Occurrence of Waxes as Plant Exudates[1]

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Abstract: Plant exudates may be identified as waxes according to criteria obtained from carbon-13 nuclear magnetic resonance spectra of bulk, solid samples and from proton (hydrogen) nuclear magnetic resonance spectra of the samples dissolved in chloroform-*d*. From a study set of almost 1500 modern exudates, we have identified 17 samples that are waxes by these criteria. They all are spermatophytes, predominantly eudicots and cycads, but also a single magnoliid. Two samples were a blend of wax materials with phenolics, one a eudicot and one a ginkgo. Exudate gums occasionally contain small admixtures of wax.

Key Words: exudate, gum, nuclear magnetic resonance spectroscopy, phenolic, resin, wax

Plant waxes have found uses for centuries if not millennia as food, cosmetics, and medicinal materials (Bianchi 1995, Lan 2019). Waxes in Nature also may be derived from animal sources and from minerals such as coal, petroleum, and oil shale, which ultimately derive from plants. Waxes can be a complex mixture of hydrocarbons, alcohols, aldehydes, ketones, esters, and carboxylic acids in varying proportions depending on the source. For example, paraffin wax, a petroleum fraction, generally contains unfunctionalized hydrocarbons with 20-40 carbon atoms. In his textbook, Noller (1957), however, defines waxes as a specific class of organic compounds composed of "esters of high molecular weight monohydric (one hydroxyl group) alcohols with the common higher fat acids," but more generally as "anything with a waxy feel and a melting point above body temperature." Fat(ty) acids are carboxylic acids with unbranched hydrocarbon chains and an even number of carbon atoms. By Noller's chemical definition, waxes have the general formula of an organic ester, R(CO)OR', in

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which the groups R and R' are long hydrocarbon chains. He adds that the chains have variable lengths according to the source and that waxes may contain purely hydrocarbon molecules (paraffin) as well as esters. Natural waxes also can contain more unusual components such as terpenes.

Waxes are insoluble in water but soluble in nonpolar, organic solvents such as chloroform. They may be found on the surface of the plant (epicuticular) or embedded in the cutin polymer (intracuticular) (Baker 1982, Seigler 1998, Zeisler-Diehl et al. 2018). They serve as a means for the plant to control evaporation, wettability, and hydration, to reflect ultraviolet light, to protect against invasion by foreign organisms, and to serve as a lubricant, for example, for emerging leaves (Lambert et al. 2021a). Thus, they provide general environmental protection. Epicuticular wax often is visible as a whitish film on leaves, fruit, or elsewhere. Waxes may be harvested by scraping, brushing, or beating the surface of a plant part, by washing with a nonpolar solvent, or even by boiling the plant part, although this last process may bring in non-wax constituents.

Common plant waxes include carnauba or palm wax from the leaves of the Brazilian palm (*Copernicia prunifera*), candellila wax from the leaves of *Euphorbia cerifera*, rice bran from the husks of rice (*Oryza sativa*), sunflower wax from the seeds and seed hulls of the sunflower (*Helianthus annuus*), and jojoba wax from the seeds of *Simmondsia chinensis*. Commercial products often involve chemical processing. In their natural state as found on plants, waxes may be confused visually with other chemical types of exudates, such as gums, resins, and phenolics (Lambert, et al. 2008, Lambert et al. 2021b).

In our studies of monocot exudates, we identified spectral patterns of eight samples that suggested they are waxes (Lambert et al. 2015, 2021a). The pattern was a dominant broad peak in the saturated region of the carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectrum, which is consistent with the long hydrocarbon chains present in waxes. Such an interpretation, however, is tentative and not unique, as the data also could be consistent with other saturated hydrocarbon natural products, such as terpenes, as present in plant exudates called resins. A spectral distinction between waxes and other functionalities draws from the homogeneity of the carbons in the long chains R and R', whereas terpenes can contain branching, unsaturation, and electron-withdrawing substituents, all of which lead to broader and more complex saturated ^{13}C resonances. Thus, we have endeavored to develop further criteria using ^{1}H NMR spectroscopy to identify wax exudates and to distinguish them definitively from other classes such as resins.

During the last 25 years, the first and last authors have developed a study collection of over 2000 fossilized and modern plant exudates for the purpose of obtaining a world view of these materials according to their molecular structures.

Most of the modern samples were epicuticular and were harvested directly from the plants without processing. We have examined the spectra of the entire collection and have identified 11 additional samples with the ^{13}C pattern that suggests wax. We report herein the examination of the ^{13}C NMR spectra of these samples and, in addition, the proton (^{1}H) spectra of all 17 wax candidates to confirm the identity of the exudates. Although ^{13}C has been used to study the structure of waxes in solution (Vlahov 2008), there are no previous surveys of solid waxes as harvested from Nature. Whereas all previous materials with ^{13}C spectra that suggested waxes were monocots, we report herein a wide variety of flowering plants that produce wax exudates.

Methods

The Trinity University collection of plant exudates, consisting of more than 1500 specimens, has been developed from numerous sources including samples harvested by co-author JASB at botanical gardens in North America, samples provided by other field workers, and samples drawn from museum collections. Whereas the samples provided by JASB were harvested under controlled conditions from specific plant parts, those provided by others and from museums often were not associated with plant parts and lacked information on chemical or physical processing. Table I lists the samples that have been identified as waxes.

Table 1. Plant sources of wax exudates.

Sample number	Clade	Order	Family	Genus	Species	Source
720	magnoliid	Magnoliales	Myristicaceae	*Virola*	sp.	Field Museum F612262
948	monocot	Poales	Poaceae	*Saccharum*	*officinarum*	Field Museum 614514
1000	monocot	Poales	Poaceae	*Stipa*	*tenacissima*	Field Museum 615096
1007	monocot	Arecales	Arecaceae	*Cocos*	*syagrus*	Field Museum F620960
1009	monocot	Arecales	Arecaceae	*Copernicia*	*prunifera*	Field Museum 613578
1135	monocot	Arecales	Arecaceae	*Copernicia*	*prunifera*	Harvard 1549 00200336
1192	monocot	Zingiberales	Musaceae	*Musa*	*acuminata*	Nat. Herb. Nederland
723	eurosid I	Fagales	Myricaceae	*Myrica*	*cerifera*	Field Museum 16480

729	eurosid I	Fagales	Myricaceae	*Myrica*	*pubescens*	Field Museum F1970391
877	eurosid I	Fabales	Fabaceae	*Coursetia*	*glandulosa*	Harvard, no acc. no.
918	eurosid I	Fabales	Fabaceae	*Pterocarpus*	*marsupium*	Harvard 7785 00211665
1196	eurosid I	Fagales	Myricaceae	*Myrica*	*cordifolia*	Nat. Herb. Nederland
1779	eurosid I	Fabales	Fabaceae	*Maackia*	*amurensis*	Arnold Arboretum
755	eurosid II	Sapindales	Anacardiaceae	*Rhus*	sp.	Field Museum F268299
794	eurosid II	Sapindales	Anacardiaceae	*Melanochyla*	sp.	R. Gianno
1088	core asterid	Ericales	Theaceae	*Camellia*	*sinensis*	Harvard 3976 00205568
1794	core asterid	Ericales	Styracaceae	*Styrax*	*japonicus*	Arnold Arboretum
1693	core eudicot	Saxifragles	Crassulaceae	*Tylecodon*	*paniculatus*	U. Calif., JASB
1678	ginkgo	Ginkgoales	Ginkgoaceae	*Ginkgo*	*biloba*	JASB

Solid state ^{13}C data were recorded on a 400 MHz Varian NMR System at Northwestern University with a 5 mm T3 PENCIL probe or on a 400 MHz Bruker Avance III HD NMR Spectrometer at Northwestern University with a 4 mm HX probe. The magic angle spinning rate was set to 5000 Hz. The cross polarization (CP) pulse sequence was used for normal proton decoupling on both spectrometers. For interrupted decoupling (dipolar dephasing), a 50 μs (Varian) or a 48 μs (Bruker) delay was applied in the ^{1}H channel just before the 180° pulse in the ^{13}C channel. We used adamantane (Varian) or glycine (Bruker) to adjust the Hartmann-Hahn matching condition for normal CP experiments and to set the observation pulse and the delay time for dipolar dephasing. A typical parameter set was as follows: spectrum frequency 100.544 MHz (Varian) or 100.524 MHz (Bruker), spectral width 296 ppm, pulse width 3.4 μs for the 90° pulse for both ^{1}H and ^{13}C (Varian) or 2.5 μs for ^{1}H and 4.0 μs for ^{13}C (Bruker), pre-delay time 5 s, contact time 5 ms, acquisition time 50 ms, scan number 256, carrier frequency 110 ppm, and a ramped pulse with 83 Watts used in the ^{1}H channel during contact time. Solid state ^{13}C spectra were referenced to an external adamantane peak at δ 38.3 (Varian) or to an external glycine methylene peak at δ 43.4 (Bruker) and were referenced to tetramethylsilane at δ 0.0. Proton spectra were obtained at 500 MHz on a Varian Inova-500 spectrometer at room temperature without spinning at Trinity University. Spectra were referenced in $CDCl_3$ to TMS at δ 0.0. Typical

1D parameters were as follows: spectral width 12,000 Hz, pulse width 60°, delay time 1.0 s, acquisition time 1.0 s, and scan number 4.

Results and Discussion

Figure 1 displays a typical ^{13}C spectrum for a wax, sample 723, from the bayberry or wax myrtle shrub, *Myrica cerifera* (the species name translates from Latin as *wax bearing*). The wax appears on the fruit and normally is extracted by boiling the fruit in water and skimming the exudate froth off the surface. This sample was number 16480 in the historical exudate collection of the Field Museum, Chicago, IL, and was labeled "from fruit of *Myrica mexicana*" (a synonym of *M. cerifera*). The sample was a powder, which may have been extracted from the fruit by the traditional method. The dominant carbon resonance occurs at δ 33 with a shoulder at δ 25. The overall structure of waxes is, broadly speaking, R(CO)OR', in which R and R' represent long, unbranched hydrocarbon chains of varying length. The many structurally very similar methylene (CH_2) groups contained within such chains produce this dominant resonance. The sharp peak at δ 15 comes from the methyl (CH_3) groups that terminate these long chains. In general, methyl resonances occur at lower frequency than methylene resonances (Lambert, Mazzola, and Ridge 2019). Waxes typically contain many different R and R' groups of varying length, which creates the broadness of the peak. The small peak at δ 67 is from methylene groups adjacent to oxygen in the ester functionality [R(CO)—O—**C**H_2—]. Methylene groups adjacent to the carbonyl group [—**C**H_2(CO)OR'] resonate within the dominant peak. The carbonyl groups themselves resonate at δ 174 in the characteristic region for esters (the peaks at δ 124 and 224 are spinning sidebands of this resonance, artifacts of the experimental procedure). Thus the spectrum corresponds exactly to the expected wax ester structure. The spectrum of sample 720, the exudate from *Virola* sp., reproduced as Figure 5 in Lambert et al. (2015), is identical peak by peak, demonstrating the commonality of structure. Whereas *M. cerifera* is from the clade eurosid I, part of the large group related to roses, *Virola* sp. is from the clade magnoliid, composed of plants related to magnolias and taxonomically distinct from the rosids.

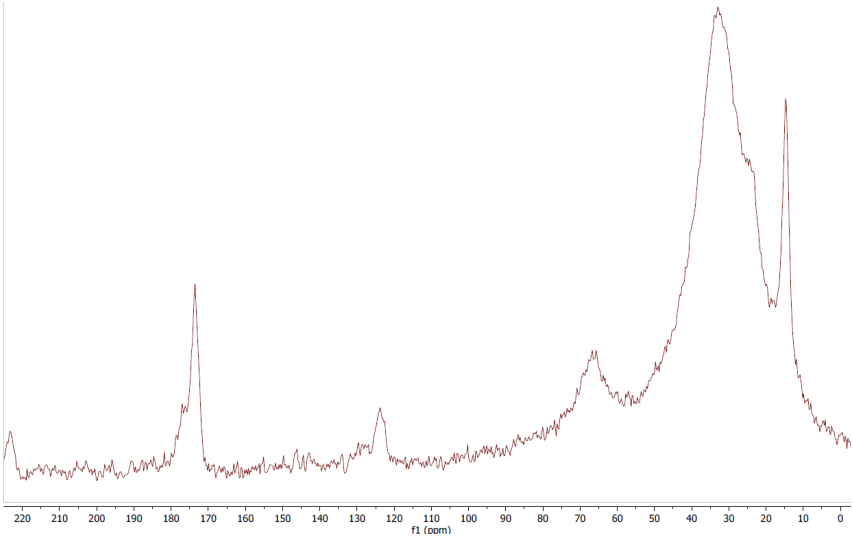

Figure 1. The 100 MHz solid state ^{13}C spectrum of sample 723, *Myrica cerifera*.

The proton (^{1}H) NMR spectrum provides an entirely different perspective on the molecular structure of exudate 723 (Figure 2). Such spectra must be taken in a suitable solvent, in this case chloroform-*d* ($CDCl_3$), so that they represent only the material that has dissolved (waxes are highly soluble). In Figure 2 the small peak at δ 7.3 is from residual undeuterated $CHCl_3$. The peak at δ 0.0 is of the standard, tetramethylsilane (Me_3Si). The spectrum is dominated by a peak at δ 1.2, which intentionally is well off scale to allow viewing of other peaks and hence is larger than the apparent intensity indicates. This resonance is from the many methylene groups in the wax. Similarly, the second tallest peak at δ 0.8 is from the methyl groups that terminate the R and R' chains. Both peaks have fine structure and contain many very similar components. The methyl resonance is much smaller because each chain terminates in a single methyl group but contains many methylene groups. The peak at δ 1.6 represents methylene groups beta to oxygen (—C**H**$_2$CH$_2$—O—) or possibly methine (CH) groups if there is any chain branching. Methylene groups adjacent to a carbonyl group [—C**H**$_2$—(CO)—] resonate at δ 2.3, and those adjacent to an ester oxygen atom [—C**H**$_2$—O(CO)—] are at δ 4.1, shifted to higher frequency because of an electronic effect between oxygen and the adjacent carbonyl group. The overall pattern strongly supports the wax structure and complements the ^{13}C data.

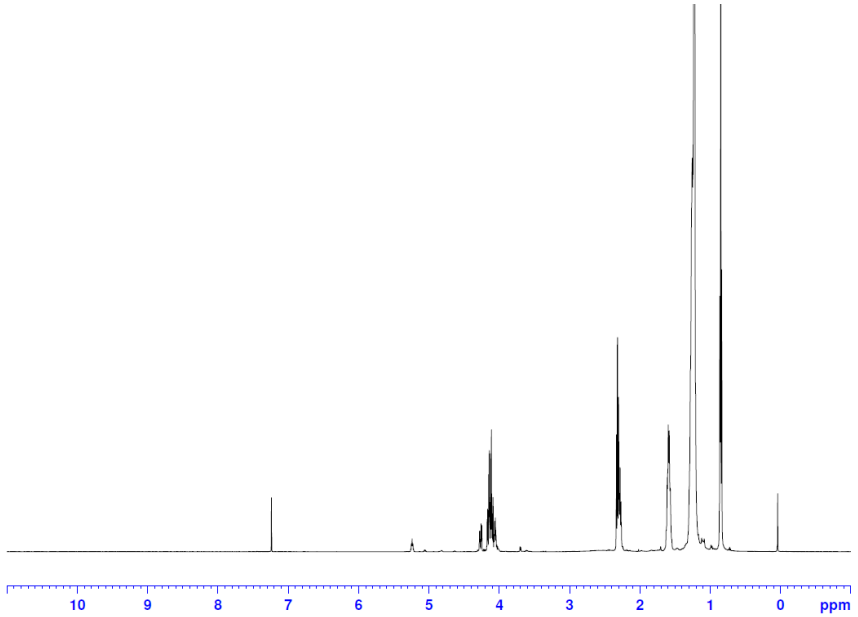

Figure 2. The 500 MHz ^{1}H spectrum of sample 723, *Myrica cerifera*, in $CDCl_3$.

Most of the samples in Table 1 have ^{13}C and ^{1}H spectra that follow this general pattern, including samples 720, 723, 729, 755, 948, 1000, 1088, 1135 (but with a more complex ^{1}H spectrum), 1192, and 1196. The remaining samples (794, 877, 918, 1007, 1009, 1779, and 1794) exhibit a sharper dominant peak at δ 33 in the ^{13}C spectrum along with a weaker or absent methyl peak δ 15, as illustrated in Figure 3 for sample 1009 (*Copernicia prunifera*, the carnauba palm, well known as the source of carnauba wax). The narrow nature of the dominant peak probably arises from a more homogeneous collection of R and R' chains. It is noted that the carbonyl peak is absent from the spectrum, possibly because of the long relaxation time of such carbons and the low sensitivity of this spectrum. Although its absence might be a cause for concern, the ^{1}H spectrum (Figure 4 for sample 1009) confirms all aspects of the wax structure. Indeed, many of the wax ^{13}C spectra have no carbonyl peak (948, 1000, 1007, 1009, 1135, 1192, and 1694) or a very weak one (1088, 1779, and 1794). There are more peaks in Figure 4 than in Figure 2, but they still occur at all the expected resonance positions. Figure 4, however, does exhibit some aromatic peaks in the region δ 6-8 (aside from the $CHCl_3$ peak), indicative of minor structural differences. It is noted that the other sample of *C. prunifera* (sample 1135) has the standard wax patterns for ^{13}C and ^{1}H, so these differences are within the range of normal molecular variation for waxes.

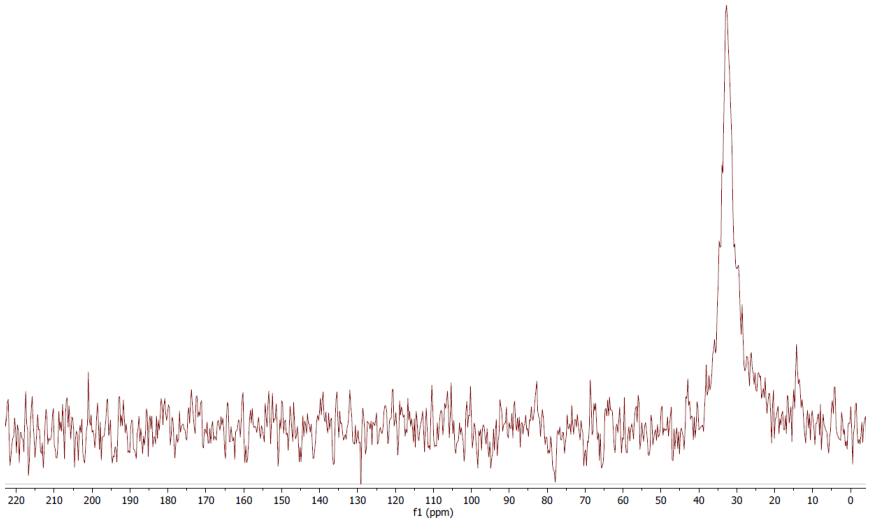

Figure 3. The 100 MHz solid state ^{13}C spectrum of sample 1009, *Copernicia prunifera*.

The ^{13}C spectrum of sample 1007 is very similar to that in Figure 3 and was published as Figure 14 in Lambert et al. (2015). This sample was labeled *Cocos syagrus* from Brazil, which is a misnomer for either *C. nucifera* (the only species in this genus) or *Syagrus romanzoffiana*. The ^{1}H spectrum is very clean, as in Figure 2, but, rather than a single, broad methyl peak at δ 15, there is a collection of many small, sharp peaks. The spectrum of sample 918 (*Pterocarpus marsupium*, a deciduous tree from India) has been placed in this category of variation because of the sharper nature of the methylene peak at δ 33. Its methyl peak at δ 15, however, is larger than normal. The size of the methyl peak may depend on the length of the R and R' chains. When they are long, the methyl peak would be weaker, and, when they are short, it would be stronger. Thus, the ratio of the two peaks is a rough measure of the lengths of the two chains. The spectra of sample 794 (*Melanochyla* sp.) are somewhat anomalous. The ^{13}C peak is of the sharper variety, with a small methyl peak and a broad carbonyl peak. In addition, there is a significant alkenic or aromatic peak at δ 131. The ^{1}H spectrum confirms that the functionality is alkenic, as there is a significant peak at δ 5.6 rather than in the aromatic region. The ^{1}H spectrum contains the methylene and methyl peaks, respectively, at δ 1.6 and 0.7. The protons on carbons adjacent to a carbonyl entity occur at δ 2.0, but there is nothing in the ester region around δ 4. Thus, this material may be a fatty acid rather than a fatty ester (wax).

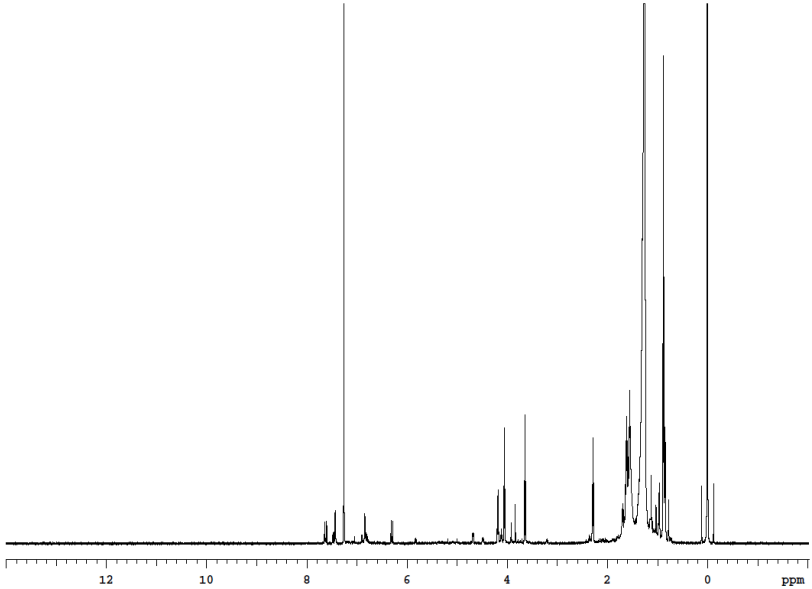

Figure 4. The 500 MHz ^{1}H spectrum of sample 1009, *Copernicia prunifera*, in $CDCl_3$.

The two groups of wax spectra, those with broad and those with sharp methylene resonances, comprise 17 samples. The other two samples, 1678 and 1693, have, in addition to the wax peaks in the region for saturated carbons, peaks that indicate the presence of phenolic constituents (unsaturation). Figure 5 of sample 1678 (*Ginkgo biloba*) illustrates these features. The saturated peak at δ 30 has the shape expected for the methylene groups in the R and R' chains of a wax, the very small peak at δ 15 represents the methyl groups, and the peak at δ 170 is from the carbonyl group. The remaining peaks, from δ 50 to 160, are indicative of phenolics. Those between δ 50 and 80 are from various saturated carbons adjacent to electron-withdrawing functionalities such as oxygen (present in both waxes and phenolics), and those between δ 100 and 130 represent unsaturated carbons generally not found in waxes. Most of these are aromatic resonances. The peak at δ 148 is the primary diagnostic of a phenolic functionality (the **C**–OH or ipso carbon of the benzene rings) (Lambert et al. 2021b). The second phenolic wax is sample 1693, *Tylecodon paniculatus*, the ^{13}C spectrum of which has been published as Figure 7 in Lambert et al. 2021b. Whereas sample 1678 is of the clade ginkgo, a gymnosperm, 1693 is a core eudicot, an angiosperm. Nonetheless, the two spectra are peak for peak nearly identical, with one qualification. The wax component is larger in 1693 in comparison with the phenolic component, so that the methylene resonance at δ 130 and the carbonyl resonance at δ 170 are proportionately larger than that for 1678.

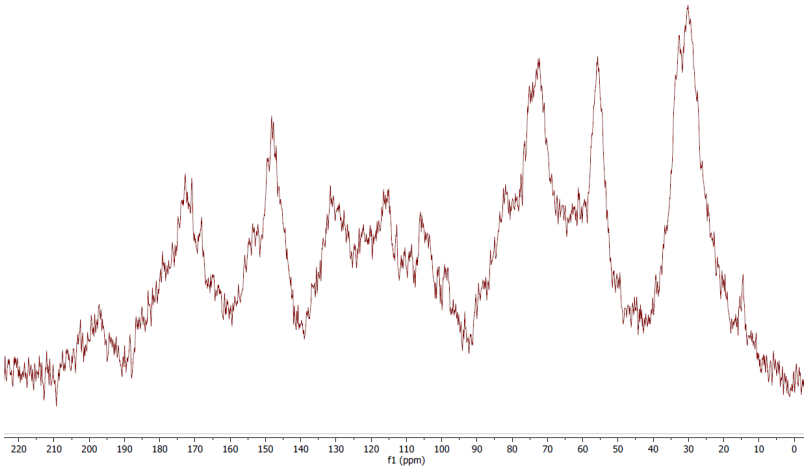

Figure 5. The 100 MHz solid state ^{13}C spectrum of sample 1678, *Ginkgo biloba*.

The ^{1}H spectrum of sample 1678 (Figure 6), contains no resonances of the phenolic component, which is insoluble in chloroform. The spectrum contains a larger resonance at δ 1.3 for the wax methylene groups, a very small resonance at δ 0.9 for the methyl resonances, small resonances at δ 2.0-2.4 for the —(CO)—C**H**$_2$— groups, and a small peak at δ 3.9 for the ester methylene group, —C**H**$_2$—O(CO)—.

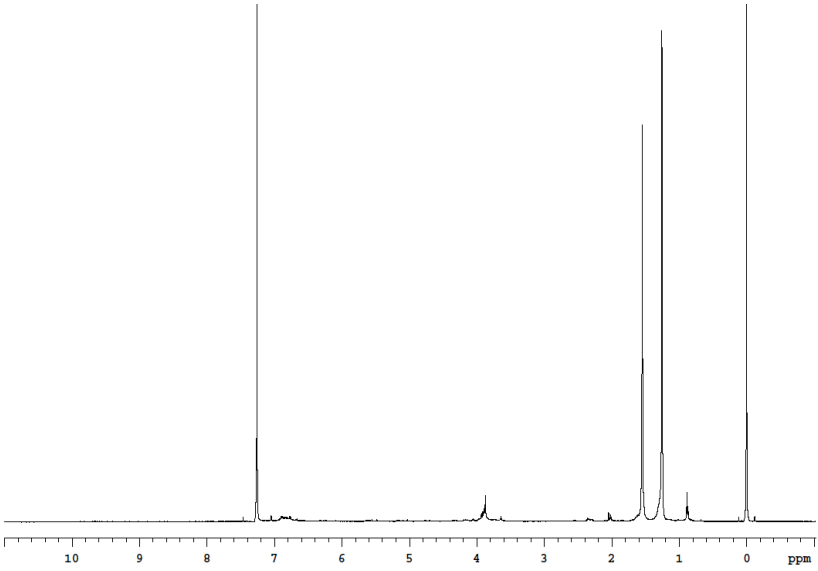

Figure 6. The 500 MHz ^{1}H spectrum of sample 1678, *Ginkgo biloba*.

Several gum samples have small resonances that suggest a wax component. Gums comprise large carbohydrate polymers that are insoluble in chloroform. Their ^{13}C spectra usually contain only two broad peaks, one at δ ca. 75 for all the C—O carbons and a smaller one at δ ca. 103 for O—C—O carbons. Any hexose sugar contains five of the former carbon atoms and one of the latter (the so-called anomeric carbon), so the peak at δ 103 always is the considerably less intense of the two. Some gum spectra have a small carbonyl peak at δ ca. 175. Figure 7 illustrates the ^{13}C spectrum of sample 627 of *Brachychiton discolor*. The two peaks δ 75 and 102 are from the carbohydrate C—O and O—C—O groups, and that at δ 174 is from a carbonyl group. The small peak at δ 124 is a spinning sideband of the carbonyl peak. The peak at δ 23 suggests the methylene groups of a wax, and the shoulder at δ 18 suggests methyl resonances from the same source. These resonances are too small to be definitive to demonstrate unambiguously that the sample has a wax component, but the result is likely. Because of its insolubility in chloroform, we could not explore the ^{1}H spectrum. We have observed hundreds of gum samples containing the two-peak gum pattern, and a few also contain this small, wax-like peak, including 628 (*Brachychiton discolor*, a eurosid II), 660 (*Marattia* sp., a pteridophyte or fern), 1346 (*B. acerifolius*), 1589 (*Sterculia apetala*, a eurosid I), 1922 (*Encephelartos hilderbrandtii*, a cycad), and 1944 (*Dioon merolae*, a cycad).

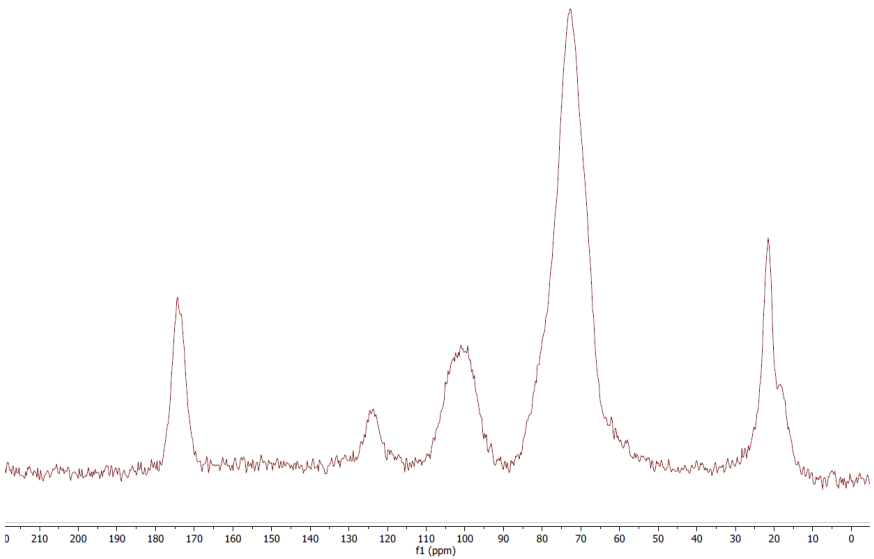

Figure 7. The 100 MHz solid state ^{13}C spectrum of sample 627, *Brachychiton discolor*.

We have established NMR criteria for identifying waxes in plant exudates harvested directly from their sources. The class of organic compounds has the general structure of an ester, R(C=O)OR', in which R and R' are long, generally unbranched, hydrocarbon chains. Additional, minor structural components such as alcohols, aldehydes, ketones, unesterified carboxylic acids, and terpenes can be present in small amounts. Waxes are signified in the solid state ^{13}C spectrum by the sharp peak at δ ca. 33 from methylene groups in the hydrocarbon chains, by a smaller, sharp peak at δ ca. 15 from methyl groups in the hydrocarbon chains, by a small peak at δ ca. 65 from methylene groups adjacent to oxygen, and by a small peak at δ ca. 175 from the carbonyl group. The structure is further supported by the ^{1}H spectra in solution, which contain the methylene resonances at δ ca. 1.2, the methyl resonances at δ ca. 0.8, methylene resonances next to carbonyl at δ ca. 2.3, methylene resonances next to the ester oxygen at δ 4.0, and any unsaturated components at δ 6-8.

The 17 samples that proved to be waxes represent a variety of plant clades, but all are spermatophytes, or seed plants. Furthermore, all are flowering plants (angiosperms), including one magnoliid, half a dozen cycads (monocots), and the remainder eudicots.

When unaccompanied by other resonances, these patterns are indicative of a pure wax. Wax materials can be accompanied by other organic classes that constitute special exudate composites. Two samples clearly contained phenolic constituents, as indicated by strong resonances in the unsaturated regions, including, in particular, the phenolic spectral diagnostic in the region δ 145 to 160 of the solid state ^{13}C spectrum. One was a core eudicot, and the other a ginkgo, which is an exotic gymnosperm. We term these exudates *phenolic waxes*. Gum exudates are composed of long carbohydrate chains, as indicated by a large resonance at δ ca. 75 and a smaller resonance at δ ca. 103. We found several samples that are composites of gum and wax components, in which the wax portion is minor. These *gum waxes* include several eudicots, a cycad, and a pteridophyte (fern).

Acknowledgments

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