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Source: Journal of Insect Science, 1(4) : 1-7

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.001.0401

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Patel S., Nelson D.R. and Gibbs A.G. 2001. Chemical and Physical Analyses of Wax Ester Properties. 7 pp. *Journal of Insect Science* , 1.4. Available online: insectscience.org

Journal of Insect Science

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Chemical and physical analyses of wax ester properties

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Received: 23 March 2001, Accepted 4 April 2001, Published April 24, 2001

Abstract

Wax esters are major constituents of the surface lipids in many terrestrial arthropods, but their study is complicated by their diversity. We developed a procedure for quantifying isomers in mixtures of straight-chain saturated and unsaturated wax esters having the same molecular weights, using single-ion monitoring of the total ion current data from gas chromatography-mass spectrometry. We examined the biological consequences of structural differences by measuring the melting temperatures, T_m, of >60 synthetic wax esters, containing 26-48 carbon atoms. Compounds containing saturated alcohol and acid moieties melted at 38-73°C. The main factor affecting T_m was the total chain length of the wax ester, but the placement of the ester bond also affected T_m. Insertion of a double bond into either the alcohol or acid moiety decreased T_m by ~30°C. Simple mixtures of wax esters with *n*-alkanes melted several °C lower than predicted from the melting points of the component lipids. Our results indicate that the wax esters of primary alcohols that are most typically found on the cuticle of terrestrial arthropods occur in a solid state under physiological conditions, thereby conferring greater waterproofing. Wax esters of secondary alcohols, which occur on melanopline grasshoppers, melted >60°C below primary esters of the same molecular weight and reduced T_m of the total surface lipids to environmental values.

Keywords: cuticular lipid, melting temperature, single-ion monitoring, wax ester

Abbreviation:

FTIR	Fourier-transform infrared spectroscopy
SIM	Single ion monitoring
TIC	Total ion current

Introduction

Wax esters, consisting of a fatty acid esterified to a fatty alcohol, serve a variety of biological functions. Many terrestrial arthropods deposit large quantities of wax esters (and other lipids) on the surface of their cuticle to reduce evaporative water loss (de Renobales *et al*., 1991; Buckner, 1993; Gibbs, 1998).

Whiteflies cover their cuticles with long-chain wax esters and dust their bodies and surroundings with waxy particles composed of long-chain aldehydes and alcohols (Nelson *et al*., 2000 and references cited therein). Wax esters are major constituents of beeswax (Blomquist *et al*., 1980), and plants such as jojoba store large quantities (Busson-Breysse *et al*., 1994). In the marine realm, dinoflagellates, pelagic invertebrates (especially krill and other crustaceans) and fishes store low-density wax esters in their swimbladders or other tissues to provide buoyancy (Phleger, 1998). Moreover, wax esters may be important dietary components for marine birds that consume the zooplankton (Roby *et al*., 1986).

Naturally occurring wax esters are chemically diverse. Most are esters of primary alcohols, although esters of secondary alcohols can be major components of the cuticular lipids of melanopline grasshoppers (Blomquist *et al*., 1972). Surface waxes of insects typically contain saturated fatty alcohol and fatty acid chains having 12 to >20 carbon atoms each (Buckner, 1993), although the giant whitefly, *Aleurodicus dugesii*, has wax esters in which the chain lengths of both moieties are up to 30 carbons in length (Nelson *et al*., 1999, 2000). A few taxa (e.g. honeybees, dragonflies, whiteflies) contain wax esters with monounsaturated fatty acid moieties, which are also major components of beeswax (Buckner, 1993; Nelson *et al*., 2000). Wax esters from marine animals usually contain high

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levels of unsaturated fatty acids and alcohols (Kattner *et al*., 1996; Phleger, 1998; Saito and Murata, 1998).

A major technical problem in the analysis of wax esters is that isomers having the same molecular weight (and retention times on gas chromatograms) are often present. Researchers typically digest the waxes and analyze the resulting fatty acids, thereby losing information about the specific molecular species of wax esters present. Despite these problems, the chemical composition of wax esters has been described in numerous species, but the biological significance of wax ester differences is unclear. In both terrestrial and marine organisms, the physical properties of wax esters may be functionally important. For example, surface lipids of insects provide a better waterproofing barrier when they are in a solid rather than fluid state (Gibbs, 1998; Rourke and Gibbs, 1999; Rourke, 2000), so one would expect insects to synthesize waxes with high melting temperatures. On the other hand, melted lipids are less dense than solid lipids, so marine organisms that use wax esters for buoyancy would gain greater lift if their waxes were in a fluid state.

Most biophysical analyses of wax esters have used complex mixtures containing waxes and other lipid components (e.g. Basson and Reynhardt, 1988a,b; Sessa *et al*., 1996). In this paper, we report a method for determining the relative amounts of isomers of straight-chain saturated wax esters, based on single-ion monitoring values from gas chromatography-mass spectrometry (GC-MS) analyses. We also describe the physical properties (melting temperatures, T_m) of over 60 pure synthetic wax esters, most of which occur naturally. We examine how structural variations (acid and alcohol chain length, ester position, unsaturation) affect T_m values. If waxes melt at ecologically relevant temperatures, then lipid phase transitions may have significant effects on insect water balance.

Methods and Materials

Wax Esters

Synthetic wax esters of primary alcohols were purchased from Nu-Check Prep or Sigma Chemical Co., or were synthesized by modification of the method previously described (Nelson *et al*., 1990), based on that of Iyengar and Schlenk (1969). Equal molar amounts (about 0.1 mmole) of the alcohol and the acid chloride were separately dissolved in 4 ml of dry diethyl ether or tetrahydrofuran. The solutions were slowly mixed together in a flask of a reflux apparatus and refluxed for 1 hr. Five 100 µl portions of dry pyridine (distilled and stored over a molecular sieve) were then added at 10 min intervals. Sufficient solvent was then added to allow refluxing overnight. The reaction mixture was transferred to a separatory funnel and washed sequentially with 6N sulfuric acid, 6N sodium hydroxide, and water. The organic phase was dried over magnesium sulfate, filtered through glass wool, the solvent removed and the wax ester weighed. The wax was dissolved in a minimum volume of chloroform and spotted on a 20x20 cm plate of 250 µm thick silica gel G. The plate was developed in hexane:diethyl ether:formic acid (80:20:1; *v/v/v*). The area containing the wax ester was removed and the esters eluted with 4 bed volumes of chloroform. The eluate was dried and the ester redissolved in 95% ethanol with heating. The solution was then cooled, and the wax ester crystals collected.

Wax esters of secondary alcohols were isolated from the grasshopper, *Melanoplus sanguinipes*, as follows. Cuticular lipids were extracted from frozen grasshoppers using a 10-minute hexane wash, followed by a 1-minute wash. Waxes were separated from other lipid constituents using silica gel chromatography. Total surface lipids from individual grasshoppers were applied to a column in a pasteur pipet. Alkanes were eluted with hexane, and wax esters were eluted with a hexane:chloroform (98:2 *v/v*) mixture. The purity was checked by thin-layer chromatography and GC, and was >98% wax ester. Base-catalyzed hydrolysis of the wax esters, followed by GC analysis of the products (Nevenzel *et al*., 1985), indicated that the major alcohol and acid components contained 16-23 carbons, as reported previously (Blomquist *et al*. 1972).

Gas Chromatography-Mass spectrometry

Gas chromatographymass spectrometry (GCMS) was performed on a Hewlett-Packard HP 5890A gas chromatograph equipped with a pressure programmable cool oncolumn injection port. The column consisted of a 1 m retention gap connected to a 12.5 m X 0.2 mm capillary column of crosslinked dimethyl silicone Ultra 1 (Hewlett-Packard) and was coupled to a HP 5970B quadrupole mass selective detector. The carrier gas was helium, and the initial column temperature was between 150 and 200 ^oC and was programmed to increase to 320^oC at 3 or 4°/min and held at 320^oC. The system was operated and data collected with a Hewlett-Packard 5970C computer. The system was calibrated, and the quality of the chromatogram monitored, with a standard mixture consisting of: methyl heptadecanoate, methyl icosanoate, 3-methyltricosane, tricosanyl acetate, octacosane, tetracontane, and tricosanyl heptadecanoate, run at six concentrations. The mass spectral data for the wax esters were analyzed by single-ion extraction of the data (Nelson *et al*., 2000). The integrated areas for the total ion current (TIC) and the single ions obtained by single-ion monitoring (SIM) of the data were then compared to obtain the factor needed to convert SIM area values to TIC areas, so that wax esters could be quantified based solely on their SIM values if present in a mixture.

Lipid Physical Properties

Melting temperatures (T_m) of lipid samples were determined using Fourier-transform infrared (FTIR) spectroscopy (Gibbs and Crowe, 1991). Approximately 50 µg was dissolved in hexane and deposited as a thin film on a CaF_2 window, which was then placed in a temperature-controlled cell holder in a Perkin-Elmer Systems 2000 FTIR spectrometer. The sample temperature was increased in increments of ~1^oC, with an infrared spectrum being collected at each temperature. As alkyl chains melted, the frequency of -CH_2-symmetric stretching vibrations increased from ~2849 cm^{-1} to ~2854 cm^{-1}, and was used as an index of lipid melting. The midpoint of the phase transition (T_m) was calculated by fitting plots of frequency *vs.* temperature to a logistic equation.

Results

Gas chromatography-mass spectrometry

We used GC-MS to confirm that all wax esters studied were greater than 98% pure and were of the predicted structures. Wax esters can be identified by the chain lengths and unsaturation of their alcohol and acid chains, respectively. For example, arachidyl

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palmitoleate, a 36-carbon wax ester having a 20-carbon, saturated fatty alcohol esterified to a 16-carbon, monounsaturated acid would be designated as C20:0-16:1. Single-ion monitoring is an effective means of analyzing mixtures because the saturated wax esters are characterized by a major diagnostic ion corresponding to the protonated acid moiety of the ester, e.g., at *m/z* 285 for stearic acid (C18:0). When the acid moiety was unsaturated, the major diagnostic ion is that of the acylium ion minus a hydrogen, e.g., at *m/z* 264 for oleic acid (C18:1) and *m/z* 262 for linoleic acid (C18:2). Also, a molecular ion is usually visible in the mass spectrum of the wax ester.

The factors for converting SIM areas to TIC areas for several wax esters are listed in Table 1. Within the range of wax quantities injected, these factors were highly repeatable for a given compound. However, despite the fact that the same diagnostic ions were used for different compounds (e.g. C14:0-18:0, C16:0-18:0 and C18:0-18:0), the factors decreased with the overall mass of the wax esters. For the same chain length, factors for unsaturated acid moieties were approximately twice as high as those of saturated fatty acids.

A closer analysis of molecular features affecting the intensity of the protonated acid fragment showed that in addition to the chain length of the wax ester, the ratio of the chain lengths of the acid and alcohol moieties also affected the intensity (Figure 1). Not only did the intensity of the protonated acid fragment decrease with increasing chain length of the wax ester, the intensity also decreased as the length of the acid moiety decreased (or as the length of the alcohol moiety increased).

Table 1. Factors to convert SIM values to TIC values for selected wax esters arranged by increasing carbon number of the alcohol moiety[1].

Wax Ester Carbon No.	Alcohol-Acid Carbon No.	Protonated Acid Ion	Factor	Standard Deviation
30	14:0-16:0	257	5.47	0.14
32	14:0-18:0	285	4.70	0.12
30	16:0-14:0	229	6.57	0.21
32	16:0-16:0	257	5.25	0.15
34	16:0-18:0	285	4.41	0.07
34	18:0-16:0	257	5.05	0.11
36	18:0-18:0	285	4.34	0.10
36	18:0-18:1	264	11.73	0.17
38	20:0-18:1	264	11.59	0.25
40	20:0-20:0	313	4.40	0.08
42	20:0-22:0	341	5.44	0.11
44	20:0-24:0	369	5.24	0.06
40	22:0-18:0	285	4.02	0.08
42	22:0-20:0	313	4.29	0.10
44	22:0-22:0	341	5.34	0.06
42	24:0-18:0	285	3.87	0.05
44	24:0-20:0	313	4.13	0.10
48	24:0-24:0	369	5.02	0.15
44	26:0-18:0	285	3.81	0.07
46	28:0-18:0	285	3.71	0.08
54	30:0-24:0	369	4.74	0.09

[1] SIM = single ion monitor values for the protonated acid fragment of the saturated fatty acid moiety or of the ion due to the acyl moiety minus a hydrogen for the unsaturated fatty acid moiety. TIC = total ion current of the wax ester peak. The factor was calculated by dividing the TIC peak area by the SIM peak area. Values were the averages of GC-MS analyses at injected amounts of 25, 50, 75 and 100 nanograms.

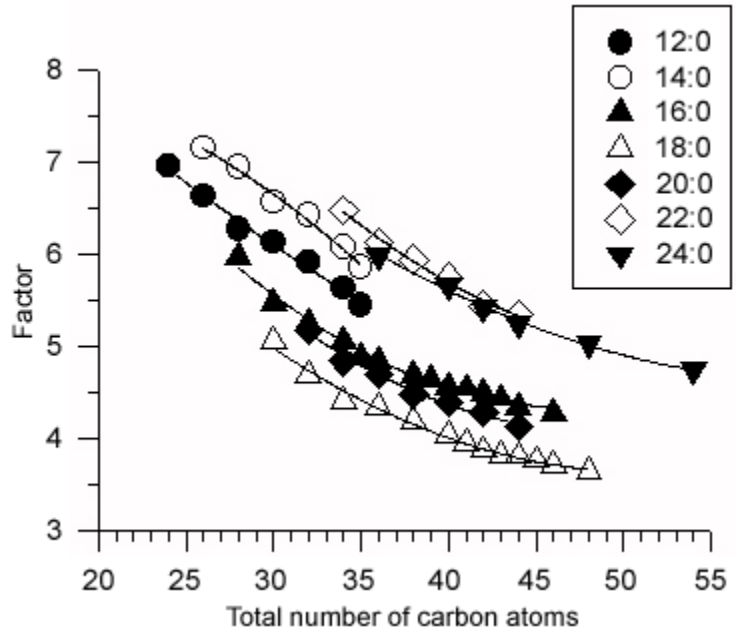

Figure 1. The effects of wax ester chain length and size of the acid and alcohol moieties on the factor needed to convert SIM values for the protonated acid fragment to TIC values for the wax ester.

Melting temperatures of synthetic wax esters

Synthetic wax esters exhibited sharp phase transitions (Figure 2). Melting temperatures of saturated waxes ranged from ~38°C for dodecyl myristate (C12:0-14:0) to >75°C for tetracosanyl tetracosanate (C24:0-24:0). The T_m increased by 1-2°C with each single-carbon increase in the total number of carbon atoms in the molecule (Figure 3).

For a given number of carbon atoms, multiple isomers containing the ester linkage in different positions were available. Saturated wax esters containing the same number of carbon atoms melted at similar temperatures, although consistent differences

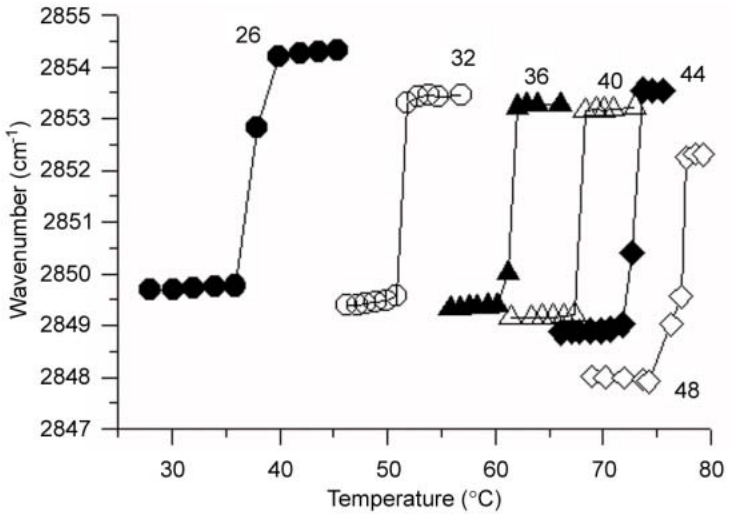

Figure 2. Melting curves for saturated wax esters. At the T_m, the symmetric stretching frequency of -CH_2- groups of acid chains increases from ~2850 cm^{-1} to ~2854 cm^{-1}. Numbers indicate the total number of carbon atoms in each molecule. Except for the shortest wax (C12:0-14:0), each compound had an equal number of carbons in the alcohol and fatty acid moieties.

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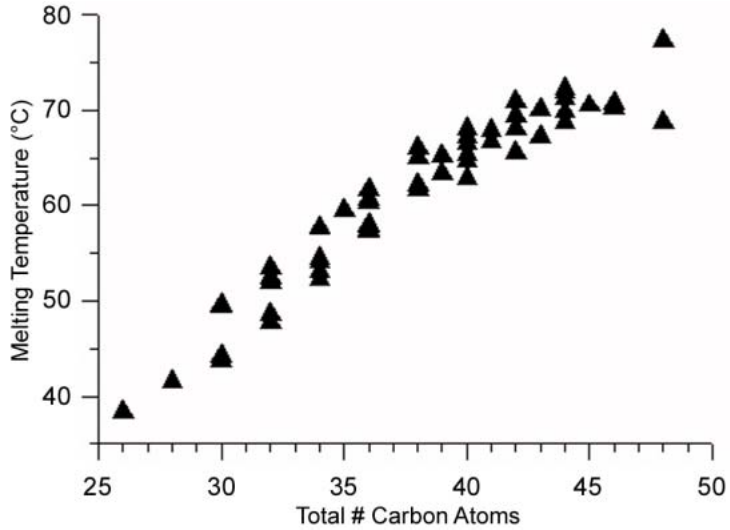

Figure 3. Effects of carbon number on melting temperatures of saturated wax esters of primary alcohols. For a given number of carbon atoms, each point represents an isomer whose ester bond is in a different position.

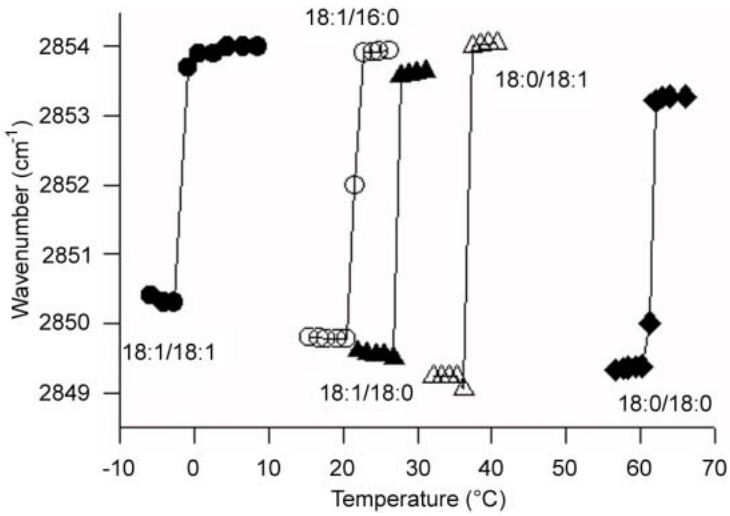

Figure 5. Effects of unsaturation on melting temperatures of wax esters.

related to the position of the ester linkage were observed. Lipid T_m values were higher for compounds having alcohol and acid chains of the same length on either side of the ester moiety. When the ester link was moved to less central locations along the molecule, the T_m decreased by 1-5°C (Figure 4).

Unsaturation significantly affected the properties of wax esters. For waxes containing 18 carbon atoms in both alcohol and acid moieties, insertion of a single *cis*-9 double bond reduced the T_m from 61°C to 27 or 37°C, depending on whether the double bond occurred in the alcohol or acid chain, respectively (Figure 5). Oleyl oleic ester, which is unsaturated in the 9-position on both chains, melted slightly below 0°C.

Wax esters of secondary alcohols

The lesser migratory grasshopper, *M. sanguinipes*, contains ~30% wax esters of secondary alcohols (Blomquist *et al*., 1972). In these, the fatty acid moiety, containing 14-22 carbons, forms an ester bond with 11-tricosanol or other secondary alcohols. These wax esters therefore have a fundamentally different geometry from the synthetic wax esters studied above, being T-shaped rather than linear. In spite of the fact that wax esters from *M. sanguinipes* are saturated and contain ~40 carbon atoms, they melted at 5-10°C (Figure 6). We note that tricosanyl 1-stearate, an isomer of the major wax ester of *M. sanguinipes*, melts at ~68°C (Figure 3).

Mixtures of wax esters with alkanes

Although wax esters are abundant on many arthropod species, the major component of the surface lipids is usually long-chain hydrocarbons (de Renobales *et al*., 1991). Interactions between lipid classes can significantly affect bulk lipid properties (Gibbs, 1995). We used FTIR to examine the properties of simple mixtures between pairs of wax esters, containing 21-30 carbons, and *n*-

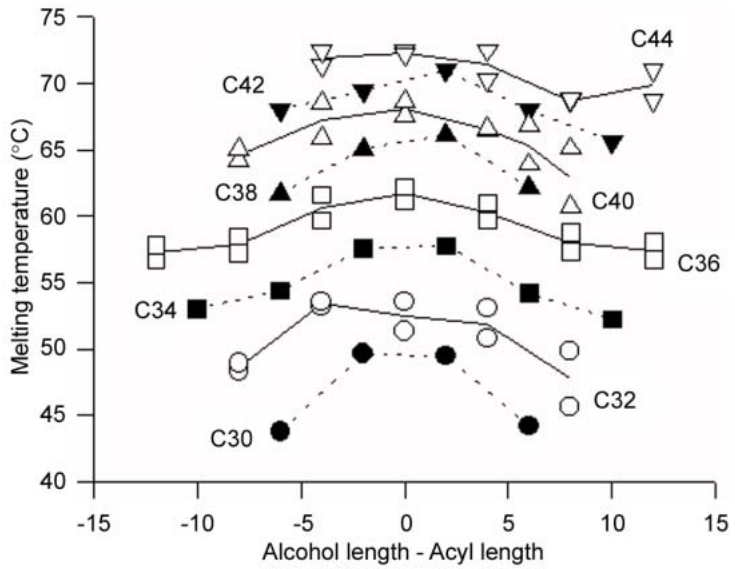

Figure 4. Effects of ester bond position on T_m. Numbers indicate the total number of carbon atoms for each series of points. Ordinal data are expressed as the difference in chain length between the alcohol and acid moieties. Each point indicates a separate determination of T_m.

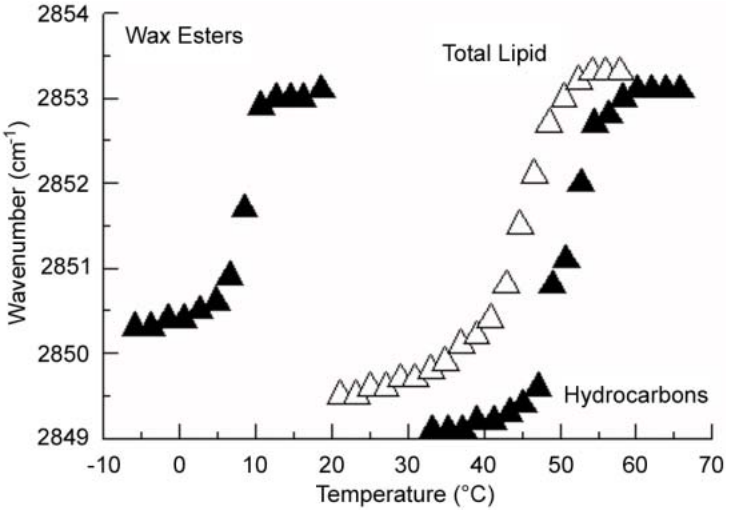

Figure 6. Melting curves for cuticular lipids isolated from *Melanoplus sanguinipes*. Wax ester and hydrocarbon fractions were separated using silica gel chromatography. These melting curves were obtained using lipids isolated from a single adult grasshopper.

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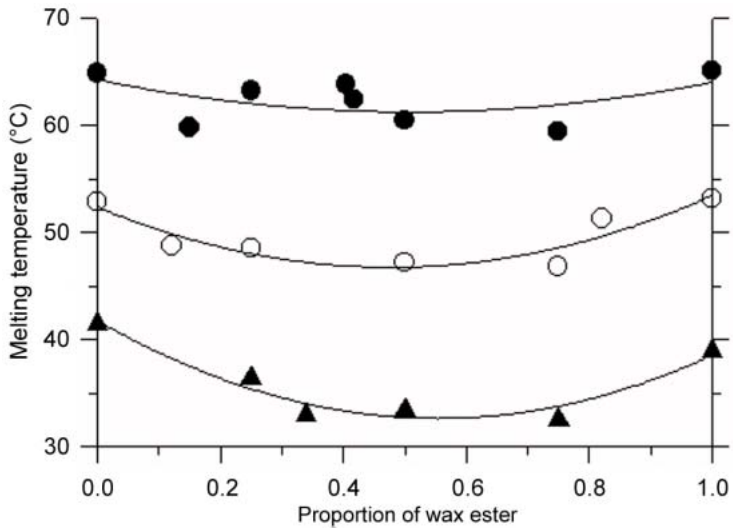

Figure 7. Melting temperatures of mixtures containing a wax ester and an *n*-alkane. Filled circles: stearyl arachidate (C18:0-20:0) and triacontane (C30); open circles: tetradecyl stearate (C14:0-18:0) and pentacosane (C25); filled triangles: dodecyl myristate (C12:0-14:0) and heneicosane (C21).

alkanes, containing 26-48 carbons. We chose pairs in which the wax ester and alkane had similar T_m values, so that the predicted T_m would be the same as that of the two components. However, these mixtures melted 3-5°C below the T_m of the component lipids (Figure 7).

Discussion

Wax esters are major lipid components in many terrestrial and marine organisms and play an important role in cuticular water-proofing, buoyancy, and energy metabolism. They may be present in very complex mixtures of isomers, e.g. in preen gland waxes (Dekker *et al*., 2000) and as minor components on the cuticle of ants (Nelson *et al*., 2001). Our objectives in this study were to develop a quantitative method for analyzing mixtures of wax esters and to assemble a systematic description of the effects of structural changes on wax ester properties. Although some data have been published (Iyengar and Schlenk, 1969; *CRC Handbook*, 1992), a more thorough account is essential in order to interpret the biological significance of variation in wax ester composition. Do wax esters always occur in either a solid or a liquid state, or do they undergo phase transitions under biological conditions? How do they interact with other surface lipid components? Once these answers are in hand, we can ask whether wax ester properties affect the function of biological systems. Our major interest here is insect water balance, but we note that lipid properties may affect other functions of wax esters.

In the analysis of cuticular lipids, the wax esters must be differentiated from hydrocarbons, aldehydes, acetate esters and laboratory contaminants. Our data indicate that single-ion monitoring appears to be a useful technique for determining the isomeric composition of wax ester mixtures, as well as for detecting and measuring them when other components interfere with TIC measurements. The factors required for converting SIM area values to wax quantities, however, differ among compounds and must be determined for each (Table 1; Figure 1). For a given compound, the conversion factors were constant over the range of wax quantities we assayed, but differed when the same diagnostic ion was used for different waxes. Some of this variation can be attributed to differences in the relative size of the acid and alcohol moieties and to differences in molecular weight. Also, differences in detector responses may cause these factors to differ from one GC-MS machine to another.

Effects of structural changes on melting points of wax esters

Our measurements of T_m values in wax esters were consistent with previous reports obtained using other techniques (Iyengar *et al*., 1969; *CRC Handbook*, 1992), and the structural effects were similar to those of other lipids. The increase in T_m of 1-2°C per additional carbon atom (Figure 3) is similar to that observed for hydrocarbons (Gibbs and Pomonis, 1995), phospholipids (Stubbs and Smith, 1984), and fatty acids and triacylglycerides (Small, 1986). The presence of an ester linkage decreased T_m by ~15°C, relative to hydrocarbons containing the same number of carbon atoms (Gibbs and Pomonis, 1995; this study). The ester linkage introduces a kink in the lipid chain and disrupts lipid packing, thus causing a decrease in T_m similar to that caused by methylbranching or unsaturation (Gibbs and Pomonis, 1995).

Our analyses of isomers containing the same number of carbon atoms, but a different position of the ester linkage, revealed that "symmetric" wax esters melted 1-5°C higher than compounds whose acid and alcohol moieties had different chain lengths (Figure 4). This is in contrast to the effects of unsaturation and methylbranching. More internally-located double bonds and methyl groups tend to decrease T_m more than the same structural changes near the ends of hydrocarbons (Gibbs and Pomonis, 1995) or phospholipids (Stubbs and Smith, 1984).

Melting temperatures of wax esters from *M. sanguinipes* were much lower than expected, based solely on the number of carbon atoms. These compounds are wax esters of secondary alcohols (Blomquist *et al*., 1972), rather than of primary alcohols as in the synthetic compounds. Figure 8 depicts a space-filling, energy-minimized model of the most abundant wax ester in *M. sanguinipes*. The reason for the low T_m values of grasshopper waxes is clear. These T-shaped molecules will not pack closely, and the reduced van der Waals forces will cause these compounds to melt at much lower temperatures than linear wax esters of the same molecular weight.

Introduction of a double bond decreased T_m by ~30°C (Figure 5), similar to results obtained for other types of lipids (Stubbs and Smith, 1984; Small, 1986; Gibbs and Pomonis, 1995). In other lipid classes, the placement of the double bond is critical in determining its effects on T_m; internal unsaturation reduces T_m more than insertion of a double bond near the end of the molecule. Addition of a second double bond lowers T_m further, but not by as much as the first one (Stubbs and Smith, 1984). Limited data (Figure 5) from this study suggest that the T_m-lowering effects of unsaturation are greater when a double bond is placed in the alcohol moiety (T_m decreased by ~35°C) than in the acid chain (T_m decreased by ~25°C). Insertion of two double bonds had an additive effect (T_m decreased by ~60°C). The overall decrease in T_m is consistent with that seen in comparisons of hydrogenated carnauba waxes with native wax esters, which are also diunsaturated (Sessa *et al*., 1996).

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Wax ester properties and insect water balance

Wax esters are important components of the surface lipids of many terrestrial arthropods (de Renobales *et al*., 1991; Buckner, 1993). Most cuticular wax esters are saturated and contain at least 30 carbon atoms, so they will melt above 50°C (Figure 3). This is above the range of body temperatures typically experienced in nature (Heinrich, 1993), so these compounds will tend to remain in a solid state under physiological conditions.

Dragonflies, honeybees, and a few other species contain significant quantities of monounsaturated cuticular wax esters (Jacob and Hanssen, 1979; Blomquist *et al*., 1980), having a total of 36-50 carbon atoms. Saturated wax esters of this size melt at 60-80°C (Figure 3), so we can calculate that the unsaturated waxes will melt above 30°C (Figure 5). We note, however, that most of the waxes on these species are saturated, so the overall T_m of the wax esters will probably be at least 45°C. Thus, deposition of wax esters on these insects will tend to raise T_m above environmental temperatures and maintain the surface lipids in a solid, impermeable state, despite the presence of some unsaturated components.

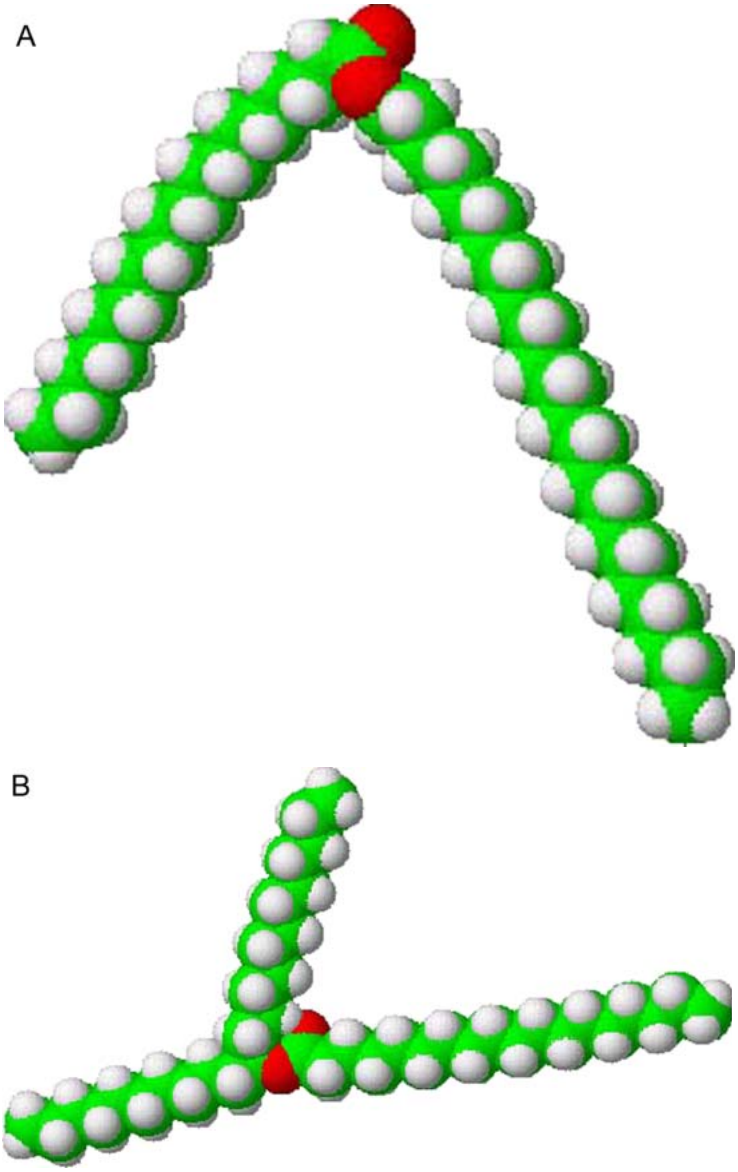

Figure 8. Space-filling models for (A) tricosanyl 1-stearate and (B) tricosanyl 11-stearate. The latter is the major wax ester species found on the cuticle of *M. sanguinipes* (Blomquist et al., 1972). Green atoms are carbons, red atoms are oxygens, and gray atoms are hydrogens.

An exception to the apparent waterproofing benefits of wax accumulation is provided by the secondary wax esters of grasshoppers from the genus *Melanoplus*. These melt at 5-10°C, and their presence reduces the T_m of the total surface lipids by ~10°C (Figure 7). Cuticular lipids from *M. sanguinipes* melt at 35-50°C (Gibbs *et al.*, 1991; Gibbs and Mousseau, 1994; Rourke and Gibbs, 1999; Rourke, 2000), within the ecologically relevant temperature range for this species (Chappell, 1983; Rourke, 2000). Thus, deposition of wax esters of *M. sanguinipes* may increase rates of evaporative water loss. These compounds do not have any known function in communication or predator deterrence, so their presence is problematic. It may be that some lipid melting is desirable, for example to aid in dispersal of lipids over the surface of the cuticle. Alternatively, the additional thickness of the lipid layer provided by wax esters may offset the effects of lower T_m.

Arthropod cuticles contain numerous other lipids in addition to wax esters, particularly hydrocarbons. These may interact with wax esters to affect the properties of the overall lipid mixture (Riederer and Schneider, 1990; Gibbs, 1995; Dodd and Afzal-Rafii, 2000). Our data suggest that interactions between wax esters and hydrocarbons reduce lipid T_m by no more than 5°C (Figure 7). Thus, the effects of cuticular wax esters on insect water balance are clear. Even in species having unsaturated waxes, these compounds will reduce cuticular permeability by maintaining lipid T_m values above environmental temperatures. The novel wax esters of melanopline grasshoppers are an exception to this rule.

Acknowledgements

This work was supported by an Undergraduate Research Opportunity Program award to S. Patel and NSF award IBN-9317471 to A.G. Gibbs. We thank Charlotte Fatland (ARS, Fargo) for the GC-MS data, Cheryl Baduini for discussions of marine waxes, and Michael S. Singer for comments on an early version of the manuscript. Mention of a commercial or proprietary product does not constitute endorsement by the U.S. Department of Agriculture.

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