Technical

Saturated and Unsaturated Wax Esters Produced by *Acinetobacter* sp. HO1-N Grown on C_{16}-C_{20} *n*-Alkanes

S. DEWITT, J.L. ERVIN, D. HOWES-ORCHISON, D. DALIETOS, S.L. NEIDLEMAN and J. GEIGERT[1], Cetus Corporation, 600 Bancroft Way, Berkeley, CA 94710

ABSTRACT

The wax ester compositions produced by the action of *Acinetobacter* sp. HO1-N on *n*-alkanes (C_{16} through C_{20}) were analyzed using capillary gas chromatography/mass spectrometry (GC/MS). The wax esters contained, surprisingly, a large percentage of mono- and diunsaturated components. The acyl and alkoxy segments are reported for each wax ester component. Also, the positions of the carbon-carbon double bonds in the wax esters produced from the C_{16} and C_{20} *n*-alkanes are reported. These microbial-produced wax ester mixtures bear a close chemical similarity to those of sperm whale and jojoba oils.

INTRODUCTION

Gas chromatography (GC) has been used to analyze the wax ester compositions resulting from the metabolic action of *Acinetobacter* sp. HO1-N (also known as *Micrococcus cerificans*) on C_{14} through C_{18} *n*-alkanes. At no time were any wax ester components detected that contained carbon-carbon double bonds (Table I). Other microorganisms examined by GC also were reported to produce only saturated wax ester components from *n*-alkane substrates (3,5,6).

By use of capillary GC, we have found, surprisingly, that *Acinetobacter* sp. HO1-N metabolizes *n*-alkanes into wax esters containing 0, 1 or 2 carbon-carbon double bonds. By means of capillary GC/mass spectrometry (GC/MS) the acyl and alkoxy chain lengths and the positions of unsaturation in the wax ester components were determined.

EXPERIMENTAL PROCEDURES

Materials

All solvents used were of highest purity available (Burdick and Jackson, Muskegon, MI; and MCB Reagents, Cincinnati, OH). Silica gel was from E. Merck (Darmstadt, W. Germany); osmium tetroxide and *n*-alkanes from Aldrich Chemical (Milwaukee, WI); and Tri-Sil from Pierce (Rockford, IL). Hydrogen chloride and hydrogen sulfide gases were of highest purity available (Matheson, Lyndhurst, NJ). Wax ester standards were purchased from Sigma Chemical (St. Louis, MO) and Pfaltz and Bauer (Stamford, CN). Jojoba oil (Jojoba Marketing, Bakersfield, CA) was purchased from a local health food store.

Microbial Wax Esters

Formation. Several procedures for production of wax esters by *Acinetobacter* sp. HO1-N (ATCC 14987) have been published which may be used (2,7).

All of our production studies were done by first growing the organisms on a mineral medium consisting of (in g/L): K_2HPO_4, 5; $(NH_4)_2HPO_4$, 10; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.04; KCl, 1; $MgSO_4$, 0.1; $MnSO_4 \cdot H_2O$, 0.04; Na_2SO_4, 0.05; NaCl, 0.02; Na acetate, 5; Na propionate, 5; pH 7.2. Cultures were grown in Erlenmeyer flasks (50 mL medium/250 mL size) on a rotary shaker (200 rpm) at 25 C for 18 hr.

Portions (25-mL) of the starter culture were used to inoculate Erlenmeyer flasks (1 L) containing 200 mL of modified mineral medium. The mineral medium was modified by replacing the sodium acetate and propionate with *n*-alkane (0.1% final concentration). To facilitate dispersion in the medium, the *n*-alkanes were dissolved in the minimal amount of carbon disulfide and then added to the medium. The flasks were placed on a rotary shaker (250 rpm) at 25 C for 24 hr.

Isolation

Lipid material was extracted from the fermentations with chloroform (2 x 50-mL portions/fermentation flask). The chloroform layer was separated from the aqueous layer

[1] Corresponding author.

TABLE I

Previous Reports of Wax Esters Produced by the Metabolism of *n*-Alkanes by *Acinetobacter* sp. HO1-N

n-Alkane	Wax esters produced		Ref.
	Saturated wax esters	Unsaturated wax esters	
n-Tetradecane (C_{14})	$CH_3(CH_2)_{14}CO_2(CH_2)_{13}CH_3$	none reported	1
n-Hexadecane (C_{16})	$CH_3(CH_2)_{14}CO_2(CH_2)_{15}CH_3$	none reported	2,3
n-Heptadecane (C_{17})	8:1:1 Mixture of $CH_3(CH_2)_{15}CO_2(CH_2)_{16}CH_3$ $CH_3(CH_2)_{14}CO_2(CH_2)_{16}CH_3$ $CH_3(CH_2)_{13}CO_2(CH_2)_{16}CH_3$	none reported	4
n-Octadecane (C_{18})	1:1 Mixture of $CH_3(CH_2)_{16}CO_2(CH_2)_{17}CH_3$ $CH_3(CH_2)_{14}CO_2(CH_2)_{17}CH_3$	none reported	1

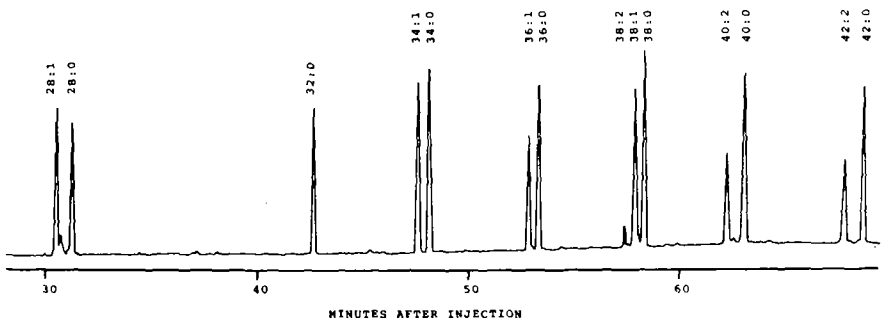


FIG. 1. Capillary GC/FID chromatogram of wax ester standards (100 μg/mL each component; 200 μL/mL jojoba oil added): decyl oleate ($C_{28:1}$); decyl stearate ($C_{28:0}$); cetyl palmitate ($C_{32:0}$); oleoyl palmitate ($C_{34:1}$); palmityl stearate ($C_{34:0}$); oleoyl stearate ($C_{36:1}$); stearyl stearate ($C_{36:0}$); jojoba oil component ($C_{38:2}$); arachidyl oleate ($C_{38:1}$); arachidyl stearate ($C_{38:0}$); jojoba oil component ($C_{40:2}$); arachidyl arachidate ($C_{40:0}$); jojoba oil component ($C_{42:2}$); arachidyl behenate ($C_{42:0}$).

and then evaporated off under vacuum at 40 C.

The wax esters were isolated from the residual starting *n*-alkane and other lipid components in the extract by loading the extract residue onto the head of a glass column (15 cm × 1 cm) packed with silica gel. Hexane (50 mL) was passed through the column to elute the residual starting *n*-alkane. Hexane/ethyl ether (90:10, 50 mL) was then passed through the column to elute the wax esters. The solvent from this fraction was evaporated off at 60 C.

Ethanolysis

Wax ester (20 mg) was refluxed in a mixture of anhydrous ethanol (5 mL) and anhydrous benzene (0.2 mL) for 2 hr with a slow bubbling of hydrogen chloride gas (1 mL/min) into the reaction mixture. The solvent and HCl were then removed by distillation.

The ethanolysis procedure cleaved the wax esters into fatty acid ethyl esters and fatty alcohols (8).

Hydroxylation and Silylation

The products from the ethanolysis procedure (10 mg) and OsO_4 (20 mg) were added to a pyridine/dioxane mixture (1:8, 2 mL). The reaction mixture was allowed to stand for 1 hr at room temperature. The solution was then evaporated off at 50 C under vacuum, and the residue dissolved in absolute methanol (5 mL). Hydrogen sulfide gas (1 mL/min) was passed into the solution for 10 min. The black precipitate was then removed by centrifugation and the supernatant evaporated to dryness at 60 C under vacuum.

The residue was silylated by reacting it with Tri-Sil (1 mL) for 30 min at room temperature. The solution was then evaporated off at 40 C under vacuum.

This procedure oxidized the carbon-carbon double bonds to vicinal diols. The diol and the primary alcohol groups were then converted into trimethylsilyl (TMS) ethers (9).

Capillary Gas Chromatography/Mass Spectrometry

Wax esters, fatty acid ethyl esters, fatty alcohols and TMS-derivatives were analyzed on a Finnigan 4021 gas chromatograph/mass spectrometer equipped with a flame ionization detection (FID). The gas chromatograph was

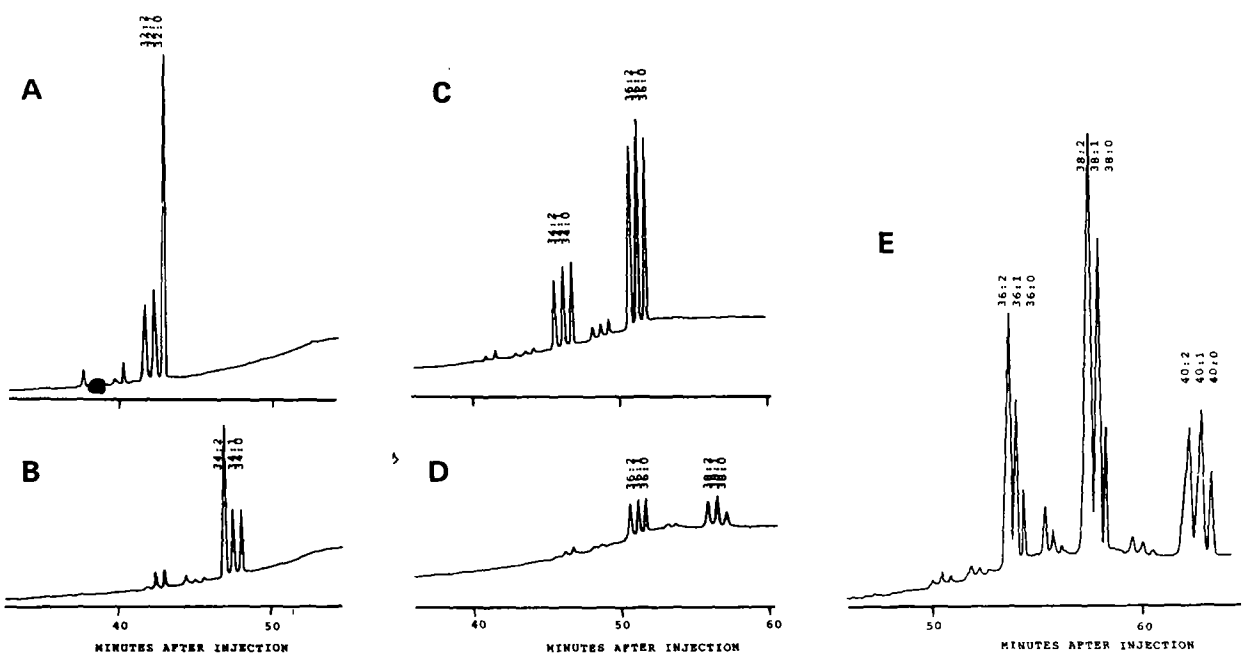


FIG. 2. Capillary GC/FID chromatograms of the wax ester composition produced by *Acinetobacter* sp. H01-N. Substrates: A—*n*-hexadecane (C_{16}); B—*n*-heptadecane (C_{17}); C—*n*-octadecane (C_{18}); D—*n*-nonadecane (C_{19}); E—*n*-eicosane (C_{20}).

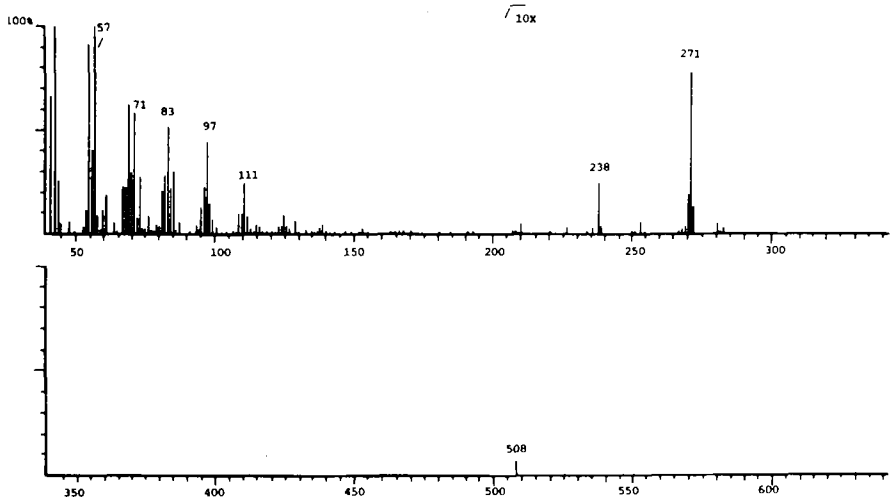


FIG. 3. Diagnostic mass spectrum of heptadecanyl heptadecanoate (17:0-17:0) produced by *Actinetobacter* sp. H01-N from *n*-heptadecane.

equipped with a fused-silica capillary column (30 m × 0.25 mm id) coated with SE-54 (J and W Scientific, Rancho Cordova, CA). The inlet port was used in the splitless mode. Samples dissolved in carbon disulfide were injected (3 μL) into the column at 40 C. After 40 sec, the splitting valve was opened, allowing the septum and injection port to be purged, and the oven temperature was increased to 325 C at 5 C/min, and held at 325 C until all components eluted. The injection temperature was 325 C; the FID detector was 350 C; the GC/MS transfer line interface was set at 330 C. Helium was used as the carrier gas with the flow rate set at 2 mL/min. The quadrupole mass spectrometer was operated in the electron impact mode at an ionizing potential of 70 eV. Data acquisition and processing were done on-line using the Finnigan Incos data system.

RESULTS AND DISCUSSION

Capillary GC has a resolving capability far superior to that of traditional GC. Although traditional GC provides little, if any, resolution, capillary GC provides near-complete resolution of wax ester components by their degree of carbon-carbon double bond unsaturation (Fig. 1) (10). Because saturated and unsaturated components have roughly the same FID response factor, approximate concentrations of each component can be obtained from the GC peak heights.

Wax esters ranging from C_{30} to C_{40} were detected. The chain lengths of the wax esters formed were a function of the *n*-alkane used as substrate:

$$C_n\ n\text{-alkane} \rightarrow C_{2n},\ C_{2n-2} \text{ and } C_{2n-4} \text{ wax esters}$$

TABLE II

Composition of Wax Esters Produced from *n*-Alkanes by *Acinetobacter* sp. H01-N

n-Alkane substrate	Capillary GC peak ID (ref. 1)	Characteristic MS peaks (m/z)					Acyl-alkoxy assignment
		M^+	$RCO_2H_2^+$	RCO^+	RCO-1$^+$	R′-1$^+$	
C_{16}	32:2	476	–	237	236	222	16:1-16:1
	32:1	478	257	237	236	222,224	16:1-16:0+16:0-16:1
	32:0	480	257	–	–	224	16:0-16:0
C_{17}	34:2	504	–	251	250	236	17:1-17:1
	34:1	506	271	251	250	236,238	17:1-17:0+17:0-17:1
	34:0	508	271	–	–	238	17:0-17:0 (Fig. 3)
C_{18}	34:2	504	–	237	236	250	16:1-18:1
	34:1	506	257	237	236	250,252	16:1-18:0+16:0-18:1
	34:0	508	257	–	–	252	16:0-18:0
	36:2	532	–	265	264	250	18:1-18:1
	36:1	534	285	265	264	250,252	18:1-18:0+18:0-18:1
	36:0	536	285	–	–	252	18:0-18:0
C_{19}	36:2	532	–	251	250	264	17:1-19:1
	36:1	534	271	251	250	264,266	17:1-19:0+17:0-19:1
	36:0	536	271	–	–	266	17:0-19:0
	38:2	ND[a]	–	279	278	264	19:1-19:1
	38:1	562	299	279	278	264,266	19:1-19:0+19:0-19:1
	38:0	564	299	–	–	266	19:0-19:0
C_{20}	36:2	532	–	237	236	278	16:1-20:1
	36:1	534	257	237	236	278,280	16:1-20:0+16:0-20:1
	36:0	536	257	–	–	280	16:0-20:0
	38:2	ND	–	265	264	278	18:1-20:1
	38:1	562	285	265	264	278,280	18:1-20:0+18:0-20:1
	38:0	564	285	–	–	280	18:0-20:0
	40:2	ND	–	293	292	278	20:1-20:1
	40:1	ND	313	293	292	278,280	20:1-20:0+20:0-20:1
	40:0	592	313	–	–	280	20:0-20:0

[a]ND—ion abundance too small to detect.

Contrary to literature reports, unsaturated (monoene and diene) components were observed (Fig. 2). The ratio of unsaturated to saturated components increased with increasing chain length of the *n*-alkane substrate.

MS of wax esters produces characteristic spectra which reveal the chain length and degree of unsaturation in the acyl and alkoxy segments. Using the general formula RCO_2R' for a wax ester, where R and R' equal the alkyl chains in the acyl and alkoxy segments, respectively, saturated acyl segments yield the $[RCO_2H_2]^+$ mass ion, whereas unsaturated acyl segments yield the $[RCO]^+$ and $[RCO\text{-}1]^+$ mass ions. The $[R'\text{-}1]^+$ ion is formed from both saturated and unsaturated alkoxy segments. Using this approach, the mass spectrum of each microbial-produced wax ester component was unambiguously assigned.

Figure 3 illustrates the characteristic spectrum of C_{34} (17:0-17:0) wax ester. A quantitative analysis of wax esters has been developed utilizing the ion abundance of these characteristic fragment ions, but unfortunately, the method is not applicable for wax esters having different molecular weights or unsaturated components (11). The ion abundances therefore have to be used in a qualitative sense.

The chemical compositions of the wax ester mixtures produced by *Acinetobacter* sp. H01-N from C_{16} through C_{20} *n*-alkanes are presented in Table II. The carbon number of each acyl segment was either equal to the carbon number of the *n*-alkane substrate or 2 and 4 carbons less than that number. The carbon number of the alkoxy segment was always equal to the carbon number of the *n*-alkane substrate. This result supports a mechanism in which the *n*-

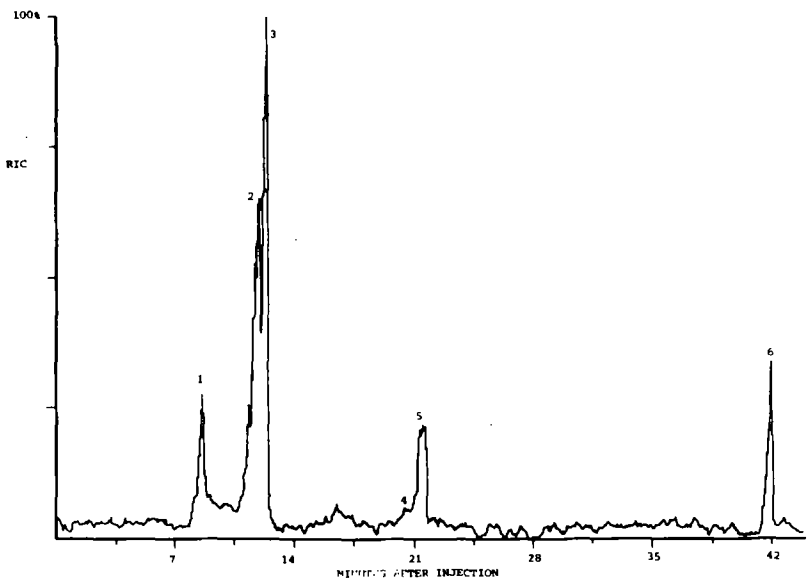


FIG. 4. Capillary GC/MS chromatogram illustrating separation of substrates and products for all steps in the derivatization procedure used for determining sites of unsaturation. Peaks: 1—hexadecanol, 2—hexadecenoic acid ethyl ester, 3—hexadecanoic acid ethyl ester, 4—tris-TMS ether derivative of hexadecenol, 5—bis-TMS ether of hexadecenoic acid ethyl ester, and 6—cetyl palmitate.

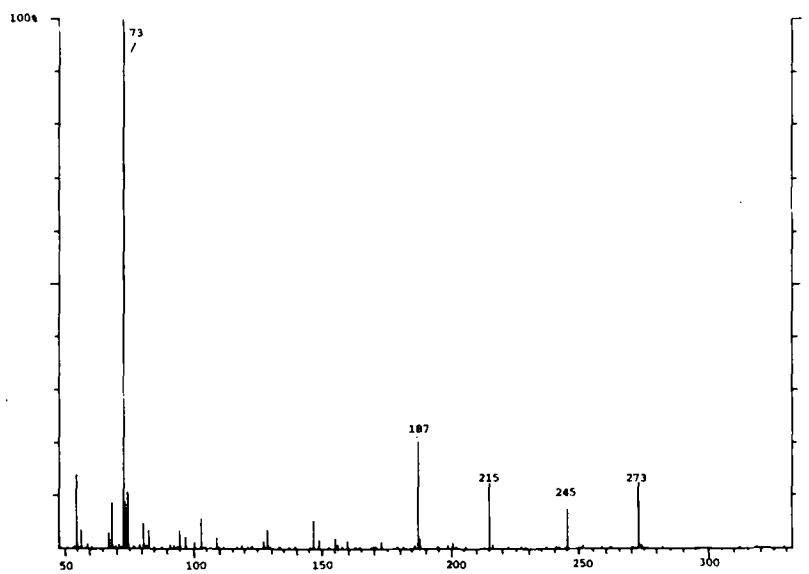


FIG. 5. Mass spectrum of the bis-TMS ether derivative of hexadecenoic acid ethyl ester obtained from wax esters produced from C_{16} *n*-alkane. Δ7 and Δ9 positional isomers predominate.

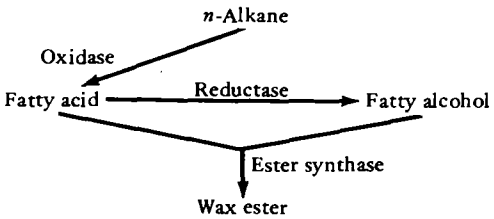
TABLE III

Unsaturation Sites in the Wax Esters Produced from *n*-Hexadecane (C_{16})

Chain length of TMS-derivatives	Characteristic MS peaks (m/z)				Approximate proportions	
	Ion a	Ion b	Ion c	Ion d	Δ7	Δ9
16 EE[a]	187,215	245,273	–	–	40	60
16 A	–	–	275	215	100	–

[a]EE = bis-TMS ether of fatty acid ethyl ester; A = tris-TMS ether of fatty alcohol.

alkane is incorporated intact, via fatty acid and fatty alcohol derivatives, into the wax ester (12):



Another feature of these wax ester components was the presence of only 0 or 1 carbon-carbon double bond per segment. Even in the diene wax ester components, 1 unsaturation was in the acyl segment and the other in the alkoxy segment.

As a result of the ready migration of the double bonds in the molecular ions, positional isomers of the wax esters give indistinguishable mass spectra. However, the carbon-carbon double bond position can be established by forming a suitable derivative to lock it in place. Many good derivatives are known (13). The method that we used for determining the positions of unsaturation in the wax esters produced from C_{16} and C_{20} *n*-alkanes involved ethanolysis to cleave the wax esters into fatty acid ethyl esters and fatty alcohols, followed by oxidation of the carbon-carbon double bonds to vicinal diols. The diols and the primary alcohol groups were then converted into trimethylsilyl (TMS) ethers. This approach was used because it was adaptable to small amounts of sample (10-mg quantities) and because the products at each step in the derivatization could be analyzed directly by the GC/MS method. However, we do not recommend this approach for quantitative measurement of the unsaturation sites. Each step of the derivatization was monitored by means of capillary GC/MS. Figure 4 illustrates this separation.

There was no attempt to optimize either the injection procedure or the temperature programming for each compound class. The fragmentation ions of the TMS-derivatives permitted unequivocal assignment of the double bond positions:

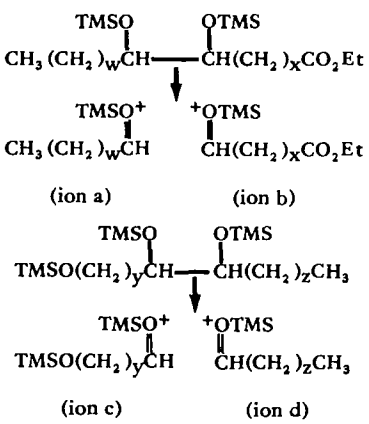


Figure 5 illustrates the key fragments arising from the derivatized C_{16} unsaturated acyl segment of the C_{32} wax ester components.

The positions of unsaturation are presented in Tables III and IV. The wax ester acyl segments contained positional isomers predominantly at the Δ7, Δ9 and Δ11 sites; or when numbering from the terminal methyl carbon, the ω7 and ω9 sites. The wax ester alkoxy segments contained positional isomers predominantly at the Δ7 and Δ11 sites; or when numbering from the terminal methyl carbon, the ω7 site only. The presence of these various positional isomers poses some interesting questions about the biosynthesis of the microbial wax esters. It would be of interest to study, for example, the desaturase system and chain shortening system in this microorganism.

The following comparison illustrates the close chemical similarity of these microbial-produced wax esters with those found in sperm whale and jojoba oils:

	Sperm whale oil (ref. 14)	Microbial-produced wax esters	Jojoba oil (ref. 15)
Carbon number of intact wax esters	28-40	32-40[a]	36-44
Acyl segments			
Carbon number	14-22	16-20[a]	16-24
Number of unsaturations	0,1	0,1	1
Alkoxy segments			
Carbon number	16-20	16-20[a]	18-24
Number of unsaturations	0,1	0,1	1
Predominant sites of unsaturation	ω7, ω9 and ω11	ω7 and ω9	ω9

[a]Carbon numbers dependent on *n*-alkane used as substrate.

Wax esters compositions such as these are finding utility as high-pressure and -temperature lubricant additives (16).

ACKNOWLEDGMENT

This work was supported by Standard Oil Company (Indiana).

REFERENCES

1. Stewart, J.E., and R.E. Kallio, J. Bacteriol. 78:726 (1959).
2. Stevens, N.J., J.W. Frankenfeld and J.D. Douros, Jr., U.S. Patent 3,409,506 (1968).
3. Makula, R.A., P.J. Lockwood and W.R. Finnerty, J. Bacteriol. 121:250 (1975).
4. Stevenson, D.P., W.R. Finnerty and R.E. Kallio, Biochem. Biophys. Res. Commun. 9:426 (1962).
5. Raymond, R.L., and V.W. Jamison, Adv. Appl. Microbiol. 14:93 (1971).
6. Krasil'mikov, N.A., and T.V. Koronelli, Mikrobiologiya 38:757 (1969).
7. Makula, R., and W.R. Finnerty, J. Bacteriol. 95:2101 (1968).
8. Miwa, T.K., JAOCS 48:259 (1971).
9. Janssen, G., and G. Parmentier, Biomed. Mass Spectrom. 5:439 (1978).
10. Geigert, J., D. Dalietos and S.L. Neidleman, J. High Resol. Chromatogr. Col. Chromatogr. 3:473 (1980).

TABLE IV

Unsaturation Sites in the Wax Esters Produced from *n*-Eicosane (C_{20})

Chain length of TMS-derivatives	Characteristic MS peaks (m/z)				Approximate proportions				
	Ion a	Ion b	Ion c	Ion d	Δ7	Δ8	Δ9	Δ10	Δ11
16 EE[a]	187,215	245,273	–	–	65	–	35	–	–
18 EE	201,215	273,284	–	–	–	–	90	10	–
20 EE	215,229,243	273,287,301	–	–	–	–	25	15	60
20 A	–	–	331	215	–	–	–	–	100

[a]EE = bis-TMS ether of fatty acid ethyl ester; A = tris-TMS ether of fatty alcohol.

11. Aasen, A.J., H.H. Hofsteller, B.T.R. Iyengar and R.T. Holman, Lipids 6:502 (1971).
12. Hankin, L., and P.E. Kolattukudy, J. Gen. Microbiol. 51:457 (1968).
13. Knapp, D.R., "Handbook of Analytical Derivatization Reactions," Part II, Chapt. 4, 1979.
14. Spencer, G.F., and W.H. Tallent, JAOCS 50:202 (1973).
15. Spencer, G.F., R.D. Plattner and T. Miwa, Ibid. 54:187 (1977).
16. Gisser, H., J. Messina and D. Chasan, Wear 34:53 (1975).

[Received July 17, 1981]

Kinetics of Bleaching of Vegetable Oils[1]

U.I. BRIMBERG, Food Division, Process Laboratory, Alfa-Laval AB, Tumba, Sweden

ABSTRACT

It was shown in a recent paper that the concentration c of remaining chlorophyll or carotene in rapeseed oil during the bleaching process follows a rate formula, $\log c/c_0 = -k\sqrt{t}$, characteristic of flocculation of colloids. Thus, the pigments are particulate and colloidally dispersed in the oil. The rate constant k was proportional to the added amount of clay. This paper reports experiments with palm oil. The same kinetics as those with rapeseed are valid. The effects of various parameters on the rate have been studied. The mechanism of the process is discussed.

INTRODUCTION

The bleaching of vegetable oils with bleaching clays in industry has been reviewed by Norris (1) and Kaufmann and Mukherjee (2). Extensive references to the literature are also given. There are a few papers (3-8) on the kinetics of the process that show an initial period of rapid bleaching, a later period of slow bleaching and a final period of constant color which is reached in 10-20 min. Unfortunately, the rate data are too few for quantitative treatment and deduction of rate formulae. We have, therefore, undertaken experiments to this purpose.

Results with rapeseed oil have already been published (9). Rate formulae were derived from the data and applied to the study of various parameters, such as amount of bleaching clay and water content. The formulae were discussed in terms of principles developed at Alfa-Laval by Berg (10).

The experiments with rapeseed oil showed that the bleaching process follows the rate formula:

$$\ln c/c_0 = -k\sqrt{t} \qquad [I]$$

where t is the time from addition of the clay, c is the concentration of pigment at time t, c_0 the concentration at t = o and k is the rate constant. The rate formula and the rate constant were the same whether the bleaching was followed by measuring the concentration of chlorophyll or that of carotene (Fig. 1).

[1]Presented at the AOCS meeting, New Orleans, May 1981.

Equation I pertains to the destabilization of a colloid by removal of the stabilizer, in this case by adsorption onto the bleaching clay. Thus, chlorophyll and carotene are particulate substances in stable dispersion in the oil. The stabilizer is a surface-active lipid among the components of the oil. When enough stabilizer with attached pigments has been removed, the pigment particles aggregate among themselves and settle out until an equilibrium is reached.

This communication reports experiments with neutralized palm oil. The effects of the water content, the method of adding the clay, and the stirring rate were studied. Palm oil differs from rapeseed oil essentially by its lack of chlorophyll, by its much higher content of carotene and, perhaps, by containing a different set of surface-active lipids or different concentrations of them.

EXPERIMENTAL PROCEDURES

Materials

Palm oil. The oil was a neutralized oil of Malaysian origin containing 0.02% water and about 400 ppm of carotene, calculated as β-carotene.

Bleaching clay A. Tonsil Optimum from Süd-Chemie, Germany. It was sieved to remove particles larger than 50 μm to facilitate observations in the microscope, especially to avoid confusing large structures formed during the bleaching with large particles of the bleaching clay. The clay contained 7.7% water as determined according to Karl Fischer. In one experiment, the clay was dried for 5 hr at 130 C, after which it contained 1.97% of water according to Karl Fischer.

Bleaching clay B. Tonsil Standard FF with 7.3% water.

Bleaching clay C. Tonsil L 80 (moisture content not determined).

Methods

Two series of bleaching experiments were done: series I—1 kg of oil was treated with 1% of clay A at 90 C for periods up to 3 hr. The experimental technique was the