R.; Wessels, P. L. *J. Chem. Soc., Chem. Commun.* **1984**, 122.
Gelderblom, W. C. A.; Thiel, P. G.; van der Merwe, K. J.; Marasas, W. F. O.; Spies, H. S. *Toxicon* **1983**, *21*, 467.
Jakubowski, A. A.; Guziec, Jr., F. S.; Sugiura, M.; Tam, C. C.; Tishler, M.; Omura, S. *J. Org. Chem.* **1982**, *47*, 1221.
Li, M.-H.; Lu, S.-H.; Ji, C.; Wang, Y.; Wang, M.; Cheng, S.; Tian, G. "Genetic and Environmental Factors in Experimental and Human Cancer"; Gelboin, H. V.; MacMahon, B.; Matsushima, T.; Sugimura, T.; Takayama, S.; Takebe, H., Eds.; Japanese Science Society Press: Tokyo, 1980; pp 139–148.
Marasas, W. F. O.; Wehner, F. C.; vanRensberg, S. J.; van-Schalkwyk, D. J. *Phytopathology* **1981**, *71*, 792.
Mareci, T. H.; Freeman, R. *J. Magn. Reson.* **1982**, 293.
Marshall, J. L. "Methods in Stereochemical Analysis, 2: Carbon-Carbon and Carbon-Proton NMR Couplings: Applications to Oranic Stereochemistry and Conformational Analysis"; Marchand, A. P., Ed.; Verlag Chemie International Inc.: Deerfield Beach, FA, 1983; pp 33–38.
Pfeffer, P. E.; Valentine, K. M.; Parrish, F. W. *J. Am. Chem. Soc.* **1979**, *101*, 1265.
Wat, C.-K.; McInnes, A. G.; Smith, D. G.; Wright, J. L. C.; Vining, L. C. *Can. J. Chem.* **1977**, *55*, 4090.
Wiebe, L. A.; Bjeldanes, L. F. *J. Food Sci.* **1981**, *46*, 1424.
Yang, C. S. *Cancer Res.* **1980**, *40*, 2633.

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Surface Wax of Coastal Bermuda Grass

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The surface cuticular wax of Coastal Bermuda grass was separated into hydrocarbons (10%), esters (33%), free alcohols (28%), free aldehydes (3%), and free acids (12%) by column chromatography. The composition of the individual fractions was determined by gas liquid chromatography (GLC) by comparison with known compounds. The major hydrocarbon was identified as tritriacontane. The ester fraction was hydrolyzed and the principle alcohols were identified as docosanol, tetracosanol, hexacosanol, and octacosanol. The major acids contained in the esters were identified as eicosanoic and docosanoic acids. The principle free constituents were found to be alcohol (unknown triterpenol), aldehyde (hexadecanal), and acid (hexadecanoic). The presence of tritriacontane (C33) as the major hydrocarbon is unusual as it is rarely a major component of plant waxes.

INTRODUCTION

The significance of plant waxes in the control of important biological phenomena as water loss, agricultural spray efficiency, and mechanical leaf damage has been well established (Eglinton and Hamilton, 1967). However, the literature contains very few reports on the analysis of surface waxes of forage plants. Ryegrass leaf wax (*Lolium perenne*) has been investigated by several workers (Pollard et al., 1931; Hamilton and Power, 1969; Allebone et al., 1970; Allebone and Hamilton, 1972). The surface wax of sorghum (*Sorghum bicolor*) (Bianchi et al., 1978; Wilkinson and Cummins, 1981) and corn (*Zea mays*) (Bianchi et al., 1975) has been studied. The surface wax composition has also been determined on wheat varieties by Tulloch and co-workers (Tulloch and Weenink, 1969; Tulloch and Hoffman, 1973), millets (Tulloch, 1982), and on sweet clover (*Metilotus alba*) by Emery and Gear (1969). There have been no reports on the composition of the surface wax of warm season grasses. Coastal Bermuda grass (*Cynodon dactylon*) is an important warm season forage crop in the southern United States that is dehydrated, grazed, hayed, and prepared as silage for use in animal feeds. In our continued investigation of the physical, chemical, and structural differences between cool and warm season grasses, the composition of the surface wax of Coastal Bermuda grass has been determined.

MATERIALS AND METHODS

Extraction of Surface Wax. Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers] was harvested after 24–30 days regrowth. The grass was fertilized with 380/112/112 Kg (N/P/K) per hectare in split applications during the growing season. The fresh grass was dipped twice in 1 liter of hexane for 20 s at room temperature. Fresh solvent was used for each dipping. Care was taken not to get the cut ends into the hexane solution. The hexane extracts were combined and filtered, and the solvent removed under vacuum to yield the crude wax as a light yellow solid. Infrared spectra were run on a Beckman IR-8 in Nujol or in carbon tetrachloride solution. Silica gel 60 was used for thin-layer chromatography and developed with chloroform containing 1% ethanol (Tulloch and Hoffman, 1971).

Separation of Wax Components. The extracted wax components were separated by column chromatography by using stepwise elution as described by Tulloch and Weenink (1969). Crude wax was applied to an unconditioned silica gel 60 column (2 × 18 cm) and 50 (100 mL) fractions were collected. The hydrocarbons were eluted in fractions 1–7 with hexane. Hexane–chloroform (75:25, v/v) eluted the esters in fractions 8–15. The same solvents 25:75, v/v) eluted the alcohols in fractions 20–26 followed by the aldehydes in fractions 27–34. Free acids were eluted with chloroform in fractions 39–42 and unidentified materials were eluted by methanol in the remaining fractions. The recovery was 97% of the crude wax applied to the column.

Fresh Coastal Bermuda grass was extracted in 1-kg batches. A total of 3–4 kg was extracted in this manner. Typical yields of surface wax were 0.8–1.0% on a dry weight basis. Infrared spectra of the crude wax gave a broad carbonyl absorption at 1710–1730 cm^{-1} and a broad hydroxyl absorption of 3500–3600 cm^{-1}. Thin-layer chromatography showed the presence of hydrocarbons, esters, alcohols, and free acids in addition to some unknown material in the crude wax. GLC system B was also used

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to identify similiar components. In preliminary experiments the infrared spectra of the alcohol fraction showed carbonyl absorption at 1725 cm^{-1} indicating the presence of aldehydes. In later runs, these aldehydes were separated from the alcohols. In a typical separation the crude wax (600 mg) was separated into hydrocarbons (60 mg), esters (200 mg), alcohols (170 mg), aldehydes (20 mg), and free acids (70 mg). The components in the final fractions (63 mg) were not identified.

Identification of Fractions. Gas liquid chromatographic (GLC) analyses were performed with a Perkin-Elmer Model 900 gas chromatograph equipped with flame ionization detectors. Columns were (A) 0.9 m by 3.2 mm i.d. stainless steel packed with Gas Chrom Q (80–100 mesh) coated with 2% Dexsil 300 and (B) 1.8 m × 3.2 mm i.d. stainless steel packed with Gas Chrom Q (80–100 mesh) coated with 3% SE-30. A Perkin-Elmer Model 2000 gas chromatograph equipped with flame ionization detector and split and splitless injector was also used with a 10 m × 0.25 mm capillary column coated with methyl silicone (C). The oven was programmed from 200–360 °C at 6 °C min for column A and B and 150–325 °C at 6 °C min for column C. The injector and detector were operated at 300 and 360 °C for column A and at 260 and 300 °C for column B and C. Nitrogen was used as carrier gas and the flow rate was 80 mL/min for column A and B and 20 mL/min for column C. Air and hydrogen flow rates were 200 and 35 mL/min, respectively. A Varian Aerograph Model 1740 GLC with flame ionization detectors was used to determine long chain fatty acids obtained from the crude wax as their methyl esters. Column D was 2.4 m × 3.2 mm i.d. stainless steel packed with Chromosorb W (AW-DMCS) 100–120 mesh coated with 10% EGSS-X. The oven was operated at 195 °C and the injector and detector were operated at 225 and 260 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 30 mL/min, respectively. Air and hydrogen flow rates were 245 mL/min and 25 mL/min, respectively. Hewlett Packard Model 3390A recording integrators were used to quantitate peak areas. Mass spectra (MS) were measured with a Hewlett-Packard 5985B GC/MS system. The capillary GC/MS system was an open split design with the following operating conditions: scan range, 40–400 AMU; scan rate, 400 AMU/s; electron energy, 70 eV; ion source temperature, 200 °C; electron multiplier voltage, 2200 V.

The wax esters (40 mg) were saponified by refluxing in 10 mL of 10% methanolic potassium hydroxide containing a trace of water for 24 h. The basic reaction mixture was diluted with water (about 20 mL) and extracted with chloroform to separate the alcohols from the acids. The basic reaction mixture was neutralized with dilute hydrochloric acid to pH 4 and the acids removed with chloroform. After solvent removal, the fatty acids were dissolved in methanol (2 mL) and esterified with 3 mL of 14% BF_3/methanol. The alcohols were acetylated with pyridine (2 mL) and acetic anhydride (2 mL) at 100 °C. The aldehydes were reduced with $LiAlH_4$ in anhydrous ether.

The hydrocarbons were identified by using GLC systems A and C. The alcohols were identified as free alcohols and acetates with GLC systems B and C. The unknown triterpenol was separated from C_{30} alcohols by using system C. The fatty acids were identified by using system D. All peaks were identified by comparison of retention times with those of known compounds.

Small amounts of the unknown triterpenol were collected from column C after detection with a hot wire detector. The unknown compound has a longer retention time than α- or β-amyrin or lanosterol. Mass spectra were determined for the unknown "neat" and its trimethylsilyl ether (Me_3Si) derivative. Neat MS, m/z (relative intensity) 426 (M^+, 5), 393 (1), 341 (5), 329 (3), 287 (2), 269 (5), 253 (4), 207 (12), 205 (14), 175 (7), 159 (5), 149 (11), 137 (17), 123 (77), 121 (29), 109 (37), 95 (62), 81 (74), 69 (73), 55 (100), 41 (74). Me_3Si MS, m/z 498 (M^+, 3), 483 (3), 408 (4), 341 (17), 269 (7), 253 (4), 231 (5), 219 (5), 210 (12), 205 (28), 195 (18), 189 (16), 169 (34), 157 (32), 143 (32), 123 (93), 109 (58), 95 (89), 73 (100), 55 (55).

Table I. Composition of the Surface Wax of Coastal Bermuda Grass

component	weight, %
hydrocarbons	10
esters	33
free alcohols	28
free aldehydes	3
free acids	12
unknown	10

Table II. Compositions of Wax Fractions from Coastal Bermuda Grass (%)[a]

		hydrolysis products of esters		free		
no. of C	hydrocarbons	acids	alcohols	alcohols	aldehydes[b]	acids
12		2				4
14		3			9	4
16:0		14	+		76	38
16:1		7				+
18:0		7	2		10	14
18:1		9				11
20		20	+	+	5	4
21						
22		24	22	4	+	22
23						
24	1	10	28	+		+
25	+[c]					
26	+		19	+		
27	5					
28	2		20	35		
29	11					
30	2		5	3		
30 (unknown)				59		
31	22					
32	+		4			
33	39					
34	+					
35	18					

[a] Based on total GC volatile material in fractions. [b] Analyzed as alcohols. [c] Present at levels less than 1%.

RESULTS AND DISCUSSION

The composition of the whole plant wax is shown in Table I. The esters were the major components followed closely by the free alcohols with smaller amounts of the hydrocarbons, free aldehydes, and free fatty acids.

The composition of the individual wax fractions from Coastal Bermuda grass is shown in Table II. The major hydrocarbons separated were identified as hentriacontane, tritriacontane, and pentatriacontane with tritriacontane being the principle hydrocarbon. The cool season grass *Lolium perenne* L. (perennial ryegrass) major wax hydrocarbons have been identified as nonacosane, hentriacontane, and tritriacontane with nonacosane being the principle hydrocarbon (Hamilton and Power, 1969). However, the ryegrass studied was only 14 days old.

The esters were hydrolyzed and the alcohols separated from the acids. The composition of the hydrolyzed esters are shown in Table II. The major ester alcohols found in Coastal Bermuda grass wax were docosanol, tetracosanol,

hexacosanol, and octacosanol. Tetracosanol was the principle alcohol present in the wax esters. Coastal Bermuda grass contains a much broader spectrum of alcohols in its ester fraction than does ryegrass esters which contain 79% hexacosanol (Allebone and Hamilton, 1972).

Coastal Bermuda grass was found to contain octacosanol as its principle identified free alcohol. However, the major alcohol (Table II) has not been identified. The mass spectra of the unknown alcohol indicated a molecular weight of 426. It formed a monotrisilyl ether derivative indicating the presence of a single hydroxyl group. Its mass spectrum was similar in appearance to that of the triterpenol lanosterol. The degree of similarity suggests that it is a triterpene with a similar elemental composition as lanosterol. In ryegrass hexacosanol accounts for 93% of the free alcohols (Allebone and Hamilton, 1972).

The fatty acids were identified as their methyl esters. The major acids freed from the esters were eicosanoic and docosanoic acids with smaller amounts of tetracosanoic, octadecanoic, and hexadecanoic acids (Table II). The free fatty acids isolated contained the same broad spectrum of acids as found associated with the esters. However, the major free acid was hexadecanoic acid with lesser amounts of docosanoic, octadecanoic, and 9-hexadecenoic acids (Table II). Ryegrass wax esters have been reported to contain octadecanoic acid as its major acid with lesser amounts of eicosanoic and hexadecanoic acids (Allebone and Hamilton, 1972). The aldehyde fraction was reduced and the aldehydes identified as their corresponding alcohols. Hexadecanal was the principle aldehyde isolated along with lesser amounts of octadecanal, tetradecanal, and eicosanal (Table II).

The hydrocarbon components of Coastal Bermuda grass wax are unusual, since tritriacontane is rarely a major component of plant waxes (Tulloch, 1974). The major hydrocarbons of most plant waxes are heptacosane, nonacosane, or hentriacontane (Herbin and Robins, 1967; Kolattukudy, 1970). Plant waxes with tritriacontane as the major hydrocarbon have been isolated from members of the family Crassulaceae and Gymnosperms (Herbin and Robins, 1967). Tulloch (1974) has also identified tritriacontane as the major hydrocarbon in the common annual weed (*Portulaca oleracea* (L.)).

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Registry No. Hentriacontane, 630-04-6; tritriacontane, 630-05-7; pentatriacontane, 630-07-9; nonacosane, 630-03-5; octacosanol, 557-61-9; docosanoic acid, 112-85-6; lanosterol, 79-63-0; 9-hexadecenoic acid, 2091-29-4; hexadecanal, 629-80-1; octadecanal, 638-66-4; tetradecanal, 124-25-4; eicosanal, 2400-66-0; octadecanoic acid, 57-11-4.

LITERATURE CITED

Allebone, J. E.; Hamilton, R. J. *J. Sci. Food Agric.* **1972**, *23*, 777.
Allebone, J. E.; Hamilton, R. J.; Knights, B. A.; Middleditch, B. S.; Power, D. M. *Chem. Phys. Lipids* **1970**, *4*, 37.
Bianchi, B.; Avato, P.; Bertorelli, P.; Manini, G. *Phytochemistry* **1978**, *17*, 999.
Bianchi, B.; Avato, P.; Salamini, F. *Maydica* **1975**, *20*, 1965.
Eglinton, G.; Hamilton, R. J. *Science (Washington, D.C.)* **1967**, *156*, 1322.
Emery, A. E.; Gear, J. R. *Can. J. Biochem.* **1969**, *47*, 1195.
Hamilton, R. J.; Power, D. M. *Phytochemistry* **1969**, *8*, 1771.
Herbin, G. A.; Robins, P. A. *Phytochemistry* **1968**, *7*, 257.
Kolattukudy, P. E. *Lipids* **1970**, *5*, 259.
Pollard, A.; Chibnall, A. C.; Piper, S. H. *Biochem. J.* **1931**, *25*, 2111.
Tulloch, A. P. *Lipids* **1974**, *9*, 664.
Tulloch, A. P. *Phytochemistry* **1982**, *21*, 2251.
Tulloch, A. P.; Hoffman, L. L. *Phytochemistry* **1971**, *10*, 871.
Tulloch, A. P.; Hoffman, L. L. *Phytochemistry* **1973**, *12*, 2217.
Tulloch, A. P.; Weenink, R. O. *Can. J. Chem.* **1969**, *47*, 3119.
Wilkinson, R. E.; Cummins, D. G. *Crop Sci.* **1981**, *21*, 397.

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Fruit Development and Growth Regulator Effects on Normal Alkanes of "Washington" Navel Orange Fruit Epicuticular Wax

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The *n*-alkanes as percent of total wax were highest in the very young "Washington" navel orange (*Citrus sinensis* (L.) Osbeck) fruit, but *n*-alkanes per unit area were highest in maturing and senescing fruits. Odd-numbered alkanes dominated with a minimum of 81.5% in Sept. C_{25} dominated in the immature fruit. At maturity and during senescence, C_{31} was highest, followed by C_{29} and C_{27}. During fruit growth and maturation, a shift of *n*-alkanes from lower to higher molecular weight was observed, but the reverse of that occurred during the postmaturation phase. Gibberellic acid (GA_3 = 10 ppm) alone or in combination with (2,4-dichlorophenoxy)acetic acid (2,4-D = 16 ppm) reduced *n*-alkane accumulation, but 2,4-D alone did not produce any significant effect until the senescence stage, where it decreased the rate of *n*-alkane accumulation. GA_3, either alone or in combination with 2,4-D, reduced total percent of odd-numbered hydrocarbons in favor of the even-numbered fractions. Treatment effect on individual *n*-alkanes and possible relationships between wax *n*-alkanes and wax fine structure are discussed.

Plant surfaces are characteristically covered by several layers of hydrophobic material, the outermost being the epicuticular wax. Functional and physiological roles of epicuticular wax include: reduction in transpiration and maintenance of water balance; regulation of gas exchange; protection against pathogens and mechanical damage; protection against UV radiation; protection against atmospheric pollutants; and resistance to freezing. The appearance and quantity of epicuticular waxes vary greatly

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