Chemical and physical analyses of wax ester properties

Authors: Patel, Sejal, Nelson, Dennis R., and Gibbs, Allen G.

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

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.001.0401
Chemical and physical analyses of wax ester properties

Sejal Patel¹, Dennis R. Nelson² and Allen G. Gibbs³*

¹ Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Room 2-310, Cambridge, Massachusetts 02139; ² Biosciences Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, 1605 Albrecht Boulevard, Fargo, North Dakota 58105; ³ Center for Insect Science & Department of Ecology and Evolutionary Biology 1041 E. Lowell St., University of Arizona, Tucson, Arizona 85721. agibbs@arl.arizona.edu

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
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 chromatography-mass spectrometry (GCMS) was performed on a Hewlett-Packard HP 5890A gas chromatograph equipped with a pressure programmable cool on-column 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 °C and was programmed to increase to 320°C at 3 or 4°/min and held at 320°C. 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°C, 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
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.

<table>
<thead>
<tr>
<th>Wax Ester Carbon No.</th>
<th>Alcohol-Acid Carbon No.</th>
<th>Protonated Acid Ion</th>
<th>Factor</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>14:0-16:0</td>
<td>257</td>
<td>5.47</td>
<td>0.14</td>
</tr>
<tr>
<td>32</td>
<td>14:0-18:0</td>
<td>285</td>
<td>4.70</td>
<td>0.12</td>
</tr>
<tr>
<td>30</td>
<td>16:0-14:0</td>
<td>229</td>
<td>6.57</td>
<td>0.21</td>
</tr>
<tr>
<td>32</td>
<td>16:0-16:0</td>
<td>257</td>
<td>5.25</td>
<td>0.15</td>
</tr>
<tr>
<td>34</td>
<td>16:0-18:0</td>
<td>285</td>
<td>4.41</td>
<td>0.07</td>
</tr>
<tr>
<td>34</td>
<td>18:0-16:0</td>
<td>257</td>
<td>5.05</td>
<td>0.11</td>
</tr>
<tr>
<td>36</td>
<td>18:0-18:0</td>
<td>285</td>
<td>4.34</td>
<td>0.10</td>
</tr>
<tr>
<td>38</td>
<td>18:0-18:1</td>
<td>314</td>
<td>11.13</td>
<td>0.17</td>
</tr>
<tr>
<td>40</td>
<td>20:0-18:0</td>
<td>344</td>
<td>11.59</td>
<td>0.25</td>
</tr>
<tr>
<td>42</td>
<td>22:0-18:0</td>
<td>374</td>
<td>4.40</td>
<td>0.08</td>
</tr>
<tr>
<td>44</td>
<td>24:0-18:0</td>
<td>369</td>
<td>5.24</td>
<td>0.06</td>
</tr>
<tr>
<td>40</td>
<td>22:0-20:0</td>
<td>313</td>
<td>4.29</td>
<td>0.10</td>
</tr>
<tr>
<td>42</td>
<td>24:0-20:0</td>
<td>341</td>
<td>5.44</td>
<td>0.11</td>
</tr>
<tr>
<td>44</td>
<td>26:0-20:0</td>
<td>369</td>
<td>5.24</td>
<td>0.06</td>
</tr>
<tr>
<td>46</td>
<td>28:0-18:0</td>
<td>313</td>
<td>4.13</td>
<td>0.10</td>
</tr>
<tr>
<td>48</td>
<td>30:0-18:0</td>
<td>369</td>
<td>5.02</td>
<td>0.15</td>
</tr>
<tr>
<td>50</td>
<td>32:0-18:0</td>
<td>313</td>
<td>3.81</td>
<td>0.07</td>
</tr>
<tr>
<td>48</td>
<td>34:0-18:0</td>
<td>369</td>
<td>3.71</td>
<td>0.08</td>
</tr>
<tr>
<td>50</td>
<td>36:0-18:0</td>
<td>369</td>
<td>4.74</td>
<td>0.09</td>
</tr>
</tbody>
</table>

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.

---

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 in the melting temperatures were observed.
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, *Melanoplus 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-
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).
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.

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 melanoplusine 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.

References

University of Nebraska Press.