Chemical Characterization of Cuticular Extracts of *Sitobion avenae* (Hemiptera: Aphididae)

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ABSTRACT Data on the chemical composition of the aphid epicuticle are scarce and often incongruent depending on the methodological parameters of the extraction and chemical analysis. This study aims to provide a chemical characterization of the epicuticular lipids of the English grain aphid, *Sitobion avenae* (F.) (Hemiptera: Aphididae). First, our results showed that the adsorption chromatography on a silica gel column is suitable to separate the hydrocarbons from more polar constituents found in the extract (e.g., wax esters, alcohols, and aldehydes). Then, we showed that other compounds not detected in previous analysis are likely to be constituents of the extract. Through a transesterification reaction, we demonstrated that the extracts produced numerous fatty acid methyl esters that can come either from long chain wax esters or fatty acid parts of cuticular acylglycerols. The possible semiochemical value of the wax esters and other long chain compounds is discussed. This study opens new insight on the identification of host recognition chemical cues by the parasitoids.

KEY WORDS aphid epicuticule, cuticular hydrocarbons, wax esters, *Sitobion avenae*, exuviae

The main function of cuticular lipids is to minimize transpiration and to protect terrestrial insects from desiccation, but because the lipids cover the body surface, some of the components also are involved in chemical communication between species and between individuals of the same species (Lockey 1988). Epicuticular lipids of insects vary in composition and may include the following chemical classes: fatty acids, alcohols, esters, glycerides, sterols, aldehydes, ketones, and hydrocarbons (Blomquist and Dillwith 1985). Knowledge on the chemical composition of the epicuticle of insects is useful for the design of insecticide molecule as well as for the understanding of communication between individuals.

Papers dealing with chemical composition of the aphid cuticle are not strictly comparable, mostly because of the variety of biological material and extraction and chemical analysis methods used. Despite methodological heterogeneity, numerous studies have yielded critical insight into the characterization of the cuticular lipids of the aphid cuticle (Stransky et al. 1973, Bergman et al. 1990, Hebanowska et al. 1989, Szafranek et al. 2001, Borth and Deslippe 2004, Raboudi et al. 2005). Cuticular lipids of most aphids include normal and branched alkanes. However, the *n*-alkanes in aphid species are consistently the predominant component of the cuticular hydrocarbons (Lockey 1988). Cuticular polar lipids also have been found on the cuticle of pea aphid, *Acyrthosiphon pisum* (Harris), such as 1) wax esters (WE) consisting of C_{16}, C_{18}, and C_{20} saturated fatty acids esterified to C_{26}, C_{28}, and C_{30} primary alcohols; 2) long-chain aldehydes; 3) alcohols; 4) triacylglycerols; and 5) free fatty acid (Stransky et al. 1973). Beside lipids, compounds of other nature such as amino acids (Brey et al. 1985) have been identified from methanolic cuticular extracts of *A. pisum*, but these compounds have rarely been reported as chemical cues for recognition in insects.

The English grain aphid, *Sitobion avenae* (F.) (Hemiptera: Aphididae), is a major pest of cereal field in northern Europe. Colonies of this aphid species are found on winter wheat, *Triticum aestivum* L.; barley, *Hordeum vulgare* L.; oats, *Avena sativa* L.; and rye, *Secale cereale* L.

Among aphid natural enemies, parasitoids are promising biological control agents. Recently, we showed that *Aphidius rhopalosiphi* De Stefani Perez reacts positively to methanol:*n*-hexane extracts of *S. avenae* exuviae cuticles (Muratori et al. 2006), demonstrating for the first time that Aphidiine wasps were stimulated by chemical cuticular cues. The use of exuviae reduces the risks of contamination of the extract by noncuticular compounds (Gilby 1980). Gas chromatography-mass spectrometry (GC-MS) analyses of crude extracts revealed the occurrence of *n*- and methyl-branched alkanes but also aldehydes, alcohols, and wax esters. These latter compounds have been

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reported only once in a study dealing with the aphid cuticle (Stransky et al. 1973).

The aims of this study were 1) to characterize the chemical composition of *S. avenae* cuticular extracts and 2) to produce an easy and valuable method to fractionate the extract for the identification of minor constituents.

Materials and Methods

Biological Material and Extraction. Colonies of *S. avenae* were maintained on winter wheat 'Windsor' under the following conditions: 19.5 ± 0.6°C, 40–50% RH, and a photoperiod of 16:8 (L:D) h. Sheets of paper were placed for 1–3 d under aphid colonies. Exuviae falling from the colony were collected and carefully separated from dead aphids and waste material. They were weighed with a 0.1-mg precision balance (Ohaus explorer, Pine Brook, NJ). Exuviae cuticular constituents were extracted in glass flasks by shaking (100 rpm) with a mixture of *n*-hexane:methanol (1:2, vol:vol) for 15 min. The exuviae per solvent ratio was of 1 mg of exuviae to 10 ml of solvent. The extracts have been filtered by passing through a Pasteur pipette on a plug of silanized glass wool. The extracts were than concentrated to dryness at 35°C under reduced pressure (R110, Büchi rotavapor, BÜCHI Labortechnik AG, Zurich, Switzerland) in preweighted vessels.

Fractionating of Total Extract. Twenty milligrams of exuviae was extracted as described above to obtain a concentrated extract in ≈2 ml. This latter extract was deposited on 200 mg of silica gel (G60, 70–230 mesh, VWR, from Merck, Darmstadt, Germany) in a glass petri dish (the silica gel was first rinsed with *n*-hexane:methanol to avoid contamination), and it was allowed to dry for 20 min at room temperature. The silica gel containing the extract was placed at the top of a glass micro-column made of a Pasteur pipette filled with a 4-cm height of silica gel layer. The following solvent series was used to elute the different fractions of interest: *n*-hexane (V_e = 3 ml), *n*-hexane:diethyl ether 1:1, vol:vol (V_e = 3 ml), and diethyl ether (V_e = 3 ml). The three fractions were concentrated to dryness under a gentle stream of nitrogen and finally diluted in 200 μl of *n*-hexane:methanol (1:2, vol:vol) for further analysis.

Transesterification of Cuticular Extract. The *n*-hexane:methanol extract of 20 mg of exuviae was obtained as described above. The lipid residue was solubilized into 0.2 ml of *n*-hexane before addition of the transesterification reagent (0.5 ml of methanol BF_3:methanol:*n*-hexane) (Christie 1993). The solution was heated in sealed duravit tubes at 70°C for 1.5 h. When solution cooled down, 0.2 ml H_2SO_4 (10%) and 0.5 ml of saturated NaCl solution were added, allowing the recovery of the upper hexane phase, which was injected into the GC-MS (1 μl).

Chemical Analysis. GC-MS investigations were performed on a Hewlett–Packard HP 5973 mass spectrometer coupled with an HP 6890 gas chromatograph equipped with an HP-5 (crosslinked 5% phenyl-methylpolysiloxane) column (30 m × 0.25 mm i.d.; film thickness, 0.25 μm). The operating conditions were fixed as follows: split-splitless injector (splitless mode) at 280°C; carrier gas, helium at 1 ml min^{-1}; temperature program, from 50 to 300°C at 15°C min^{-1}, and finally hold at 300°C for 25 min. The mass spectra were recorded in the electron impact mode at 70 eV (source temperature, 230°C; scanned mass range, 35–700 amu). The detected peaks were identified on the basis of their retention time (RT), whenever possible compared with RT of pure *n*-alkanes taken as references, and finally by interpretation of their characteristic fragmentation patterns. The mass spectra of the compounds also were compared with those of the NBS75K.L and Wiley275K.L computer databases.

Results

Exuviae Total Lipid Content. Total cuticular extract counted for up to 39.0 ± 6.1% (mean ± SE; n = 4) of the initial exuviae weight. Nevertheless, after complete evaporation of the solvent, the solid extract cannot be easily redissolved in small volumes of *n*-hexane:methanol.

Fractionating of Exuviae Extract. Classes of compounds can be efficiently separated by adsorption chromatography on the silica column (Table 1). Indeed, concentrated mixtures of *n*-alkanes and branched alkanes were isolated in the first fraction, whereas alcohols, aldehydes, and wax esters were found exclusively in the *n*-hexane:diethyl ether, a more polar eluent. The GC-MS analysis of the third fraction did not reveal any constituent. This indicates that all exuviae products that could be chromatographed have been totally eluted. Indeed, compared with the whole extract, the chromatographic profiles from the two fractions revealed no discrepancies. The process afforded concentrated fractions and, therefore, allowed their complete characterization (higher signal-to-noise ratio). Abundance of the predominant compound (n-C_{27}) was ≈3 times higher in fraction 1 than in the whole extract. Twelve additional hydrocarbons were identified from this analysis (Table 1). Finally, two compounds detected at RT = 13.43 min and RT = 15.58 min, respectively, were found in all of the three fractions, and they were, therefore, not assigned to the cuticular extract (probably contaminants form the final solvent).

In fraction 1, *n*-alkanes from C_{23} to C_{33}, with a predominance of four homologous compounds (n-C_{25} [8.95%], n-C_{27}[29.16%], n-C_{29}[27.26%], and n-C_{31} [9.50%]), were the major constituents, whereas the second class of apolar compounds (11.46%) correspond to monomethyl-branched saturated hydrocarbons. Dimethyl-alkanes represented only 1.56% of the total. The analysis of fraction 2 revealed the occurrence of long-chain aldehydes that were unambiguously identified as *n*-octacosanal (RT = 18.89 min; 6.74% of fraction 2) and *n*-triacontanal (RT = 20.61 min; 10.73% of fraction 2). The occurrence of *n*-octacosanal was corroborated by coinjection of a pure reference. Moreover, two alcohols have been identified as 1-eicosanol (RT = 19.37 min) and 1-docosanol

Table 1. Compounds identified in fractions of the cuticular extract

RT (min)	Compound name	Abbreviation	% area			
			Fraction 1	Fraction 2	Fraction 3	Whole extract[a]
14.80	*n*-Tricosane	*n*-C_{23}	0.14	-	-	-
15.35	x-Methyltetracosane	x-MeC_{24}	0.08	-	-	-
15.90	*n*-Pentacosane	*n*-C_{25}	8.95	0.12	-	3.08
16.07	11-Methylpentacosane	11-MeC_{25}	0.09	-	-	-
16.19	4-Methylpentacosane	4-MeC_{25}	0.29	-	-	0.55
16.27	3-Methylpentacosane	3-MeC_{25}	0.48	-	-	0.31
16.40	*n*-Hexacosane	*n*-C_{26}	1.65	0.07	-	0.70
16.71	4-Methylhexacosane	4-MeC_{26}	0.36	-	-	0.17
16.92	*n*-Heptacosane	*n*-C_{27}	29.16	-	-	11.22
17.06	11-Methylheptacosane	11-MeC_{27}	0.36	-	-	0.33
17.10	7-Methylheptacosane	7-MeC_{27}	0.16	-	-	-
17.14	5-Methylheptacosane	5-MeC_{27}	0.30	-	-	0.25
17.27	3-Methylheptacosane	3-MeC_{27}	0.25	-	-	0.21
17.40	*n*-Octacosane	*n*-C_{28}	1.31	-	-	0.61
17.58	10-Methyloctacosane	10-MeC_{28}	0.39	-	-	-
17.74	4-Methyloctacosane	4-MeC_{28}	0.25	-	-	-
17.97	*n*-Nonacosane	*n*-C_{29}	27.26	-	-	10.99
18.13	11-Methylnonacosane	11-MeC_{29}	1.54	-	-	1.06
18.23	5-Methylnonacosane	5-MeC_{29}	0.13	0.19	-	0.12
18.32	11,13-Dimethylnonacosane	11,13-diMeC_{29}	0.34	-	-	0.23
18.57	*n*-Triacontane	*n*-C_{30}	0.97	0.15	-	0.67
18.78	x-Dimethylnonacosane	x-diMeC_{29}	0.56	-	-	0.33
18.89	Octacosanal	C_{28}-al	-	6.74	-	0.92
19.30	*n*-Hentriacontane	*n*-C_{31}	9.50	-	-	4.57
19.37	1-Eicosanol	C_{20}-1-ol	-	17.22	Trace	14.61
19.53	11-Methylhentriacontane	11-MeC_{31}	0.94	-	-	1.02
19.75	11,15-Dimethylhentriacontane	11,15-diMeC_{31}	0.66	-	-	-
20.14	HC n.i.[b]		0.32	-	-	-
20.41	*n*-Dotriacontane	*n*-C_{32}	0.39	-	-	-
20.61	Triacontanal	C_{30}-al	-	10.73	-	1.27
21.11	*n*-Tritriacontane	*n*-C_{33}	1.57	-	-	0.92
21.27	1-Docosanol	C_{22}-1-ol	-	16.17	-	13.57
21.46	11-Methyltritriacontane	11-MeC_{33}	5.84	-	-	-
22.26	HC n.i.		3.42	0.63	-	-
22.69	HC n.i.		2.37	-	-	-
24.88	Hexadecanoic acid octadecyl ester	$C_{15}CO_2C_{18}$	-	4.12	-	2.50
29.42	Hexadecanoic acid eicosyl ester	$C_{15}CO_2C_{20}$	-	34.47	-	20.97
34.03	Trihexadecanoin[c]		-	4.67	-	5.32
35.66	Hexadecanoic acid docosyl ester	$C_{15}CO_2C_{22}$	-	4.71	-	3.51

-, corresponding compound was not detected.
[a] From Muratori et al. (2006).
[b] Nonidentified hydrocarbon.
[c] Tentative interpretation.

(RT = 21.27 min). By applying an adapted temperature program, high-molecular-weight waxy compounds have been detected and identified as hexadecanoic acid octadecyl ester (RT = 24.88 min; 4.12%), hexadecanoic acid eicosyl ester (RT = 29.42 min; 34.47%), and hexadecanoic acid docosyl ester (RT = 35.66 min; 4.71%). To our knowledge, these molecules are reported for the first time in *S. avenae* extracts. Trihexadecanoin also was tentatively identified (RT = 34.03 min).

Transesterification of Cuticular Extract. The transesterification reaction allowed us to corroborate the occurrence of long chain wax esters in *S. avenae* exuviae: the major detected fatty acid methyl ester (methyl-hexadecanoate, RT = 12.55, %peak area = 26.89) being the acyl moiety of the high-molecular-weight compounds eluted at 24.88, 29.42, and 35.66 min. We identified up to 14 different fatty acid methyl esters ranging from C_{10} to C_{30} of which the predominant compounds were the hexadecanoic acid methyl ester 9-octadecenoic acid methyl ester (RT = 13.70 min, %peak area = 19.58) and triacontanoic acid methyl ester (RT = 21.64 min, %area peak = 3.39). Along with the transesterification products, GC-MS analyses revealed the presence of the major compounds of the typical hydrocarbons series of the whole extract, *n*-C_{25} (RT = 15.94 min), *n*-C_{27} (RT = 16.96 min), *n*-C_{29} (RT = 18.01 min), and *n*-C_{31} (RT = 19.36 min), which accounted for 3.37, 6.45, 6.34, and 2.71% of the total detected peaks area, respectively. None of the wax esters present in the whole extract was found in the reaction products.

Discussion

This study investigated the cuticular composition of *S. avenae* by using GC-MS analyses. First, we showed that the *n*-hexane:methanol cuticular extracts contain different lipid classes (e.g., wax esters, long-chain aldehydes, and alcohols) that can be isolated from the cuticular hydrocarbons fraction. WE occurrence in the cuticular lipids of an aphid species was only re-

ported once (Stransky et al. 1973). Stransky et al. (1973) identified 12 different wax esters (from C_{32} to C_{54}) from cuticles of *A. pisum* and showed that the wax esters mass count for 2.6% of the total lipid mass. In *Sitobion avenae*, we found that WE represented the third class of compounds and counted for 27% of the total lipid mass. Besides the species factor, the great difference in the gravimetric results comes from the difference in extraction methods between studies. Stransky et al. (1973) treated fresh whole aphids for >8 h in seven successive extractions with *n*-pentane (250 ml); therefore, they were likely to extract inner lipids, in particular, glycerides (27.1% of the total lipid mass). This would reduce the relative importance of the WE in the total lipids but more generally would make noncuticular lipids occur in the analysis. Moreover, this could lead to report as "cuticular", compounds that are not really exposed on the surface of the cuticle. Brey et al. (1985) showed the occurrence of several alcohols (from C_8 to C_{22}) in methanol: chloroform cuticular extract of *A. pisum*. Nevertheless, they stated that contamination from the aphid inner body cannot be excluded. In the current study, the original use of exuviae reduces the risk of contamination (Gilby 1980) and led us to confirm the presence of alcohols and aldehydes on the cuticle of *S. avenae*. The same C_{28} and C_{30} aldehydes have already been reported in *A. pisum* cuticular lipids (Stransky et al. 1973).

The transesterification reaction products were more numerous than if they were produced from the three wax esters identified in whole extract analysis (Table 1). For example, the 9-octadecenoic acid methyl ester revealed the occurrence of unsaturated fatty acid ester and the triacontanoic acid methyl ester revealed long-chain fatty acid ester. Hypothetical compound origins that can be put forward to explain these results are wax esters (including unsaturated compounds) of higher molecular masses not detected in the analytical conditions described herein, or triacylglycerols on the aphid cuticle. All of these compounds are susceptible to undergo a transesterification to produce fatty acid methyl esters, but they could have been undetected through GC-MS conditions used to investigate the whole extract. This suggests that the polarity properties of the mixture of *n*-hexane and methanol gave rise to the extraction of other lipid compounds.

Typical branched and *n*-alkanes were found in the *S. avenae* cuticular extract. Table 2 compares the hydrocarbons identified in the *n*-hexane:methanol cuticular extracts in the current study with other aphid cuticle studies. It highlights the heterogeneity of the profiles, but one cannot conclude that these differences are linked to the extraction methods or from species characteristics. For example, Borth and Deslippe (2004) found 13 *n*-alkanes only in cuticular extracts of *Aphis gossypii* Glover, whereas Hebanowska et al. (1989) identified up to 50 different saturated hydrocarbons (ranging from *n*-C_{23} to *n*-C_{35}) in *A. pisum* cuticular extracts. Hebanowska et al. (1989) used extreme extraction treatments (maceration of the insects during two weeks in methylene chloride) that allowed extraction of a detectable amount of numerous compounds. It is thus likely that these extra compounds are present in other aphid species (including *S. avenae*) but not extracted in detectable quantities.

Finally, we showed that cuticular compounds can be separated according to the class of chemicals they belong. The first elution with *n*-hexane eluted the hydrocarbons, whereas the hexane:diethylether mixture eluted polar lipids (WE, alcohols, and aldehydes). This method allowed us to detect and identified several hydrocarbons not reported in previous studies (Muratori et al. 2006).

Cuticular lipids profile is an indicator of insect species (Lockey 1988, Raboudi et al. 2005), biotype (Dillwith et al. 1993), or nutritional state (Howard and Baker 2003). Thus, the ability to recognize these precise profiles on its host should bring a great competitive advantage for the parasitoid. It has been shown that exuviae cuticular extracts of *S. avenae* trigger recognition and oviposition attempts by the parasitoid *A. rhopalosiphi* (Muratori et al. 2006). Development of alternative and cost-effective methods for mass rearing of biological control agents, by using artificial diets, is a promising way to extend the use of biological control to open fields. Manipulation of the female behavior is a key step in the development of the oviposition substrate. In this frame, chemical characterization of semiochemicals can help us developing such systems.

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