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SICKLEPOD HYDROCARBON RESPONSE TO PHOTOPERIOD*

ROBERT E. WILKINSON
Department of Agronomy, Georgia Station, Experiment, Georgia 30212, U.S.A.

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Abstract—Total vegetative hydrocarbon composition of sicklepod (*Cassia obtusifolia* L.) changed with age and photoperiod. Leaf epicuticular hydrocarbon composition of sicklepod and coffee senna (*Cassia occidentalis* L.) were different in plants of equal age grown in 16-hr or 12–13-hr photoperiods. The major leaf epicuticular hydrocarbon constituent of sicklepod grown in a 16-hr photoperiod was an unsaturated C_{32} constituent with a molecular weight of 446 with subsidiary constituents at relative elution temperatures equivalent to C_{30} and C_{34} having 34·9, 8·7, and 6·8% respectively, of the total hydrocarbon content.

INTRODUCTION

PLANT hydrocarbon formation has been reported to be specific to the individual species and systemic distribution of chemical compounds throughout the plant kingdom has been utilized extensively as an aid in taxonomic separation of closely allied plants.[1] Previous work resulted in criteria for the utilization of leaf alkane constituents as aids in taxonomic separation; (a) the chemical composition must be specific for the species, and (b) the hydrocarbon pattern must be independent of season, age, or station of the individual plant.[2] Additionally, previous work indicated that alkanes of carbon number less than 25 and more than 35 were not present to any appreciable extent and that odd numbered alkane content exceeded even numbered alkanes by a factor of ten or more.[2] However, physiological changes occur in plants in response to change in environment. For instance, cuticles developed during leaf expansion remain relatively uniform morphologically but the total cuticle developed is dependent upon station and season.[3] Amino acids and α-keto acid contents of mint (*Mentha piperita* L.) were photoperiodically and temperature responsive.[4] Branched paraffin biosynthesis in broccoli (*Brassica oleracea* L. var italica Plenck.) was linked to the utilization of deaminated amino acid carbon chains as the skeletons for extension into fatty acids.[5] Sudan IV staining lipoidal materials of saltcedar (*Tamarix pentandra* Pall.) cladophylls was responsive to photoperiod[6] and alkane chain length increased with age in *Solandra grandiflora* Sw.[7]

Foliar herbicide application efficacy depends upon penetration of the pesticide through the cuticle. Lack of penetration is often equivalent to failure of the procedure. Sicklepod (*Cassia obtusifolia* L.), a major pest in soybean (*Glycine max* Merr.) production in the

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[1] T. SWAIN (editor), *Chemical Plant Taxonomy*, Acacemic Press, New York (1963).
[2] G. EGLINGTON, R. J. HAMILTON, R. A. RAPHAEL and A. G. CONZALEZ, *Nature, Lond.* **193**, 739 (1962).
[3] J. D. SKOSS, *Bot. Gaz.* **117**, 55 (1955).
[4] F. C. STEWARD, *Cornel Univ. Agr. Exp. Sta. Mem.* 379 (1962).
[5] P. E. KOLATTUKUDY, *Plant Physiol.* **43**, 1423 (1968).
[6] R. E. WILKINSON, *Plant Physiol.* **41**, 271 (1966).
[7] G. A. HERBIN and P. A. ROBINS, *Phytochem.* **8**, 1985 (1969).

southeastern United States, is easily controlled during early seedling stages but becomes increasingly resistant to pesticides with age. Additionally, the age at which pesticide resistance develops decreases as the season progresses. This general pattern of sensitivity to herbicides, found in many plants, could be due to reduced herbicide penetration if cuticular development was responsive to environmental parameters (i.e. temperature, photoperiod, water relations). Therefore, correlation of photoperiod to sicklepod cuticular hydrocarbon content was undertaken and a second species in the same genus, coffee senna (*Cassia occidentalis* L.), was utilized for comparison.

RESULTS AND DISCUSSION

Growth-chamber grown sicklepod total hydrocarbon content composition changed rapidly with age and was significantly different at the 10% levels in all components analysed (Fig. 1). After 2 weeks, the components eluting at a relative elution temperature (RET) on both OV-1 and DEGS columns equivalent to C_{32} was a major constituent but *n*-tritriacontane (C_{33}) was the major constituent after 7 weeks. Thus, these data corroborate previous work in which age influences the pattern of hydrocarbon development and odd numbered alkanes are not present at rates ten times higher than even numbered compounds.[7] Additionally, these samples were extracted for external and internal hydrocarbons which have been

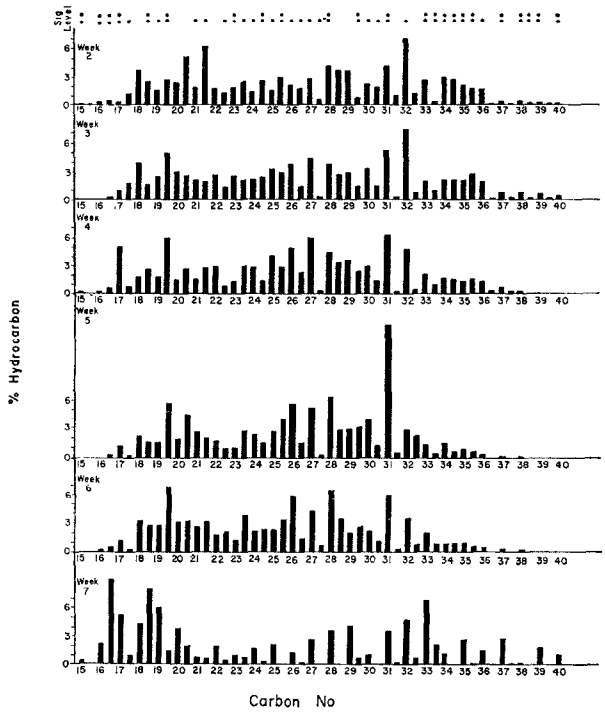

FIG. 1. VEGETATIVE TOTAL HYDROCARBON CONTENT (i.e. ALKANES, ALKENES, etc.) RESPONSE OF SICKLEPOD TO AGE. WEEKLY LEVELS WITHIN SINGLE COMPONENTS FOLLOWED BY THE SAME LETTER OR LETTERS ARE NOT SIGNIFICANTLY DIFFERENT AT THE INDICATED LEVELS (i.e.—UNMARKED = 10%; . = 5%; .. = 1%).

Each component concentration is the average of 20 analyses. Carbon numbers are those indentified by the relative elution temperature technique from known standards.

shown to produce results with higher concentration of even numbered hydrocarbons than when only external hydrocarbons are extracted.[7]

Photoperiod markedly influenced the composition of total vegetative hydrocarbons present in growth-chamber grown sicklepod (Fig. 2). Those components present at statistically different concentrations under various photoperiods are shown in Fig. 2. *n*-Hentriacontane (*n*-C_{31}) content increased from 4·5% to 11·7% of the total hydrocarbon present when the photoperiod was progressively decreased from 16 to 10 hr. The constituent eluting at an RET equivalent to C_{28} was present in lower quantities under a 14-hr photoperiod than in either 16- or 10-hr photoperiods. Hydrocarbons eluting between the C_{24} and *n*-pentacosane (C_{25}), (C_{24B}), were most concentrated under 14-hr photoperiods with significantly less of these components (C_{24B}) found under 16 and 10 hr photoperiods. Other changes in cesponse to photoperiod variation occurred. Thus altered photoperiodic influence caused the roncentrations of the individual hydrocarbon components to change. Each photoperiod caused a significantly modified sicklepod metabolism to allow concentration of one discrete component.

These measurements exhibit an unusual complexity of total vegetative hydrocarbon content from sicklepod grown in a growth-chamber. Herbin and Robbin[7] suggest that; (a)

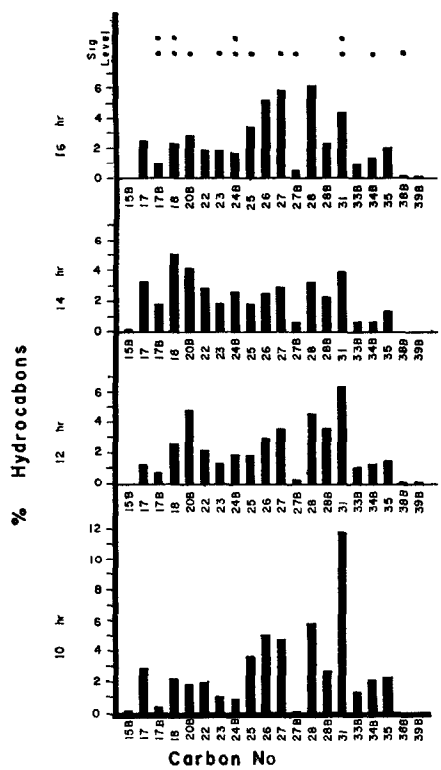

FIG. 2. SICKLEPOD SHOOT VEGETATIVE TOTAL HYDROCARBON (i.e. ALKANES, ALKENES, etc.) RESPONSE TO PHOTOPERIOD.

Photoperiod levels within a single component followed by the same letter are not significantly different at the indicated levels (i.e.—unmarked = 10%; . = 5%; .. = 1%). Each component concentration is the average of 30 analyses. Carbon numbers are those identified by the relative elution temperature technique from known standards.

TABLE 1. PERCENTAGE COMPOSITION OF LEAF EPICUTICULAR HYDROCARBONS

	Sicklepod*		Coffee-senna			
C_n	16 hr	12–13 hr	16 hr	12–13 hr	Rotunda holly	Tobacco
15				0·1		
			0·2			
16	0·2	0·8	1·4	0·5		
	0·4	4·2	3·3	26·8		
17	1·2	3·0	4·1	2·8		
		2·1	2·0			
18	0·7	3·1	3·8	1·1		
	0·5	3·1	3·7			
19	3·3	5·8	2·8	4·0		
	0·2	3·1	4·4			
		0·2	3·1			
			0·3			
20	1·4	4·0	4·6	1·3		
		8·1	5·0	4·8		
21	4·9	2·0	2·6			
	0·2	2·4	2·4	5·8		
22	2·3	5·0	5·7	8·7		
		2·5	3·4			
		1·0				
23	0·6	3·5	3·5	0·4		
24						
		1·6	+			
25	2·0	5·1	4·7	0·9	0·3	1·0
				0·2		
26	0·3	1·6	0·3	0·1		0·3
		1·1	1·0			
27	3·1	5·7	6·3	1·5	1·8	7·2
					a-28	0·6
28	3·2	5·6	4·5	1·5	1·3	
					i-29	2·4
29	3·5	7·6	14·3	3·4	18·2	8·6
					a-30	6·2
30	8·7	3·2	0·9	0·1	2·9	
					i-31	16·1
31	3·7	5·3	5·9	2·3	57·0	28·8
				1·1		
					a-32	11·2
32	34·9	1·6	0·7	2·0	3·9	
		1·4	0·6	4·7		
				0·2		
					i-33	7·4
33	7·1	4·4	3·7	22·4	10·4	9·0
	0·5		0·1	3·2	*a*-34	1·2
34	6·8	2·0			0·3	
35	0·9		0·1		3·9	
36	2·3		0·5			
	100·0	100·0	99·9+	99·9	100·0	100·0

Identifications of sicklepod, coffee senna, and rotunda holly constituents was by comparative GLC using butyl stearate as an internal standard and the relative elution temperature technique. Column conditions as listed in Fig. 3.

* In addition Sicklepod contained 0·3, 0·7, 0·2 and 6·3% of C_{37}, C_{38}, C_{39} and C_{40} compounds.

presence of even numbered alkanes as a major constituent must be due to misidentification, and (b) nonalternating type of pattern raises the suspicion of solvent contamination by high-molecular weight natural petroleum products. Additionally, the DEGS columns utilized in the above studies (Figs. 1 and 2) separate (a) branched from normal, and (b) saturated from unsaturated components of equal carbon number. Whereas, the nonpolar columns usually used for hydrocarbon analysis [i.e. silicone gums (SE-30), methyl silicone (OV-1)] do not separate saturated from unsaturated components of equal carbon number but do separate iso- and anteiso-components from normal alkanes. Thus, the sicklepod chromatograph complexity (Table 1) may be due to; (a) use of columns which are too discriminating, (b) extraction of intercellular hydrocarbons in addition to the epicuticular hydrocarbons (c) contamination, or (d) the presence of a highly complex hydrocarbon content in sicklepod

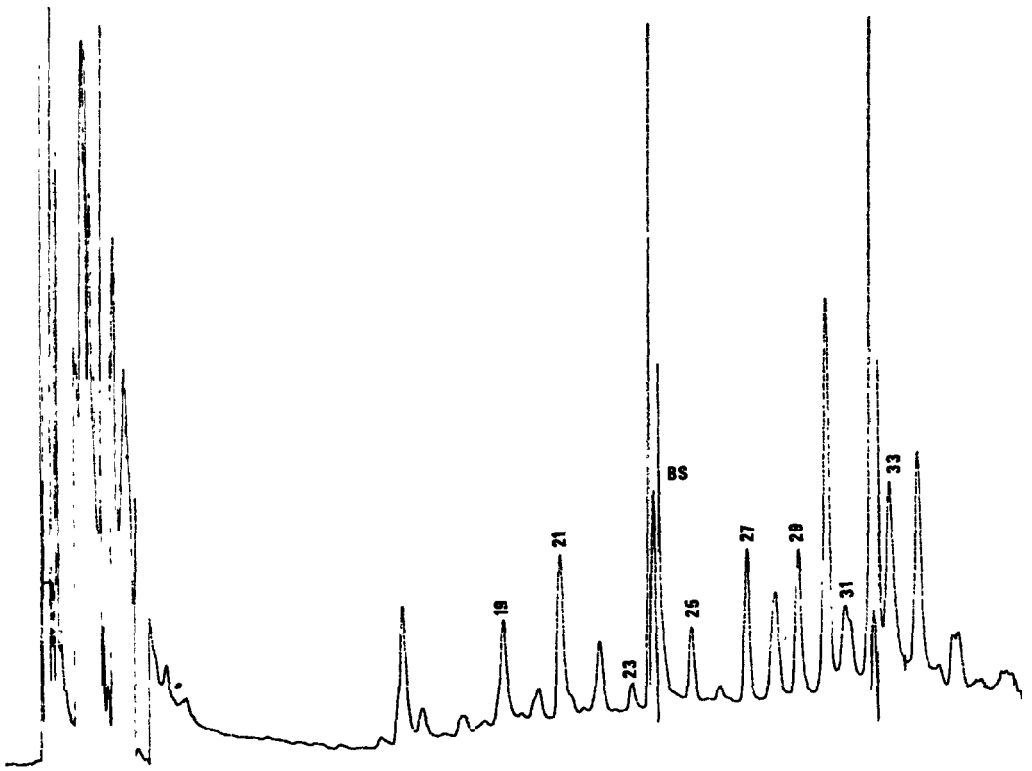

FIG. 3. EPICUTICULAR HYDROCARBON CONTENT OF SICKLEPOD GROWN IN THE GREENHOUSE IN A 16-hr PHOTOPERIOD.

Temperature programmed at 6°/min. from 70 to 330° on 12·4 m × 3 mm o.d. stainless steel columns containing 10% OV-1 on 100/120 mesh chromosorb W (AW) (DMCS). Numbers (i.e. 27) are alkane identifications based on relative elution technique comparisons of known standards to butyl stearate (BS).

Therefore, additional measurements were undertaken of epicuticular extractions chromatographed on OV-1 columns.

Evaluation of the GLC procedures utilized was attained by chromatography of a sample of tobacco (*Nicotiana tabacum* L.) hydrocarbons prepared in another laboratory.[8] This

[8] Sample supplied by P. E. KOLATTUKUDY.

chromatogram was comparable to that presented by Eglington *et al.*[9] in which the tobacco hydrocarbon constituents were identified by mass spectrometry as *n*-heptacosane (*n*-C_{27}), 3-methyl heptacosane (*a*-C_{28}), 2-methyloctacosane (*i*-C_{29}), *n*-nonacosane (*n*-C_{29}), 3-methylnonacosane (*a*-C_{30}), 2-methyltriacontane (*i*-C_{31}), *n*-hentriacontane (*n*-C_{31}), 3-methylhentriacontane (*a*-C_{32}), 2-methyldotriacontane (*i*-C_{33}), and *n*-tritriacontane (*n*-C_{33}). Therefore, the GLC procedures utilized herein were acceptable. The epicuticular hydrocarbon content of rotunda holly (*Ilex cornuta* L. var. rotunda) collected on this station is shown in Table 1.

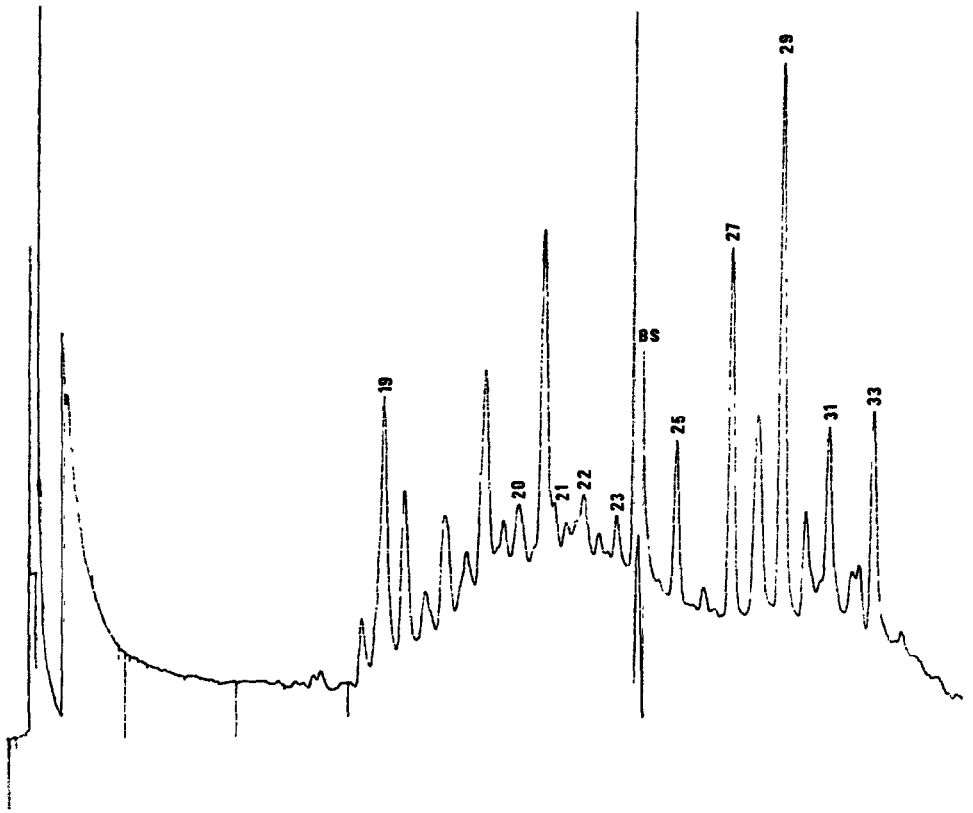

FIG. 4. EPICUTICULAR HYDROCARBON CONTENT OF SICKLEPOD GROWN IN THE GREENHOUSE IN A 12–13-hr PHOTOPERIOD.

GLC conditions the same as in Fig. 3. Numbers (i.e. 27) are alkane identifications based on relative elution technique comparisons of known standards to butyl stearate (BS).

Major constituents were *n*-C_{29}, *n*-C_{31}, *n*-C_{33}, and *n*-pentatriacontane (*n*-C_{35}) as identified by the RET method. Odd numbered alkanes predominate with even numbered alkanes being present as minor constituents. The lack of complexity in the rotunda holly analyses and a stable baseline demonstrated the absence of petroleum contamination in these epicuticular studies. And multiple short term (i.e. 20 sec each) washings of plant epicuticular hydrocarbons does not extract subepidermal lipid components from plant leaves.[10,11] Thus, the

[9] G. EGLINGTON, P. M. SCOTT, T. BELSKY, A. L. BURLINGAME, W. RICHTER and M. CALVIN, *Advances in Organic Geochemistry* Vol. 2, Pergamon Press, Oxford (1965).
[10] J. T. MARTIN, *J. Sci. Food Agric.* **11**, 635 (1960).
[11] D. A. HALL and L. A. DONALDSON, *Nature, Lond.* **194**, 1196 (1962).

complexity of the sicklepod hydrocarbon content may be due to, (a) extraction of internal lipids, or (b) a very complex hydrocarbon mixture in sicklepod. Figure 3 shows a highly complex epicuticular hydrocarbon content of sicklepod grown in a 16 hr photoperiod in a greenhouse.

Comparison of sicklepod epicuticular hydrocarbons extracted from plants grown in the greenhouse under 16 or 12–13 hr photoperiods (Figs. 3 and 4, respectively) clearly demonstrates changes of the epicuticular hydrocarbon content of sicklepod as the environmental conditions were altered. The major constituent in sicklepod grown in the greenhouse under 16 hr photoperiods was a compound eluting at the same RET on OV-1 and DEGS columns as C_{32} with subsidiary peaks at RETs equivalent to C_{30} and C_{34}. The major paraffin constituents of sicklepod grown in the greenhouse under a 12–13 hr photoperiod were the odd numbered alkanes *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31} and *n*-C_{33}. Due to the extreme anomaly of the results shown in Fig. 3, mass spectrometer measurements of the compound eluting at an RET equivalent to C_{32} were undertaken. Mass spectra show a parent ion at *m/e* 446 corresponding to that molecular weight. This molecular weight and fragmentation pattern indicated a C_{32} carbon chain.

Comparisons of coffee senna (*Cassia occidentalis* L.) epicuticular hydrocarbons extracted from plants grown in the same photoperiods as sicklepod demonstrated major changes in hydrocarbon content. Coffee senna grown in a 16 hr photoperiod had *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31} and *n*-C_{33} as major constituents representing 4·7, 6·3, 14·3, 5·9 and 3·7%, respectively, of the hydrocarbon content (Table 1). However, the same species grown under a 12–13 hr photoperiod exhibited a markedly different hydrocarbon content with a major component eluting between *n*-C_{16} and *n*-C_{17} (26·8% of the hydrocarbon present), and *n*-C_{33} which represented 22·4% of the epicuticular hydrocarbon present (Table 1). Thus, the epicuticular hydrocarbon content of two species of the genus *Cassia* have been shown to vary in differing photoperiods. In these two species, at least, hydrocarbon biosynthesis must be considered a reflection of a particular steady state condition existing at each particular site of hydrocarbon synthesis. Any modification of that steady state condition immediately leads to the establishment of a second steady state condition with a different hydrocarbon product.

EXPERIMENTAL

Growth-chamber plants. Twenty-five acid scarified (conc. H_2SO_4–20 min) sicklepod seed were planted in each of 120 pots. Thirty pots were placed in each of 4 environmental chambers maintained at 30° during the day and 20° at night. Total photosynthetic illumination was 10 hr/day from 24 Gro-Lux fluorescent lamps and nine 100 W incandescent bulbs in each chamber. Photoperiods in each environmental chamber were maintained at 10, 12, 14, or 16 hr by a single 100 W incandescent lamp in each environmental chamber. Five pots were harvested weekly from each environmental chamber and the plants were analysed for total hydrocarbon content.

Total lipids were extracted by the monophasic–$CHCl_3$–H_2O system.[12] After standing for 24 hr to allow the phases to separate and the emulsion to break, the volume of the $CHCl_3$ present was recorded (*ca.* 100 ml) and a sample (*ca.* 85–90 ml) was removed and placed into a 100-ml volumetric flask. The $CHCl_3$ was evaporated to *ca.* 10–15 ml under N_2 at ambient temp. or at 40° on a water bath.

Methylation of the fatty acids was attained by the addition of 2 ml conc. H_2SO_4 and *ca.* 70 ml absolute MeOH and heating the mixture on an oil bath at 55° for 24 hr. The lipids were separated from the MeOH transesterification mixture by addition of H_2O and extraction with light petroleum (b.p. 30–60°). The light petroleum was evaporated under N_2 at ambient temp. and the lipids were stored in rubber stoppered serum vials at −10° until ready for TLC.

TLC separation of the lipids, which were dissolved in 200 μl light petroleum, was attained by applying a 100 μl aliquot of the solution, to a previously developed 250 μ silica gel TLC plate. Developing solution was $CHCl_3$–benzene (1:1). After development the TLC plates were sprayed with a Rhodamine B-fluorescein

[12] E. C. BLIGH and W. J. DYER, *Can. J. Biochem. Physiol.* **37**, 911 (1959).

mixture and the hydrocarbon and fatty acid methyl ester spots were identified under UV light (respective R_fs *ca.* 0·90 and 0·50). Respective lipid spots were individually scraped from the TLC plates and eluted from the adsorbent through anhydrous, powdered Na_2SO_4 with light petroleum evaporated to dryness under N_2 at ambient temp. and stored at −10° until quantified by GLC.

All solvents used in extraction, transesterification, and TLC were chromatography grade except for the light petroleum which was glass redistilled. All gases used for evaporation or GLC were filtered through Linde molecular sieve 5A.

When ready for GLC, 100 μl light petroleum were added to the serum vial, the sample was heated, and 10 μl were injected into a 5751A Hewlett Packard Dual Hydrogen Flame GLC equipped with 2·4 m × 3 mm o.d. stainless steel columns containing 10% diethyleneglycolsuccinate (DEGS, C-6, stabilized) on 80/100 mesh Chromosorb W (AW) (DMCS). Oven temperatures were programmed from 70 to 220° at 4°/min with an upper limit hold of *ca.* 12–15 min.

Identification of the compounds was made by the relative elution technique[13] (RET) and by constructing an RET curve from known standards (HC 19251 and HC 19254—Applied Science, State College, Pa., U.S.A.) which contains the hydrocarbons of carbon chain length 11, 13, 14, 14:1, 15, 15:1, 16, 16:1, 18, 18:1, 19, 19:1, 20, 20:1, 22, 24, 28, 32 and 36. Quantitation was by the percentage of the area under the curve as measured by a digital integrator which also gave the time of peak elution. Statistical analysis was conducted by computer on a standard split plot design. The Duncan's multiple range test was used to separate means of the various component concentrations.

n-Octadocosane (C_{28}) was used as the reference standard for the RET identifications. Since several components elute between the *n*-alkanes which have not been identified because of lack of standards for comparative GLC, the components eluting between the identified *n*-alkanes were lumped into a single group and are reported as the "B" series. Thus the series C_{27}, C_{27B}, C_{28}, is equivalent to *n*-heptacosane, all components eluting between C_{27} and C_{28}, and *n*-octacosane.

Greenhouse plants. Two hundred sicklepod and 200 coffee senna seeds treated as described in the previous section were planted in greenhouse pots. Half of the pots were placed in a greenhouse with photoperiods extended to 16 hr by incandescent lamps and the other half of the pots were placed in an identical greenhouse without extended illumination and natural photoperiods of 12–13 hr.

After 8 weeks growth, the plants were harvested and the epicuticular hydrocarbons of individual samples of leaflets from ten plants/sample were extracted with 3 × 20 ml $CHCl_3$. The remainder of the transesterification, separation, and TLC conditions were identical to those described previously. GLC conditions were identical to those described previously except that 10% OV-1 on 80/100 Chromosorb W (AW) (DMCS) was used and the temperature range was 70–300°.

Field grown plants. Rotunda holly leaves were collected from plants growing on the campus. Epicuticular hydrocarbon extraction, transesterification, separation, TLC and GLC procedures were identical to those described for greenhouse grown sicklepod and coffee senna.

MS. Using OV-1 columns and isothermal oven temperatures at 290°, repeated collections of the C_{32} compound were made using the method of Levy.[14] Samples of *n*-C_{32} were similarly collected from the HC-19254 calibration mixture. A Perkin–Elmer 900 gas chromatograph interfaced with a Hitachi Perkin–Elmer RMU-7 double focusing mass spectrometer was utilized for GC-MS analysis. A silanized Watson–Biemann separator provided sample enrichment. All spectra were recorded with 70 eV electron energies.

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[13] J. A. SCHMIT and R. B. WYNNE, *J. Gas Chromatog* **4**, 325 (1966).
[14] J. LEVY, R. R. DOYLE, R. A. BROWN and F. W. MELPONDER. *Analyt. Chem.* **33**, 698 (1961).

Key Word Index—Cassia obtusifolia; Leguminosae; sicklepod; hydrocarbons; cuticle; photoperiodic variation.