea of solid injection and the creation of a special injection needle, which does not require any modification of the gas chromatograph. To assess this analytical tool, the ant *Cardiocondyla wroughtonii* was used. Individuals of this species are between 1 and 2 mm in length and were therefore suitable. The construction of the needle was done on the basis of the SPME fiber, an already existing analytical tool. The design and the principle of operation of the needle have been accurately described by Turillazzi et al. [70]. They compared this method with two currently accepted methods of analytical extraction: SPME and solvent extraction. Solid injection proved to be a superior method of extracting hydrocarbons when compared with headspace SPME and solvent extraction. By use of the solid injection method, 44 hydrocarbons were identified on the basis of their mass spectra. Solvent extraction and SPME proved to be unsatisfactory, with insufficient hydrocarbons being extracted in many cases.

## Column chromatography

One of methods for separating cuticular lipids, especially hydrocarbons, is liquid chromatography. The lipid fractions are further analyzed by GC and GC-MS.

The lipid extracts of larvae of *Attagus megatoma* were fractionated on a Florisil® column [71]. The Florisil® was heated for 90 min at 260 °C and deactivated with water (7%, v/w). The column contents were eluted with the following solvents: ether, diethyl ether/hexane (1:19, v/v), diethyl ether/hexane (3:17, v/v), diethyl ether/hexane (1:3, v/v), diethyl ether/hexane (1:1, v/v), methanol/diethyl ether (1:49, v/v), and acetic acid/diethyl ether (1:49, v/v). Each solvent wash was dried and the fraction was determined. The

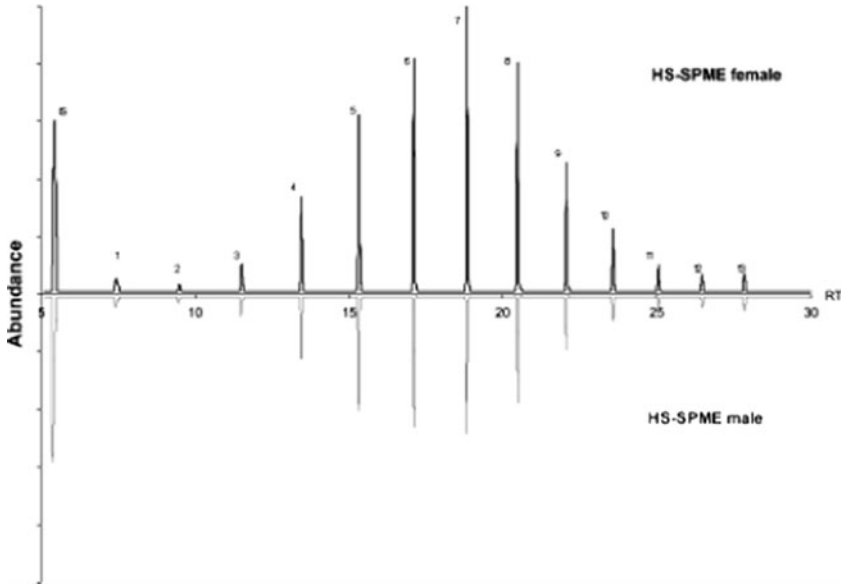


**Fig. 1** Representative chromatograms obtained from female and male *Bagrada hilaris* cuticular extracts using the headspace solid-phase extraction (*HS-SPME*) method (fiber PA, sample holder 22-ml vial, extraction temperature 150 °C). Samples were analyzed by gas chromatography–mass spectrometry with an HP5-MS column. *IS* internal standard, *RT* retention time. (Reprinted from [67] with the permission of Springer. All rights reserved)

cuticular lipids of *A. megatoma* (separated on column) contained sequentially hydrocarbons, non-glyceryl esters, triacylglycerols, sterols, diacylglycerols, monoacylglycerols, and fatty acids.

Also cuticular lipids from both larvae and adults of *Lasioderma serricorne* were fractionated on a Florisil® column [72]. The Florisil® was heated for 180 min at 260 °C and then deactivated by the addition of water (7%, v/w). The lipid fractions were eluted sequentially with hexane, 50% diethyl ether/hexane, 2% methanol/diethyl ether, 2% glacial acetic acid/diethyl ether. The following fractions were separated: hydrocarbons, wax esters, triacylglycerols, and free fatty acids.

The cuticular lipids in many insects consist of numbers of hydrocarbons, which are mainly responsible for the water balance. Moreover, the hydrocarbons ensure chemical communication between species as well as between insects of a single species. As a result, only this group of compounds was often separated and analyzed. For example, the hydrocarbons of the larvae of *Trogoderma granarium* were separated from the lipids by elution with hexane on a column filled with silica gel activated at 150 °C for 24 h [73]. The hydrocarbons of *Apis mellifera* were separated on a column of BioSil A impregnated with 20% (w/w) silver nitrate. Alkanes were eluted with hexane and alkenes with hexane/diethyl ether (50:50, v/v) [74].

## High-performance liquid chromatography

HPLC is a very common method used for many analytical problems; however, it has been slow to gain ground for lipid analysis. During the last few years, HPLC has been increasingly applied to the analysis of lipids.

In discussing lipid class separations, the approach used will depend largely on the nature of the available detection systems. For example, this determines the nature of the solvents used in the mobile phase and whether gradient elution is possible. Some relevant separations are therefore described below in terms of specific detectors, as examples of what is possible. Most lipids lack chromophores of value in spectrophotometric detection, but the absorbance of isolated double bonds (and some other functional groups) at about 205 nm in the UV range can be used successfully [75]. However, using UV detection at low wavelengths is limited. For example, only a few solvents are transparent and can be applied in the appropriate range (e.g., hexane, methanol, acetonitrile, 2-propanol, and water). Refractive index detectors also have several applications in lipid analysis. They are "universal" detectors, but lack sensitivity and are sensitive to minor fluctuations in temperature. Apart from this, their readouts change with changes in the mobile phase composition, thus preventing application of the gradient mode, a prerequisite in the separation of lipids with different polarities [5, 76]. A possible alternative is laser light scattering detection (LLSD): the readouts from this technique are proportional to the amount of analyzed solute, not its chemical type [77, 78]. Laser light scattering detectors are especially attractive for determining nonvolatile compounds which do not absorb above 200 nm (e.g., saturated hydrocarbons, steroids, surfactants, sugars) as their responses are independent of their optical characteristics. Therefore, no chromophores need be present in the molecules and no derivatization is required during the analytical process—which minimizes the risk of some product stability. With such a detector, the eluent from the column passes into a heated chimney, where the solvent is

evaporated in a stream of compressed air. The solute does not evaporate, but the remaining solutes are nebulized in an inert gas. The amount of scattered light can be measured and bears a relationship to the amount of material being eluted. It can therefore be termed a universal detector as it is not dependent on particular chromophores, and it can be used with gradients and a wide variety of different mobile phases. It is relatively inexpensive and rugged, but has limitations in quantitative analysis. Although the sample is lost during detection, it is possible to insert a stream splitter between the end of the HPLC column and the detector, so that a high proportion of the eluent is diverted to a fraction collector. The most common stationary phase used in lipid class analysis is silica gel [79, 80]. The heterogeneous surface of silica gel leads to variable adsorptive properties, irreversible adsorption of solutes, and a long reconditioning time. This kind of stationary phase appears to be very attractive for lipid class analysis. Several polar stationary phases as an alternative to silica have been studied for lipid class analysis, such as diol [81–83], cyano [84] and amino grafted silica [85]. A polymerized poly(vinyl alcohol) on silica gel was recently used for lipid class analysis. Numerous solvent combinations for the isocratic elution have been described in the literature [86–97]. However, it is necessary to cover the whole lipid polarity range from neutral lipids to phospholipid mobile phase gradients.

The utility of HPLC-LLSD methods for separation of the insect cuticular lipids has been described by Gołębiowski et al. [5–7, 11]. Analytical parameters identical to those described below were applied. The cuticular lipid extracts were separated into several classes of compounds using HPLC in the normal phase using a Shimadzu LP-6A binary pump in gradient mode equipped with a 250 mm×4.6-mm inner diameter analytical column filled with Econosil silica (particle size 5 μm). The mobile phase consisted of petroleum ether (solvent A) and dichloromethane with the addition of 15% acetone and 1.5% 2-propanol (solvent B). For lipid separation, gradient elution from 100% solvent A to 100% solvent B within 20 min was applied. LLSD was used as the detection system. The detector evaporation temperature was 42 °C, and the carbon dioxide pressure was 0.1–0.2 MPa. Extracts of cuticular lipids from *A. obtectus* were separated by HPLC–LLSD into fractions containing general groups of chemical entities. It was found that cuticular lipids of *A. obtectus* contain the following classes: hydrocarbons, aldehydes, methyl and ethyl esters of fatty acids, triacylglycerols, free fatty acids, alcohols, and sterols. In another study, LLSD followed by GC and GC-MS techniques was applied to the characterization of the epicuticular lipids in three insect species representing various susceptibilities to fungal infection: the pine-tree moth *D. pini*, the blowfly *C. vicina*, and the wax moth *G. mellonella*. In extracts from the three insect species, three lipids classes—hydrocarbons, triacylglycerols, and free fatty acids—were found. In extracts obtained from *G. mellonella* and *D. pini*, triacylglycerols were identified as the main compounds, whereas in *C. vicina*, extracts were dominated by fatty acids making up 79.40% of all lipids (Fig. 2). In contrast, fatty acids in *D. pini* extracts were only 29.96% of cuticular lipids. HPLC-LLSD in the normal phase was also applied to the separation of *D. pini* exuviae extracts.

HPLC-MS is a useful tool for studying insect lipids [91, 92]. Despite the method development in recent years, insect lipids are still mostly studied by traditional approaches requiring separation by HPLC and then GC-MS analyses. However, an HPLC-MS method for the analysis of insect triacylglycerols has been developed and applied to several species [93]. Several papers reported excellent separation of triacylglycerols using columns packed with a 4-μm Nova-Pak $C_{18}$ phase (Waters) [94–97]. An interesting application of two conventional Nova-Pak $C_{18}$ columns connected in series, for a total length of 45 cm, for insect triacylglycerol analysis was described by Kofronová et al. [92]. For triacylglycerol separation a mobile phase gradient consisting of acetonitrile and 2-propanol was used. Triacylglycerols were detected by atmospheric pressure chemical ionization (APCI) MS. The method was applied to analysis of triacylglycerols isolated from the fat body of the bumblebee *Bombus lucorum*.

Analysis of triacylglycerols from the fat bodies of 11 species of male bumblebee using HPLC/APCI-MS and matrix-assisted laser desorption/ionization (MALDI) MS was also reported [98]. The major aims of this study were to compare two analytical techniques, one highly informative but rather slow (HPLC/APCI-MS) and the other rapid but less informative (MALDI-MS), and to evaluate the inter- and intraspecific differences in triglycerides to confirm that fat body lipids are species-specific.

## Thin-layer chromatography

Cuticular lipid classes of insects have often been determined by thin-layer chromatography (TLC). TLC can be used to separate lipid classes and to isolate positional isomers of a single lipid class. In most cases, the lipid extract was spotted on high-performance silica gel plates with hexane/diethyl ether/formic acid (80:20:1 v/v/v) as the developing solvent. The lipid bands were visualized by charring plates after spraying with a solution of 5% concentrated sulfuric acid in 95% ethanol followed by heating to 180-200 °C [3, 13, 99–104]. The cuticular lipid classes of many insects were separated by TLC (Table 1) [3, 4, 13, 99–105].

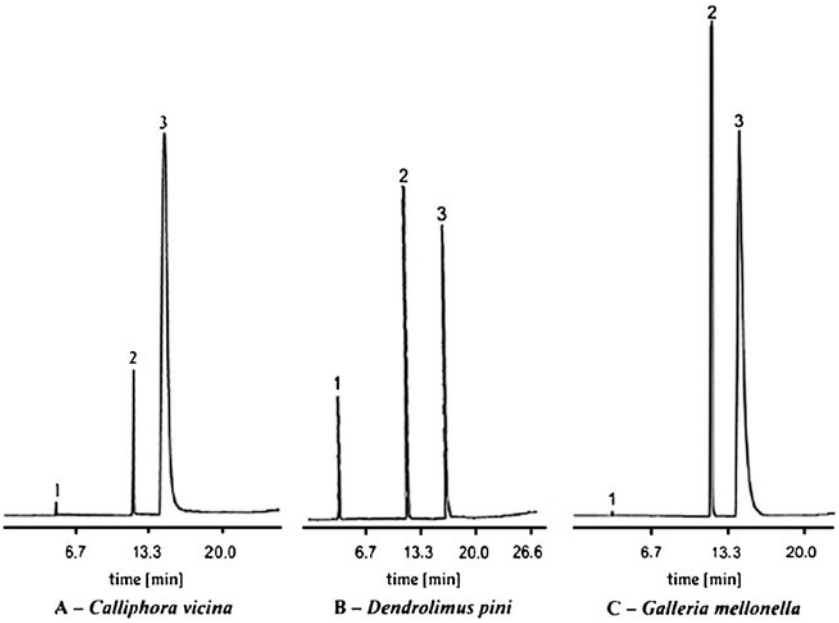


**Fig. 2** Chromatograms HPLC of cuticular lipids extracted from **a** *Calliphora vicina*, **b** *Dendrolimus pini*, and **c** *Galleria mellonella*. *1* hydrocarbons, *2* triacylglycerols, *3* fatty acids. (Reprinted from [5] with the permission of Elsevier B.V. All rights reserved)

For TLC separation of 11-oxoalcohol and 12-oxoalcohol acetate esters and 11-oxoaldehydes and 12-oxoaldehydes from cuticular lipid extracts of *Manduca sexta* pupae, silica plates with dichloromethane/acetonitrile (98:2 v/v) were used. The 12-oxoaldehydes migrated ahead of the 11-oxoaldehydes and 11-oxoalcohol and 12-oxoalcohol acetate esters [106].

The separated compounds or chemical classes were scraped from the developed TLC plate and extracted with various solvents. Most of the isolated chemical classes have to be modified before they can be analyzed by GC and GC-MS.

## Gas chromatography and gas chromatography–mass spectrometry

GC and GC-MS have been the primary tools for cuticular lipid analysis. Identification of analyzed lipids was carried out on the basis of characteristic mass spectra and retention times of native compounds, e.g., hydrocarbons and methyl esters of fatty acids. Other compounds, such as alcohols and fatty acids, were identified as derivatives. Identification of the *n*-alkanes, alcohols, free fatty acids, aldehydes, ketones, and sterols was made by direct comparisons with authentic standards and published mass spectra.

Mass spectra of the hydrocarbons were interpreted as previously described [107–112]. The methyl-branched alkanes were identified on the basis of equivalent chain lengths, retention indices, or Kovats indices coupled with mass-spectral fragmentation patterns. Particularly, equivalent chain length combined with the mass-spectral fragmentation pattern is currently commonly used for identification.

Fatty acids were analyzed by GC-MS as their methyl esters obtained, e.g., in the reaction with diazomethane [5]. The molecular ion was present in the mass spectra. The presence of a double bond was identified by a reduction in molecular mass of 2 amu. Ions M-31 and M-43 in the mass spectra of fatty acid methyl esters (FAMEs) correspond to the loss of the methoxy and propyl groups. The characteristic ions (the base peak) *m/z* 74 and 87 arise through McLafferty rearrangement.

Also trimethylsilyl ethers were prepared for identification of fatty acids [11]. The mass spectrum of trimethylsilyl ethers of fatty acids showed the following ions: $M^{+\cdot}$ (molecular ion), $[M\text{-}15]^+$, *m/z* 73 and *m/z* 75 corresponding to $[(CH_3)_3Si]^+$ and $[(CH_3)_2SiOH]^+$, and fragment ions at *m/z* 117, 129, 132, and 145. The *m/z* 132 and 145 ions arises through McLafferty rearrangement.

Alcohols were identified on the basis of silyl derivative ions $[M\text{-}15]^+$ and *m/z* 103 [113].

Wax esters have been isolated from the surface lipids of a number of insects (Table 2). The molecular ions of wax esters were not always present in the mass spectra. The wax esters were identified on the basis of characteristic ions $[RCO_2H+H]^+$ (produced by cleavage of the alkyl oxygen bond), $[C_nH_{2n+1}C{=}O]^+$, and $[C_nH_{2n}]^+$ [113].

Sterols were most frequently analyzed as the free compounds, acetate, or the trimethylsilyl ether derivative. Table 3 shows the six most abundant high-mass ions in the mass spectra of a series of sterol trimethylsilyl ethers [114]. Insects are unable to biosynthesize the steroid de novo, but they need the sterols for their growth and development. Cholesterol found in insects may originate from their food source and insects convert phytosterols to cholesterol [115, 116].

**Table 1** Cuticular lipids of insects separated by thin-layer chromatography

| Stage/species | Compounds | Developing solvent | Visualization | References |
|---|---|---|---|---|
| Pupae of *Heliothis virescens* | Hydrocarbons, wax esters, aldehydes, alcohol acetate esters, free fatty acids, alcohols | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [99] |
| Pupae of *Helicoverpa zea* | Hydrocarbons, wax esters, fatty alcohols | Hexane/diethyl ether/formic acid (66:33:1 v/v/v) | Iodine vapors | [99] |
| | Aldehydes, acetate esters of alcohols | Benzene | | |
| | Oxoalcohols, diols | Hexane/diethyl ether/formic acid (50:50:1 v/v/v) | | |
| Adult and exuviae of *Aleyrodes singularis* | Wax esters, aldehydes, and/or acetate esters and alcohols | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [100] |
| Nymphs and exuviae of *Bemisia argentifolii* | Hydrocarbons, wax esters, aldehydes, and *n*-alcohols | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [3] |
| Adult of *Aleurotithius timberlakei, Dialeurodes citri, Dialeurodes citrifolii,* and *Parabemisia myricae* | Aldehydes, alcohols, wax esters | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [101] |
| Adult of *Pogonomyrmex barbatus* | Hydrocarbon and wax ester fractions | Hexane/diethyl ether (98:2 v/v) | An iodine tank | [4] |
| Pupae of *Heliothis virescens* | Hydrocarbons, wax esters, aldehydes, free fatty acids, primary alcohols | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [102] |
| Nymphs and pupae of *Aleurodicus dugesii* | Alcohols, wax esters, and aldehydes and/or acetate esters | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [10] |
| Adult of *Melanoplus sanguinipes* and *Melanoplus packardii* | Alkanes, secondary alcohol wax esters, normal and sterol wax esters, triglycerides, aliphatic alcohols, sterols, and free fatty acids | Hexane/chloroform (50:50 v/v) | Rhodamine 6 G under UV light | [104] |
| Adult and nymphs of *Zygogramma exclamationis* | Hydrocarbons, triacylglycerols | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [13] |
| Larvae of *Eurosta solidaginis* | Hydrocarbons, cholesterol esters and/or wax esters, triacylglycerols, free fatty acids and/or long-chain alcohols, and cholesterol and/or diacylglycerols | Hexane/diethyl ether/formic acid (80:20:1 v/v/v) | 5% concentrated sulfuric acid in 95% ethanol | [105] |

## Derivatization

The FAMEs were obtained as follows. Free fatty acids were dissolved in anhydrous diethyl ether, to which small portions of a diazomethane solution were subsequently added. Diazomethane was synthesized as follows. *N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide was diluted in diethyl ether. The solution was cooled and KOH dissolved in ethanol was added. The ether solution of diazomethane was distilled over a water bath to yield a solution containing diazomethane [5]. The free fatty acids can also be methylated by the addition of 14% $BF_3$ in methanol, the mixture is kept at 60 °C for 1 h, water is added, and the solution is extracted with hexane [6].

The trimethylsilyl ethers of acids were obtained by the addition of 100 μl of a mixture of 85% bis(trimethylsilyl) acetamide and 15% chlorotrimethylsilane to 1 mg of cuticular extract. The mixture obtained was kept at 100 °C for 1 h [11].

**Table 2** The cuticular lipids of insects analyzed by gas chromatography and gas chromatography–mass spectrometry

| Insects | Compounds | Derivatization | References |
|---|---|---|---|
| Adult, nymphs, and exuviae of *Bemisia argentifolii* | Wax esters, long-chain aldehydes, hydrocarbons, long-chain alcohols | Alcohols—reacted with acetic anhydride/pyridine and the acetate esters<br>Free fatty acids—reacted with $BF_3$/methanol | [3] |
| Adult of *Pogonomyrmex barbatus* | Hydrocarbons, wax esters | – | [4] |
| Larvae of *Calliphora vicina, Dendrolimus pini*, and *Galleria mellonella* | Hydrocarbons, triacylglycerols, fatty acids | Free fatty acids—reacted with diazomethane | [5] |
| Adult and larvae of *Frankliniella occidentalis* | Hydrocarbons, fatty acids | Free fatty acids—reacted with $BF_3$/methanol | [6] |
| Adult of *Acanthoscelides obtectus* (Say) | Hydrocarbons, aldehydes, methyl and ethyl esters of fatty acids, triacylglycerols, free fatty acids, alcohols and sterols | Free fatty acids—reacted with a mixture of 85% bis(trimethylsilyl) acetamide and 15% chlorotrimethylsilane | [7] |
| Adult of *Diaprepes abbreviatus* (L.) | Hydrocarbons | – | [8] |
| *Liposcelis bostrychophila* | Hydrocarbons, aldehydes, fatty acids, and fatty acid amides | Free fatty acids—reacted with (1,1)-*N*,*N*-dimethylhydrazine | [9] |
| Male, female, and nymphs of *Periplaneta brunnea, Periplaneta fuliginosa Periplaneta australasiae*, and *Periplaneta americana* | Hydrocarbons | – | [10] |
| Exuviae of *Dendrolimus pini* | Free fatty acids | Free fatty acids—reacted with a mixture of 85% bis(trimethylsilyl) acetamide and 15% chlorotrimethylsilane<br>Free fatty acids—reacted with diazomethane | [11] |
| Male and female of *Tenodera sinensis, Tenodera angustipennis, Mantis religiosa, Stamomantis carolina,* and *Bruneria borealis* | *n*-Alkanes, aldehydes, alcohol | – | [12] |
| Adult and nymphs of *Zygogramma exclamationis* | Hydrocarbons, wax ester | – | [13] |
| Female of *Osmia lignaria* Say and *Megachile rotundata* (F.) | Hydrocarbons, wax ester | – | [14] |

**Table 3** Mass spectra of sterol trimethylsilyl ethers. (Reprinted from [114] with the permission of ACS Publications. All rights reserved)

| Sterols | $M^+$ | Fragment ions | | | | | |
|---|---|---|---|---|---|---|---|
| Cholesterol | 458 | 129 (100%) | 329 (87%) | 368(52%) | 145(38 %) | 121(36%) | 353(32%) |
| Cholestanol | 460 | 215 (100%) | 216 (59%) | 217 (41%) | 147 (37%) | 121(32%) | 355 (25%) |
| Brassicasterol | 470 | 125 (100%) | 129(98%) | 380(84%) | 255(75%) | 145 (58% | 147(55%) |
| Ergosta-7,22-diene-3β-ol | 470 | 255 (100%) | 147 (68%) | 145 (55%) | 159 (52%) | 229 (49%) | 343 (47%) |
| 24-Methylenecholesterol | 470 | 129 (100%) | 119 (38%) | 386 (36%) | 380 (34%) | 341 (30%) | 296 (25%) |
| Campesterol | 472 | 129 (100%) | 121 (55%) | 119 (44%) | 147 (32%) | 343 (25%) | 382 (19%) |
| Stigmasterol | 484 | 129 (100%) | 255 (63%) | 133 (38%) | 394 (35%) | 145 (32%) | 484 (22%) |
| Fucosterol | 484 | 129 (100%) | 386 (73%) | 296 (62%) | 119 (41%) | 145 (33%) | 355 (27%) |
| Stigrnasta-5,25-diene-3β-ol | 484 | 129 (100%) | 119 (43%) | 121 (37%) | 145 (29%) | 147 (27%) | 394 (26%) |
| β-Sitosterol | 486 | 129 (100%) | 357 (98%) | 396 (66%) | 121 (58%) | 145 (45%) | 215 (42%) |
| Stigmastanol | 488 | 215 (100%) | 216 (52%) | 121 (40%) | 147 (32%) | 217 (30%) | 305 (22%) |

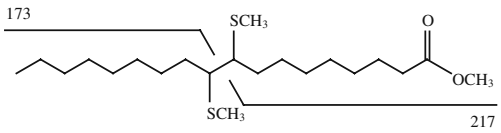


**Fig. 3** Characteristic ions of the dimethyl disulfide adduct of methyl oleate [(*Z*)-9-octadecenoate]

The cuticular triacylglycerols were hydrolyzed by heating them in a methanolic solution of 0.5 M KOH for 3 h at 70 °C in sealed ampoules. The mixture was dried under a stream of $N_2$ and the dried sample was then silylized with a mixture of 85% bis(trimethylsilyl)acetamide and 15% chlorotrimethylsilane for 1 h at 100 °C [6].

To locate the double bonds in the fatty acid alkyl chains, the acid was reacted with diazomethane. The methyl esters were then reacted with dimethyl disulfide [5, 117]. The sample was dissolved in hexane, dimethyl disulfide spiked with iodine was added, and the mixture was dissolved in dimethyl ether (6% v/v). The reaction mixture obtained was left for 15 h at room temperature. The aqueous phase was extracted twice with hexane. The combined organic extracts were evaporated and subjected to GC and GC-MS analysis. For example, the mass spectrum of the (*Z*)-9-octadecenoic acid derivative revealed the molecular ion *m/z* 390 with the characteristic ions *m/z* 173 and 217 (Figs. 3, 4).

For determination of the double-bond positions, the carboxyl group was derivatized. For preparation of pyrrolidide derivatives [113], the free fatty acids were dissolved in pyrrolidine, glacial acetic acid was added, and the mixture was heated for 1 h at 100 °C. The reaction mixture dissolved in dichloromethane was washed with 2 M hydrochloric acid and water. In the spectrum of pyrrolidine derivatives, the base peak was related to the McLafferty rearrangement ion. The mass spectrum of pyrrolidine derivatives showed the following ions: [M-1] and a series of ions separated by 14 Da.

For preparation of diols, FAME was dissolved in a mixture of pyridine and dioxane, $OsO_4$ (in dioxane) and

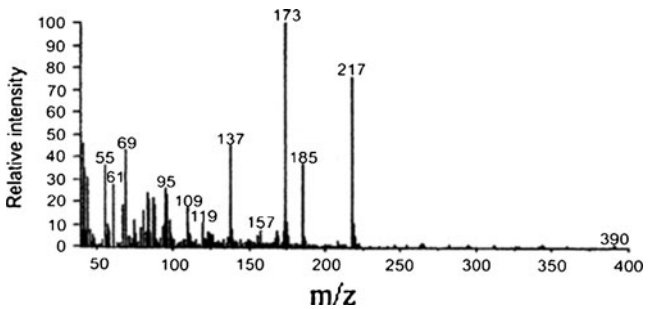


**Fig. 4** Mass spectrum (70 eV) of (*Z*)-9-octadecenoic acid (methyl ester) with dimethyl disulfide. (Reprinted from [5] with the permission of Elsevier B.V. All rights reserved)

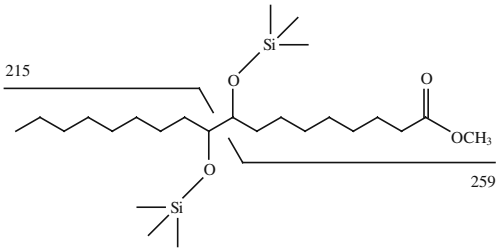


**Fig. 5** Structure of methyl 9,10-bis(trimethylsiloxy)octadecenoate

$Na_2SO_4$ (in methanol) were added, then the solvent was evaporated, and the dried residue was silylized. To locate the double bonds in the fatty acids, the fragment ions of the trimethylsilyl ethers of the diols of FAME in the spectrum were used. For example, in the mass spectrum of methyl 9,10-bis(trimethylsiloxy)octadecenoate characteristic ions at *m/z* 215 and 259 were present (Fig. 5) [113].

The positions of double bonds in alkenes can be determined by a sodium borohydride reduction of their methoxymercuration products. This method was used for double-bond location in alkenes from the cuticular lipids of the honeybee *A. mellifera* L. [118].

## Conclusions

The outer layers of the epicuticle in insects contain free lipids consisting mainly of aliphatic polar and nonpolar compounds. Chemically, epicuticular lipids consist of wax esters (esters of long-chain alcohols and long-chain acids), and also esters, ketones, aldehydes, oxoaldehydes, alcohols, free fatty acids, and acylglycerols. These layers are responsible for the water balance in insects but can also affect conidia germination of entomopathogenic fungi. Many different methods of lipid analysis have been established and among these methods liquid chromatography and GC coupled with mass-spectrometric techniques have the most significant potential.

In this work, the strategy for the treatment in the lipid composition analysis with the application of chromatographic techniques was reviewed. For qualitative and quantitative analysis of surface lipid insect composition it is necessary to carry out several stages as follows: extraction of analytes from biological material, lipid class separation by TLC, column chromatography, HPLC-LLSD, derivatization, and final determination by GC and GC-MS. The possible role of cuticular fatty acids in preventing fungal infection was discussed.

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