PLANT LEAF-SURFACE LIPIDS:

AGE VARIATION PATTERNS

IN

COFFEA ARABICA L. AND *THEA SINENSIS* L.

by

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A thesis submitted in part fulfilment for the Degree of Master of Science in the University of Nairobi.

1977

MEMORANDUM

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with my approval as University supervisor.

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SYNOPSIS

As early as 1973, an antifungal compound was reported in a fraction from the cuticular wax of *Coffea arabica* L. leaf and berry, and an attempt to correlate the activity of this fraction with the resistance of the plant species to Coffee Berry Disease in the field was made. The current study reports the results of examination of this "active fraction" by TLC. Its activity is attributed to caffeine and either long-chain primary alcohols or triterpenoids.

The qualitative and quantitative composition of tissue surface of both *Coffea arabica* L. and *Thea sinensis* L. is reported. Caffeine (and possibly its analogues) is present in the chloroform extracts of both species. Triterpenoids are absent in the tea leaf and very young coffee berry extracts.

An age variation in the relative concentrations and chain length distribution patterns in the leaf wax alkanes, primary alcohols and free fatty acids of both coffee and tea has been established. The use of these wax constituents in delimiting plant taxa without due regard to the uniformity of the physiological age of the particular tissue examined would therefore appear misleading. In all three wax fractions of both coffee

and tea, the results indicate that homologs of increasing chain length are deposited as the leaf ages. The alkane distribution patterns of both species are such that the coffee leaf may be regarded as a *n*-nonacosane accumulator and the tea leaf a *n*-hentriacontane accumulator.

From a biosynthetic point of view, very low coefficients of linear correlation exist between the *n*-alkanes and their "parent" fatty acids in the case of coffee and fatty acids and primary alcohols in the case of tea. Except for the senescent leaf, the very young and expanded tea leaf alkanes and fatty acids are highly correlated. So are the fatty acids and primary alcohols of the coffee leaf at all stages of development.

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SPECIAL ABBREVIATIONS USED

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CHAPTER 1

INTRODUCTION

1.1 General

The tea plant (*Thea Sinensis*) is one of the most economically important of the 500 species in the family Theaceae composed of 16 genera [Perry and Greenwood, 1972]. Tea planting is practised in several countries, including South-East Asia and East Africa, for both local consumption and export. According to Eden (1965) the cultivation of tea is a horticultural operation carried out on an agricultural and industrial scale.

The genus *Coffea* is of economic importance and belongs to the Rubiaceae family, one of the largest families of dicotyledonous plants composed of about 500 genera and 6000 species in the form of trees, shrubs and herbs [Perry and Greenwood, 1972], one such species being the coffee tree (kept to shrub dimensions by pruning) from whose berries is processed the coffee bean (and in turn, the drink) only second to petroleum in terms of trade in the world market. Amongst the four physiological groups of coffee species [Rodrigues *et al.*, 1975] the *Eucoffea* section, subgroup *Erythrocoffea*, comprises, inter alia, the species *Coffea arabica* L (Arabican Coffee) and *C. caniphora* Pierre (Robusta Coffee) both of which are

widely cultivated in Tanzania, Kenya and Uganda. Uganda grows more of the robusta type than either Kenya or Tanzania. *Coffea arabica*, in its various varieties, is especially marked for its best liquoring quality.

1.2 Berry Disease of *Coffea arabica*

Like many other plants, the coffee tree is not immune to attack by fungal pathogens. While the resistance of Arabican coffee to leaf rust and control of the causal fungus, *Hemileia vestatrix*, remains the object of dynamic research in the worst-hit areas of South America [Rodrigues *et al.*, 1975 and Schieber, 1975], Coffee Berry Disease (CBD) is yet another aspect of concern particularly in agricultural East Africa since under conditions of severe attack total loss of crop may be experienced.

McDonald (1925a) was the first to recognize the economic implications of the onset of CBD in Kenya. He observed considerable damage caused by *H. vestatrix* resulting in defoliation of coffee leaves [McDonald, 1925b]. The author described the symptoms of CBD [McDonald, 1926] as a small dark-brown spot which gradually enlarges in area, becoming slightly sunken and eventually involving the whole berry, the pulp of which becomes brown, hard and brittle. CBD is

attributed to a virulent strain of the fungus *Colletotrichum coffeanum* Noack which also causes an anthracnose of leaves. The nature of the host-parasite relation became a subject of intensive laboratory and field experimentation, the results of which were reviewed by Rayner (1952).

Nutman and Roberts (1960) observed variations in resistance to the pathogen of different varieties of *C. arabica* and the differing degree of susceptibility of the crop at different stages of maturity. The author reported that, while the flowers, very young berries and fully ripe fruit are extremely susceptible, the pre-ripening stage (hard green berry) "is a lengthy period during which the endosperm matures and develops without appreciable change in the size of the green berry, and during this stage a phase of semi-resistance to the fungus occurs". From the results obtained by these workers and similar ones by Firman (1964), preliminary observations by Hocking (1967) led him to believe that a heat-labile antifungal compound may be present in coffee berries and that a phytoalexin response (Section 2.3) may be operative in determining the resistance of *C. arabica* to CBD.

1.3 Resistance of *Coffea arabica* varieties to CBD

Lampard and Carter (1973) investigated the possibility that resistance to CBD was associated

with the presence of a substance or substances either present in the berry/leaf as a consequence of natural growth or secreted in response to attack by the fungus. These workers reported the presence of an antifungal activity in coffee berry and leaf (epi-) cuticular wax fractions which was inhibitory to the CBD causal microorganism. Although they could not identify the chemical nature of the active principle, they correlated its activity with the reported field resistance of Rume Sudan, Hybrido de Timor, Blue Mountain, Geisha 10, Harrar, SL_{28} and N_{39} varieties to CBD but failed to do the same for varieties K_7 and SL_{34}. The cuticular wax (obtained by 5 min. chloroform refluxing) was partitioned between water (fraction H) and chloroform, the latter evaporated (red. pressure, 30°C) to low volume and precipitated (fraction B) with nitromethane. Following filtration, the filtrate was again evaporated to dryness, taken up in chloroform and precipitated (fraction CA) with n-hexane. Filtration afforded them the active fraction CH. The activity of CH was determined by direct bioautography on Silica Gel F_{254} thin layers *[Homans and Fuchs, 1970]* with chloroform or n-hexane-ethyl acetate (1:1) as developing solvents. The authors noted that the fungus could not sporulate at a zone hRf 50 (developing solvent not specified). A slight inhibition zone at lower hRf was observed though not consistently.

These workers reported that the leaf cuticular wax contained this fungitoxic principle but at a lower level than the berry, while, on the other hand, they stated that under Kenyan conditions the leaves are not normally attacked. In the wake of this statement it would therefore appear that the reported principle is unimportant in the defense of the plant to attack by the fungi or else the level of the active principle would have been higher in the leaf than the berry, all other things being equal. The presence of this inherent resistance activity in the waxes of varieties K_7 and SL_{34} and the lack of correlation between the levels of this activity and the reported field resistance led Lampard and his colleague to the thought that a phytoalexin-type response (internal resistance) may be operative in these varieties.

1.4 Chemical composition of the antifungal fraction in Coffee leaf wax

Using the epicuticular wax obtained by refluxing expanded (see Plate 3,A) coffee leaves (from field observations the most resistant stage to CBD in the life cycle) for 5 min. in chloroform, the fractionation procedure adopted by Lampard and Carter (1973) was repeated by us.

The non-active fractions B and CA, and the active fraction CH were monitored on pre-activated (see Section 3.3.3) Silica Gel PF_{254} thin layer films (250 μm thick). These fractions were spotted along with expanded coffee leaf and hard green berry whole waxes, standard n-eicosan-1-ol, n-docosanoic acid, authentic caffeine (1, 3, 7-trimethylxanthine) and a triterpenoid-rich fraction obtained by PLC of the cuticular wax. A total of four plates spotted in the same format were developed in ascending direction in solvent-vapour saturated tanks, two in chloroform and the other set in n-hexane-ethyl acetate (1:1) solvent system. In each set, one chromatogram was spray-visualized with 1 per cent methanolic phosphomolybdic acid reagent and the other with the Liebermann-Burchard reagent formulated by addition of concentrated sulphuric acid (1 ml) to an ice-cold mixture of acetic anhydride (20 ml) in chloroform (50 ml).

The major components of CH, judged from their spot intensities as very strong (vs) or strong (s), were identified as caffeine (vs) [hRf = 4 (chloroform), 5 (compound solvent)], triterpenoids (vs) [hRf = 9 (chloroform), 60 (compound solvent)], alkanes (vs) [hRf = 97 (chloroform), 100 (compound solvent)] and primary alcohols (s) [hRf = 39 (chloroform), 85 (compound solvent)] ___ Plate 1A, B, C and D.

PLATE 1

TLC of antifungal fraction from expanded leaf epicutioular wax of Coffea arabica

Adsorbent = Silica gel PF_{254}; Solvent = $CHCl_3$

Location: A = Phosphomolybdic acid reagent
B = Liebermann-Burchard reagent

Samples:
1 = expanded coffee leaf wax;
9 = hard-green berry wax; 8,7,6 and 5 = CH, CA, B and triterpenoid-rich fractions respectively; 4 = authentic caffeine; 3 = docosanoic acid; 2 = eicosanol.

PLATE 1

TLC of antifungal fraction from expanded leaf epicuticular wax of Coffea arabica

Adsorbent = Silica gel PF_{254};

Solvent = n-hexane-ethyl acetate (1:1)

Location: C = Phosphomolybdic acid reagent
D = Liebermann-Burchard reagent

Samples:

1 = expanded coffee leaf wax; 9 = hard-green berry wax; 8,7,6 and 5 = CH, CA, B and triterpenoid-rich fractions respectively; 4 = authentic caffeine; 3 = docosanoic acid; 2 = eicosanol.

Judging from these results and the sample bioautographic plate published by Lampard and Carter (1973), it can reasonably be deduced that the main fungal inhibition zone was due to primary alcohols and the low hRf zones due to both caffeine and triterpenoids (chloroform as developing solvent) while if n-hexane-ethyl acetate (1:1) was used as the developing solvent the main inhibition zone may be attributed to triterpenoids and the lower zones exclusively to caffeine. While it seems unlikely that long chain primary alcohols could be fungistatic, caffeine and the triterpenoids may indeed be fungistatic in view of their high concentrations in both the CH fraction and whole waxes from berry and leaf (Section 4.2).

With the aim of assessing the fungitoxicity of the individual CH constituents, this fraction is currently being investigated by us in conjunction with the Coffee Research Station, Ruiru, Kenya and the outcome will be reported elsewhere.

1.5 Objectives of the present study

While a number of chemical constituents present in chloroform extracts of plant surfaces are reported to possess fungistatic activity, for example long chain fatty acids (Martin and Juniper, 1970),

triterpenoids [Harborne, 1973] and caffeine, all of which are present in the chloroform leaf-extracts of coffee and tea (with exception of triterpenoids in the latter) [current report], their importance in determining the resistance of *Coffea arabica* to CBD has not been established. The phytoalexin 5-hydroxytryptophan is reported to accumulate in some members of the Leguminosae in response to attack by larvae of some army worm species [Bell *et.al.*, 1976], but the long chain fatty acid derivatives of this acid; 5-hydroxytryptamides of eicosanoic, docosanoic and tetracosanoic acid, are, however, reported as natural constituents of the superficial wax of *Coffea arabica* beans [Harms and Wurziger, 1968]. The possibility that 5-hydroxytryptophan is released from its long chain amide when hydrolysed by enzymes secreted by a pathogen is worth further investigation in the case of *Coffea arabica*. On the other hand, the wax of the tea leaf was reported to contain some fractions that stimulate the germination of the spores and the growth of the germ tubes of *Pestalotia theae* and others that completely inhibit spore germination [Venkata Ram, 1962].

The inconclusive results obtained by Lampard and Carter (1973) are curious enough to warrant a detailed investigation into the chemical constitution

of the leaf and berry waxes of *Coffea arabica*. There are observed differences in susceptibility to CBD between *C. arabica* varieties, and within the same variety, at different stages of maturity of a particular tissue. The current study aims, initially, at establishing any differences in the composition of the wax constituents of both coffee and tea at different stages of maturity of the leaf with the ultimate purpose of establishing if a correlation exists between the presence or absence of active chemical components in the coffee leaf surface wax and the plant's resistivity to CBD at a particular stage of development.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure and Composition of the plant cuticle

The aerial surface of the cells of most Angiosperms and Gymnosperms are covered by a protective non-cellular membrane, the cuticular membrane, whose chief components are cutin and waxes. By virtue of its chemical composition, cutin, a relatively rigid mesh-work of cross-esterified fatty and hydroxy fatty acids chiefly of the C_{16} - and C_{18} - families (Figure 1), is regarded as the main structural frame-work of the cuticular membrane. The wax embedded within the cuticular membrane, the cuticular or occluded wax, is considered distinct from that exuded to its surface, namely the superficial or epicuticular wax. The region where the membrane merges into the outer walls of the epidermal cells is an admixture of cutin, cellulose and a class of complex polysaccharides giving positive staining-tests for pectin (Martin, 1964). Electron microscopy techniques have been used to demonstrate the structures of leaf cuticular membranes and observations of the electron micrographs obtained have shown remarkable structural variations from one plant species to another (Martin and Juniper, 1970), the specific structure of the cuticle being influenced by the genetic background and ploidy of the plant, climate, humidity

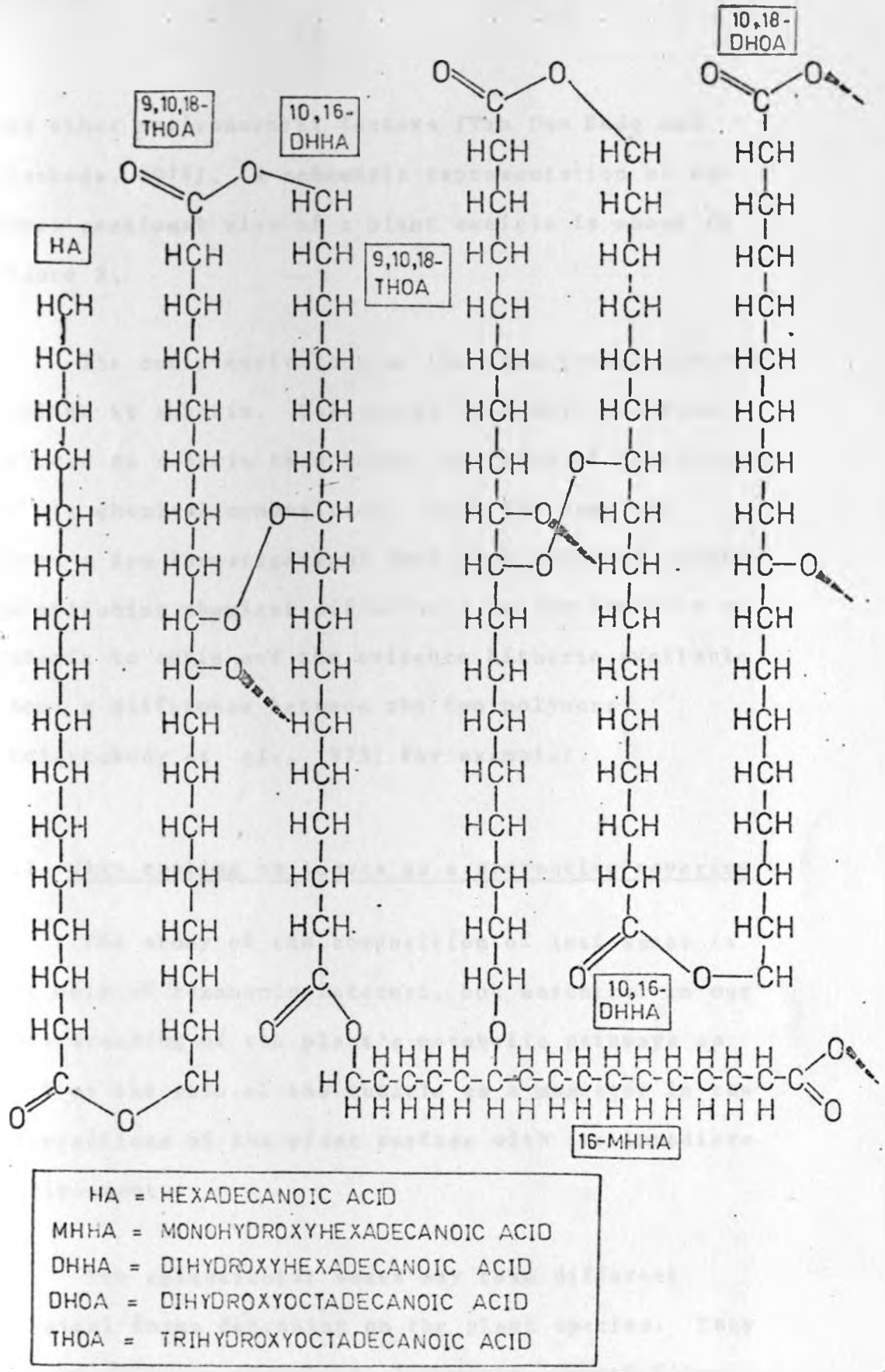


Figure 1 Arrangement of the Cutin framework

[Van Den Ende and Linskens, 1974]

and other environmental factors [Van Den Ende and Linskens, 1974]. A schematic representation of the cross-sectional view of a plant cuticle is shown in Figure 2.

The cutin-equivalent of the underground parts of a plant is suberin. Relatively less work has been devoted to suberin than cutin, in terms of elucidation of its chemical composition. Over the past few years a few investigations have been directed towards establishing chemical differences or similarities of suberin to cutin and the evidence hitherto available shows a difference between the two polymers [Kolattukudy *et al.*, 1975; for example].

2.2 Waxy coating on leaves as a protective covering

The study of the composition of leaf waxes is not only of taxonomic interest, but essential in our understanding of the plant's metabolic pathways as well as the role of the cuticle as a mediator in the interactions of the plant surface with its immediate environment.

The epicuticular waxes may take different physical forms depending on the plant species. They may be rodlets, platelets, filaments, smooth films or crystals for example [Martin and Juniper, 1970]. That on the surface of the coffee leaf was found to

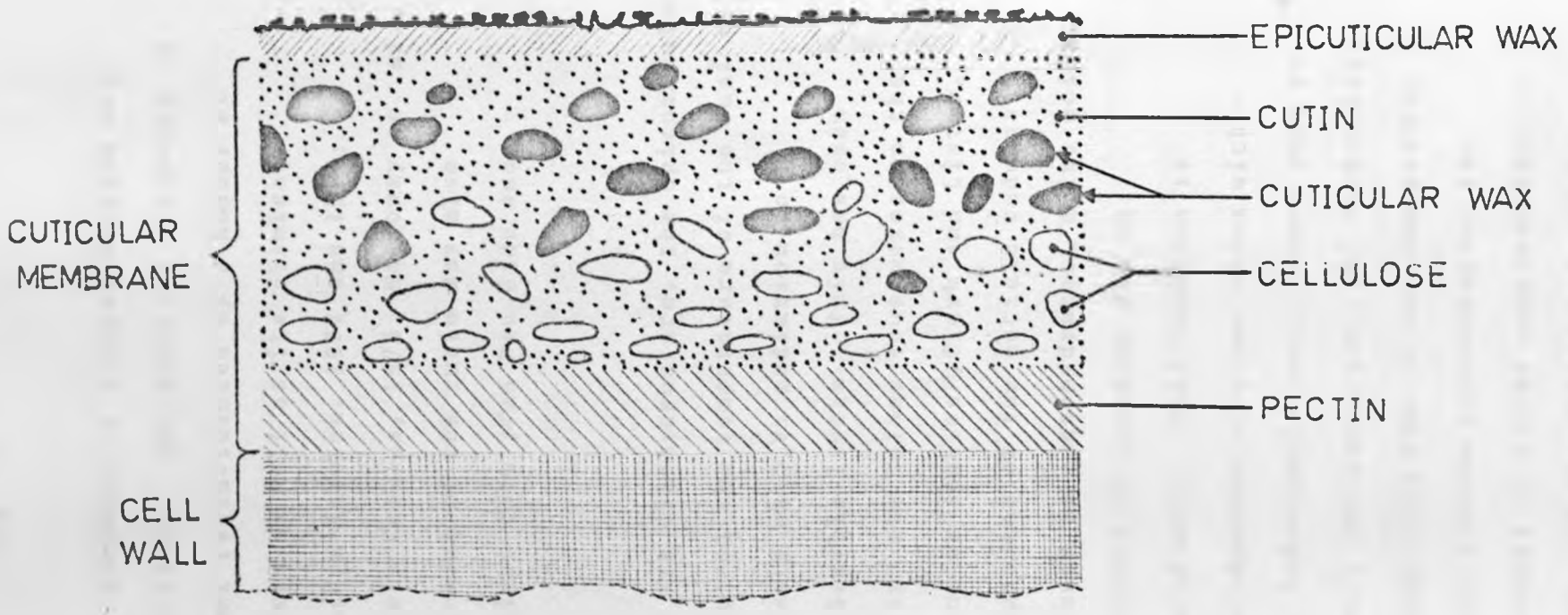


Figure 2 Schematic representation of the plant cuticle
[Van Den Ende and Linsken (1974)]

be granular and no evidence of crystallization was observed (Kabaara, 1973). The physical structure of the epicuticular wax is influenced by a number of environmental factors as well as its chemical composition (Wettstein-Knowles, 1972 and 1974; Netting and Wettstein-Knowles, 1973; Holloway *et. al.*, 1976). The arrangement of the cutin may also determine its physical form (Van Den Ende and Linskens, 1974).

Depending on its ultrastructure, the epicuticular wax deposit may control the temperature of the leaf by reducing the intensity of U.V. radiation to physiologically tolerable levels. This has been shown to be true with the filamentous waxes on the needles of some conifer species (Clark and Lister, 1975). The wax component of the cuticle reduces loss of nutrients such as carbohydrates, amino acids and inorganic nutrients by leaching due to precipitation, dew or mist. This function is facilitated by the presence of a wax bloom with a high proportion of hydrophobic constituents such as alkanes which greatly influence the leaf wettability properties. Although this type of wax composition presents yet another problem necessitating the addition of surfactants to foliar nutrient sprays, it has a role to play in the defense of the plant against fungal pathogens (Martin, 1964). Before a

fungal pathogen can establish an infection peg, the water innoculum bearing it must have landed successfully on the surface of the host-plant. In addition to the phyllosphere microflora, resident on the host's surface, that may suppress the fungal invasion (Martin and Juniper, 1970), hydrophobic constituents in the superficial wax may repel the water-droplets carrying the invader. Chemical substances inhospitable to phytopathogenic fungi occur on plant surfaces either as components of the natural wax or as exudates from the internal cellular tissues of the cuticle.

2.3 Phytoalexins and disease resistance

The failure of a fungus to establish a post-infection peg, which is a prerequisite for development of the disease, is attributed to the ability of the plant to respond by accumulating chemical substances inimical to the growth and development of the pathogen.

These antimicrobial metabolites, called phytoalexins, are absent or present in very low concentrations in healthy plants. Their accumulation in or around the cells of the damaged tissue is regarded as a defensive reaction of the plant to the invading parasite. The role of

phytoalexins in relation to plant diseases has been reviewed by various authors [Hare, 1966; Kosuge, 1969; Deverall, 1972; Ingham, 1972; Kuć, 1972a, b].

A great majority of the known phytoalexins are isoflavonoids [McClure, 1975]. VanEtten (1973) identified another antifungal isoflavan from *Phaseolus vulgaris* tissues as the 2′ - methyl ether of phaseollinisoflavan. Sativan [R(-)-7-hydroxy-2′,4′-dimethoxy isoflavan] and vestitol [(-)-7, 2′-dihydroxy-4′-methoxy isoflavan] may also function as phytoalexins in the leaves of *Lotus corniculatus* [Bonde *et al.*, 1973]. The induced isoflavan from the leaves of *Medicago sativa* was reported as (-)-2′-methyl vestitol [Ingham and Millar, 1973]. The phytoalexin in sugar beet leaves, *Beta vulgaris*, was identified by Geigert *et al.* (1973) as 2′-methyltlatlancuayin. The innoculation of the roots of *Pastinaca sativa* with several fungal species resulted in accumulation of xanthotoxin [Johnson *et al.*, 1973]. Sesquiterpenoid aldehydes act as phytoalexins in some species of the family Malvaceae [Bell *et al.*, 1975]. Ingham (1976) reported the phytoalexin from ground nuts, *Arachis hypogaea*, as 3, 5, 4′-trihydroxystilbene. Several pterocarpans and other isoflavonoids have been implicated as phytoalexins. Their antifungal activity in relation to the 3-dimensional shape of

the molecules was examined by VanEtten (1976). Ingham (1977) isolated two new phytoalexins from diseased leaves of *Anthyllis vulneraria* and five *tetragonolobus* species and identified them as 7, 4'-dihydroxy-2'-methoxy - and 7, 2', 4' - trihydroxyisoflavans.

The plant's susceptibility to a particular disease is influenced by a number of environmental factors including temperature, soil moisture, atmospheric humidity, light duration and its intensity, the pH of the soil and its nutrient content (Colhoun, 1973, Pratt and Mitchell, 1976). The major elements of plant nutrition are nitrogen, phosphorus and potassium. It is, however, the form of nitrogen available to the host or pathogen that affects disease severity or resistance rather than the amount of nitrogen (Huber and Watson, 1974).

2.4 The Chemistry of plant waxes

2.4.1 General distribution and type

Waxes are common to the surfaces of leaves, stems, flowers, pollen and fruits (Eglinton and Hamilton, 1963; Douglas and Eglinton, 1966); and possibly to grain testa (for example Briggs, 1974). They are end-products of the plant's metabolic processes (Martin and Juniper, 1970), the nature and

composition of which varies considerably with the evolutionary state of the plant, the type of tissue under consideration and the age or stage of development of the particular tissue from which the wax is extracted [Herbin, G.A.: personal communication].

Generally the composition of plant waxes is of a complex nature but the more common constituents are long-chain hydrocarbons, esters, ketones, aldehydes, alcohols and acids. Table 1 shows the general spectrum of the compounds that may be expected from plant waxes as well as the form in which each of these constituents may occur.

2.4.2 Hydrocarbons

Amongst the constituents of the waxy coating on the surface of plants, hydrocarbons, saturated or unsaturated may form an appreciable percentage of the wax.

Of the unsaturated hydrocarbons, polyacetylenes have been reported mostly from the plant family Compositae [Robinson, 1967] of which they are characteristic [Bu'lock, 1966]. Lam *et al.*, (1968) identified their presence in *Dahlia coccinea* strains (Compositae) and more recently Lam and Drake (1973) reported their presence in the light petrol flower-extracts of *Senecio jacobea* (Compositae)

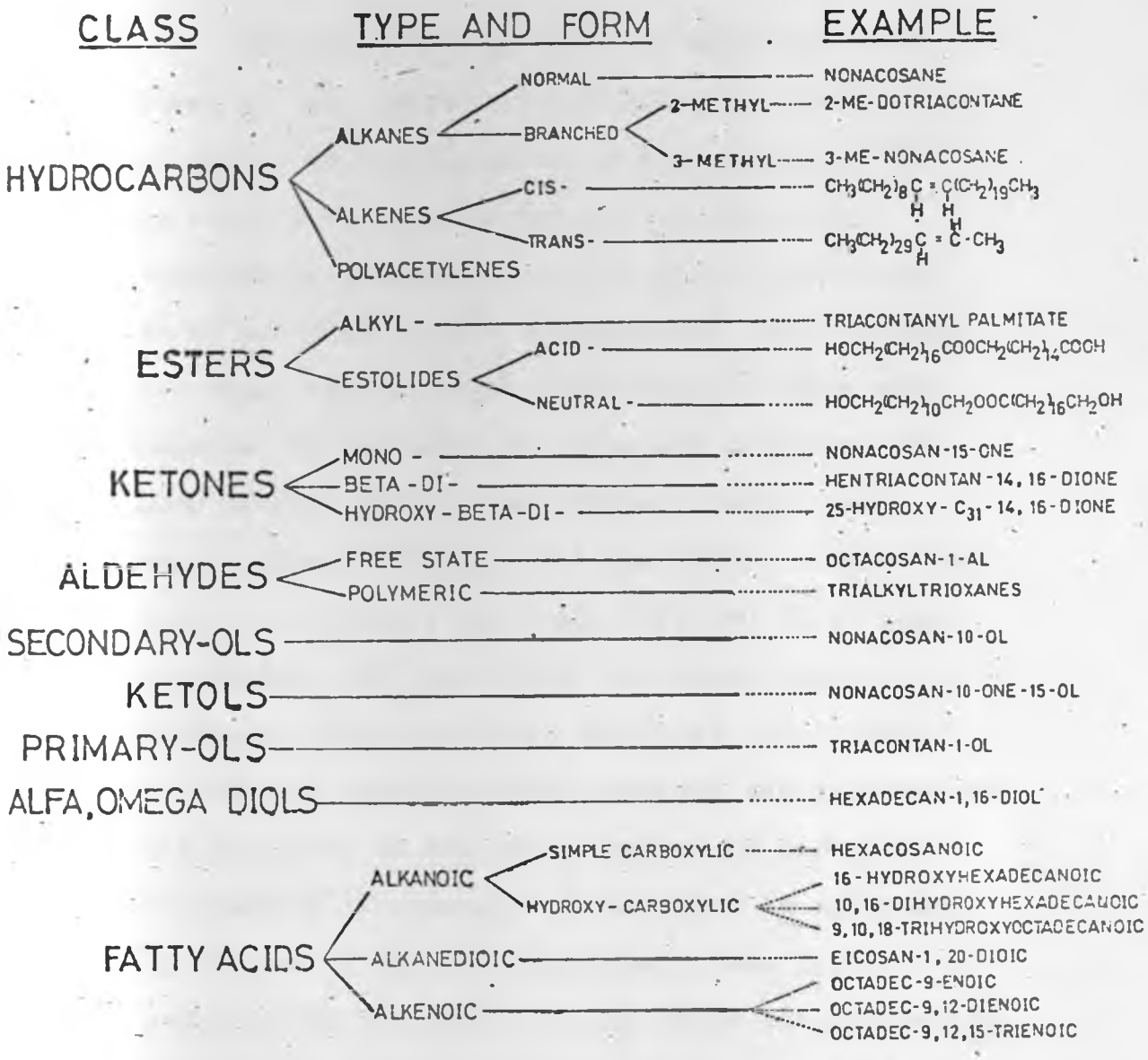


Table 1 Common Constituents of plant waxes

and established their absence in the roots and leaves of that species.

Alkenes, first detected in sugar-cane wax [Šorm *et al.*, 1964] occasionally occur among the cuticular hydrocarbons, as an homologous series. An even to odd carbon number preference was reported in *Lycopodium* species [Lytle and Sever, 1973] and citrus fruit epicuticular waxes [Nordby and Nagy, 1977]. Their occurrence has also been reported in the waxes of style and filaments of *Aloe* species [Herbin and Robins, 1968c], some *Rosa* species [Wollrab, 1968 and 1969], citrus fruit juice sacs [Nordby and Nagy, 1972 and 1975; Nagy and Nordby, 1973 and 1972a, b], Mosci spores [Karunen, 1974] and ferns [Lytle *et al.*, 1976]. Tulloch and Hoffman (1976) reported the presence of cis-9-alkenes in the wax of spikes of *Agropyron intermedium* (Graminae). In addition to aplotaxene (1, 8, 11, 14-heptadecatetraene), Yano (1977) reported the isolation for the first time (from the hydrocarbon fraction of the fresh-root oil of *Cirsium japonicum* (Compositae) obtained by ether extraction of the plant tissue) of tetrahydroaplotaxene (1, 8-heptadecadiene), reportedly a biogenetically important intermediate in the conversion of oleic acid to the C_{17}-acetylenic compounds; dihydroaplotaxene (1, 8, 11-heptadecatriene) and hexahydroaplotaxene (1-heptadecene).

Both cis- and trans- alkenes occur in waxes but the cis- forms are the most frequently encountered.

Paraffinic hydrocarbons were first identified by crude chemical methods when they were isolated from ether extracts of expressed cabbage leaf fluids (Chibnall and Channon, 1927) and their identity reported as n-nonacosane (Channon and Chibnall, 1929) and n-hentriacontane (Chibnall *et al.*, 1934). With the advent of more sophisticated instrumentation such as GLC and GLC-MS combination, these early investigations were confirmed and expanded (for example. Purdy and Truter, 1963c; Horn *et al.*, 1964; Kollatukudy, 1965; Hill and Mattick, 1966; Laseter *et al.*, 1968).

Normal alkanes are universally present as components of cuticular waxes of higher plants (Douglas and Eglinton, 1966). More often than not, they occur as an homologous series, the common range being C_{21} to C_{37} although some homologous series extend down to very low carbon numbers (Eglinton and Hamilton, 1967) and individual chain lengths may be present in overwhelmingly higher concentrations than others. In such a series, the odd carbon number homologs, C_{29} or C_{31} or C_{33} are frequently the major (Eglinton and Hamilton, 1963) although

cases where C_{27}, C_{25} or C_{23} chain lengths predominate are now known [for example: Carnduff *et al.*, 1966; Corrigan *et al.*, 1973, Suzuki *et al.*, 1973].

Quantitatively, the normal alkane portion of the total hydrocarbon fraction in leaf waxes obtained by 30 sec. immersion in chloroform may vary from a fraction of 1 percent as in *Prosopis spicigera* (Leguminosae) [Jewers *et al.*, 1976], *Eucalyptus* (Myrtaceae) and *Pinus* (Pinaceae) species [Herbin and Robins, 1968a, b] to over 90 percent as in *Solandra grandiflora* (Solanaceae) [Herbin and Robins, 1969].

Since, with only a few exceptions [for example: Jackson, 1971, Agarwal and Rastogi, 1974, Jewer *et al.*, 1976], a plant wax fraction is almost always composed of an homologous series, it is evident that the absolute dependence on one or more of such physico-chemical methods as elemental analysis, m.p., m.m.p., TLC, co-TLC, IR and NMR in establishing and characterizing the constituent members of the wax fraction, with absolute disregard to such exact methods as GLC or GLC-MS combination, can lead to dubious and unexpected results. For one thing, an homologous series of a pure wax-constituent would not be expected to give sufficiently discriminating results, under those methods, from an authentic

fatty homolog of the series. It is therefore highly unexpected that Dominguez and Gutierrez (1972) identified octacosene (m.p.) from the light petrol extract of *Chiranthodendron pentadactylon* (Sterculiaceae); Iyer *et al.* (1972) reported n-dotriacontane (elemental analysis, m.p.) as the only n-alkane in the hydrocarbon fraction of *Gisekia pharnaceoides* (Aizoaceae); Domínguez *et al.* (1972) reported n-triacontane (m.p., IR, NMR) from *Conyza filaginoides*; Ulubelen and Baytop (1973) identified the hydrocarbons of the light petrol/chloroform leaf-extract of *Euonymus latifolius* (Celastraceae) as n-octacosane and n-triacontane (TLC, IR, m.p., NMR); Abramson and Kum (1973) reported n-nonacosane (m.p.) as the only hydrocarbon in the light petrol leaf-extract of *Gymnocladus dioica* (Leguminosae) while Gupta *et al* (1977) identified n-triacontane (m.p., m.m.p., TLC, co-TLC) from the ether extract of the seeds of *Psoralea corylifolia* (Leguminosae); Domínguez *et al.* (1974) characterized n-dotriacontane (m.m.p., co-TLC, IR, NMR) from petrol extract of the leaves and twigs of *Eupatorium perfoliatum* (Compositae) whereas Mathur and Torres (1976) reported n-octacosane from the whole-plant wax of *Eupatorium ballotaefolium* (Compositae) and Mathur and Bejarane (1976) reported n-triacontane from whole-plant wax of *Acanthospermum hispidum* (Compositae); Aynehchi and Eshaghzadeh (1974)

quoted n-nonacosane (m.p., m.m.p., TLC, IR, NMR) from petrol extracts of the roots, stems, leaves and flowers of *Acroptilon picris* (Compositae) and Agarwal *et al.* (1974) identified triacontane (m.p., m.m.p.) from petrol extract of seeds of *Bunium cylindricum* (Umbelliferae).

The presence of methyl-branched alkanes as constituents of the cuticular wax hydrocarbon fraction was first detected in tobacco leaf [Carruther and Johnstone, 1959]. Using mass spectrometry, Waldron *et al.*, (1961) reinvestigated some plant waxes and recognized the presence of isoparaffins in tobacco leaf and rose petal waxes although they could not identify them. Eglinton *et al.* (1962a), utilizing GLC, examined the surface wax of leaves of certain species of the genera *Monanthes*, *Greenovia*, *Aichryson* and *Aeonium* of the sub-family Sempervivoideae (Crassulaceae) and found, in addition to n-paraffins, significant amounts of 2-methyl alkanes; those with odd numbers of carbon atoms predominating. Later, using GLC and MS, Eglinton *et al.* (1966) confirmed the presence of 2-methyldotriacontane in *Aeonium lindleyi* leaf wax and concluded that the compounds accompanying odd carbon number n-alkanes on GLC were 2-methyl alkanes.

Purdy and Truter (1961) investigated the leaf waxes of ten monocotyledonous species by thin layer chromatography and concluded that a brief qualitative analysis of the surface lipids of plants (even if the identities of the components have not been established) may provide a useful taxonomic data. In their detailed plant alkane-distribution analyses covering, in addition, some New Zealand plant species [Eglinton *et al.*, 1962b] Eglinton and his colleagues noted that, despite slight seasonal and geographic variations, the plant hydrocarbon-distribution patterns were species specific. Further, in the light of the ubiquitous occurrence of these compounds and their position as end-products insulated from the internal metabolic pools of the plant, their distribution pattern in higher plants were sufficiently complex that they might provide a fingerprint by which plant taxa could be delimited [Eglinton *et al.*, 1962c; Eglinton and Hamilton, 1963 and 1967]. This provided an impetus to others to survey plant cuticular waxes for their taxonomic value thus adding to the number of plant products already under investigation in the expanding field of chemotaxonomy. Since then several chemotaxonomic reviews have appeared including those by Swain (1963, 1966) and Weete (1972). Amongst those contributions referring directly to

the alkane distribution patterns as a possible taxonomic character, the following are worth noting:- Mecklenburg (1966) on some Solanaceae species, Martin-Smith *et al.* (1967) on Graminae species; Borges del Castillo *et al.* (1967) on the Gymnosperms, Herbin and Robins (1968b) on members of the Cupressaceae and Pinaceae; Herbin and Robins (1968c) on *Aloe* species, Herbin and Robins (1968a) on Agavaceae, Crassulaceae and Myrtaceae species; Dyson and Herbin (1968, 1970) on the cypresses grown in Kenya; Herbin and Robins (1969) on patterns of variations and development in leaf wax alkanes, Nordby and Nagy (1972, 1975, 1977), Nagy and Nordby (1973, 1972a, b) on citrus fruit juice sacs; Bachelor *et al.* (1972) on *artemisia* species of the family Compositae; Holman and Nichols (1972) on the lipids of some orchids; Gaskel *et al.* (1973) on Norwegian Lichens; Corrigan *et al.* (1973) on some Mosci species; Lytle and Sever (1973) on *Lycopodium* species, Matsuo *et al.* (1974) on some liverworts and Lytle *et al.* (1976) on ferns.

Mold *et al.* (1963a) used molecular sieve (5Å, Linde) to achieve a clean separation of branched from normal paraffins in tobacco leaf wax and, using GLC and MS, established the presence of homologous series of normal, 2-methyl and 3-methyl

alkanes in the wax. The homologs with odd number of carbon atoms predominated for the normal and 2-methyl series while the even carbon number ones were present in large amounts in the 3-methyl series. A more efficient separation of normal from branched alkanes was later achieved by Gülz (1968) who employed Linde Molecular sieve type 5Å, GLC and MS to identify and characterize long-chain normal and branched alkanes in the leaves and chloroplast preparations of *Antirrhinum majus* (Scrophulariaceae). Except for the 3-methyloctacosane and 3-methyltriacontane which could not be detected, both 2-methyl and 3-methyl isomers with the same carbon number were found in equal amounts in the branched-chain alkane fraction. Hunter and Brogden (1966), using GLC-MS, identified branched chain paraffins from Valencia orange oil as 2-methyl (odd) and 3-methyl (even). The normal and branched C_{23} and C_{25} predominated, with n-C_{23} alkane being the major component. Subsequent investigations on the hydrocarbon profiles of citrus fruit juice sacs revealed the presence of, in addition to n-alkanes, monounsaturated hydrocarbons (straight and branched) and saturated methyl branched paraffins. In all cases investigated, the major saturated hydrocarbon was either n-C_{23} [Nagy and Nordby, 1973] or n-C_{25} [Nordby and Nagy, 1972, 1975; Nagy and Nordby, 1972a,b]

while in the monoene fraction the principal chain length was either C_{25} [Nagy and Nordby, 1973] or C_{29} [Nordby and Nagy, 1975]. The 2-methyl (odd) and 3-methyl (even) hydrocarbons formed up to 50 percent of the saturated fraction (with odd to even preponderance) and only up to 20 percent in the monoene group where no odd/even dominance was discernible. Mecklenburg (1966) examined the inflorescence hydrocarbons of some *Solanum* L (Solanaceae) species by GLC and reported branched alkanes in the range C_{25} to C_{32}, in addition to n-paraffins C_{25} to C_{31}. However, Knapp *et al.* (1972) could not observe any branched alkanes in the tops of *Fabiana imbricata* (Solanaceae) although normal alkanes were present in the range C_{23} to C_{31} with an odd to even carbon number preference.

Sorm *et al.* (1964) detected monomethyl (2-methyl and 3-methyl) and dimethyl alkanes in sugar-cane wax hydrocarbon portion. Hendriks *et al.* (1977), using GLC, identified n-alkanes (C_9 to C_{39}), 2-methyl, 3-methyl and dimethyl alkanes in the hydrocarbon fraction of the herb and essential oil of *Cannabis sativa* (Cannabinaceae), which results could, in future, find use in forensic medicine. A branched C_{15}-hydrocarbon accounted for more than 70 percent of the alkanes of *Mylia taylorii* (Hepaticae) [Benešová *et al.*, 1973].

The presence of multi-methyl alkanes, particularly the isoprenoids, in waxes and other sources, is not unknown. Thus pristane (2, 6, 10, 14-tetramethyl-pentadecane was reported as a constituent of the hydrocarbons of zooplankton [Blumer *et al.*, 1963], fish and whale oils [Hallgren and Larsson, 1963; Lambertsen and Holman, 1963], wool wax [Mold *et al.*, 1963b] and Precambrian sediments [Eglinton *et al.*, 1964]. Rather recently both pristane and phytane (2, 6, 10, 14-tetramethylhexadecane) were reported present in the hydrocarbon fractions of the lipids of *Lycopodium* species [Lytle and Sever, 1973] and ferns [Lytle *et al.*, 1976] in which they represent up to 27 percent of the alkanes.

Low molecular weight hydrocarbons occur more frequently as part of volatile constituents in essential oils obtained by steam distillation of plant tissues [see, for example: Kami *et al.*, 1972; Milles *et al.*, 1973; Mody *et al.*, 1974a and b; Thomson *et al.*, 1974]. In such distillates which usually contain, *inter alia*, mono- and sesquiterpenes, the aliphatic hydrocarbons, saturated normal, mono- and di- methyl branched isomers may be present though in small amounts.

2.4.3 Esters

The most common wax esters are alkyl esters which contain a long chain primary alcohol combined with a monocarboxylic fatty acid.

Less common are the estolides, two types of which are distinguishable: the ACID forms resulting from interesterification of ω -hydroxy alkanoic acids, and the NEUTRAL forms resulting from interesterification of ω -hydroxy acids with α, ω -diols. Some members of the Gymnospermae seem to be particularly rich sources of estolide waxes [Herbin and Robins, 1968b and refs. cited therein, Loveland and Laver, 1972].

That alkyl esters are part of the constituents of leaf wax of cabbage, *Brassica oleraceae* var. capitata, was reported by Purdy and Truter (1963a) who found n-eicosanoic acid and n-hexacosanol as the major moieties of the saponified esters [Purdy and Truter, 1963b]. Horn et al. (1964) found that both primary and secondary alcohols (with OH group at position 2) were present in the saponified waxes of *Eucalyptus globulus* and *E. risdoni*. Odd carbon number secondary alcohols (C_9 - C_{15}) predominated for this series while even carbon number homologs were predominant in the primary alcohol (up to C_{30}) series.

Several reports, particularly those of Tulloch and his fellow workers, refer to alkyl esters in the leaf waxes of wheat (*Triticum*) varieties. Tulloch and Weenink (1966) reported their presence in *Triticum compactum* var. Little Club. The composition was reported as mainly octacosanol esters of C_{14} to C_{32} fatty acids with lesser amount of esters of trans-2-tetracosenoic and trans-2-docosenoic acids [Tulloch and Weenink, 1969]. *T. durum* varieties Pollisier and Stewart 63 were found to contain C_{36} - C_{56} esters of C_{20} - C_{28} alcohols and C_{16} - C_{30} saturated acids [Tulloch and Hoffman, 1971]. Octacosanol was the principle alcohol in the esters of trans-2-tetracosenoic and trans-2-docosenoic acids. Octacosanyl esters of C_{14} - C_{32} acids, and C_{20} and C_{22} alcohol esters of trans-2-docosenoic and tetracosenoic acids were present in the Selkirk and Manitou varieties of spring wheat, *T. aestivum* [Tulloch and Hoffman, 1973]. Diol and trans-2-docosen-1-ol were also present in both varieties. The wax esters of both lemhi wheat and its stripe rust fungus, *Puccinia striiformis*, contain mainly C_{20}, C_{22}, C_{16} and C_{18} ester-bound fatty acids and C_{24}, C_{28} and C_{22} primary alcohols [Jackson *et al.*,1973].

A mixture of even carbon numbered alkyl esters (C_{48} - C_{56}) consisting of C_{22} - C_{32} fatty acids and C_{24} - C_{30} primary alcohols was reported from the stem and leaf wax of *Larrea divaricata* (Zygophyllaceae) [Seigler *et al.*, 1974]. The leaf and stem wax esters of *Trifolium repens* (Leguminosae) were reported by Body (1974) as mainly of di- and tri-unsaturated C_{18} fatty acids and C_{30} alcohol.

The leaf wax of rye, *Secale cereale*, was found to contain diesters and alkyl esters (C_{28} - C_{58}) of mainly hexacosanol with 58 percent of the ripe rye esters being of trans-2, 3- unsaturated acids [Tulloch and Hoffman, 1974]. The same authors reported a similar range of alkyl esters in *Triticale hexaploide* with octacosanol as the major esterified alcohol. Bianchi and Corbellini (1977) examined the variation of the epicuticular wax composition with age of bread wheat, *Triticum aestivum* var. Demar 4, and noted that octacosanol was the major esterified alcohol after 30 and 130 days of germination, with shorter chain homologs being present in the 130- and 190- days old plants. The esterified acids in the range C_{16} - C_{28} had C_{16}, C_{18}, C_{20} and C_{22} chain lengths predominating.

Ismail *et al.* (1977) reported the alkyl esters of Golden egg, *Prunus domestica* (Rosaceae) as

forming about 8 percent of the wax with C_{40} (ca. 45 percent) and C_{42} (ca. 21 percent) being the major esters in the range C_{34} - C_{50}. The esterified alcohols (C_{18} - C_{30}) were mainly C_{24} (ca. 65 percent) while the ester-bound fatty acids (C_{12} - C_{32}) were mainly C_{16}, C_{18} and C_{24}. *Rosa virgo* (Rosaceae) wax ester fraction gave, upon saponification, saturated primary alcohols (C_{16} - C_{30}), secondary alcohols and a minor amount of unsaturated primary alcohols mainly $C_{24:1}$ and $C_{28:1}$ [Mladenova *et al.*, 1977].

2.4.4 Ketones

2.4.4.1 Mono-carbonyl ketones

Symmetrical and unsymmetrical ketones occur in plant waxes. Amongst the most widely investigated ketone fractions are those from waxes of *Brassica* species. Initially, Channon and Chibnall (1929) identified nonacosan-15-one from cytoplasmic fluids of cabbage leaf and later the same highly symmetrical ketone was found in Brussels sprout [Sahai and Chibnall, 1932]. In addition to the symmetrical C_{29} ketone, nonacosan-10-one was also observed by Purdy and Truter (1963c) in the wax of *Brassica oleraceae* (var. capitata, Winnigstadt). According to Channon and Chibnall (1929) the biosynthesis of the symmetrical C_{29}-15-one would require a head to

head condensation of two pentadecanoic acid monomers followed by decarboxylation. However, odd carbon number fatty acids such as C_{15} had not been detected in plants by then. Nevertheless, when the cabbage ketone was subjected to a Beckmann-rearrangement of its oxime [Horn *et al.*, 1964], pentadecanoic acid was the major product with traces of n-C_{12}, n-C_{14} and n-C_{18} acids. From its mass spectrum, Laseter *et al.* (1968) believed the ketone from *B. oleraceae* (var. Pound Dutch) was pure C_{29}-15-one but when their spectrum was reexamined by Netting and Macey (1971) the ketone fraction was found to comprise of 85 percent C_{29}-15-one and 15 percent nonacosan-14-one.

Hentriacontan-9-one was reported as the major ketone component of *Rosa damascena* bud and flower waxes in addition to smaller amounts of branched C_{32} and 2-methyl-C_{30}, -C_{28}, -C_{27} and -C_{26} ketones [Stoianova-Ivanova *et al.*, 1969; 1971]. Nonacosan-10-one was the prevalent component of the long chain fatty ketones (C_{21} - C_{29}) from skin-waxes of Bouhavitsa and Tetovka varieties of *Pyrus malus* (Rosaceae) [Dodova-Anghelova and Ivanov, 1973].

Octadecan-2-one and eicosan-2-one were identified from the leaf wax of *Euonymus latifolius* (Celastraceae) [Ulubelen and Baytop, 1973] and nonacos-1-en-4-one from *Trianthema pentandra* (Aizoaceae) [Misra and

Tiwari, 1973]. The ketone fraction of *Prunus domestica* fruit wax was reported as pure nonacosan-10-one [Ismail *et al.*, 1977]. More recently, a gamma-hydroxy ketone viz. 13-hydroxyhentriacontan-16-one was isolated from *Neolitsea sericea* (Lauraceae) leaf acetone-extract [Takaoka *et al.*, 1977].

2.4.4.2 Beta- and Hydroxy beta- diketones

The occurrence of β-diketones as components of natural waxes was first reported by Horn and Lamberton (1962) who isolated them, as insoluble copper complexes, from the leaf and stem waxes of *Eucalyptus* (Myrtaceae) and *Acacia* (Leguminosae) species; non-cereal grass, *Festuca glauca* (Graminae) and the carnation, *Dianthus caryophyllus* (Caryophyllaceae). Tritriacontan-16, 18-dione, n-tritriacontan-12, 14-dione, n-hentriacontan-14, 16-dione, n-hentriacontan-12, 14-dione and n-nonacosan-12, 14-dione were characterized and estimated by spectroscopy (IR and UV) and GLC of their alkaline hydrolysis products, namely methyl ketones and fatty acids [Horn *et al.*, 1964]. Except for two species in which shorter chain lengths predominated, the most common β-diketone in *Eucalyptus* leaf waxes was found to be n-tritriacontan-16, 18-dione.

Tulloch and Weenink (1966, 1969) examined the composition of the leaf wax of Little Club wheat, *Triticum compactum* (Graminae) and identified n-hentriacontan-14, 16-dione in the β-diketone fraction and reported for the first time the occurrence of hydroxy-β-diketones, an equal mixture of 8- and 9-hydroxyhentriacontan-14, 16-diones. The glaucousness of wheat was shown to correlate with a high content of β-diketones in the wax [Barber and Netting, 1968; Netting and Wettstein-Knowles, 1973]. Leaf waxes from varieties Polissier and Stewart 63 of durum wheat, *Triticum durum* (Graminae) contain 36 percent β-diketone, all of which is n-hentriacontan-14, 16-dione, and 9 percent hydroxy-β-diketone identified as 25-hydroxyhentriacontan-14, 16-dione [Tulloch and Hoffman, 1971]. Hentriacontan-14, 16-dione was again found in the glaucous lines of barley [Jackson, 1971] along with hydroxy-β-diketones, which formed about 1 percent of the barley surface-lipids. The substituted diketones were identified as a mixture of 8- and 9-hydroxyhentriacontan-14, 16-diones. Identical β- and hydroxy β-diketones were found to varying extents in leaf waxes of varieties Selkirk and Manitou of spring wheat, *Triticum aestivum* [Tulloch and Hoffman, 1973].

Dierickx and Buffel (1972) identified 5-, 6- and 7-hydroxyhentriacontan-14, 16-diones at relative

concentrations of 20, 50 and 30 percent respectively in the β-ketonic fraction of the wax from *Avena sativa* (Graminae).

Tulloch (1973) GLC - examined the variation in composition of whole cuticular waxes of spring wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) with age and part of the plant and concluded that the glaucousness of the leaf sheath was due to a high β-diketone content.

The leaves of dark-fired tobacco, *Nicotiana tabacum* (Solanaceae), previously subjected to a natural fermentation process for a period of 50 days prior to air-drying and cold-hexane extraction, yielded two isomers of a diketone [Zane, 1973] which was isolated and characterized as 11-isopropyl-4, 8-dimethyl-3, 7, 12-pentadecatriene-2, 14-dione. From the leaf wax of *Buxus sempervirens* (Buxaceae), Dierickx (1973) isolated and characterized n-hentriacontan-8, 10-dione which, according to Mass Spectral data, formed 81 percent of the β-diketone mixture which also contained n-nonacosan-6, 8-dione (14 percent) and n-tritriacontan-10, 12-dione (5 percent).

Jackson *et al.* (1973) reported for the first time the occurrence of β-diketones in fungal surface

lipids. The surface lipids extract of the uredospores of wheat stripe rust, *Puccinia striiformis*, contained 33 percent β-diketones composed of greater than 97 percent hentriacontan-14, 16-dione and less than 3 percent heptacosandione.

Dierickx and Compernolle (1974) isolated and identified hentriacontan-14, 16-dione from the whole-fresh-plant wax of rye, *Secale cereale* (Graminae). They also isolated 25-hydroxyhentriacontan-14, 16-dione from the wax. The waxes of eight non-cereal grasses in the Graminae were examined on TLC for β-diketones but the results were negative; so the authors concluded that β-diketones cannot be considered as chemotaxonomic markers in the Graminae. The same β-diketone and hydroxy-β-diketone were found as constituents of 60 - 70 day old-leaf epicuticular waxes of *Secale cereale* and the synthetic species, *Triticale hexaploide*, the amphiploid obtained by crossing durum wheat (var. Stewart 63) with rye (var. Prolific) [Tulloch and Hoffman, 1974]. In their extensive investigations on the composition of the spike, stem and leaf waxes of intermediate wheatgrass, *Agropyron intermedium* (Graminae), Tulloch and Hoffman (1976) identified an unusual variety of substituted β-diketones. The wax on the leaves contained, *inter alia*, 25-oxohentriacontan-14, 16-dione (17 percent), 10-oxohentriacontan-14,

16-dione (5 percent), 25-hydroxyhentriacontan-14, 16-dione (12 percent) and 26-hydroxyhentriacontan-14, 16-dione (2 percent). The wax on spikes contained 25-hydroxy-(17 percent) and 26-hydroxy-(3 percent) hentriacontan-14, 16-diones; 10, 25-dioxohentriacontan-14, 16-dione (1 percent), 4-hydroxy-25-oxo-(2 percent), 25-hydroxy-10-oxo-(1.3 percent) and 26-hydroxy-10-oxo-(0.7 percent) hentriacontan-14, 16-diones.

The leaf wax of western wheatgrass, *Agropyron smithii* (Graminae), was analysed by Tulloch (1976). It was found to be composed of 68 percent β-diketones consisting of 17 percent hentriacontan-14, 16-dione, 3 percent 25-oxohentriacontan-14, 16-dione, 30 percent 25-hydroxyhentriacontan-14, 16-dione and 18 percent 26-hydroxyhentriacontan-14, 16-dione. The proportions of the isomeric hydroxy-β-diketones were estimated by a novel method involving ^{13}C NMR spectroscopy of the hydroxy acids obtained upon hydrolysis.

The β-ketonic fraction (ca. 38 percent) of the epicuticular wax of bread wheat, *Triticum aestivum* (var. Demar 4) was found to contain hentriacontan-14, 16-dione (95 percent) and nonacosan-12, 14-dione (5 percent) in the β-diketone portion while the hydroxy-β-diketones comprised 8- and 9-hydroxyhentriacontan-14, 16-diones [Bianchi and Corbellini, 1977].

β-diketones were detected in the leaf wax of *Hyperrhenia rufa* (Graminae) and both β-diketones and hydroxy-β-diketones in that of *Pennisetum setaceum* (Graminae) [Herbin and Mndewa, unpublished observations].

2.4.5 Aldehydes

Aldehydes are among the least investigated of the wax constituents. They were first reported as the major component of sugar-cane wax [Lamberton and Redcliffe, 1959; 1960] and examined on GLC by Kranz *et al.* (1960) who found that even carbon number homologs predominated with C_{28} being the major. Using NMR, Lamberton (1965) suggested that the sugar-cane aldehydes probably occur in hemihydrate form in the wax. The wax from Sultana grapes afforded aldehydes (C_{18} - C_{32}) with C_{26} and C_{28} being the major chain lengths [Radler and Horn, 1965].

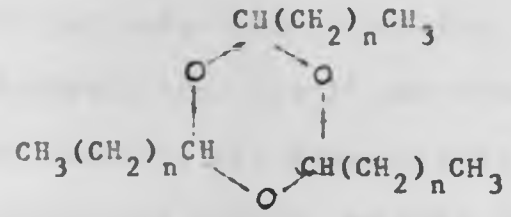
In agreement with the results obtained by Macey and Barber (1970a), Kollatukudy (1970) reported the C_{26} and C_{28} chain lengths as the principal aldehydes in the wax of Pea leaves, normal *Pisum sativum* (Leguminosae). The data published by Macey and Barber (1970 a) showed that the C_{27} aldehyde is amongst the major aldehydes in

the *ras* mutant [viz: C_{26} (30.8 percent), C_{27} (25.1 percent), C_{28} (25.3 percent) and C_{30} (18.5 percent)] while in the *wsp* mutant the C_{29} chain length was also appreciable amongst other members in the range C_{26} to C_{32} [viz: C_{26} (20.6 percent), C_{28} (22.4 percent), C_{29} (10.8 percent) and C_{32} (30.8 percent)]. The presence of aldehydes in the waxes of normal and mutant lines of *Brassica oleraceae* was first reported by Macey and Barber (1970b). The aldehyde composition of the normal-form was C_{26} (10.2 percent), C_{28} (49.3 percent), C_{29} (4.4 percent) and C_{30} (36.1 percent).

A small percentage of aldehydes (mostly C_{28}) was present in the Manitou variety of spring wheat [Tulloch and Hoffman, 1973].

In a comparative study of the neutral constituents in the waxes of decorative roses, Mladenova and Stoianova-Ivanova (1975) reported the presence of aldehydes in the flower waxes of *Rosa americana, R. imperial* and *Rosa virgo*. In addition to unsaturated C_{18} aldehydes which were present in the first two species, a homologous series of FREE state n-aldehydes in the range C_{14} to C_{28} was present in all three roses. In their POLYMERIC state they were identified

[Mladenova *et al.*, 1976] as trialkyltrioxanes:



In both free- and bound- forms the major aldehydes were, in each case, C_{22} and C_{24} at relative concentrations (in the case of *R. virgo*) of 30.1 and 15.5 percent (free-form) and 27.3 and 20.0 percent (bound-form) respectively.

The epicuticular wax of bread wheat var. Demar 4 contained aldehydes composed of 99 percent octacosanal [Bianchi and Corbellini, 1977]. The epicuticular wax aldehyde fraction (1.1 percent) from the fruit of *Prunus domestica* (Rosaceae) contained 49.5 percent nonanal (major component) for which the overall aroma of the plum owes [Ismail *et al.*, 1977]. Except for this example cited, the literature survey indicates that most of the low molecular weight aldehydes occur as constituents of plant tissue homogenates or essential oil extracts. Thus cis-cis-3, 6-nonadienal and cis-6-nonenal (flavor components of water melon and musk melon respectively), trans-2-nonenal and trans, cis-2, 6-nonadienal (flavor components of cucumber and off-flavour compounds developing in hydrogenated vegetable oils upon storage) have been

reported [Sekiya *et al.*, 1977; Kemp *et al.*, 1972 and 1974 and refs. cited therein]. The so termed leaf aldehydes viz. cis-3- and trans-2-hexenal and leaf alcohol, cis-3-hexen-1-ol, have attracted the attention of several workers [for example: Major *et al.*, 1972; Major and Thomas, 1972, Saijo and Takeo, 1975], particularly on their photochemistry and biosynthesis [for example: Hatanaka and Harada, 1973; Hatanaka *et al.*, 1976 and refs. cited therein].

2.4.6 Secondary alcohols

Like alkanes, monocarbonyl ketones, or beta-diketones, free secondary alcohols are predominantly of odd carbon numbers. When both ketones and secondary alcohols are constituents of the same wax, the functional group of the dominant homolog of each appears to be located at a position identical to that of the other.

Earlier studies on apple wax showed that it contained nonacosan-10-ol [Chibnall *et al.*, 1931; Wollrab, 1969] but Dodova-Anghelova and Ivanov (1973) showed that other members from C_{21} to C_{28} inclusive were also present in the Bouhavitsa variety of Bulgarian apple.

Sahai and Chibnall (1932) found nonacosan-15-ol in brussels sprout (*Brassica oleraceae* var. gemmifera) whereas Holloway *et al.* (1976) identified homologs of chain lengths C_{27} to C_{31} with C_{29} positional isomers predominating. Nonacosan-15-ol, nonacosan-14-ol and nonacosan-13-ol were present at relative abundances of 60.1, 36.3 and 0.9 percent respectively. Other varieties of *B. oleraceae* were shown to contain, in addition to nonacosan-15-ol, nonacosan-10-ol [Purdy and Truter, 1963c] or nonacosan-14-ol and small amounts of nonacosan-13-ol and nonacosan-12-ol [Macey and Barber, 1970b; Netting and Macey, 1971]. Nonacosan-10-ol was found in *Chelidonium majus* (Papaveraceae) [Seoane, 1961; Holloway *et al.*, 1976] and black spruce (*Pisea mariana*) and balsam fir (*Abies balsamea*) [Beri and Lemon, 1970].

Wollrab (1969) found a homologous series (C_{20} - C_{33}) of secondary alcohols in rose flower waxes and identified hentriacontan-9-ol (88.4 percent) as the principal member with smaller amounts of nonacosan-7-ol and nonacosan-10-ol. *Rosa virgo* flower wax was shown to contain C_{25} to C_{33} secondary alcohols with the hydroxyl group at positions 4, 5 and 6, the 5-OH isomer being dominant in each homolog [Mladenova *et al.*, 1977].

Using GLC and MS, Kolattukudy (1970) identified hentriacontan-16-ol as the chief secondary alcohol

from *Pisum sativum* with minor amounts of nonacosan-15-ol, triacontan-15-ol, dotriacontan-16-ol and tritriacontan-17-ol; and Macey and Barber (1970a) subjected the secondary alcohol fraction of this species to a Beckmann-rearrangement and showed it to consist of principally hentriacontan-16-ol but with appreciable amounts of hentriacontan-15-ol and traces of other homologs. These assignments were generally in agreement with those by Holloway *et al.* (1976) who found, in addition, the 13-, 14- and 15- OH isomers of the C_{29} alcohol. Holloway and his colleagues also reported the structures of secondary alcohols from epicuticular waxes of various angiosperms and gymnosperms.

The secondary alcohol fraction (C_{27} - C_{31}) of *Prunus domestica* (cv. Golden Egg) contained principally nonacosan-10-ol and smaller amounts of the 9- and 11- OH isomers of C_{29} and 7-, 8- and 9-OH isomers of the C_{27} alcohol [Holloway *et al.*, 1976]. Only the 9-OH isomers among the C_{28}, C_{30} and C_{31} alcohols were present. These results were in harmony with those reported by Ismail *et al.* (1977).

Brassica oleraceae var. capitata (Winnigstadt) contained a ketol fraction [Purdy and Truter, 1963a] which was shown to be a mixture of nonacosan-10-ol-15-one and nonacosan-10-one-15-ol [Purdy and Truter, 1963b].

2.4.7 Primary alcohols

Free primary alcohols, as do other long chain wax constituents, invariably occur as an homologous series. In this class, members with even number of carbon atoms usually predominate over those with odd. Frequently a C_{26}, C_{28}, C_{30} or C_{32} homolog may be the major.

A report by Olaniyi *et al.* (1975) claiming to have isolated separately pure hexacosanol and pure triacontanol from light-petrol extract of the leaves of lemon grass, *Cymbopogon citratus* (Graminae), by successive elutions from basic alumina column with light petrol-benzene and benzene-chloroform mixtures respectively, should be treated with some reserve. These authors identified their eluates by IR, m.p., MS and NMR although they did not publish the relevant spectra. Other primary alcohol chain lengths eluted from columns and identified by one or more of the above physical methods include hentriacontanol (elemental analysis, m.p., m.m.p., co-TLC, IR) from *Trianthema pentandra* (Aizoaceae) [Misra and Tiwari, 1972], octacosanol (m.p., m.m.p., TLC, IR) from light petrol leaf-extract of *Calycopteris floribunda* (Combretaceae) [Gupta *et al.*, 1973], heptacosanol (m.p., IR, NMR) from light petrol leaf-extract of *Gymnocladus dioica* (Leguminosae) [Abramson and Kum, 1973], octacosanol and hentriacontanol (m.p., IR, MS)

from benzene heartwood- and leaf-extracts respectively of *Dichrostachys cinerea* (Leguminosae) [Joshi and Sharma, 1974].

The importance of GLC and MS analyses in structural assignment and homogeneity determination of long chain wax components should be stressed judging from the results by Starratt (1969) who identified hexacosanol, by physical data (IR, m.p., m.m.p., and m.p. of its acetate), from *Euphorbia polygonifolia* (Euphorbiaceae) but later, using GLC confirmed the fraction to be a mixture of mainly octacosanol with minor amounts of hexacosanol and other homologs [Starratt, 1972].

2.4.8 Diols

The various sources from which α, ω-diols have been reported are reviewed by Martin and Juniper (1970). Recently a homologous series of gamma-diols (C_{17} - C_{33}) was reported from the blossom wax of *Rosa damascena* (Rosaceae), with major homologs containing an odd number of carbon atoms [Stoianova-Ivanova *et al.*, 1974]. The principal homolog was nonacosan-5, 8- diol.

2.4.9 Fatty acids

The present-day literature on fatty acids, like that of hydrocarbons, is rather large. However, one may distinguish between the hydroxy- and hydroxyepoxy-fatty acids (mainly of C_{16} and C_{18} families) found in polymers of cutins and suberins of angiosperms and gymnosperms (for example: Baker and Holloway, 1970; Holloway and Deas, 1971; Hunneman and Eglinton, 1972; Walton and Kolattukudy, 1972, Holloway and Deas, 1973; Deas *et al.*, 1974 and Kolattukudy *et al.*, 1975), and those unhydroxylated monobasic monomers occurring in superficial waxes.

Of the unsaturated long chain monobasic acids occurring in epicuticular waxes, the $\omega 9$, $\omega 6$ and $\omega 3$ families of polyunsaturated methylene-interrupted fatty acids (so designated by numbering the positions of the double bonds from the methyl end of the chain) are the most important, the first members of these families being octadec-9-enoic, octadec-9, 12-dienoic and octadec-9, 12, 15-trienoic acids respectively (Gurr and James, 1971). In the higher carbon number ranges, the saturated monomers usually predominate and show a clear even/odd dominance with the C_{26} or C_{28} acid the principal homolog. The common and less common acids reported from plant cuticles have been reviewed by Martin and Juniper (1970).

The closely related triterpenoids, betulinic, ursolic and oleanolic acids occasionally occur in the waxy coating of leaves and fruits where they may serve a protective function in suppressing microbial attack (Harborne, 1973).

2.5 Previous work on Coffee and Tea leaf cuticles

Holloway *et al.* (1972) determined the composition of cutin from young (3rd leaf pair) and mature (6th leaf pair) *Coffea arabica* L. leaves. Positional isomers of dihydroxyhexadecanoic acid (mainly 9, 16- OH and 10, 16 -OH) comprised more than 60 percent of the total acids. No major differences were found in the composition of cutin from the two leaf ages or from the adaxial and abaxial surfaces. Generally the coffee leaf cutin was found to resemble those of other angiosperms.

The quantitative wax-composition of coffee leaves was determined by Kabaara (1973) as part of a general investigation of the physical and chemical properties of the leaf cuticle in relation to the penetration of foliar sprays. Alkanes were the principal class of constituents (Table 2). Nonacosane was the major alkane and triacontanol the principal alcohol in both young and mature leaf wax-fractions.

Status of leaf	CLASS OF CONSTITUENT (PERCENT)			
	Alkanes	Primary - ols	Triterpenoids	Remainder
Young (3rd pair)	41.3	16.9	26.5	15.3
Mature (6th pair)	54.3	10.2	18.2	17.3

Table 2 Major constituents of coffee leaf epicuticular waxes as determined by densitometry (Kabaara, 1973; with permission).

In the work herein reported the stata of leaves considered are essentially different from those quoted in Table 2 in which the young leaf approximates to the expanded leaf while the mature leaf is a little younger than the senescent leaf herein analysed (see Section 3.2, Plate 3A).

Stocker and Wanner (1975) reported changes in composition of *Coffea arabica* L (cv. Bourbon) leaf waxes with age of the plant. These investigators worked on the oldest of the leaf pairs from 66-, 81- and 112- day old GREENHOUSE-CULTIVATED plants at which stages the germinated plants had 1, 2 and 5 leaf pairs respectively. The results obtained by Kabaara (1973) are substantially different from those reported by Stocker and his colleague . The principal homologs of the wax-fractions as published by the latter authors are quoted in Table 10 (Section 4.4)

to facilitate discussion and comparison with the results herein reported (Section 4.3).

In their analysis of sterol and lipid components of dry processed commercial green tea, Khanna *et al.* (1974) claimed to have isolated, separately, triacontanol and dotriacontanol from the neutral portion of the saponified petrol-extract. These authors reported to have eluted the two chain lengths in succession from a neutral alumina column using petrol-benzene (1:3) and petrol-benzene (1:5) mixtures respectively, and identified each of the alcohols by MS, IR, m.p. and m.m.p of their acetates which they could not purify. However from the foregoing discussion on the literature of primary alcohols and esters as well as the results obtained in the present work (Section 4.3.2.2.2), the so called "thea alcohols" characterized by Khanna *et al.* (1974) and other workers referenced by them can only be an homologous series with the C_{30} and C_{32} primary alcohols prevalent.

CHAPTER 3

MATERIALS AND METHODS

3.1 Source of materials

Coffee tissue samples were obtained from a coffee farm, situated at Loresho (Lower Kabete) near Nairobi, maintained as a field station by and for the Faculty of Agriculture of the University of Nairobi. Tea leaves were sampled from clone TRI 6/8 tea bush, at Limuru (Kenya), maintained by an individual farmer. Plates 2A and 2B are photographs of the relevant coffee and tea bushes respectively. Samples were collected as frequently as when needed in the period between October, 1975 and June, 1977; some of the coffee tissue sampling coincided with the fruit-ripening season of the tree as shown in Plate 2C. Samples for analysis were extracted from plant material immediately after collection although occasionally it was necessary to store the material in a deep freeze.

3.2 Materials

3.2.1 *Coffea arabica* L

The flower was taken to include only its petals and the sexual parts. Leaves and berries belonging to three different stages of maturity were

A

PLATE 2

A = *Coffea arabica bush at Loresho (Lower Kabete), Nairobi, Kenya.*

B = *Clone TRI 6/8 tea bush at Limuru, Kenya.*

B

C

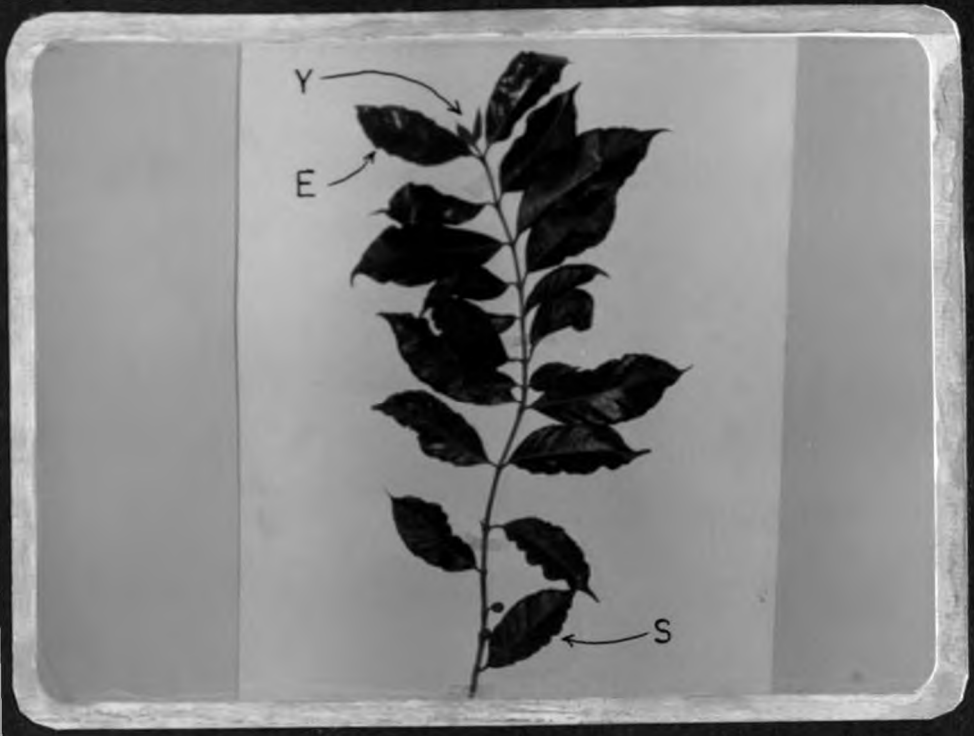
PLATE 2

C = *Fruit ripening Coffea arabica tree at Loresho (Lower Kabete), Nairobi, Kenya.*

collected. Of the berries, the fully ripe (senescent), the hard green (expanded) and the very young berries, relatively smaller in size, were picked. Leaf ages were distinguished on the basis of texture, size and position of the tissue on the branch relative to the main stem of the tree and chlorophyll content as judged crudely from the greenness of the leaf. On this basis, the oldest dark-green leaf pair nearest to the main stem, very rough to the touch and just about shedding off the branch was taken as the senescent leaf. The smooth shiny-green fully grown leaf pair very tender to the touch was taken for the expanded leaf. The very young leaves were taken as the pale-yellow or sometimes purplish leaf-twins at the very tip of the branch. In each case, only the seemingly undamaged tissues were sampled at random from the different trees and rows of the bush so that each sample taken for analysis was more or less representative of the population. Plate 3A illustrates this sampling technique.

3.2.2 *Thea sinensis* L

As with coffee, the rough oldest dark-green leaves ready to fall were removed as representing the senescent, the third alternate leaf pair (pale yellowish) after the closed bud for the expanded, and the relatively small and thinner leaf



A

PLATE 3

A = *Typical coffee branch showing the leaf ages sampled.*

Y, E, S = very young, expanded, and senescent leaf respectively.

immediately after the closed bud for the youngest stage - Plate 3, B and C.

3.3 Methods

3.3.1 Quantitative wax-isolation

Generally the fresh tissues (ca. 100 g) were quantitatively extracted for the epicuticular wax by refluxing in chloroform (ca. 200 ml) for 5 minutes. The extract was filtered on Whatman paper to remove gross earthy impurities. The filtrate was dried over sodium sulphate (anhyd.) which also removes the small amounts of materials of cellular origin such as oils and pigments. The chloroform solution was left to air-dry, giving the creamy-white solid wax.

3.3.2 Wax extraction for HCI determinations

The wax hydrocarbons used in determining the Hydrocarbon Concentration Index (HCI) on the leaf surface were obtained by a different but uniform wax extraction procedure. For each age, a rectangular or square piece was cut from the central portion of the leaf such that the main-vein of the latter was more or less symmetrically embraced by the dimensions of the sampled piece. The sample was dipped and swirled in chloroform

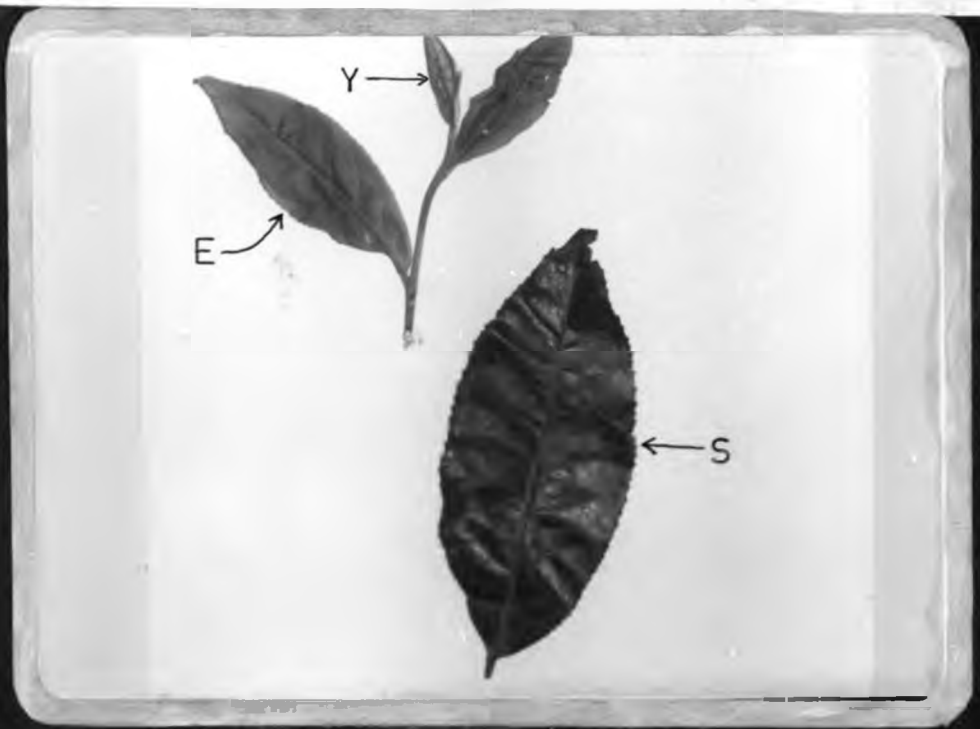
B

PLATE 3

B = *Typical tea branch showing the leaf ages sampled.*

C = *Typical sampled tea leaves dettached from the main branch.*

Y, E, S = very young, expanded, and senescent leaf respectively



C

(ca. 20 ml) for 45 seconds, solvent just evaporated, and the residue taken up in the minimum amount of petroleum ether (B.P. 60° - 80°C). The pet. ether solubles were chromatographed on a short column (80 x 8 mm) packed* with basic alumina (type H) and the hydrocarbon fraction eluted with pet. ether (ca. 10 ml). A minimum amount of celite (80 - 100 mesh) was added to the eluate and the solvent gently evaporated whilst shaking continuously to ensure homogenous adsorption of the alkanes. The whole celite sample or an accurately weighed portion of it was taken for direct gas chromatographic analysis. Since no distinction was made between the adaxial and abaxial surface-waxes of the leaf-piece sample, the area of the sample piece (in mm^2) was doubled and expressed in subsequent analyses as the total surface area (see Section 3.3.7).

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*Independent observations showed that glass wool commonly used as a plug to support adsorbents in column chromatography contained impurities that elute from silicon grease GLC columns at 240°C like long chain alkanes. Hence, glass wool used in all column chromatographic work herein was pre-purified by refluxing in chloroform for at least 5 minutes. After drying, the wool was washed with petroleum ether (B.P. 60° - 80°C) prior to use.

3.3.3 Thin Layer Chromatography

Exploratory thin layer chromatography (ELC) was employed for qualitative examination of the gross composition of the waxes and preparative thin layer chromatography (PLC) for the isolation of the individual classes of constituents.

3.3.3.1 Adsorbent, Sample Application and Solvent Systems

All thin layer films were made from Silica gel G (E. Merck, Darmstadt) accredited to Stahl, marketed under the common German name Kieselgel G nach Stahl. Clean glass plates were coated with a slurry of Si gel G made in distilled water (1:2 $^{w}/v$), 60 g of the adsorbent being just sufficient to coat ten (20 x 20 cm) or twenty (10 x 20 cm) plates with a 250 μm film (for ELC purposes) using a BTL motorised TLC-coater. For PLC use, films of 300 μm thickness on 20 x 20 cm plates were made. The coated plates were first air-dried at room temperature for one hour followed by activation at 110°C for at least two hours prior to use. For both ELC and PLC, samples were monitored on the chromatofilms as chloroform solutions. The common spot-staining technique was adopted in ELC using, in most cases, pipettes drawn-out in the laboratory from glass

capillary tubings. In PLC, the sample was streaked as a narrow band of approximately 18 cm length situated about 1.5 cm from the bottom and leaving a margin of about 1 cm on either ends of the plate.

All developments were done in ascending direction in solvent-vapour saturated tanks lined with solvent-soaked filter paper. The ELC of non-polar constituents, viz: hydrocarbons, aldehydes and primary alcohols, was carried out with benzene as developing solvent while the more polar fatty acids and triterpenoids were satisfactorily resolved in petroleum ether (B.P. 60^{o} - 80^{o}C) - diethyl ether - glacial acetic acid (70 + 30 + 2) solvent system, hereinafter abbreviated to PEA. The PLC of the above constituents, including caffeine, was effected in benzene and/or PEA solvent system depending on their polarity and extent of separation of the constituent bands. Caffeine had an hRf of zero in both solvent systems.

3.3.3.2 Location of wax constituents

3.3.3.2.1 General

A 1 percent ($^{W}/v$) methanolic solution of dodecamolybdophosphoric acid, $H_3[P(Mo_3O_{10})_4] . x\,H_2O$, was employed as the general chromogenic reagent (hereinafter abbreviated to DDMP) for all wax

constituents. DDMP was by far the most satisfactory of all location reagents tried or herein employed and hence developed films located with it were used as check-chromatograms against similar chromatograms for which visualization had been effected otherwise. After air-drying, the developed plate was sprayed with the reagent and the plate heated in oven at 120°C to bright spot formation. At least 5 minutes were sufficient for the wax components to show up as dark-blue spots against a yellowish-green background. Caffeine (1, 3, 7 -trimethylxanthine) which was found present in both coffee and tea tissue epicuticular waxes gave a yellowish-brown response to this heteropoly acid formulation, the colour showing up instantaneously upon spraying. DDMP gradually deteriorated at room temperature, its initial yellow colour going green, but kept indefinitely when stored in the deep freeze immediately after use.

3.3.3.2.2 Specific reagents and selective treatments

Long chain fatty acids obtained by PLC (developing solvent PEA) or otherwise (see Section 3.3.6) gave a red colour response to the PLDP spray reagent, a 0.1 percent (%/v) methanolic solution of phenolindo-2, 6-dichlorophenol.

Triterpenoids obtained by PLC (PEA solvent system) gave a similar response to PLDP as the long chain fatty acids. In both ELC and PLC with the PEA solvent system, triterpenoids produced, on warming at 120°C, a characteristic intense-purple colour with the Lieberman-Burchard reagent formulated as described in Section 1.4. Direct location of both acids and triterpenoids with PLDP in ELC or PLC was not possible because of the masking effect of the acetic acid in the PEA solvent system.

Long chain aldehydes were visualized by formation of their hydrazones upon treatment of the spot on Si gel G film with DNP reagent. The DNP formulation was a saturated ethanolic (absolute) solution of 2, 4-dinitrophenylhydrazine, containing a few drops of conc. hydrochloric acid. On the thin layer, the whole-wax or PLC-isolated aldehyde fraction was co-spotted with the DNP reagent and the plate heated for 15 minutes in an oven at 120°C prior to development in benzene. Sugar-cane stem wax was also used as a standard against the test-sample aldehydes. Oxidation (see Section 3.3.4) of the PLC isolated aldehyde fraction gave acids which responded positively to the PLDP reagent.

On-the-plate treatment of the whole-wax spot with a drop or two of acetyl chloride, followed by evaporation of the excess reagent by warming and subsequent development in benzene caused a change in hRfs of some of the wax constituents relative to the untreated spot. The primary alcohols or other OH-group-containing constituents had their hRfs ascertained by this on-the-plate acetylation technique. Mild oxidation (see Section 3.3.4) of PLC isolated primary alcohols yielded aldehydes which gave yellow hydrazones with the DNP reagent.

3.3.4 Chemical treatment and derivatization of samples

3.3.4.1 Oxidation of primary alcohols to aldehydes and aldehydes to acids

PLC isolated aldehydes or alcohols, dissolved in glacial acetic acid, were refluxed with a saturated solution of potassium dichromate (in glacial acetic acid) at about 70°C for 20 minutes and 5 minutes respectively. After dilution with water, the resultant acids or aldehydes were recovered by extraction with diethyl ether or chloroform respectively, the extract well washed with water, dried over calcium chloride and filtered.

3.3.4.2 Quantitative acetylation of alcohols

Two equally effective acetylation techniques were employed. In the first, the whole-wax or its PLC-isolated primary alcohol fraction in chloroform or diethyl ether was acetylated, at room temperature, by addition of a few drops of an ice-cold stock reagent [Fritz and Schenk, 1959] formulated by addition of 4 g (3.5 ml approx.) of 60 percent perchloric acid to 150 ml ethyl acetate (previously washed, successively, with an equal volume of 5 percent sodium carbonate solution and saturated calcium chloride solution, dried over sodium and freshly distilled), followed by addition of acetic anhydride (8 ml), mixture allowed to stand at room temperature for 30 minutes before cooling to $5^{o}C$ when a further 42 ml of acetic anhydride was added. The wax or wax-fraction treated with this reagent was passed through a basic alumina (type H) column (80 x 8 mm) and the acetates eluted with chloroform.

In the second technique, the chloroform solution of the pure alcohols was treated in the cold with a few drops of acetyl chloride and the excess reagent quenched by addition of a few drops of bench sodium hydroxide. The contents were diluted with water and the acetates extracted into chloroform. The extract was well washed with water, dried over calcium chloride and filtered.

3.3.4.3 Methylation of fatty acids and triterpenoids

The fatty acid or triterpenoid fraction was dissolved in the minimum volume of diethyl ether (ca. 0.5 ml) and treated with ethereal diazomethane generated by inserting a few milligrams of ice-cold N-methyl-N-nitroso-urea into a cone-shaped 50 ml quick-fit flask containing ice-cold concentrated aqueous potassium hydroxide solution (20 ml) covered with diethyl ether (10 ml). The container was submerged in warm water and the liberated diazomethane (in ether) bubbled through the ethereal solution of the sample, under a hood.

3.3.4.4 Bromination of carbon-carbon double bonds

Primary alcohols (as acetates) or fatty acids (as methyl esters) suspected to contain unsaturated members were taken up in the least amount of chloroform and a drop of liquid bromine added. The contents were passed through a basic alumina (type H) column (80 x 8 mm) and the acetates or methyl esters eluted with chloroform.

3.3.5 The cabbage-wax standard on TLC

The wax of *Brassica oleraceae* var. capitata (Cruciferae) was used consistently as a standard to aid preliminary chemical class identification of the

test wax constituents. Although the chemical composition of cabbage leaf wax has been well studied previously [see Martin and Juniper, 1970] the relative positions of the spots of the reported constituents, on our developed chromatograms, had to be established beyond doubt. The dark-green, outermost senescent leaf wax, obtained by 5 minute-refluxing in chloroform, was spotted on 250 μm thick Si gel G film and developed in benzene to a height of 16 cm. Upon location with DDMP, at least six strong spots, in addition to more polar materials remaining at the starting point, were observed at hRfs of 18, 52, 74, 85, 91 and 97 (averaged from 90 TLC separations of different waxes). Those at hRfs 18 and 97 co-chromatographed with authentic eicosan-1-ol and dotriacontane respectively. Further, on-the-plate acetylation of the whole-wax prior to development and subsequent visualization, caused disappearance of the hRf 18 and 52 spots with enhanced intensity of that at hRf 91 and appearance of another at hRf ca.65. A similarly acetylated mixed-spot of whole-wax and eicosan-1-ol caused an increased intensity at hRf 91 while that of hRf ca.65 remained unaltered. Thus the spots at hRfs 18, 52, 91 and 97 corresponded to primary alcohol, secondary alcohol, alkyl esters and alkane components of *Brassica*, in that order.

On-the-plate treatment of the whole-wax spotting with the DNP reagent prior to development in benzene, afforded three spots (Figure 3), a reddish spot at hRf 1 due to excess reagent, a yellow hydrazone only slightly removed from coincidence with those of eicosanal and octadecanal (the aldehydes were prepared as per Section 3.3.4.1) and, an orange-yellow hydrazone running faster than the yellow one. Judged from their colour, the yellow and orange-yellow hydrazones would be due to long chain fatty aldehydes and ketones respectively.

To find out which of the *Brassica* components at hRfs 74 and 85 was an aldehyde and which a ketone, a 2-Dimensional thin layer development was carried out by making two spottings, A and B, of the wax towards one corner of a 250 μm thick, 20 x 20 cm Si gel G film, at a separation of 2 cm from each other.

After development in benzene, A was first visualized with DDMP (employed here so as to produce an hRF-check chromatogram against iodine visualization of B - see Section 3.3.3.2.1) followed by insertion of the plate in iodine-vapour-saturated tank. Those spots in B corresponding to strong spots in A were marked by needle punctures, one on the centre of each. The visualized components of A were removed (and discarded) by carefully scrapping-off the adsorbent

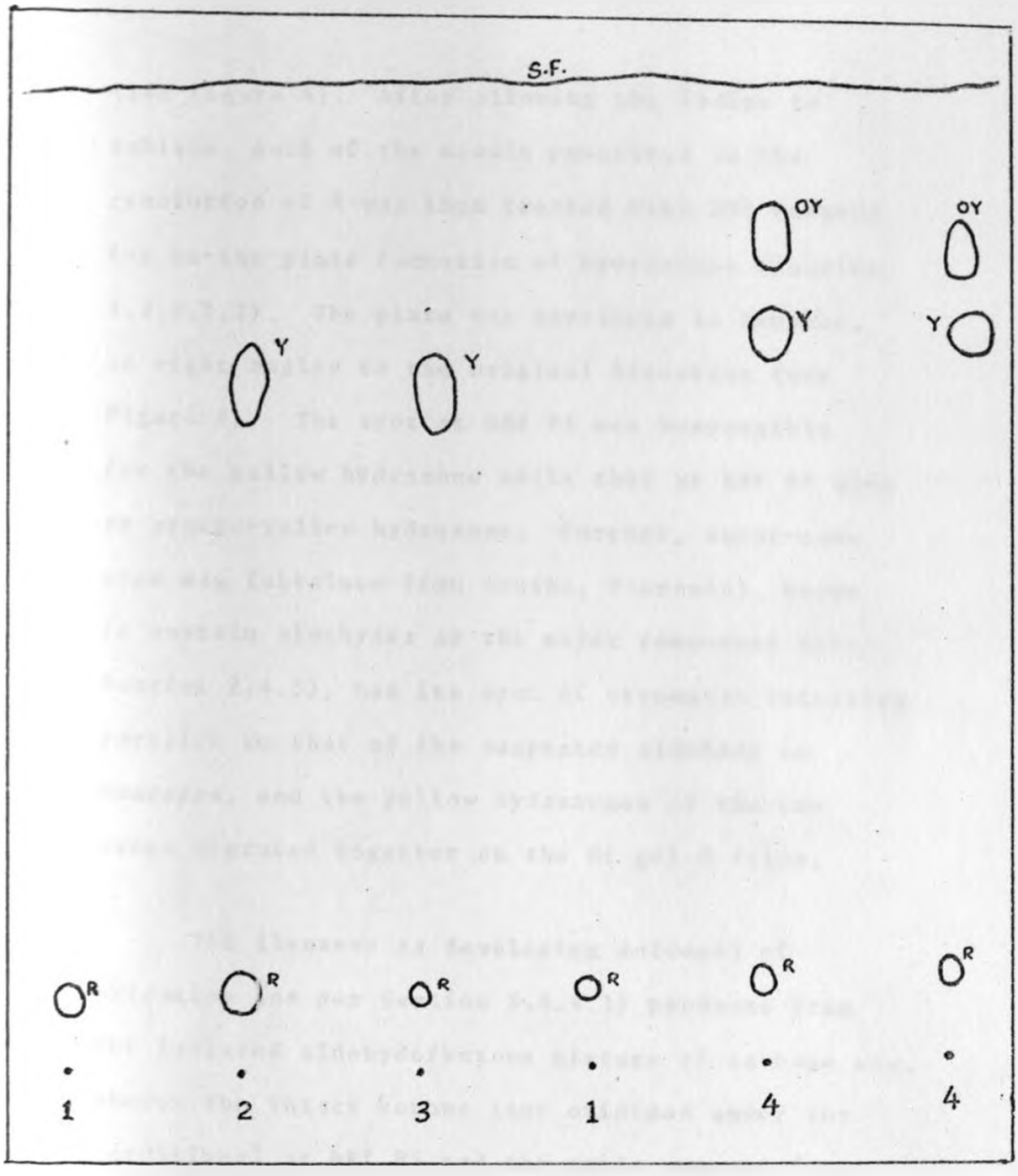

Figure 3 RESOLUTION OF 2, 4-DINITROPHENYLHYDRAZONES OF CABBAGE LEAF WAX.
Adsorbent: Silica gel G. Solvent:Benzene
Hydrazones formed by on-the-plate treatment of samples with DNP reagent.
1 = DNP reagent; 2 = Eicosanal plus 1;
3 = Octadecanal plus 1; 4 = Cabbage leaf wax plus 1.
Colour code: R = red; Y = Yellow; OY = Orange-yellow

(see Figure 4). After allowing the iodine to subline, each of the needle punctures in the resolution of B was then treated with DNP reagent for on-the-plate formation of hydrazones (Section 3.3.3.2.2). The plate was developed in benzene, at right angles to the original direction (see Figure 4). The spot at hRf 74 was responsible for the yellow hydrazone while that at hRf 85 gave an orange-yellow hydrazone. Further, sugar-cane stem wax (obtained from Arusha, Tanzania), known to contain aldehydes as the major component (see Section 2.4.5), had its spot of strongest intensity parallel to that of the suspected aldehyde in *Brassica*, and the yellow hydrazones of the two waxes migrated together on the Si gel G films.

TLC (benzene as developing solvent) of oxidation (as per Section 3.3.4.1) products from PLC isolated aldehyde/ketone mixture of cabbage wax, showed the intact ketone (not oxidized under the conditions) at hRf 85 and the acids unmoved from the starting point. The latter gave a positive response to the PLDP reagent (Section 3.3.3.2.2). The methylated acids (obtained after PLC of the oxidation products and subsequent treatment as per Section 3.3.4.3) co-chromatographed on GLC (see Section 3.3.7 for conditions) with methyl esters of the acids obtained from similarly oxidized

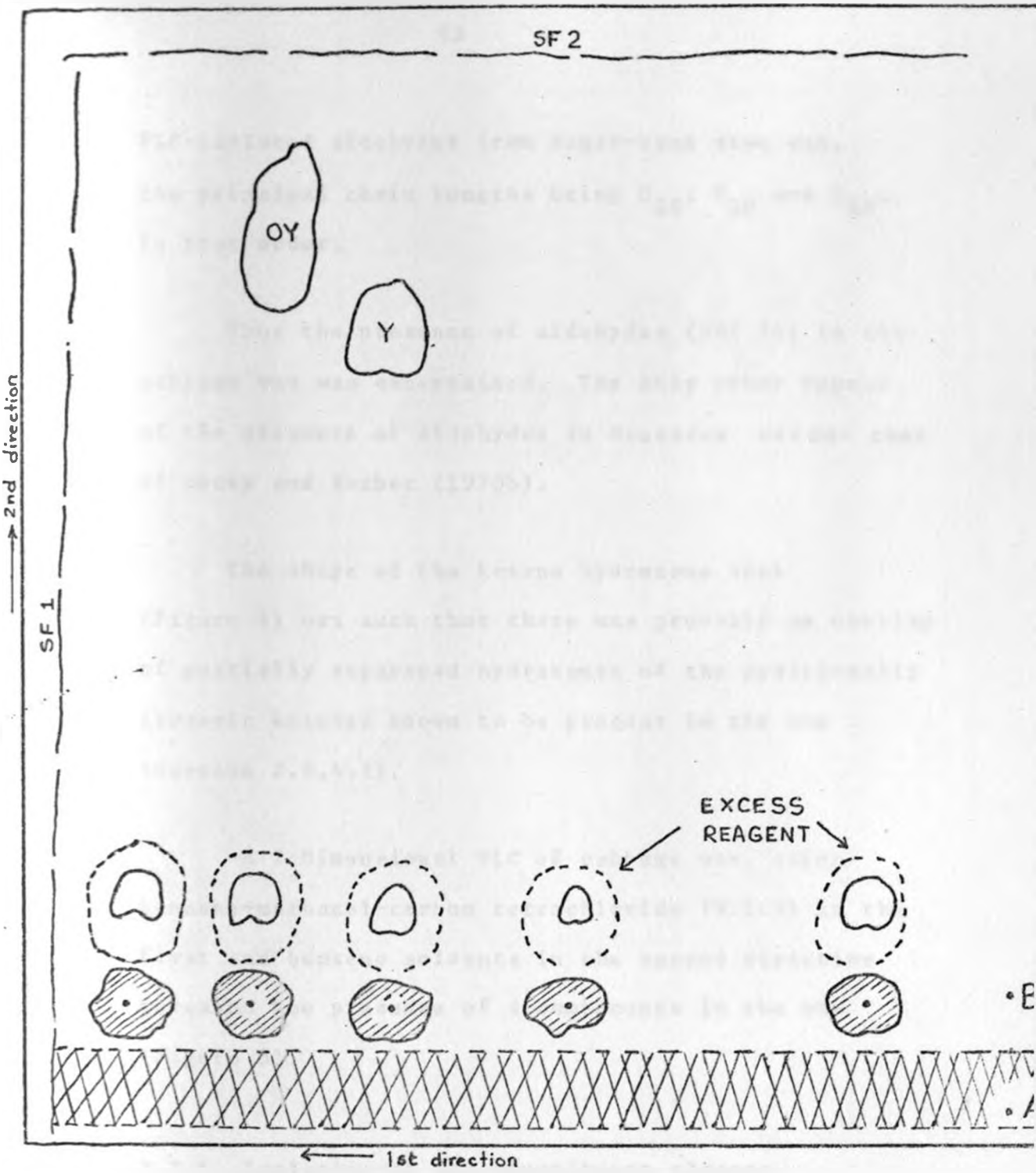

Figure 4 RESOLVED BRASSICA WAX (A and B), PRINCIPAL COMPONENTS DNP-treated, HYDRAZONES DEVELOPED IN PERPENDICULAR DIRECTION.

Adsorbent: Silica gel G; Solvent: benzene in both directions.

Location: sequential, A with DDMP then B with iodine vapour.

▨ - adsorbent scrapped off after Location sequenc

▨ - region (surrounding puncture) treated with DNP reagent.

OY = Ketone hydrazones; Y = Aldehyde hydrazones.

PLC-isolated aldehydes from sugar-cane stem wax, the principal chain lengths being C_{28}, C_{30} and C_{26}, in that order.

Thus the presence of aldehydes (hRf 74) in the cabbage wax was ascertained. The only other report of the presence of aldehydes in *Brassica* wax was that of Macey and Barber (1970b).

The shape of the ketone hydrazone spot (Figure 4) was such that there was probably an overlap of partially separated hydrazones of the positionally isomeric ketones known to be present in the wax (Section 2.4.4.1).

A 2-Dimensional TLC of cabbage wax, using benzene-methanol-carbon tetrachloride (9:1:3) in the first and benzene solvents in the second direction revealed the presence of 9 components in the wax (Figure 5).

3.3.6 Isolation of wax constituent classes

The wax sample (ca. 10 mg) was taken up in the minimum volume of chloroform (ca.0.5 ml) and the solution streaked as a band on the Si gel G film (see Section 3.3.3.1). About 1.5 cm strips on either ends of the developed band were visualized with DDMP reagent. Developed in benzene,the bands at

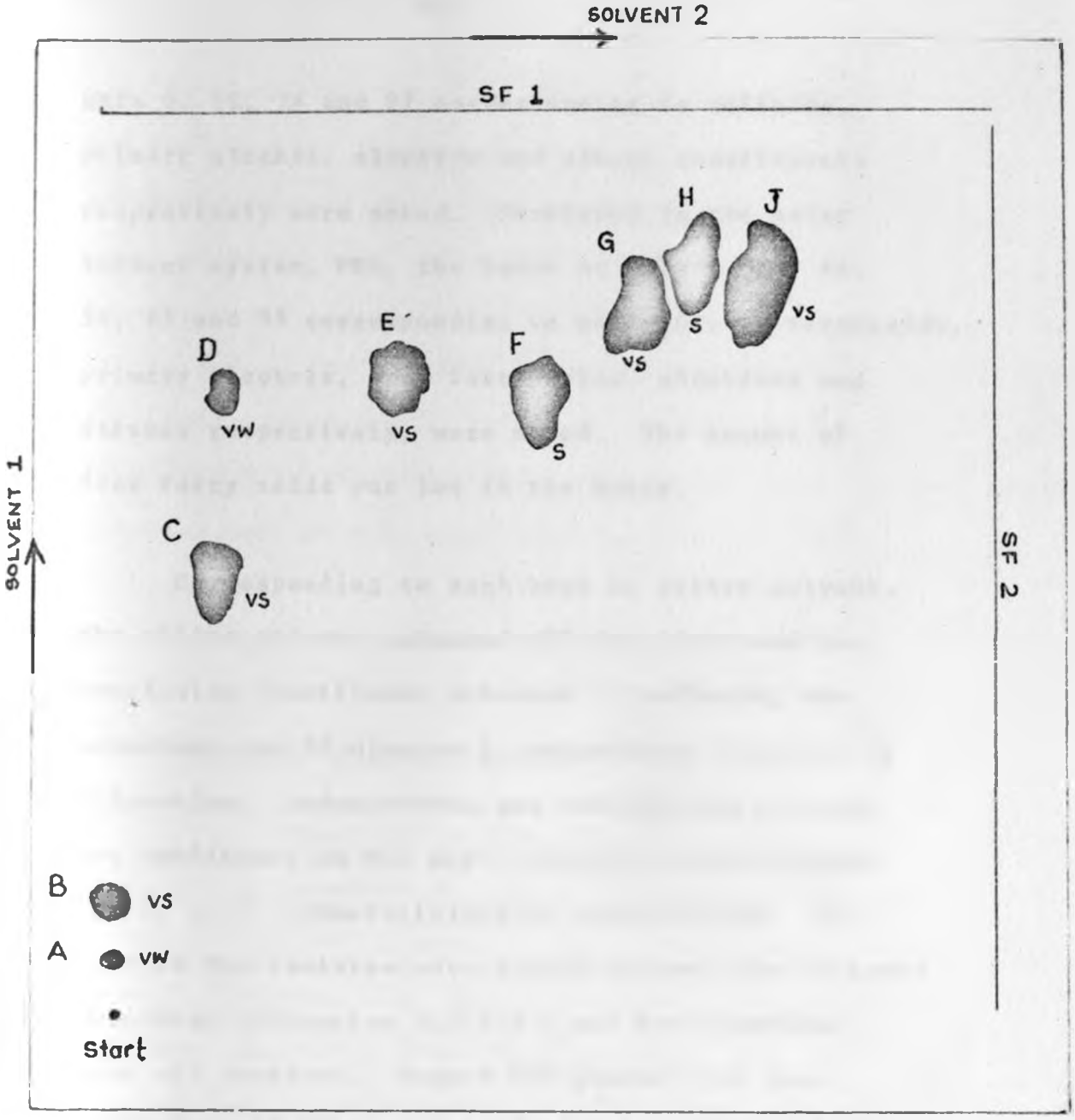

Figure 5 TWO-WAY RESOLUTION OF CABBAGE LEAF WAX STANDARD ON SILICA GEL G. Location by DDMP reagent.
Solvent 1 = benzene-methanol-carbon tetrachloride (9:1:3)
Solvent 2 = benzene
A = unknown; B = fatty acids; C = primary alcohols; D = ketols; E = secondary alcohols; F = aldehydes; G = ketones; H = esters; J = alkanes.

hRfs 0, 18, 74 and 97 corresponding to caffeine, primary alcohol, aldehyde and alkane constituents respectively were noted. Developed in the polar solvent system, PEA, the bands at hRfs 0, 34, 44, 54, 83 and 95 corresponding to caffeine, triterpenoids, primary alcohols, free fatty acids, aldehydes and alkanes respectively, were noted. The amount of free fatty acids was low in the waxes.

Corresponding to each band in either solvent, the silica gel was scrapped off the plate and the particular constituent obtained by refluxing the adsorbent for 30 minutes in chloroform, followed by filtration. Hydrocarbons and caffeine were tested for confluence on TLC with authentic dotriacontane and 1, 3, 7 -trimethylxanthine respectively. The rest of the isolates were tested against the reagents described in Section 3.3.3.2.2 and the responses were all positive. Twenty PLC plates were just sufficient to give enough of each constituent for infra-red spectroscopy on a Unicam SP 200G.

Given the high concentration of caffeine material (unmoved from the base-line by either solvent) and the limited loading capacity of thin layer films, the free fatty acids for GLC analysis were obtained quantitatively by a quicker method which allowed the use of larger quantities of the wax

samples than PLC can allow. The wax (ca. 1 g) was refluxed for 5 minutes in concentrated methanolic potassium hydroxide solution (15 ml). After dilution with water (10 ml), the non-acidic materials were extracted into chloroform (20 ml x 3). The aqueous phase was acidified with conc. hydrochloric acid and the free acids extracted into diethyl ether (20 ml x 3). The ethereal extract was dried over calcium chloride, filtered and the acids derivatized, for GLC analysis, as described under Section 3.3.4.3.

3.3.7 Gas Liquid Chromatography

A Pye Argon Gas Chromatograph (^{90}Sr detector) was employed throughout. Hydrocarbons and aldehydes were fed into the column directly. The less volatile primary alcohols, free fatty acids and triterpenoids were analysed only after derivatization (see Section 3.3.4): primary alcohols as their acetates and the latter two as their methyl esters. Samples, adsorbed on celite (80 - 100 mesh), were applied to a 30 x 0.5 cm glass column of the same celite grade coated with 1.0 percent Apiezon L grease. The stationary phase (0.1 g) was dissolved in warm chloroform and deposited, whilst continuously stirring and warming, on celite (10 g). The column was operated, for most of the time, at a temperature

of 238°C and therefore new columns had to be made as frequently as necessary to restore the composition of the stationary phase to its initial level.

With the β-emitter detector, the detector response is always proportional to the molecular concentration. Areas (in sq. mm) of individual molecular peaks were calculated by triangulation and the molecular concentrations in the sample expressed as a percentage based on the sum of the areas of all peaks under the chromatogram. For all analyses, the detector voltage was set at 1750V at an attenuation (sensitivity) x 10 and an argon gas flow rate of 15 ml/min.

For each leaf age, the concentration of the hydrocarbons on the leaf surface was taken as the ratio of the sum of the areas of all measurable peaks under the chromatogram to the total surface area (see Section 3.3.2) of the sample leaf-piece(s) considered. This ratio was referred to as the Hydrocarbon Concentration Index (HCI) - [Herbin and Robins, 1969].

The total abundances of odd to even carbon-number chain lengths in a wax fraction was expressed numerically in terms of an unbiased

Carbon Preference Index (CPI) which is the mean of two ratios determined by dividing the sum of the concentrations of odd carbon-number homologs by the sum of the concentrations of even carbon-number ones over two concentration ranges [Cooper and Bray, 1963]. Expressed mathematically for an arbitrary homologous series covering the range C_{21} to C_{33}, such an index would take the form:

$$CPI = \frac{1}{2}\left[\frac{\sum_{n=21}^{33} C_n \text{ odd}}{\sum_{n=20}^{32} C_n \text{ even}} + \frac{\sum_{n=21}^{33} C_n \text{ odd}}{\sum_{n=22}^{34} C_n \text{ even}}\right]$$

Chain lengths were assigned to molecular species by co-chromatography of the wax fraction with authentic standards (when available) or similar fractions isolated from waxes previously studied by other workers. Such waxes included, amongst others, paraffin wax (for alkanes) and

sugar-cane stem wax (for aldehydes). The authentic standards that were available included tetracosane, dotriacontane, eicosanol, hexacosanol, and the methyl esters of C_{16} to C_{26} fatty acids containing an unsaturated C_{18} member but with the C_{21} and C_{25} saturated homologs missing. Primary alcohol chain lengths were further confirmed by oxidizing the fraction to the correponding aldehydes (see Section 3.3.4.1) followed by direct co-chromatography of the latter with PLC-isolated aldehydes of sugar-cane stem wax known to contain octacosanal as the principal homolog *[Kranz et al., 1960]*.

Retention distances were frequently taken as the distance from the air-peak to the points of inflexion where the molecular peaks were at their maximum heights although distances measured to the points of ascent of these peaks yielded equivalent results. For each wax fraction, molecular species comprising an homologous series were ascertained from a plot of the logarithm of the retention distances versus carbon number, for which series the semi-logarithmic curve was a straight line.

CHAPTER 4

RESULTS AND CONCLUSIONS

4.1 Chemical identity of PLC isolates

Representative Infra-Red spectra* were done on PLC isolates from senescent coffee leaf wax except for the triterpenoids which were isolated from the wax of young leaves.

Run in solution in carbon tetrachloride, the hydrocarbons exhibited absorptions at 2920, 2845 (C-H); 1464, 1379 (CH_2, CH_3) and 715 [$(CH_2)_n$] cm^{-1}

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*In the particular double-scan Unicam SP 200G instrument used, the lower wavelength range (650 to 2000 cm^{-1}) is known to have been permanently mis-calibrated such that absorption wave-numbers registered over the region (1300 to 2000 cm^{-1}) of overlap with the upper wavelength range (1300 to 4000 cm^{-1}) are always in total disagreement. Absorptions occurring in this region have therefore been quoted from the upper range scan. The spectra herein presented as appendices or otherwise are a product of fitting together the scans over the lower and upper wavelength ranges to give a single perspective for ease of reference. The only exception is the spectrum shown in Appendix 4B which was single-scanned on a Perkin-Elmer instrument at the International Centre for Insect Physiology and Entomology (ICIPE), Nairobi, Kenya.

characteristic of long-chain alkanes. The characteristic long-chain aldehyde absorption was observed at 2725 (H-CO) cm^{-1} (Appendix 1, A and B). The aldehyde absorption spectrum was in harmony with that of PLC isolated sugar-cane wax aldehydes (Appendix 3A) and aldehydes synthesized by oxidation of long chain alcohols (see Section 3.3.4.1) of known identity. Upon acetylation, the primary alcohols showed a disappearance of the band at 3640 (O-H) with increased intensity of the band at 1050 (C-O symm. stretch) and the appearance of ester bands at 1760 (C = O str.) and 1235 (C-O asymm. str.) wave-numbers (Appendix 2, A and B). There was however, prior to acetylation, an absorption at 1735 cm^{-1} which was absent in the spectrum of the authentic C_{20} alcohol (Appendix 2, C and D). The spectrum of the free fatty acids in carbon tetrachloride had an absorption at 1735 cm^{-1} (Appendix 4A) which was shifted to 1745 cm^{-1} in chloroform (Appendix 3B). The chloroform phase spectrum had an additional band at 1620 cm^{-1} possibly due to unsaturation. The methylated fatty acid spectrum in CCl_4 (Appendix 4B) showed bands at 1739 and 1120 wave-numbers as expected for an esterified carbonyl function.

The material identified as caffeine had the following properties in common with authentic caffeine.-

i. On TLC, it was unmoved by benzene or PEA solvent systems and co-chromatographed with the authentic sample in all solvents tried, viz: chloroform, benzene-methanol (9:1), benzene-methanol-carbon tetrachloride (9:1.3) and n-hexane-ethyl acetate (1.1). Its colour reaction to the DDMP reagent (see Section 3.3.3.2.1) was identical to that of authenticated caffeine and different from that of all other wax constituents examined.

ii. The material was elutable from a short, basic alumina, column with methanol and its U.V. spectrum in that solvent (Figure 6A) showed a λ_{max} at 272 mμ (literature value in alcohol (usually ethanol) is 274 mμ).

iii. The IR spectrum (nujol mull) exhibited characteristic bands at 1700 (w), 1665 (s), 1545 (w) and 1235 (w) wave-numbers but the possibility of the presence of other caffeine analogues such as theobromine (3, 7-dimethylxanthine) and/or theophylline (1, 3-dimethylxanthine) could not be ruled out in view of the strong band observed at 1095 cm^{-1} which was absent in the spectrum of the authentic sample (Figure 6, B and C).

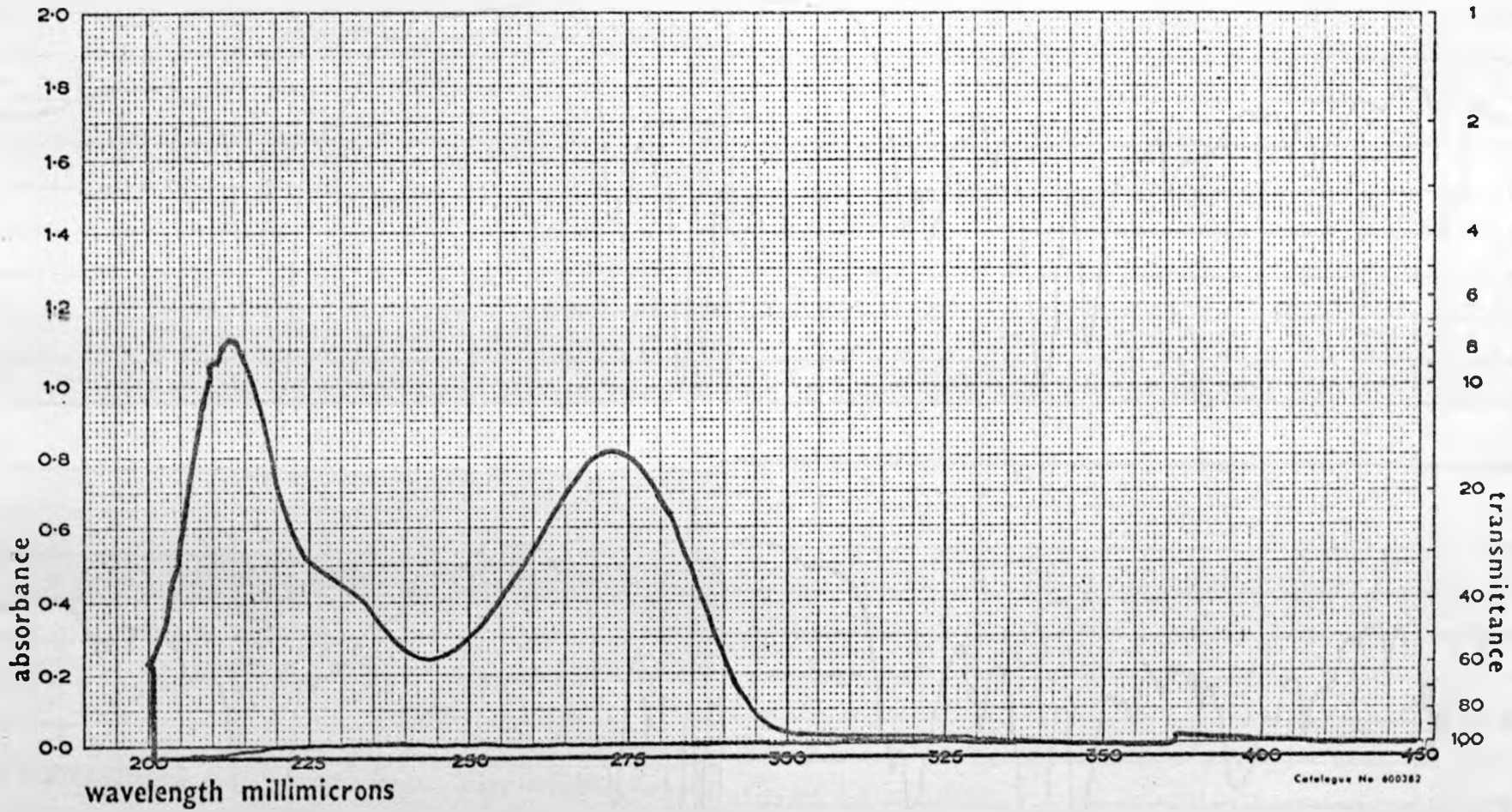


Figure 6A U.V. spectrum of caffeine material from coffee leaf epicuticular wax.

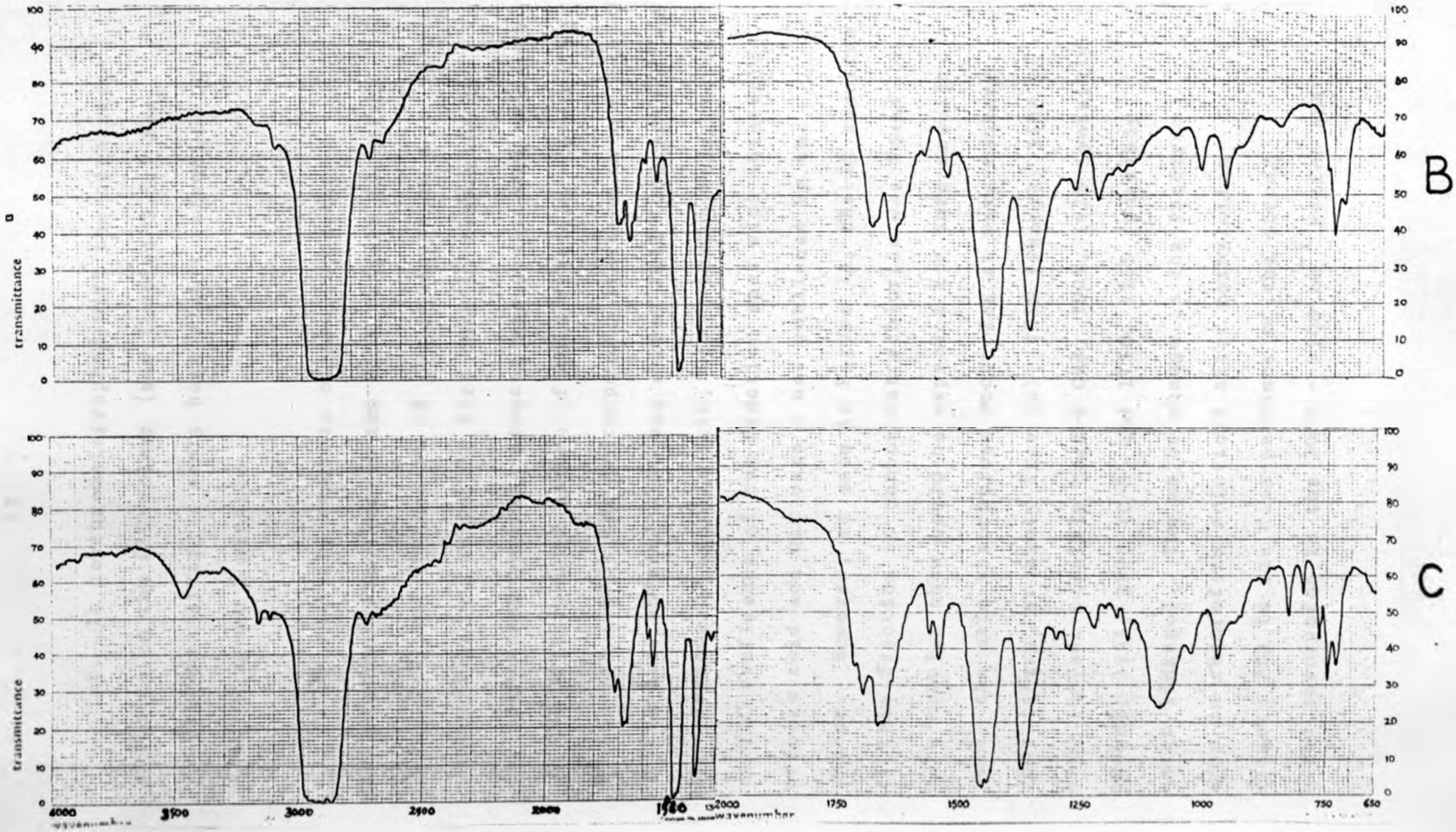


Figure 6B and C Infra-red spectra (nujol mull) of authentic caffeine (B) and caffeine material from coffee leaf epicuticular wax (C).

iv. It co-chromatographed with the authentic caffeine on the GLC column (see Section 3.3.7 for conditions) giving a sharp peak with a retention time of about 5 minutes.

The positive response of the suspected triterpenoid fraction to the PLDP reagent (Section 3.3.3.2.2) was indicative of the presence of a carboxyl group, while the fact that it responded to the Liebermann-Burchard reagent was additional evidence of the triterpenoid nature of this fraction. According to Kabaara (1973) the triterpenoid fraction from coffee leaf epicuticular wax showed "all indications" of the presence of ursolic acid, the evidence being based on TLC confluence of the fraction spot with authentic ursolic acid and GLC data - not published by the author. However, as seen in Figure 7A, the IR spectrum of this fraction in carbon tetrachloride was found more complex than could be expected for pure ursolic acid, the main absorptions occurring at wave-numbers 3680 (sharp, probably free OH); 3535 (sharp); 3480 to 3100 (broad, possibly bonded OH); 3005, 1625 (possibly >C=CH); 2945, 2895 (C-H) and 1750, 1265 (possibly ester linkage). The major change in the spectrum (run in chloroform solution) after methylation (Figure 7B) is the disappearance of the strong broad absorption in the 3000 - 3500 cm^{-1} region and

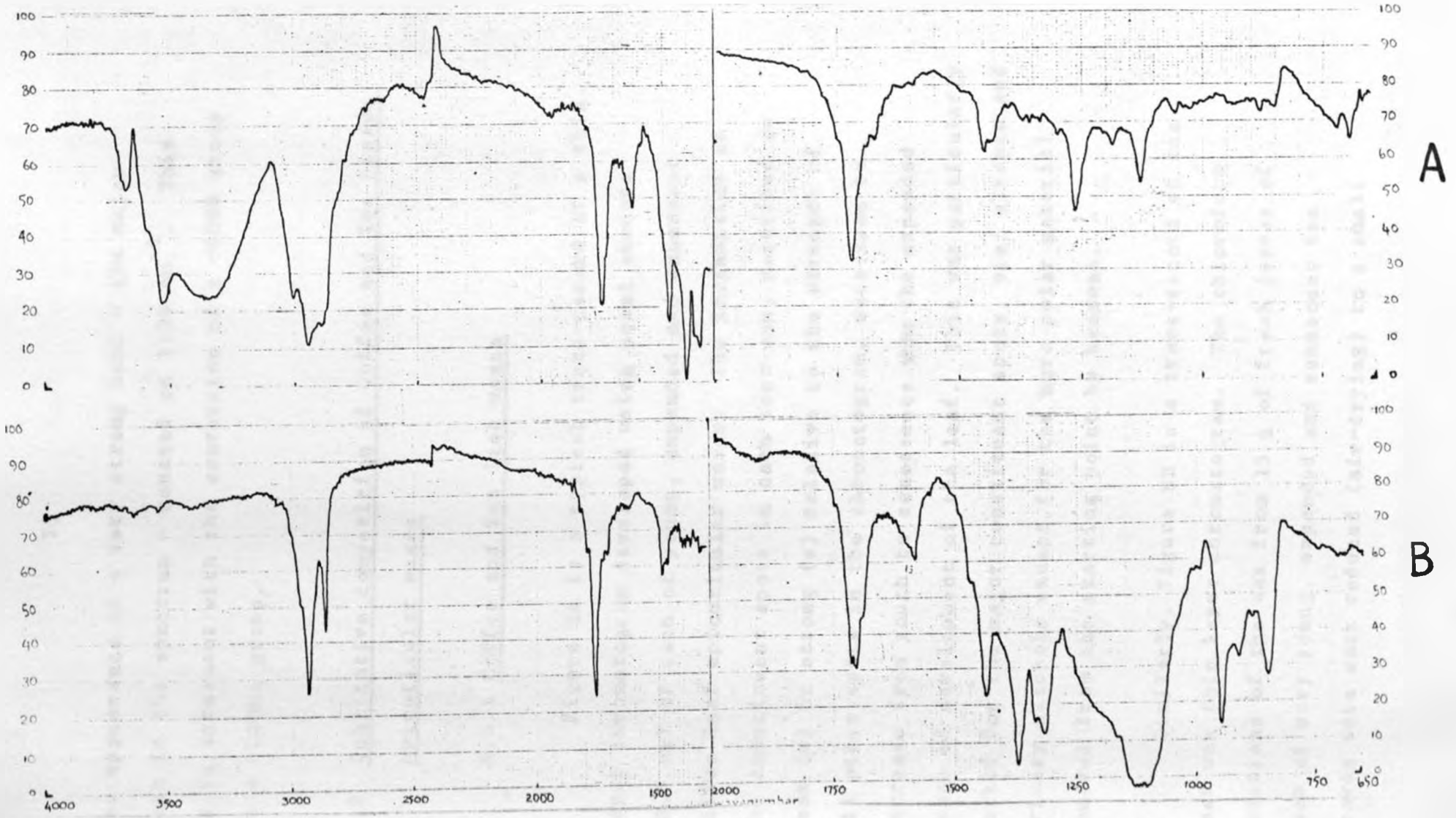


Figure 7 IR spectra of triterpenoid fraction from young coffee leaf epicuticular wax. A - in CCl_4 prior to methylation

B - in $CHCl_3$ after methylation

the appearance of a very strong band - the major peak in the spectrum - centred on 1130 cm^{-1}. This is in agreement with the conversion of a -COOH group to a -COOMe group.

4.2 Qualitative composition of Coffee and Tea tissue epicuticular waxes

4.2.1 Coffee and Tea leaf waxes

Figure 8A is a typical trace-record of a thin layer chromatogram developed using equal amounts (0.5 mg) of each of young, expanded and senescent coffee leaf epicuticular waxes. The intensities of the constituent spots in each test-wax, described as weak (w) or strong (s) relative to the strength of all other spots in the chromatogram, were seen to increase from youth to senescence with the expanded stage of development of the leaf. This was particularly marked for the major constituent spots, viz: alkanes and primary alcohols except for the more polar material unmoved from the starting point by benzene.

Similarly, Figure 8B is a trace-record of tea leaf wax thin layer chromatogram. The chloroform solutions of the wax from 15 g of fresh leaves of each of very young, expanded and senescent tea leaves were each reduced (air-drying) to a small

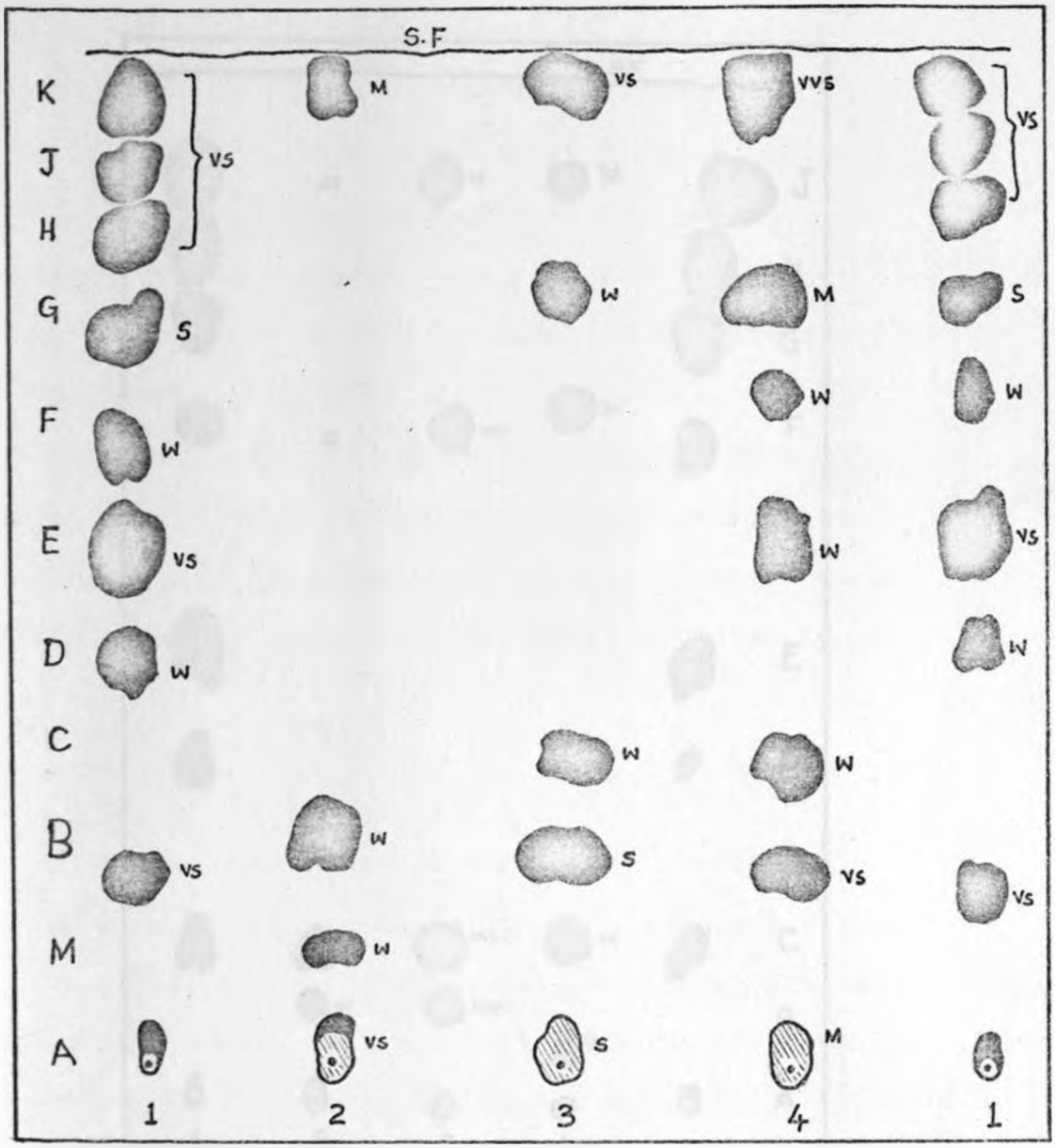


Figure 8A TLC of Coffee leaf epicuticular waxes
Adsorbent: Silica Gel G; Solvent: benzene
Location: DDMP spray reagent
Samples: 1 = Cabbage leaf wax; 2, 3 and 4 = 0.5 mg loads of very young, expanded and senescent coffee leaf waxes respectively.
A = polar materials including caffeine (shaded);
B = primary alcohols; E = secondary alcohols;
G = aldehydes; H = ketones; J = esters;
K = alkanes; D = possibly ketols;
F, C and M = unknown.

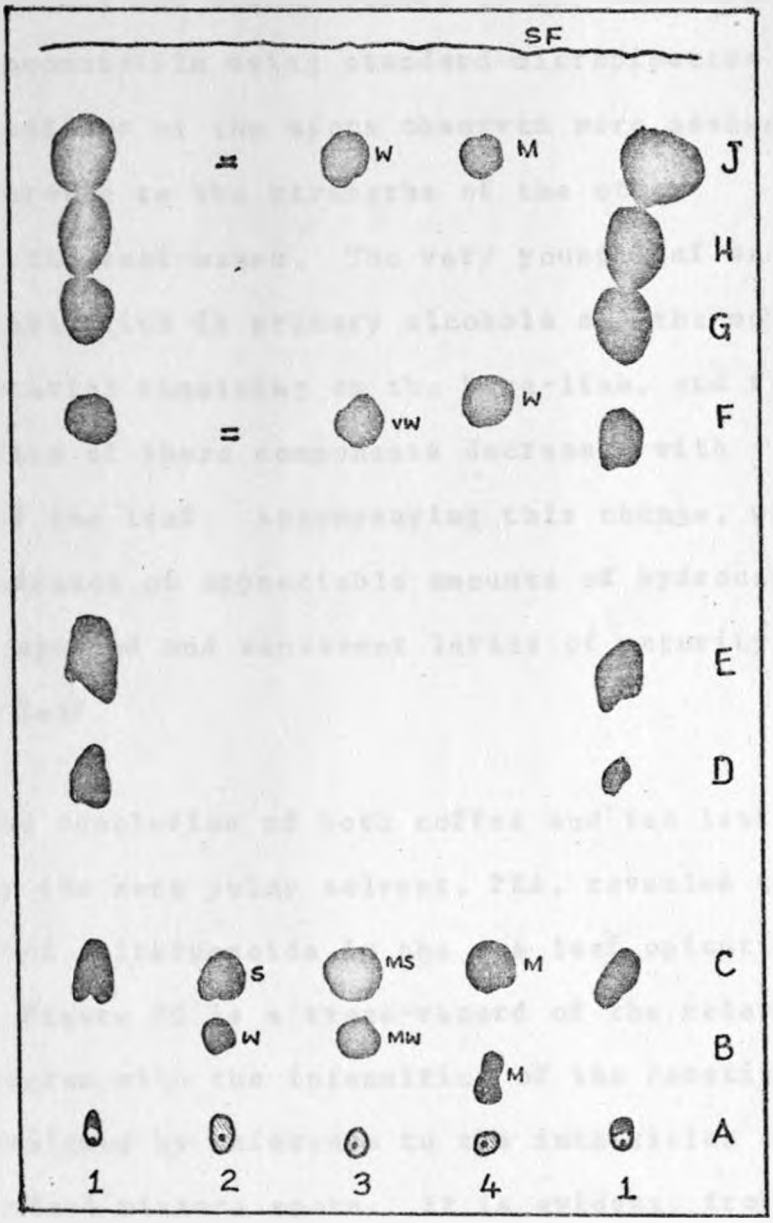


Figure 8B TLC of Tea Leaf epicuticular waxes

Adsorbent: Silica Gel G; Solvent: benzene

Location: DDMP spray reagent

Samples: 1 = Cabbage leaf wax;

2, 3 and 4 = 6 μl loads of very young, expanded and senescent tea leaf waxes respectively.

A = polar materials including caffeine (shaded);

B = unknown; C = primary alcohols;

D = probably ketols; E = secondary alcohols;

F = aldehydes; G = ketones; H = esters;

J = alkanes.

volume (10 ml) and equal volumes (6 µl) monitored on the chromatofilm using standard micropipettes. The intensities of the spots observed were assigned with reference to the strengths of the other spots in the test-waxes. The very young leaf was particularly rich in primary alcohols and the more polar material remaining on the base-line, and the intensities of these components decreased with ageing of the leaf. Accompanying this change, was the occurrence of appreciable amounts of hydrocarbons at the expanded and senescent levels of maturity of the tea leaf.

The resolution of both coffee and tea leaf waxes by the more polar solvent, PEA, revealed the absence of triterpenoids in the tea leaf epicuticular waxes. Figure 8C is a trace-record of the relevant chromatogram with the intensities of the constituent spots assigned by reference to the intensities of the standard mixture spots. It is evident, from this chromatogram, that the more polar material observed in coffee leaf wax (Figure 8A) is comprised of free fatty acids, caffeine and triterpenoids but the tea leaf wax (Figure 8B) differs only in the absence of triterpenoids.

Figure 8D is a two-dimensional separation of senescent coffee leaf wax constituents, using a high

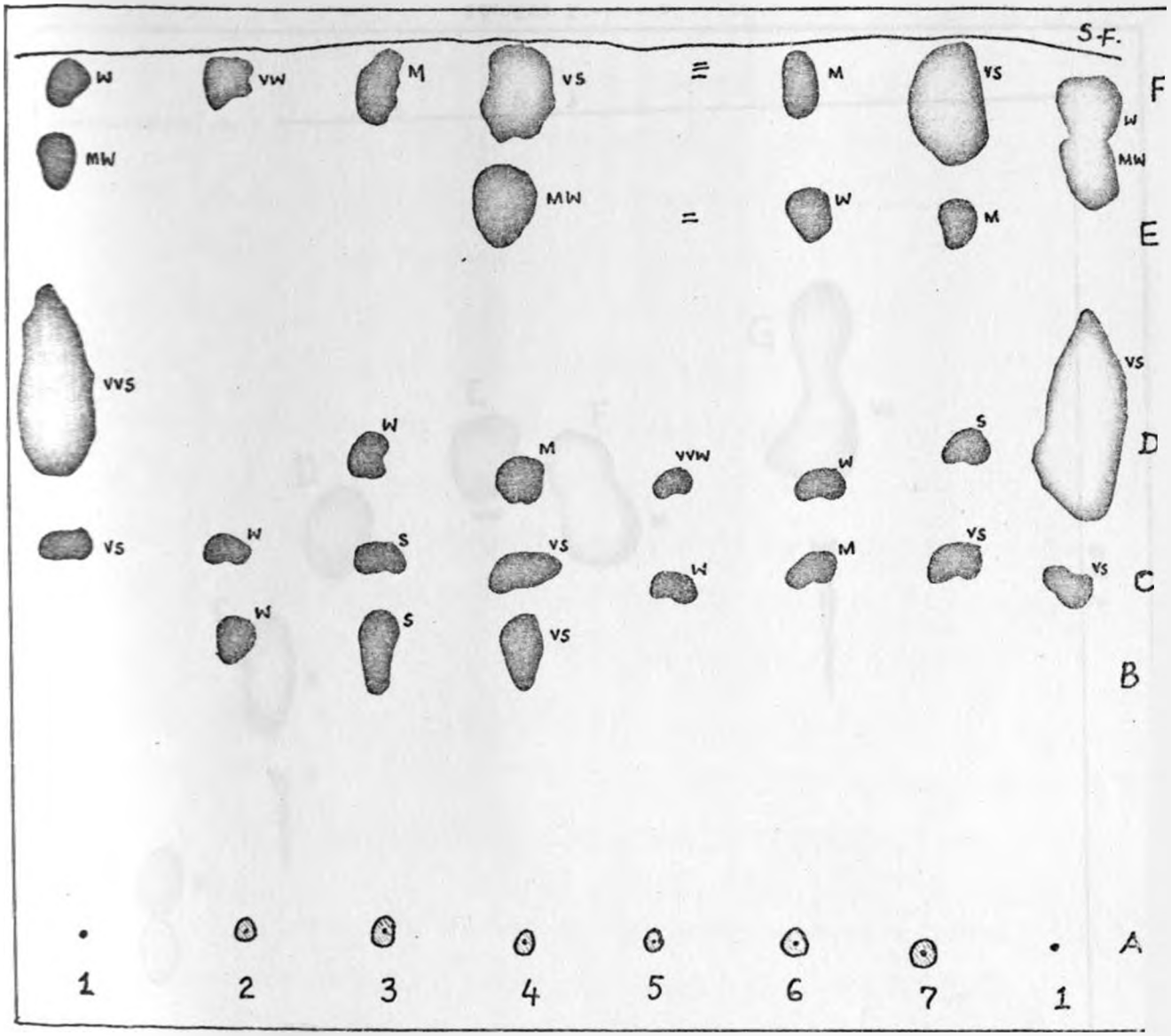


Figure 8C TLC of Coffee and Tea Leaf epicuticular waxes in polar solvent system.

Adsorbent: Silica Gel G; Solvent: PEA;

Location: DDMP spray reagent.

Samples: 1 = standards mixture comprising oleic acid, stearic acid, palmitic acid, docosanoic acid and eicosan-1-ol,

2,3 and 4 = very young, expanded and senescent coffee leaf waxes respectively,

5,6 and 7 = very young, expanded and senescent Tea leaf waxes respectively,

A = Caffeine; B = triterpenoids; C = primary alcohols

D = free fatty acids; E = aldehydes; F = alkanes.

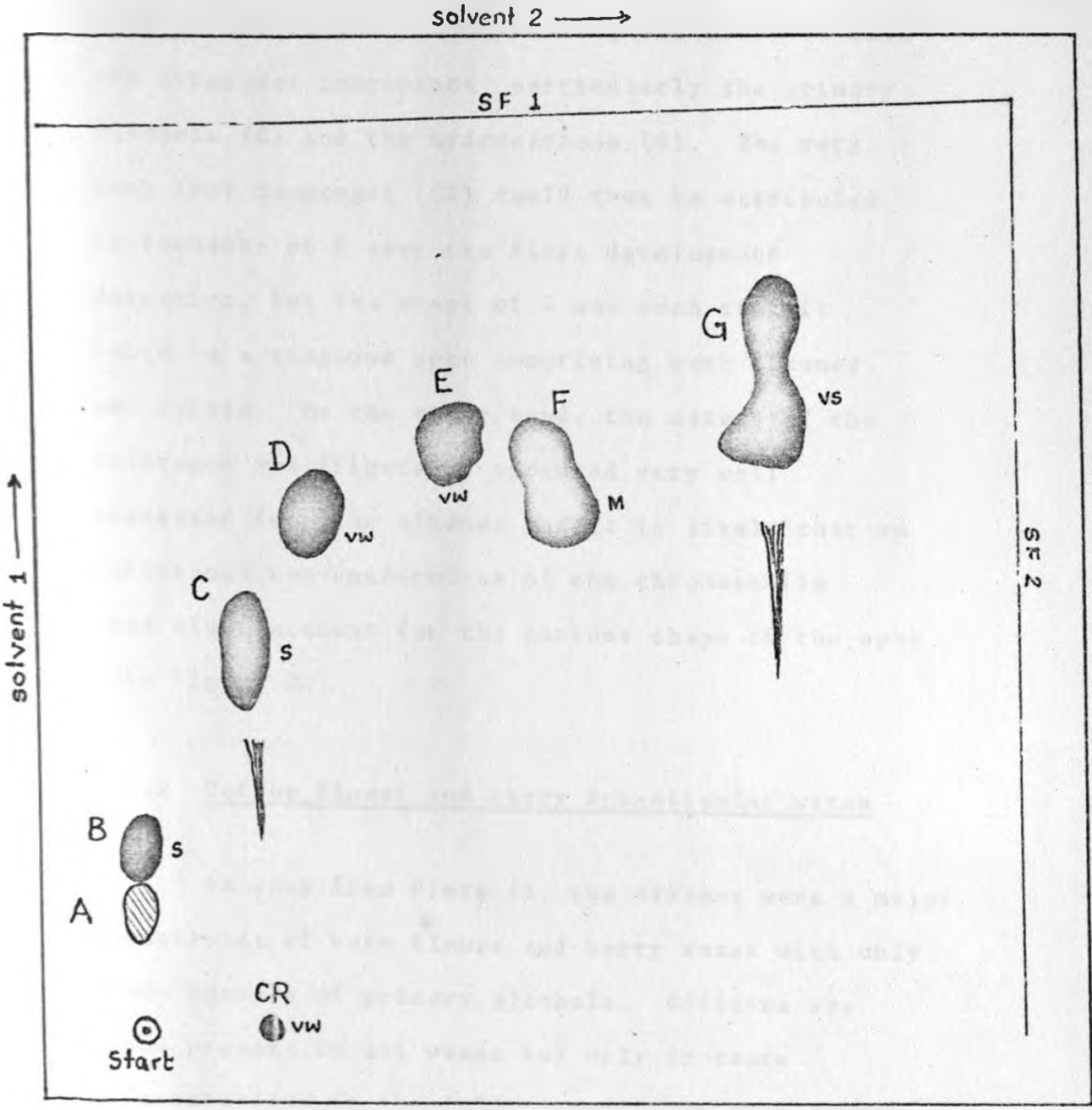


Figure 8D Two-dimensional TLC of Senescent Coffee leaf epicuticular wax.
Adsorbent: Silica Gel G; Solvent 1: benzene-methanol-carbon tetrachloride (9:1:3); solvent 2 = benzene
Sample size: 1 mg of wax; Location: DDMP spray reagent
A = caffeine; B = triterpenoids and fatty acids;
C = primary alcohols; D = unknown; E = secondary alcohols; F = Aldehydes plus unknown;
G = alkanes (possibly plus esters)

load (1 mg) of wax. Some tailing was observed with the strongest components, particularly the primary alcohols (C) and the hydrocarbons (G). The very weak (vw) component (CR) could thus be attributed to remnants of C over the first development direction, but the shape of G was such that it could be a compound spot comprising both alkanes and esters. On the other hand, the esters of the reference wax (Figure 5) appeared very well separated from the alkanes and it is likely that an unforeseen non-uniformness of the chromatofilm used might account for the curious shape of the spot G in Figure 8D.

4.2.2 Coffee flower and berry epicuticular waxes

As seen from Plate 4A, the alkanes were a major constituent of both flower and berry waxes with only trace amounts of primary alcohols. Caffeine was found present in all waxes but only in trace concentrations in the inflorescence and very young berry epicuticular waxes.

In the PEA solvent system, no triterpenoids could be detected in the young berry wax whereas these were present in large amounts in both the hard green and fully ripe berry waxes (Plate 4B). The inflorescence

PLATE 4

TLC of coffee flower and berry epicuticular waxes

Adsorbent: Silica gel G

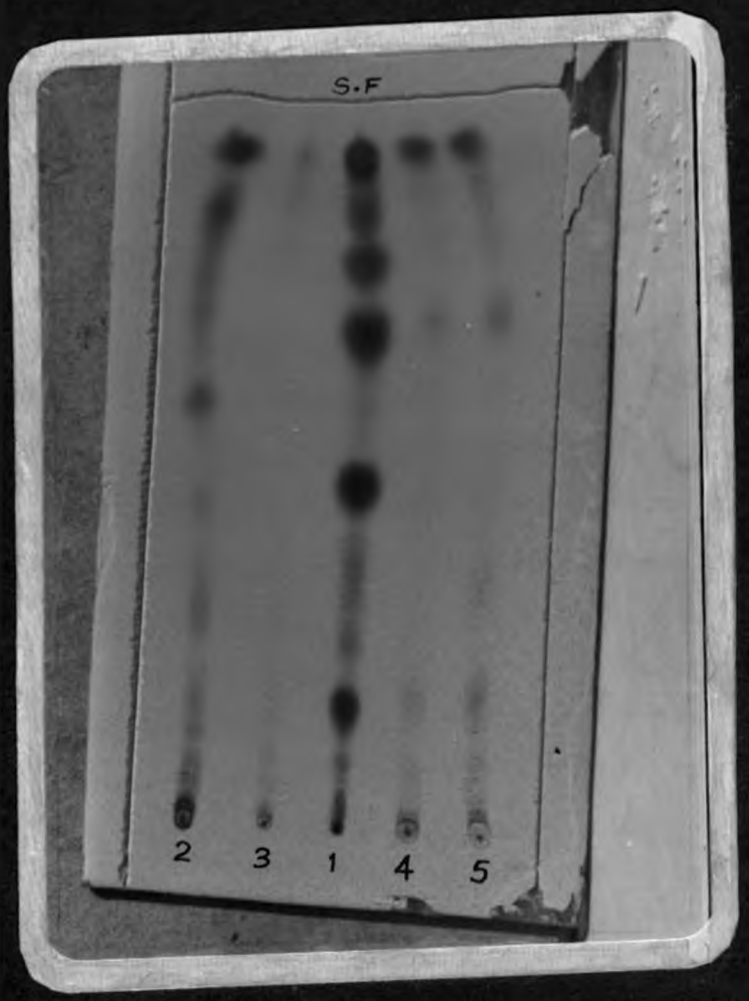
Solvents: A = benzene; B = PEA

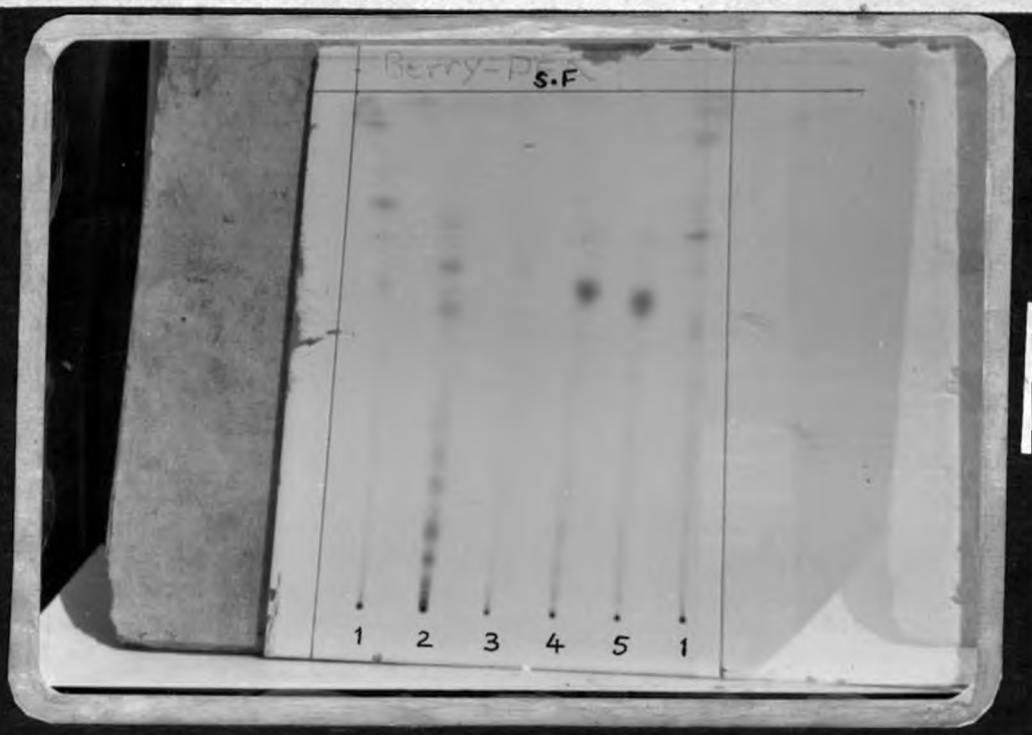
Location: A = DDMP reagent;

B = Liebermann-Burchard reagent.

Samples: 1 = cabbage leaf wax; 2 = coffee flowe wax; 3,4 and 5 = young, hard-green and ripe coffee berry waxes respectively.

A





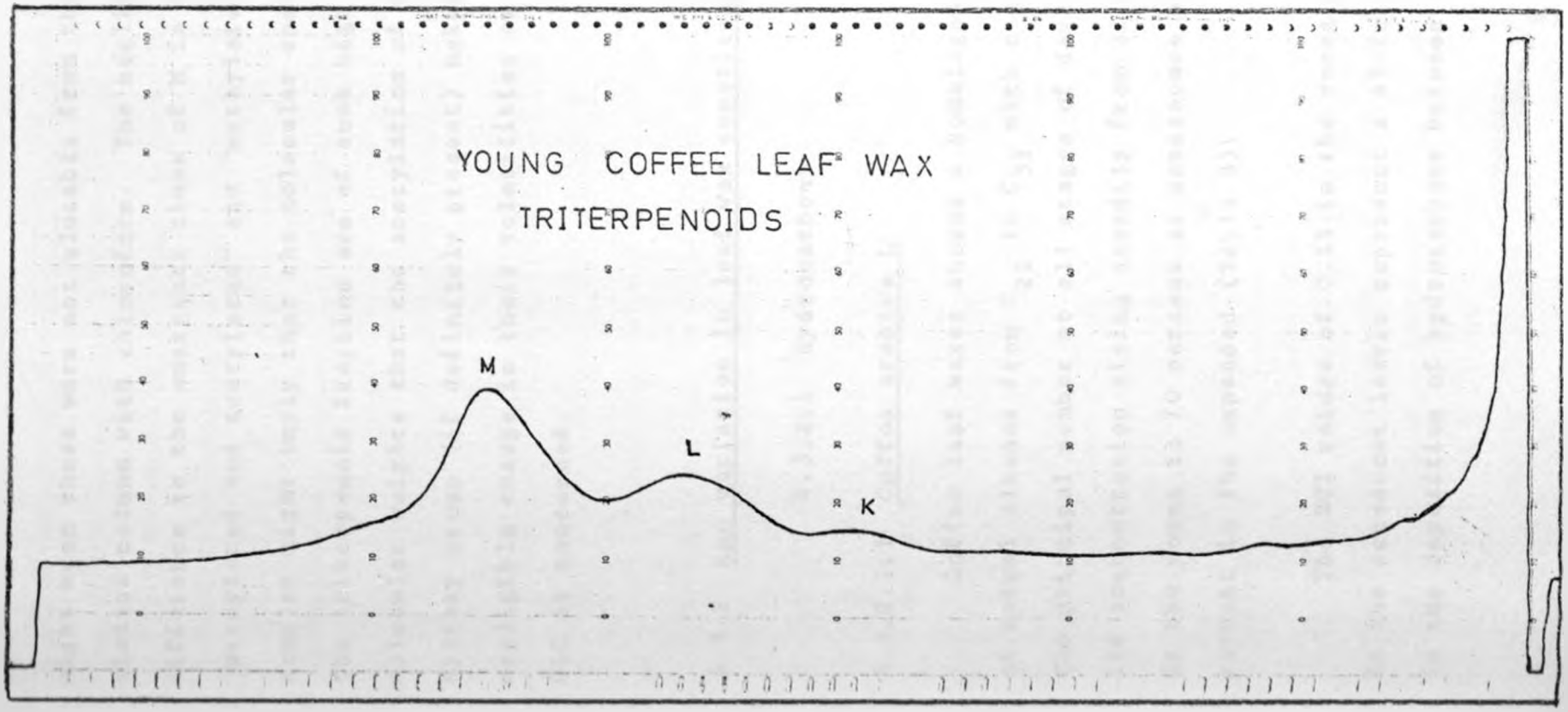
contained triterpenoids but lower in both intensity and spot-area relative to the berry waxes. Further, the flower wax triterpenoid fraction was seen to have a slightly higher mobility than those of the berry waxes. In view of this, a difference in the chemical composition of the triterpenoid fractions of the flower, on the one hand, and berry epicuticular waxes, on the other, can not be completely ruled out.

4.3 Results of GLC analyses

4.3.1 Triterpenoids from very young coffee leaf wax

On GLC, the methylated triterpenoid fraction afforded at least three major peaks, K, L and M (see Figure 9) not of the same homologous series, the major molecular species M emerging from the column after approximately 80 minutes. These results shed some light on the complexity of the infra-red absorption spectrum of the fraction. Acetylation of the methylated triterpenoid fraction, followed by column chromatography over basic alumina with chloroform as eluant, gave, on GLC, only the major peak M but with a slightly shorter retention time than was observed in the "methylated only" sample. The absence of the other components in the glc-trace

Figure 9 GLC trace of triterpenoid methyl esters (see Section 3.3.7 for conditions).



might mean these were not elutable from the basic alumina column with chloroform. The negligible difference in the emergence times of M in the "methylated and acetylated" and "methylated only" samples might imply that the molecular species of the triterpenoid fraction are of such high molecular weights that the acetylation of the hydroxy group (if definitely present) makes a negligible change in their volatilities as far as GLC is concerned.

4.3.2 Age variation in leaf wax constituents

4.3.2.1 Hydrocarbons

4.3.2.1.1 *Coffea arabica* L

Coffee leaf waxes showed a homologous series of normal alkanes from C_{22} to C_{31} with nonacosane the principal member at all stages of development, its concentration rising steadily from 38 percent in the young to 70 percent at senescence *via* 44 percent in the expanded (Table 3).

The HCI values of 0.13 in the young and 6.08 in the senescent leaves represent a 47-fold increase in the deposition of hydrocarbons between the two

extreme levels of maturity. This deposition factor was accompanied by a 3-fold growth in CPI value from youth (6.2) to senescence (18.9) indicating a high total abundance of odd carbon number chain lengths in the latter stage which abundance may however be attributed to the preferential deposition of mainly nonacosane and a relatively smaller amount of hentriacontane.

In Figure 10, a histogramatic* presentation of the data in Table 3, the strong dominance and growth of n-nonacosane with leaf ageing is evident. While n-heptacosane was the second major normal alkane in the young leaf, this position was taken by hentriacontane in the expanded and senescent leaves. Except for octacosane and hexacosane whose concentrations, like those of pentacosane and heptacosane, appeared to be continually decreasing with ageing of the leaf, the even carbon-number homologs showed concentration maxima at the expanded level of the tissue thus contributing to the CPI minimum observed at this stage.

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*In this and subsequent histograms it should be clearly understood that the heights of the bars are proportional to the percentage composition of the constituent homologs.

Carbon number	STAGE OF LEAF DEVELOPMENT					
	Coffea arabica L			*Thea sinensis* L		
	V.Young	Expanded	Senescent	V.Young	Expanded	Senescent
18				3.5	2.0	1.4
19				t	t	t
20				8.0	5.3	0.9
21				3.4	2.8	0.6
22	1.9	3.7	0.4	12.5	6.1	1.5
23	0.9	1.8	0.2	10.4	9.8	2.9
24	1.9	3.9	0.3	11.4	11.0	2.5
25	4.2	2.6	0.5	10.1	13.5	3.0
Post 25				4.0	4.7	1.2
26	2.6	2.1	0.4	8.4	10.6	3.4
27	14.3	7.2	1.[illegible]	7.7	11.8	5.7
Post 27	21.0	12.8	4.3	8.4	-	5.5
28	2.9	2.4	1.6	4.0	5.7	4.1
29	37.9	43.7	70.0	3.9	9.9	25.0
30	2.6	3.0	2.3	2.8	4.3	5.7
31	9.8	16.8	18.1	1.3	2.0	36.5
HCI	0.13	0.53	6.08	0.83	0.67	3.66
CPI	6.2	5.6	18.9	0.8	1.1	3.9

t = trace (< 0.1 percent)

Table 3 Age variation in Coffee and Tea leaf-epicuticular wax alkanes.

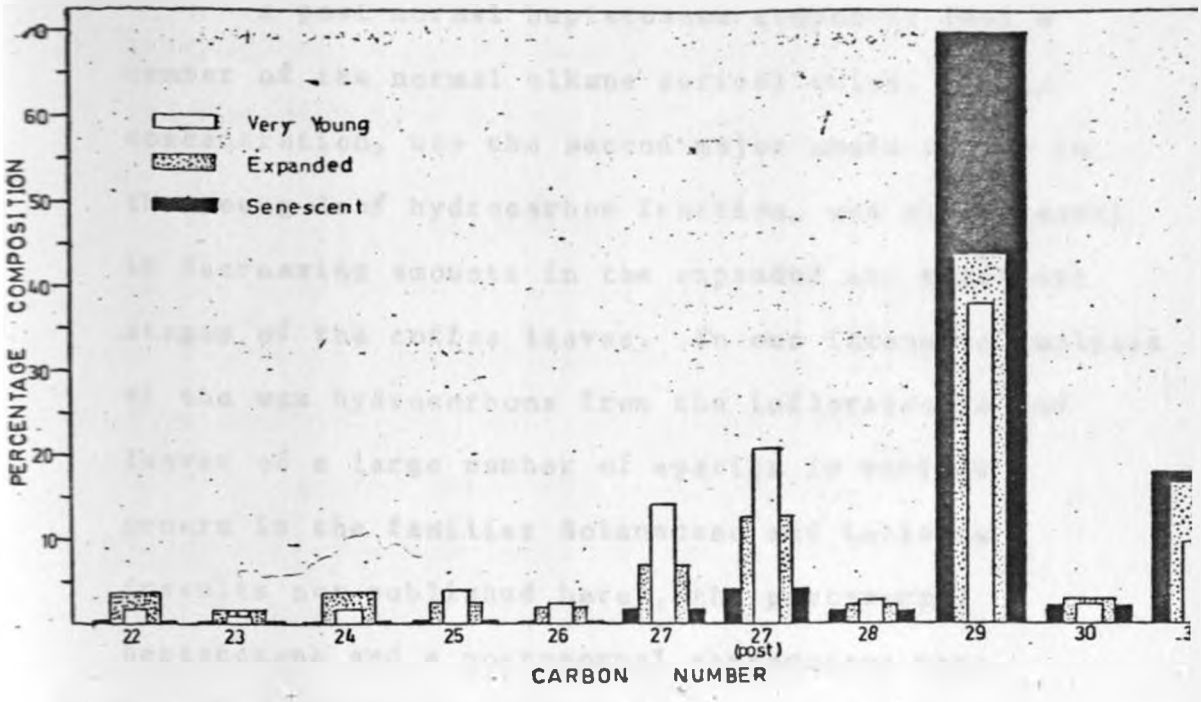


Figure 10: Histogramatic presentation of the age variation in composit of coffee leaf epicuticular wax alkanes

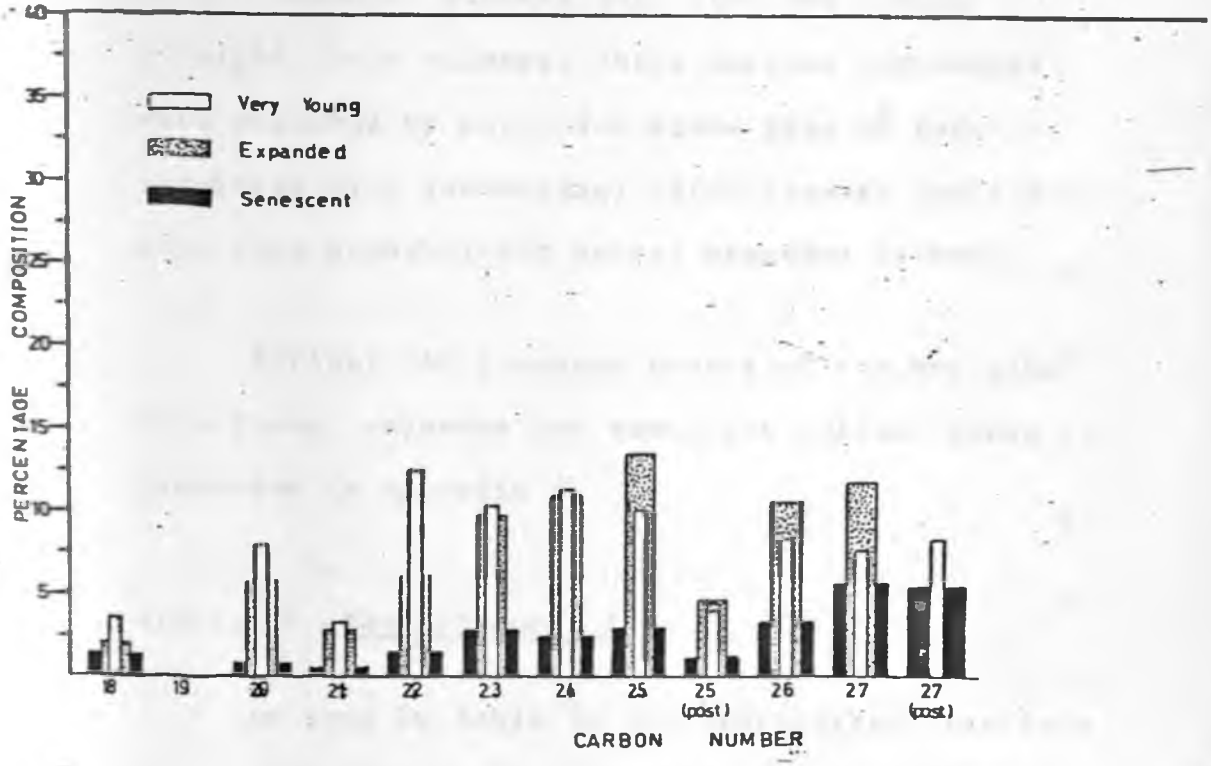


Figure 11: Histogramatic presentation of the age variation in compositi of Tea leaf epicuticular wax alkanes

A post-normal heptacosane component (not a member of the normal alkane series) which, by its concentration, was the second major chain length in the young leaf hydrocarbon fraction, was also present in decreasing amounts in the expanded and senescent stages of the coffee leaves. In our intensive analyses of the wax hydrocarbons from the inflorescence and leaves of a large number of species in various genera in the families Solanaceae and Labiatae (results not published here), the post-normal heptacosane and a post-normal pentacosane were invariably present amongst the normal - and methyl-branched alkanes but, like the normal straight chain alkanes, these curious components were absorbed by molecular sieve type 5Å (pre-saturated with iso-octane) which results indicated they were probably not methyl branched isomers.

Typical GLC recorder traces of the wax alkanes from young, expanded and senescent coffee leaves are presented in Appendix 5.

4.3.2.1.2 *Thea sinensis* L

As seen in Table 3, the hydrocarbon fractions of tea leaf waxes consisted of normal alkane homologs from C_{18} to C_{31}. In addition to a

post-normal pentacosane component, the post-normal heptacosane component was also present in all but the expanded leaf wax.

The age variation in the leaf wax alkanes as given in Table 3 is more easily seen from its histogramatic presentation in Figure 11. Whereas hentriacontane and nonacosane were strongly dominant in the senescent leaf wax fraction, the young and expanded leaves showed a rather flat distribution with large amounts of lower chain lengths and a net even to odd carbon number preponderance especially at youth where the CPI value was actually lower than unity.

Large amounts of lower chain length alkanes ($C_{25} - C_{20}$) were recently reported in the epicuticular wax of immature citrus fruits whereas C_{27} to C_{33} homologs predominated in the mature fruits (Nordby and Nagy, 1977). Herbin and Robins (1969) observed that those plant species which had low HCI values exhibited a more complex chain-length distribution pattern with even carbon-number alkanes tending to form a substantial proportion of the total. In the current study, the young tea leaf that had an HCI of 0.83 showed a very weak dominance of docosane while the expanded leaf with an HCI value of 0.67 showed a correspondingly weak dominance of n-pentacosane.

The HCI minimum occurring at the expanded stage of tea leaf is reminiscent of the findings of Herbin and Robins (1969) that during the development of wax alkanes in *Solandra grandiflora* (Solanaceae) the expansion of the leaf surface area with age and the hydrocarbon deposition do not necessarily proceed at equal rates.

Generally, the variation in chain-length concentrations with leaf ageing in Tea fell into three groups. Except for the C_{19} alkane which was present in trace quantities at all ages, each member in the C_{18} to C_{24} group was at its maximum concentration at youth, gradually falling to lower levels at senescence. In the second chain-length group (C_{25} - C_{28}), the concentration of each homolog reached a maximum at the expanded stage and thereafter fell at senescence to values lower than at youth. In the third group, the higher carbon-number alkanes, C_{29} to C_{31}, appeared to be accumulating with ageing of the leaves.

Shown in Appendix 6 are typical GLC traces for alkanes from tea leaves at the three stages of development.

4.3.2.2 Primary alcohols

4.3.2.2.1 *Coffea arabica* L

Primary alcohols of C_{26} to C_{32} chain lengths were identified in coffee leaf waxes. The variation in chain length concentrations with leaf ageing is shown in Table 4 below. Figure 12 is a histogramatic presentation of the GLC data and typical GLC recorder traces are presented in Appendix 7.

	CHAIN LENGTH						
Leaf age	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}
Very Young	4.3	-	26.7	9.4	22.9	36.7	t
Expanded	1.5	-	16.0	2.9	49.8	-	29.9
Senescent	t	-	8.1	2.5	69.8	-	19.6

Table 4 Age variation in coffee leaf epicuticular wax primary alcohols (as acetates).

The principal primary alcohol at the expanded and senescent stages of the leaf was n-triacontanol, with dotriacontanol and octacosanol the second and third major respectively. In all waxes, heptacosanol was absent or present in concentrations below the threshold of detection. A component with a GLC retention time coincident with that of n-hentriacontanol was the principal component in the alcohol fraction of the young leaf wax. This component was, however, absent in the expanded and senescent leaves. Although

a plot of log-retention distance versus carbon number indicated that the component was hentriacontanol, we have not cited as yet any reliable report quoting an odd carbon-number primary alcohol as a major chain length in plant waxes. However, as pointed out in Section 2.4.7, Joshi and Sharma (1974) claimed to have isolated and characterized n.hentriacontanol from the benzene extract of the leaves of a Leguminosae species.

Only a trace peak was observed at a retention time corresponding to that of dotriacontanol in the young leaf. Octacosanol and triacontanol were the major primary alcohols after the unusual C_{31} component.

As evident from Figure 12, the concentration of n-triacontanol increases continuously throughout the life cycle of the plant tissue, the biggest deposition occurring in the growth period from youth to expanded. At the expanded stage of the leaf, the chain-length C_{30} becomes more than twice as much as at youth, subsequently growing by a factor of about 1.5 towards senescence. Dotriacontanol was at its maximum concentration in the expanded leaf, falling off slightly at senescence. The rest of the chain-lengths including octacosanol had concentration maxima at youth, gradually falling to minimum values at senescence.

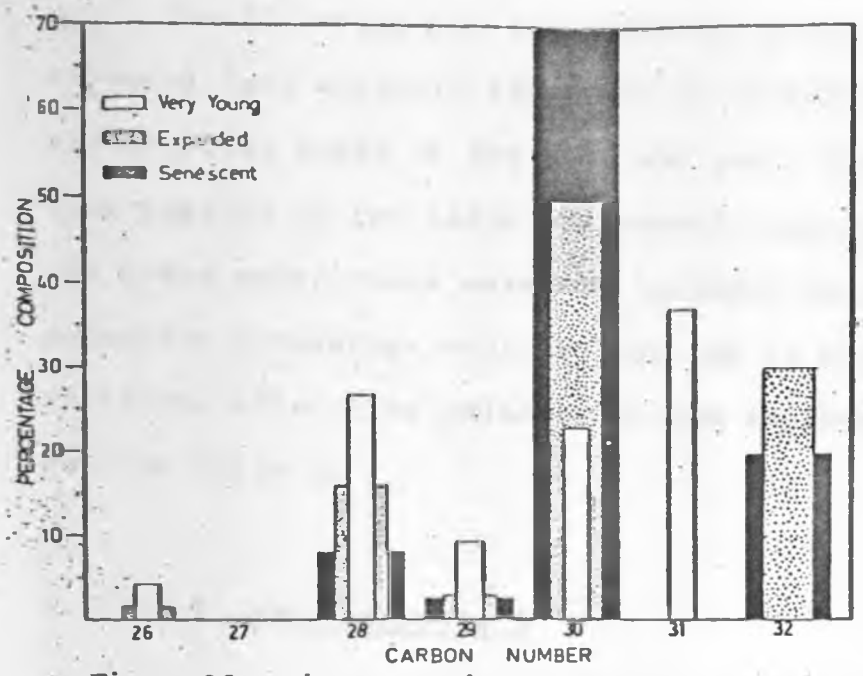


Figure 12: Histogramatic presentation of the primary alcohol profiles of coffee leaf waxes.

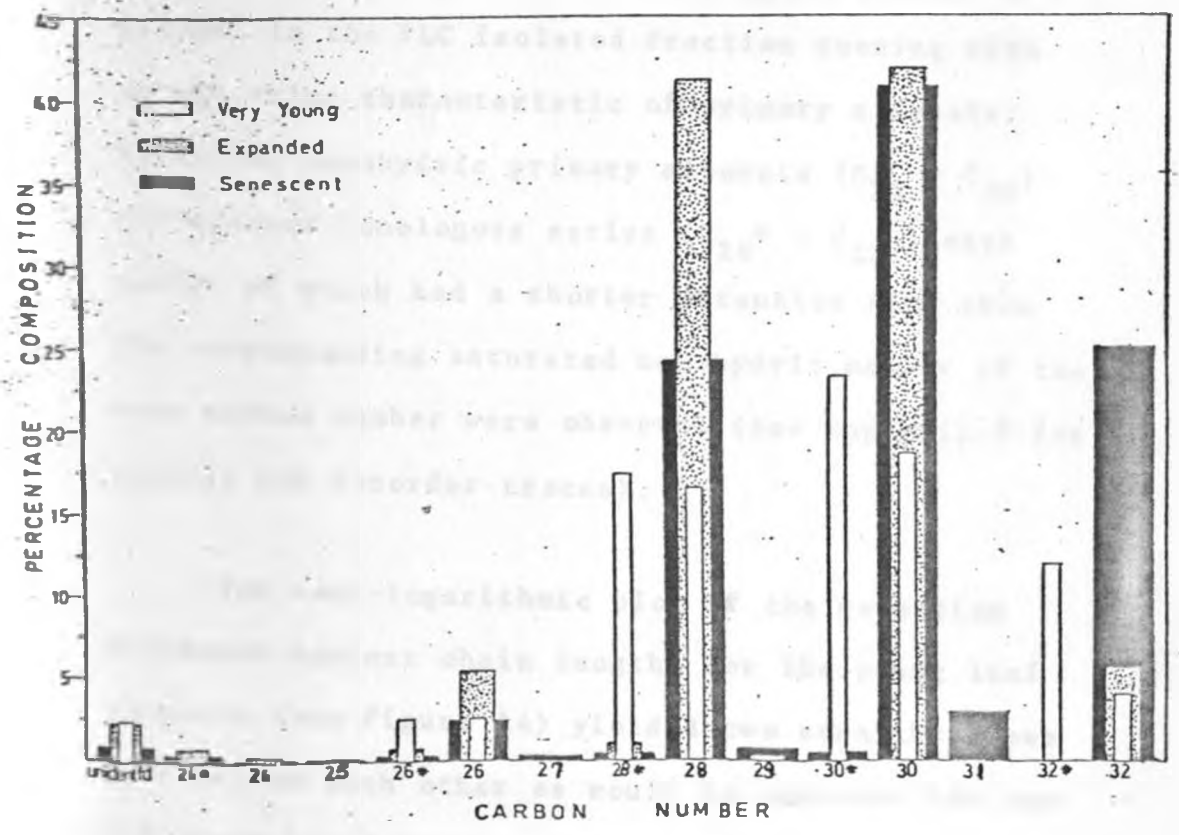


Figure 13: Histogramatic presentation of the age variation in Tea leaf epicuticular wax alcohols.

The GLC trace for the acetates of the expanded leaf alcohols (Appendix 7) showed some unidentified peaks in the pre- and post- retention time regions of the saturated normal homologs but the areas under these were not included in the molecular percentage calculations and do not therefore affect the validity of the expanded leaf data in Table 4.

4.3.2.2.2 *Thea sinensis* L

In tea leaf waxes two homologous series were present in the PLC isolated fraction running with an hRf value characteristic of primary alcohols. Saturated monohydric primary alcohols (C_{24} - C_{32}) and another homologous series (C_{24}* - C_{32}*) each member of which had a shorter retention time than the corresponding saturated monohydric member of the same carbon number were observed (see Appendix 8 for typical GLC recorder traces).

The semi-logarithmic plot of the retention distances against chain lengths for the young leaf alcohols (see Figure 14) yielded two straight lines parallel to each other as would be expected for two different homologous series. Following bromination (see Section 3.3.4.4) of the acetylated alcohol

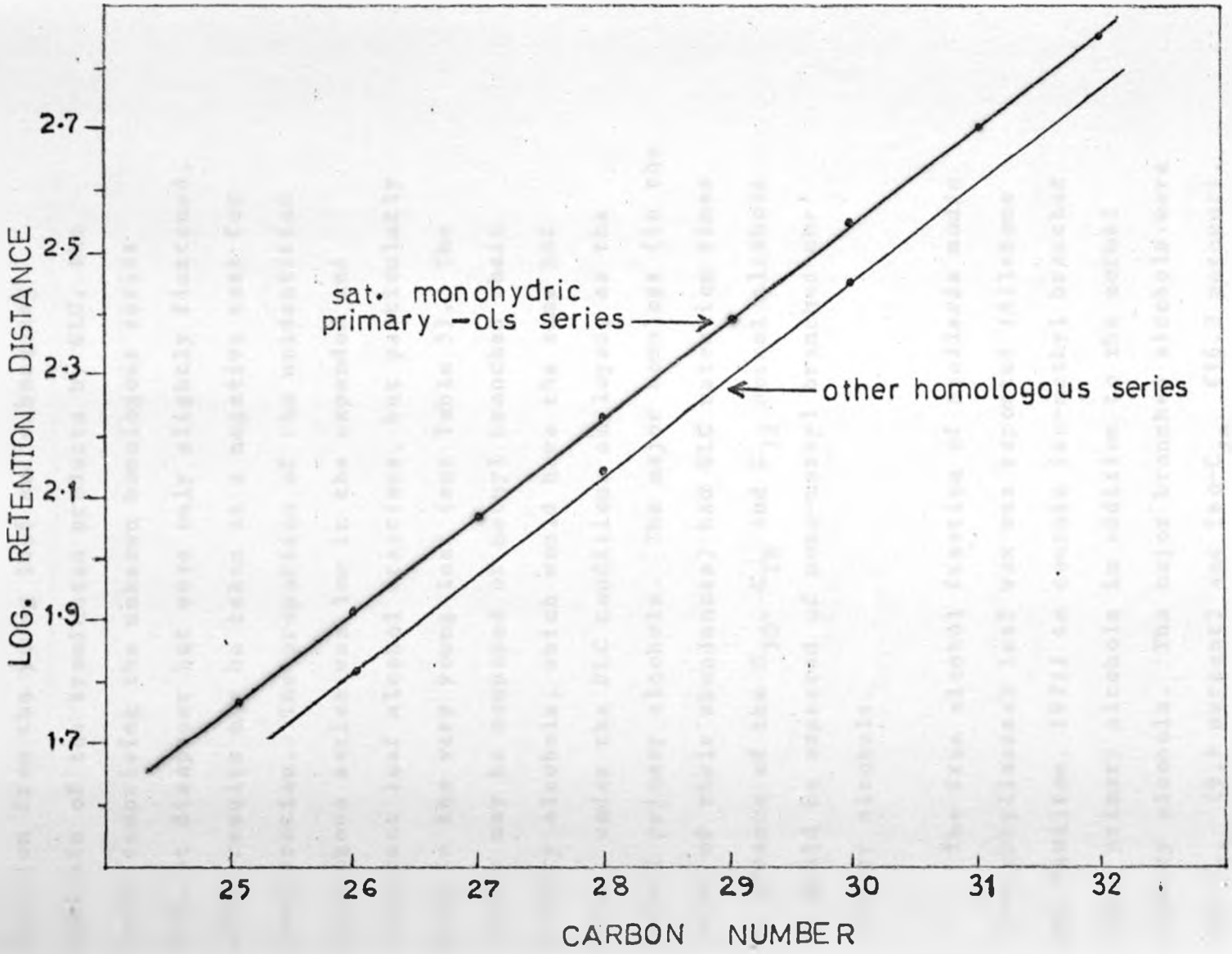


Figure 14 Semi-logarithmic plot for young tea leaf epicuticular wax alcohols.

fraction from the young leaf and subsequent analysis of the brominated products by GLC, the peaks comprising the unknown homologous series did not disappear but were only slightly flattened, which results may be taken as a negative test for unsaturation. The proportion of the unidentified homologous series was low in the expanded and senescent leaf alcohol fractions, but particularly high in the very young leaf (see Table 5). The series may be composed of methyl branched chain primary alcohols, which would have the same hRf value, under the PLC conditions employed, as the normal primary alcohols. The major homologs (in the order of their abundances) had GLC retention times in advance of the C_{30}, C_{28} and C_{32} normal alcohols as would be expected of mono-methyl branched chain primary alcohols.

The free alcohol fraction of *Stellaria media* (Caryophyllaceae) leaf wax was reported [Allebone and Hamilton, 1972] to contain iso-methyl branched chain primary alcohols in addition to the normal primary alcohols. The major branched alcohols were iso-C_{24} (9.9 percent) and iso-C_{26} (16.2 percent). Combined GLC-MS analysis would, however, be required to verify this supposition (in the case of the tea

Carbon No.	STAGE OF LEAF DEVELOPMENT		
	Very Young	Expanded	Senescent
Unidentified	2.5	2.4	0.9
24*	t	0.9	0.5
24	t	0.4	0.1
25	t	0.2	0.1
26*	2.6	0.8	0.4
26	2.6	5.5	2.5
27	t	t	0.4
28*	17.5	1.1	0.5
28	16.7	41.1	24.4
29	t	0.4	0.8
30*	23.4	t	0.6
30	18.7	41.8	40.7
31	t	t	3.1
32*	12.1	t	t
32	4.1	5.6	25.1

* = members of unidentified homologous series;

t = trace (<0.1 percent).

Table 5 Age variation in Tea leaf epicuticular wax alcohols (as acetates)

leaf) and to determine the position of methyl branching along the chain.

The chief monohydric primary alcohols in the young leaf were triacontanol and octacosanol, in that order. The principal alcohols at the expanded stage of the leaf were octacosanol and triacontanol, these being present in almost equal amounts. At senescence triacontanol, dotriacontanol and octacosanol were the chief alcohols, the latter two occurring in approximately equal amounts. As seen from Table 6 (below) and Figure 13, the biggest deposition of both octacosanol and triacontanol in the waxes occurs during the growth period from young

LEAF AGE	C_{28}/C_{30}	C_{30}/C_{32}
Very Young	0.89	4.56
Expanded	0.98	7.46
Senescent	0.60	1.62

Table 6 Variation in relative proportions of principal monohydric primary alcohols of Tea with leaf ageing.

to expanded stages. The amount of octacosanol falls in the senescent leaf relative to the expanded while triacontanol is laid down to an amount almost equal to that in the expanded leaf.

4.3.2.3 Free fatty acids

4.3.2.3.1 *Coffea arabica* L

High temperature (240°C) and low temperature (200°C) GLC of the methyl esters of free fatty acids from very young, expanded and senescent coffee

Carbon Number	STAGE OF LEAF DEVELOPMENT		
	Very Young	Expanded	Senescent
14	t	t	0.2
15	t	t	0.1
Pre-16	0.8	0.8	t
16	17.0	5.1	1.1
17	1.9	0.8	0.1
Pre-18	8.7	0.6	0.1
18	7.3	1.1	0.6
Post-18	9.7	1.2	0.2
19	2.3	0.5	0.1
20	2.7	2.7	0.8
Post-20	16.5	2.5	0.3
21	1.6	0.4	0.1
22	2.6	2.5	0.4
23	0.9	0.7	0.4
24	2.5	5.1	1.5
Post-24	-	-	0.4
25	1.0	1.7	1.5
26	5.0	36.3	56.9
27	1.0	2.4	2.9
28	11.1	21.1	18.9
29	2.2	2.0	2.1
30	5.4	8.7	7.2
31	t	1.3	0.6
32	t	2.4	3.4
CPI	0.12	0.11	0.09

t = trace (< 0.1 percent)

Table 7: Age variation in coffee leaf wax free fatty acid composition

leaf waxes afforded, in each case, a homologous series in the range C_{14} to C_{32} (see Table 7). The high even to odd carbon number fatty acid preponderance is indicated by the low CPI values, all being less than unity and representing approximately 10-fold abundance of even over odd chain lengths. The young leaf, especially, contained a high amount of low molecular weight fatty acids, C_{14} to C_{20}, with hexadecanoic acid the chief saturated homolog of the free fatty acid fraction.

Free fatty acids with retention times in the pre-hexadecanoic, pre- and post- octadecanoic and post-eicosanoic acid regions were observed, their concentrations being highest at youth and falling off rather abruptly with ageing of the leaf. While they are likely to be the unsaturated counterparts of the saturated homologs, these fatty acids were not investigated any further and their identity, therefore, remains to be established.

Figure 15 is a histogramatic view of the pattern of variation in the concentration of individual fatty acid chain lengths with ageing of the coffee leaf.

While the C_{16}, C_{18} and C_{28} homologs are the major saturated acids at youth, hexacosanoic.

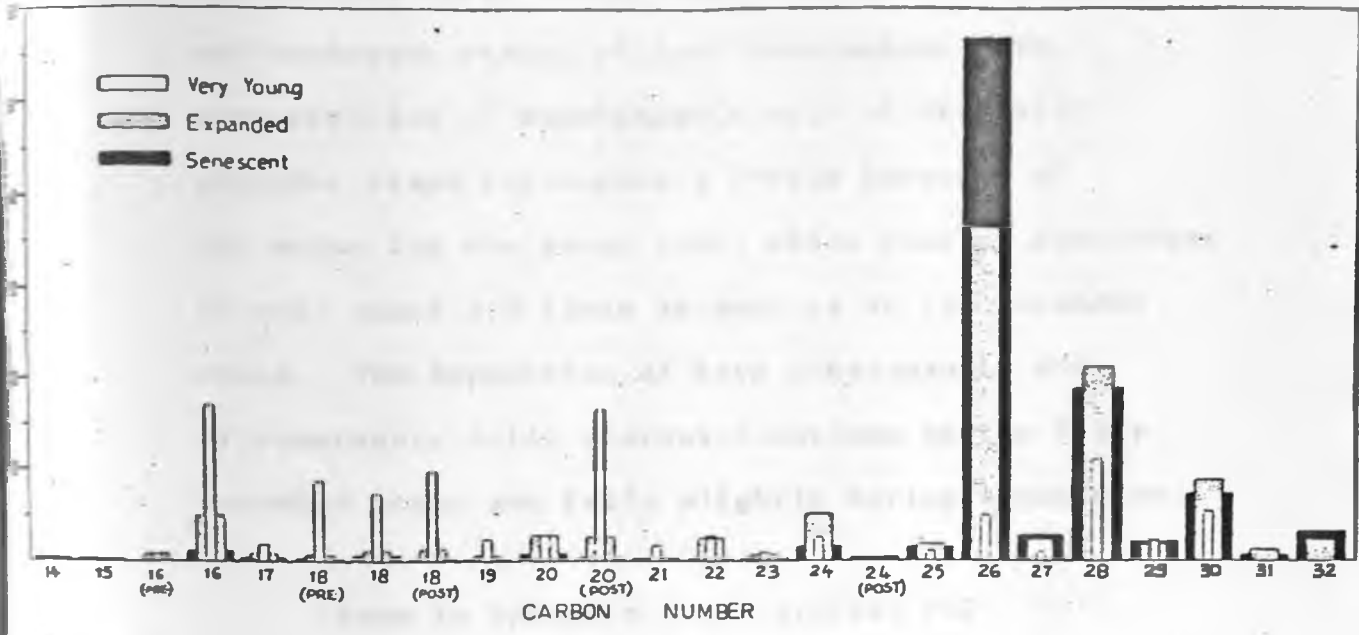


Figure 15: Histogramatic presentation of the variation in the composition of free fatty acids of Coffee leaf with ageing.

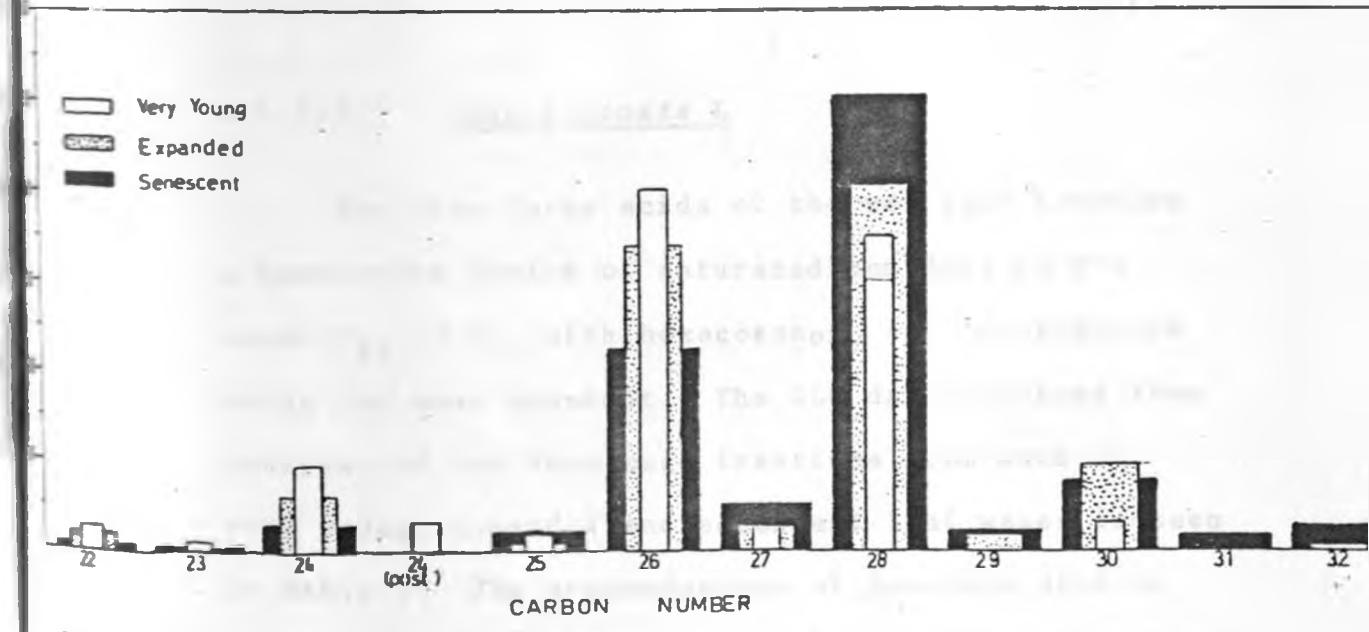


Figure 16: Histogramatic presentation of the age variation in the composition of free fatty acids from tea leaf epicuticular wax.

octacosanoic and triacontanoic acids, in that order, are the principal chain lengths at the fully expanded and senescent stages of leaf development. The concentration of hexadecanoic acid at the fully expanded stage represents a 7-fold increase of the value for the young leaf, while that at senescence is only about 1.5 times as much as at the expanded stage. The deposition of both octacosanoic and triacontanoic acids reaches a maximum at the fully expanded stage and falls slightly during senescence.

Shown in Appendix 9 are typical GLC chromatograms of the coffee leaf free fatty acid methyl esters as eluted from the Apiezon L grease column at the high temperature of ca. 240°C.

4.3.2.3.2 *Thea sinensis* L

The free fatty acids of the tea leaf comprise a homologous series of saturated homologs in the range C_{22} to C_{32} with hexacosanoic and octacosanoic acids the most abundant. The GLC data obtained from analyses of the free acid fractions from each of very young, expanded and senescent leaf waxes is seen in Table 8. The preponderance of homologs with an even number of carbon atoms to those with odd number is indicated by the very low CPI values in each of the young, expanded and senescent leaves. Seen in a

Carbon Number	STAGE OF LEAF DEVELOPMENT		
	Very Young	Expanded	Senescent
22	2.8	2.1	0.9
23	1.1	0.5	0.8
24	9.6	6.3	3.1
Post 24	3.5	-	-
25	2.1	1.0	2.3
26	40.2	34.0	22.6
27	2.7	2.7	5.2
28	35.0	40.6	50.5
29	t	1.9	2.4
30	3.1	9.8	7.8
31	t	0.4	1.9
32	t	0.6	2.7
CPI	0.07	0.07	0.14

t = trace (<0.1 percent)

Table 8 Age variation in Tea leaf wax free fatty acid composition

histogramatic form, in Figure 16, the changes in composition of the free fatty acids with ageing of the plant tissue, particularly the replacement of hexacosanoic acid as the principal homolog at youth by octacosanoic acid at the higher levels of maturity, are clearly seen. The variation is of interest in that the relative percentage of C_{26} (40.2) in the young more or less equals that of C_{28} (40.6) in the expanded leaf whilst the amount of C_{28} (35.0 percent) in the young leaf again approximates that of C_{26} (34.0 percent) in the expanded.

Rather similar, are the curious trends existing in the variation of the relative amounts of the third major chain length, namely tetracosanoic (in the young leaf) and dotriacontanoic (in the expanded and senescent leaves) acids, as the plant tissue spans its life cycle. Whereas the percentage concentration of the C_{24} acid falls gradually with leaf ageing from 9.6 (at youth) to 3.1 (at senescence), that of the C_{30} acid increases from 3.1 in the young via a maximum of 9.8 in the expanded to a slightly lower value of 7.8 in the senescent stage.

Records of typical gas-liquid chromatograms of tea leaf free fatty acid methyl esters are presented in Appendix 10. Although the tea leaf

free fatty acid fractions were not subjected to low column-temperature GLC such as was done in the case of coffee leaf fractions, examination of the GLC recorder traces in Appendix 10 indicates a possible presence of acid chain lengths lower than the C_{22} recorded at the high temperature, particularly in the very young leaf fraction.

4.3.2.4 Aldehydes from senescent leaf wax of *Coffea arabica* L

Most wax-aldehydes (in their free state) from genetically normal plant species examined by other workers contain an even carbon number homolog as the principal chain length. On the other hand, the leaf wax of the *was* mutant of *Pisum sativum*, according to the data published by Macey and Barber (1970a), appears to contain an appreciably high concentration of heptacosanal amidst the relatively weak dominance of the C_{30} and C_{28} aldehydes (see Section 2.4.5).

However the results obtained from preliminary GLC analysis of the PLC-isolated coffee leaf wax aldehydes, with chain length identities confirmed by co-chromatography with PLC-isolated aldehydes of sugar-cane (*Saccharum officinarum*) stem wax, showed heptacosanal to be the strongly dominant member

amongst C_{25} to C_{30} homologs recorded (see Table 9 below and Figure 17). In view of the presence of the aldehydes as a trace constituent in the total wax of the coffee leaf, the isolate from twenty preparative scale thin layer chromatograms proved too small for any further investigation to be pursued in respect of this unusual composition.

Chain length	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}
% composition	2.0	2.0	50.1	4.0	16.5	25.3

Table 9 GLC data obtained from preliminary examination of aldehyde fraction from senescent Coffee leaf wax

4.4 Comparative discussion on the composition of wax constituents in *Coffea arabica* leaf

In Section 2.5 the leaf sampling adopted by both Kabaara (1973) and Stocker and Wanner (1975) was mentioned. A comparison between the ages of leaf pairs sampled by Kabaara and of those analysed by us was made. While our sampling (see Section 3.2), like that by Kabaara, is based on mature, field grown coffee trees, that by Stocker and Wanner was based on greenhouse cultivated seedlings. The fact that

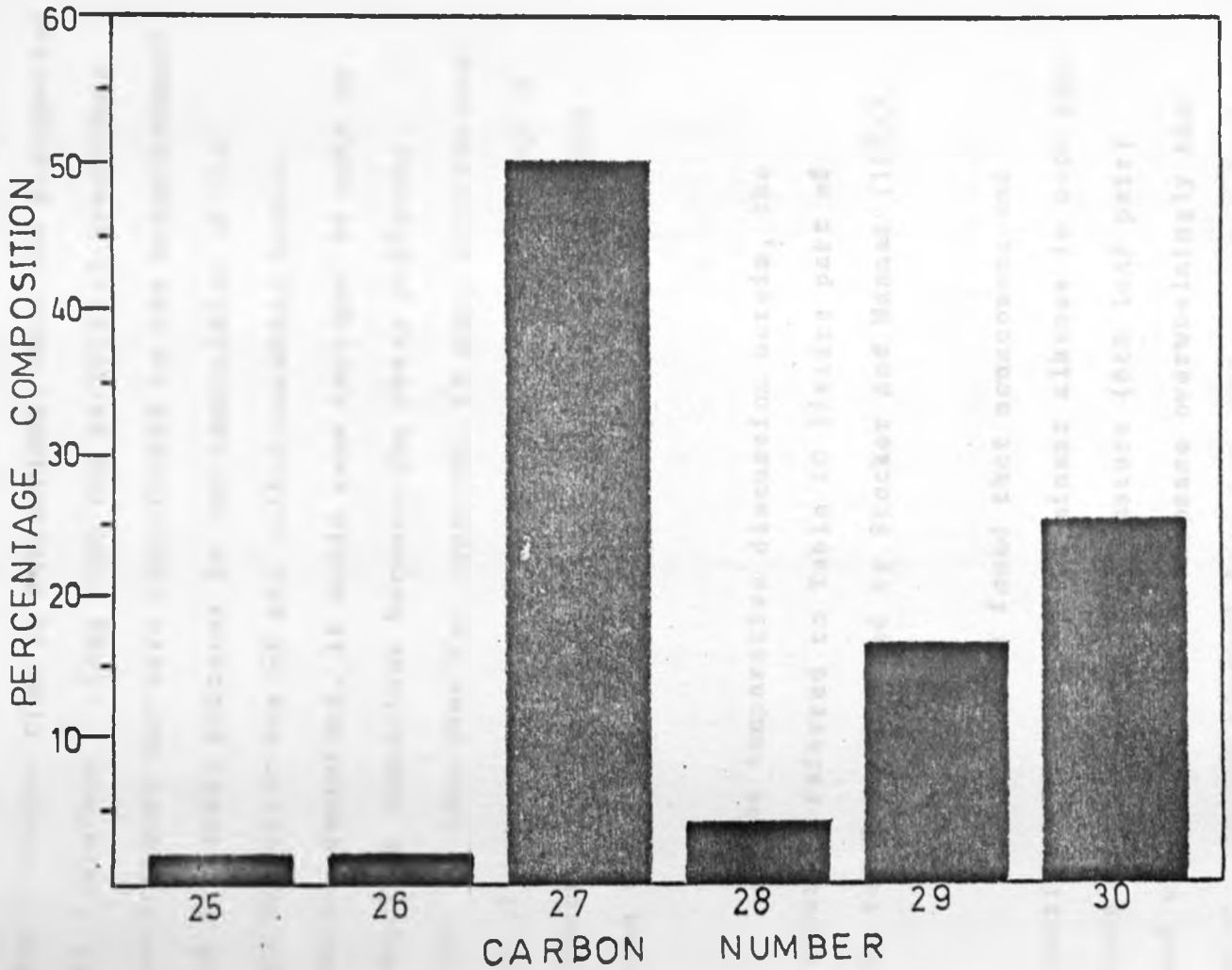


Figure 17: Histogramatic presentation of the GLC data obtained from preliminary examination of senescent coffee leaf wax aldehydes.

these were brought up during the period January to May - winter time in Switzerland, when the photoperiod is a minimum - along with the artificial greenhouse environment may have contributed to the establishment of different patterns in the composition of the epicuticular wax of the coffee seedling leaves. While, therefore, it would seem improper to make an absolute comparison between the three different sets of data thus far reported, it may, nevertheless, be of interest to look at those areas of harmony or discordance between them as they all refer to the same tissue of the same plant species.

In the comparative discussion herein, the reader is referred to Table 10 listing part of the results published by Stocker and Wanner (1975).

Kabaara (1973) found that nonacosane and hentriacontane were the dominant alkanes in both the young (3rd leaf pair) and mature (6th leaf pair) leaf waxes, but with nonacosane overwhelmingly the major. In the alcohol fraction, triacontanol (major) and dotriacontanol were the most abundant chain lengths.

Despite variations in the relative concentrations of individual chain lengths, our results on the chief n-alkanes of *Coffea arabica* leaf generally agree with

Period of germination	No. of leaf pairs on plant	ALKANES		PRIMARY-OLS			ACIDS				Unidentified
		C_{29}	C_{31}	C_{28}	C_{30}	C_{32}	C_{26}	C_{28}	C_{30}	C_{32}	
66	1	45.3	42.7	9.2	35.4	37.6	2.3	23.4	19.8	10.8	13.0
81	2	35.1	54.6	4.8	30.3	43.6	1.7	19.2	19.1	30.7	8.4
112	5	30.2	58.7	3.3	25.1	45.5	2.2	19.4	27.1	21.4	12.0

Table 10 Percentage composition of the principal chain lengths in wax-fractions from oldest of the leaf pairs of greenhouse germinated *Coffea arabica* plants (Stocker and Wanner, 1975).

those reported by Kabaara, except for the youngest leaf which, we found, contained the C_{27}-alkane as the second major rather than the C_{31}. In addition to those reported in the present work, other n-alkane analyses from coffee leaves of intermediate maturity confirm that at no stage of leaf development does hentriacontane occur at a higher concentration than nonacosane. However, Stocker and his partner found nonacosane only very weakly dominant over hentriacontane in the 66-day old leaf, the latter chain length becoming the principal homolog in the 81- and 112-day old seedlings.

In our findings, the dominance of C_{32} and C_{30} (principal) alcohols in the expanded and senescent leaves is *in* harmony with their reported order of abundance in Kabaara's young and mature leaves. In our youngest leaf, however, the C_{31} component (principal), C_{28} - and C_{30} - alcohols, in that order, were the main components of the alcohol fraction. On the other hand Stocker and his colleague reported C_{30} and C_{32} alcohol homologs as the most prevalent in the seedling leaf waxes examined, but, with the C_{32} chain length dominant.

The chief free fatty acid chain lengths according to Stocker and his fellow worker were C_{28} (principal), C_{30} and C_{32} in the 66-day old plants, C_{32} (principal), C_{28} and C_{30} (almost equally abundant) in the 81-day old plants, and C_{30} (principal), C_{32} and C_{28} in the 112-day old plants. The mount of hexacosanoic acid, according to their data (see Table 10) was relatively negligible, but saturated lower molecular weight fatty acids (their concentration highest in the 66-day old seedlings) including hexadecanoic, octadecanoic and docosanoic were reported at relative concentrations of 5.8, 7.8 and 6.7 percent respectively in the youngest seedlings. Although no unsaturation was reported by them, it is of interest to note that as much as 13 percent of the free acid fraction was quoted as unidentified. In the current report the lower molecular weight fatty acids were a major class of chain lengths in the youngest leaf with hexadecanoic acid weakly dominant over the C_{28} and C_{18} - family chain lengths. At the expanded and senescent stages of leaf development, hexacosanoic acid was predominantly major, followed by the C_{28}-, C_{30}- and C_{32}- acids in that order.

4.5 CONCLUSIONS

The chemical nature of the antifungal fraction reported present in cuticular wax of *Coffea arabica* (see Lampard and Carter, 1973) has been examined by TLC. Comparison of the thin layer chromatograms with the thin layer bioautographic plates published by Lampard and his colleague leads us to the conclusion that the resistance activity (in the wax) of *Coffea arabica* to attack by the Coffee Berry Disease causal fungus might be attributed to caffeine (and/or its analogues) and either long-chain primary alcohols or triterpenoids. Triterpenoids were present in reduced quantities in the flower wax of coffee and were not detected in the wax from very young berries. The IR absorption and Gas Chromatographic behaviour of the triterpenoid fraction (isolated from very young coffee leaf) shows it to be more complex in composition than was interpreted by Kabaara (1973) for pure ursolic acid.

The tea leaf waxes contained no triterpenoids. Caffeine (and possibly its analogues) was present in the waxes of both coffee and tea at all levels of maturity of the tissues examined.

An age variation in the relative concentrations and chain length distribution patterns in some of the leaf wax constituents of both *Coffea arabica* L and

Thea sinensis L. has been established. When cuticular wax constituents are to be used as a basis for taxonomic relationships, due regard should be taken of the nature of the organ from which the particular wax was obtained e.g. leaves, flowers, fruits, etc., how the wax was extracted, the physiological age of the organ, and the conditions of growth of the plant (e.g. greenhouse - cultivated or otherwise) from which the organ was obtained.

The principal homologs of each of the wax constituents of both coffee and tea at the three levels of leaf development (examined) are shown in Table 11. The changes in the relative concentrations of the various normal homologs in both coffee and tea leaf wax constituents can be more readily seen by comparing the ratio of the concentrations of selected chain lengths at different stages of tissue development; taking the percentage concentration of trace homologs as being 0.1 wherever they occur.

When such ratios are calculated for the coffee and tea leaf alkanes against n-heptacosane concentration (as the denominator) at each of the three stages of leaf development, certain trends are discernible:

CONSTITUENT	*COFFEA ARABICA* LEAF			*THEA SINENSIS* LEAF		
	Very Young	Expanded	Senescent	Very Young	Expanded	Senescent
Normal alkanes	C_{27}, C_{29}(d), C_{31}	C_{27},C_{29}(d), C_{31}	C_{27},C_{29}(d), C_{31}	Flat distribution C_{20} - C_{27} with C_{22} (young) & C_{25} (expd.)		C_{31}(d), C_{29}
Primary alcohols	C_{28}, C_{30}, C_{31}(d)?	C_{32},C_{30}(d)	C_{32},C_{30}(d)	C_{28},C_{30}(wd)	C_{28},C_{30}(wd)	C_{28}, C_{32}, C_{30}(d)
Unidentified -ols series				C^*_{28}, C^*_{32}, C^*_{30} (d)	Smaller quantities	
Free fatty acids	C_{16}(d),C_{28}, C_{18} - family	C_{28},C_{26}(d)	C_{28},C_{26}(d)	C_{26}(wd),C_{28}	C_{26},C_{28}(wd)	C_{26}, C_{28}(d)
Aldehydes			C_{30}, C_{27}(d)?			

d = strongly dominant; wd = weakly dominant

Table 11 The major homologs in the wax constituents of coffee and tea leaves at three stages of leaf development.

(a) All chain lengths higher than C_{27} increase in proportion to C_{27} as the leaf progressively ages and, in the case of tea, the major increase for all chain lengths above C_{27} occurs in the expanded to senescent stage.

(b) In the case of coffee the major relative changes in chain length deposition are clearly concentrated in C_{29}, C_{30} and C_{31} (by a factor of not less than 3) over the expanded-to-senescent period of leaf development. The C_{31}/C_{29} ratio indicates that nonacosane is deposited in higher concentration than hentriacontane over the same period of development. In case of tea, the major relative change is in hentriacontane and occurs over the period expanded-to-senescent stage by a factor of about 40 compared with the leaf expansion stage. This observation is also confirmed by the ratio C_{31}/C_{29}, compare 0.20 for expanded stage with 1.46 at senescence.

These observations clearly indicate a deposition of progressively longer alkane chain lengths as the plant tissue ages, and establish the coffee and tea leaf metabolic processes as geared mainly to the accumulation of nonacosane and hentriacontane respectively.

In the coffee leaf primary alcohols, the ratios of C_{30}/C_{28} and C_{32}/C_{28} increase progressively with age of the leaf. The major increase is in C_{30}/C_{28} occurring in the period expanded to senescent stage. It may be noted that, in the tea leaf alcohols, although the total concentration of C_{30} remains major even at senescence nevertheless inspection of the C_{32}/C_{30} and C_{32}/C_{28} ratios at the expanded and senescent stages shows that dotriacontanol is being deposited at a faster rate than triacontanol [compare $C_{32/C_{30}}$ = 0.13 (expanded and 0.62 (senescent) AND $C_{32/C_{28}}$ = 0.14 (expanded and 1.03 (senescent)].

Further, examination of the longer chain length ratios, notably the C_{32}/C_{28} for each of the three ages of the tea leaf shows that although both C_{32} and C_{28} acids increase progressively with leaf ageing (see Table 8 and Figure 16), dotriacontanoic acid is deposited at a faster rate than octacosanoic acid [compare $C_{32/C_{28}}$ = 0.01 (expanded) to 0.05 (senescent)]. It is of interest to note (from Figures 15 and 16) that, of the even carbon number fatty acids in both coffee and tea, it is only the C_{26} and C_{32} (in the case of coffee) AND C_{28} and C_{32} chain lengths

(in the case of tea) for which an increase in concentration occurs during the period expanded to senescent stage of leaf development. The C_{32}/C_{26} ratios of fatty acids calculated for the three stages of coffee leaf development indicate that the C_{32} and C_{26} (the major) acids are deposited at the same rate over the period expanded to senescence.

In all three wax fractions of both coffee and tea, the results indicate that homologs of increasing chain length are deposited as the leaf ages.

From a biosynthetic point of view an odd carbon-number alkane is formed by enzymatic decarboxylation of pre-formed long-chain fatty acid of one carbon atom higher than the alkane, and a long-chain primary alcohol is a product of direct reduction of the carboxyl group of a long-chain fatty acid of the same chain length (Kolattukudy *et al.*, 1976). Accordingly, linear correlation coefficients were calculated for even fatty acid chain lengths and even carbon number primary alcohols, even carbon number fatty acids and odd carbon number alkanes of one carbon atom less, for each of the three physiclogical ages of both *Coffea arabica* and *Thea sinensis* leaf. The results are shown in Table 12A and B.

		THEA SINENSIS LEAF			*COFFEA ARABICA* LEAF		
		V. Young	Expanded	Senescent	V.Young	Expanded	Senescent
	Alkanes range	n - C_{31} to C_{21} inclusive			n - C_{31} to C_{23} inclusive		
A	Free acids range	n - C_{32} to C_{22} inclusive			n - C_{32} to C_{24} inclusive		
	Corrln. Coefficient	0,713	0.797	-0.291	0.267	-0.394	-0.350
	Alcohols range	n - C_{32} to C_{26} inclusive			n - C_{32} to C_{26} inclusive		
B	Free acids range	n - C_{32} to C_{26} inclusive			n - C_{32} to C_{26} inclusive		
	Corrln. Coefficient	-0.148	0.228	-0.261	0.838	-0.831	-0.601

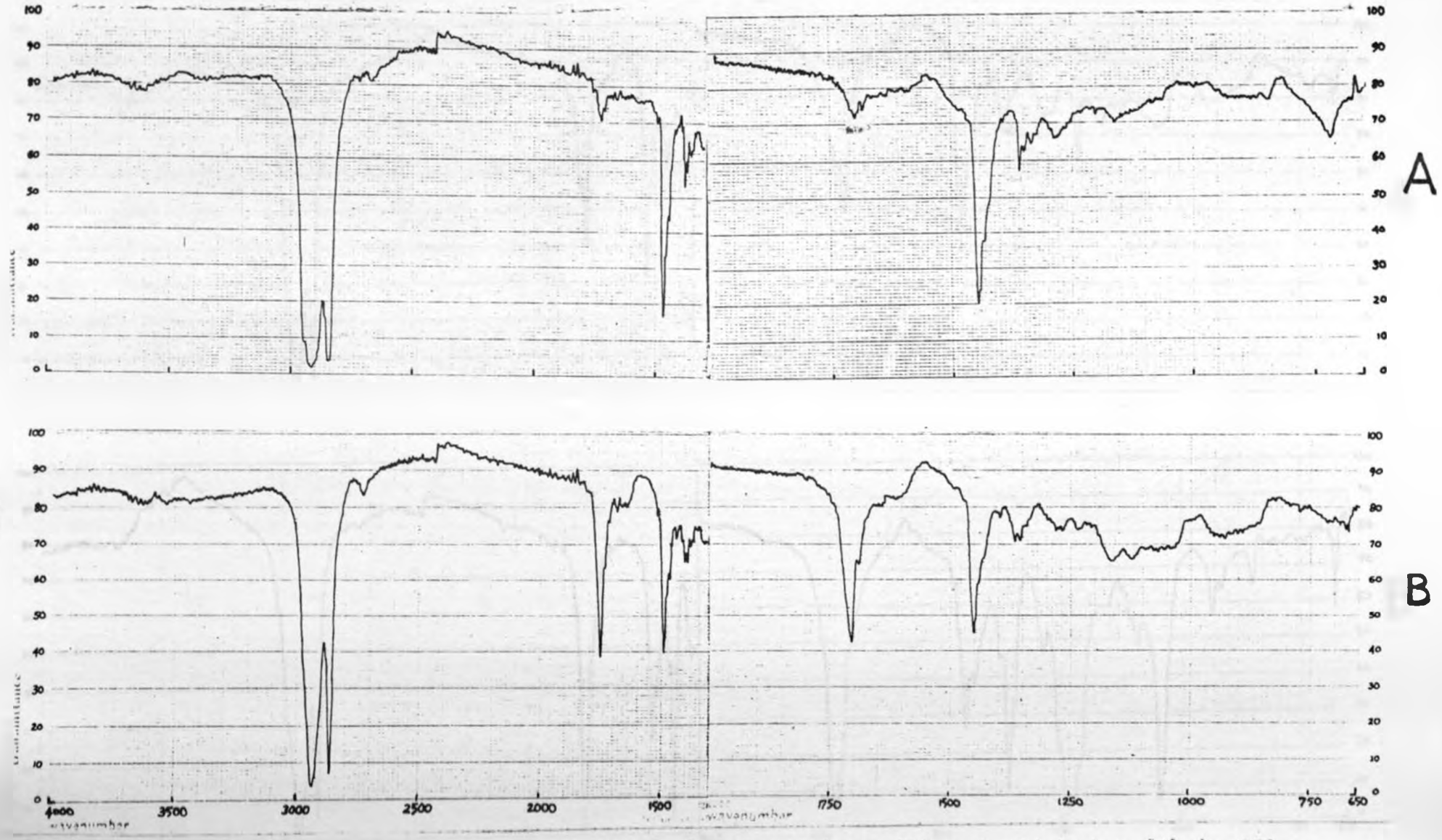
Table 12 Coefficients of linear correlation between

A: even carbon-number free fatty acids and odd carbon-number alkanes having one carbon atom less than the parent acid.

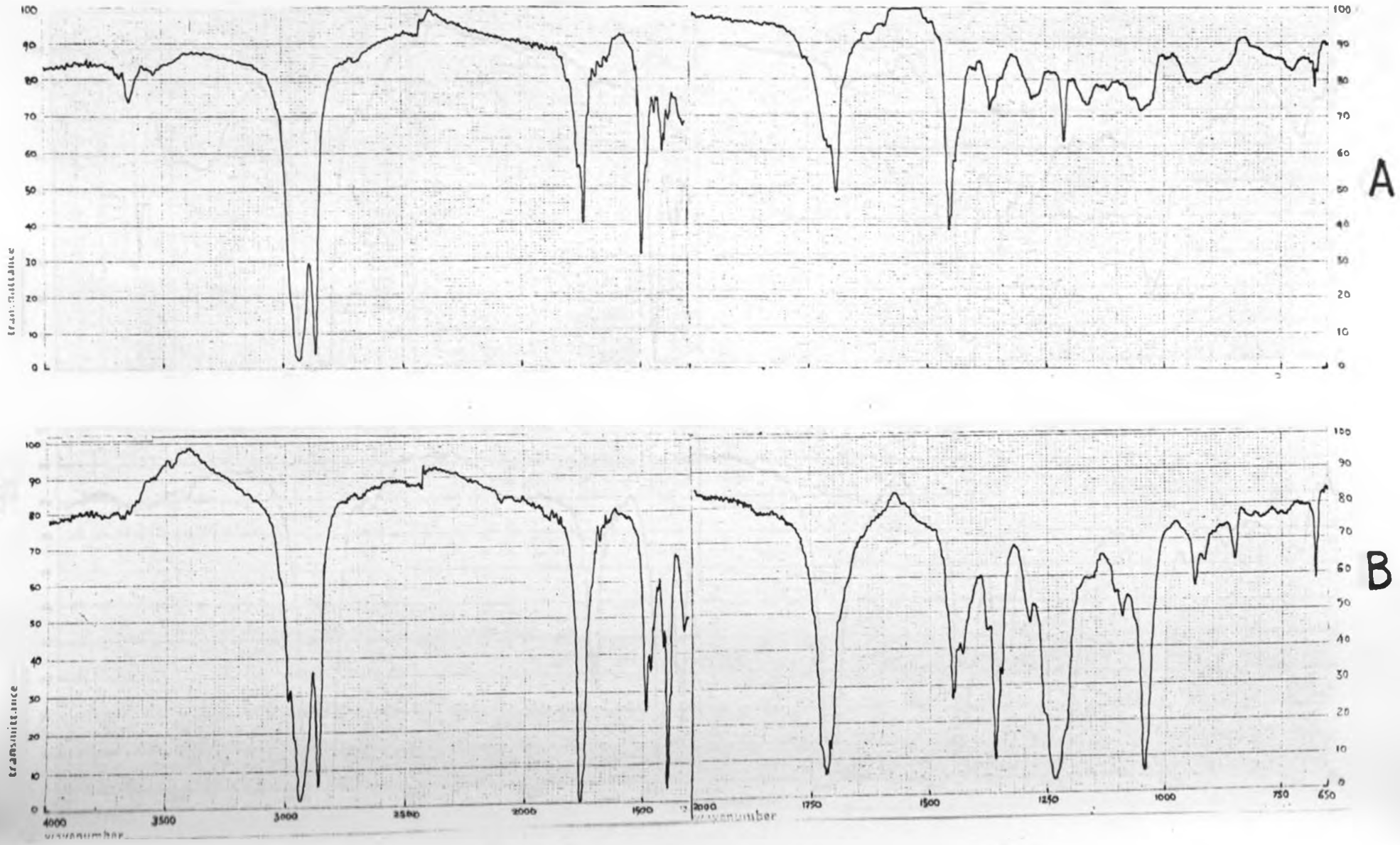
B: even carbon-number free fatty acids and primary alcohols of the same chain length.

Very low correlation coefficients were obtained between the alkanes and fatty acids of coffee and, alcohols and fatty acids of tea, at all stages of leaf development. On the other hand, high correlation coefficients were observed between the alkanes and fatty acids of the very young and expanded tea leaves. The senescent tea leaf had a comparatively very low coefficient. Appreciably high correlation coefficients were obtained for the fatty acids and primary alcohols of *Coffea arabica* at all stages of development of the leaf.

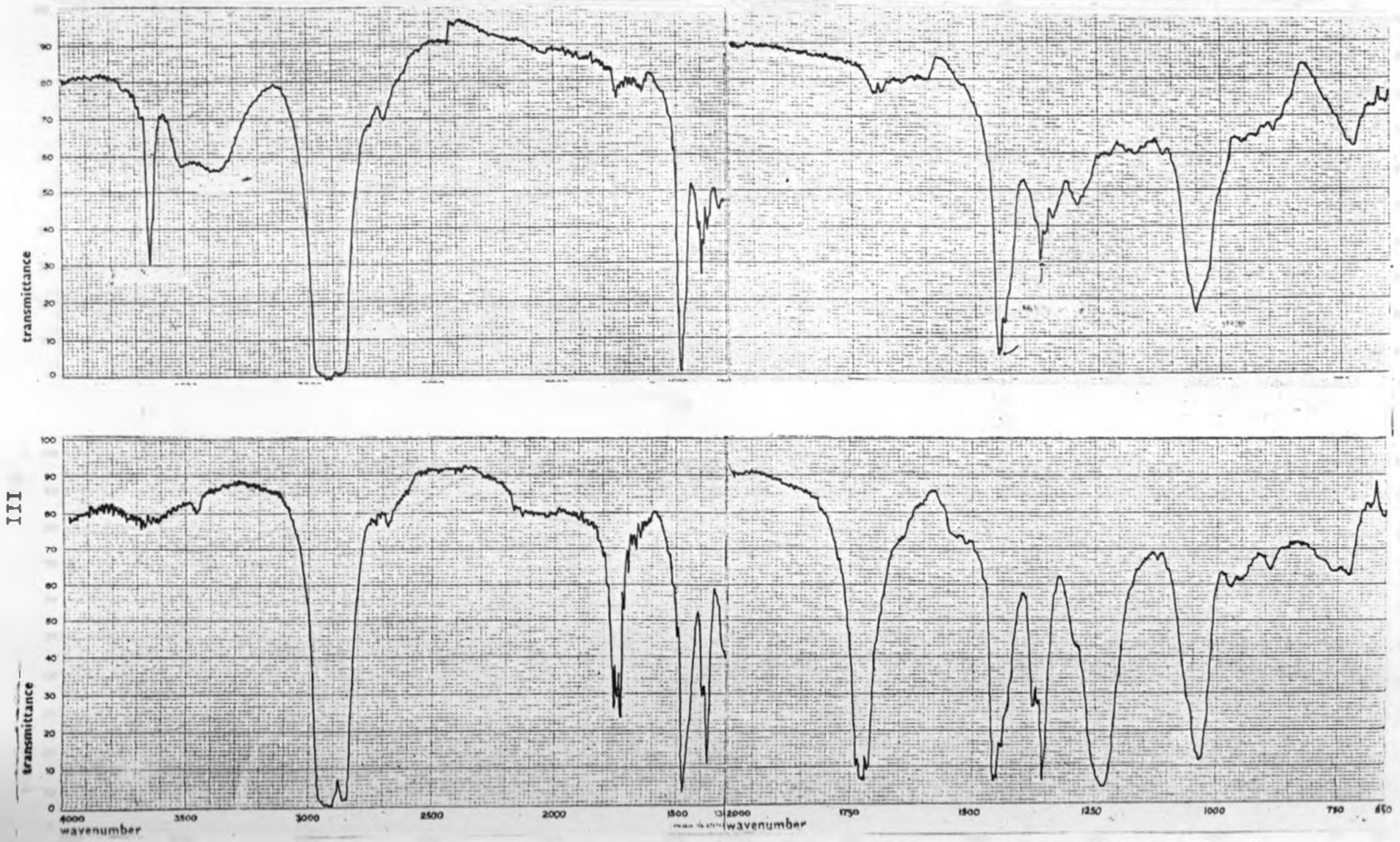
A P P E N D I C E S



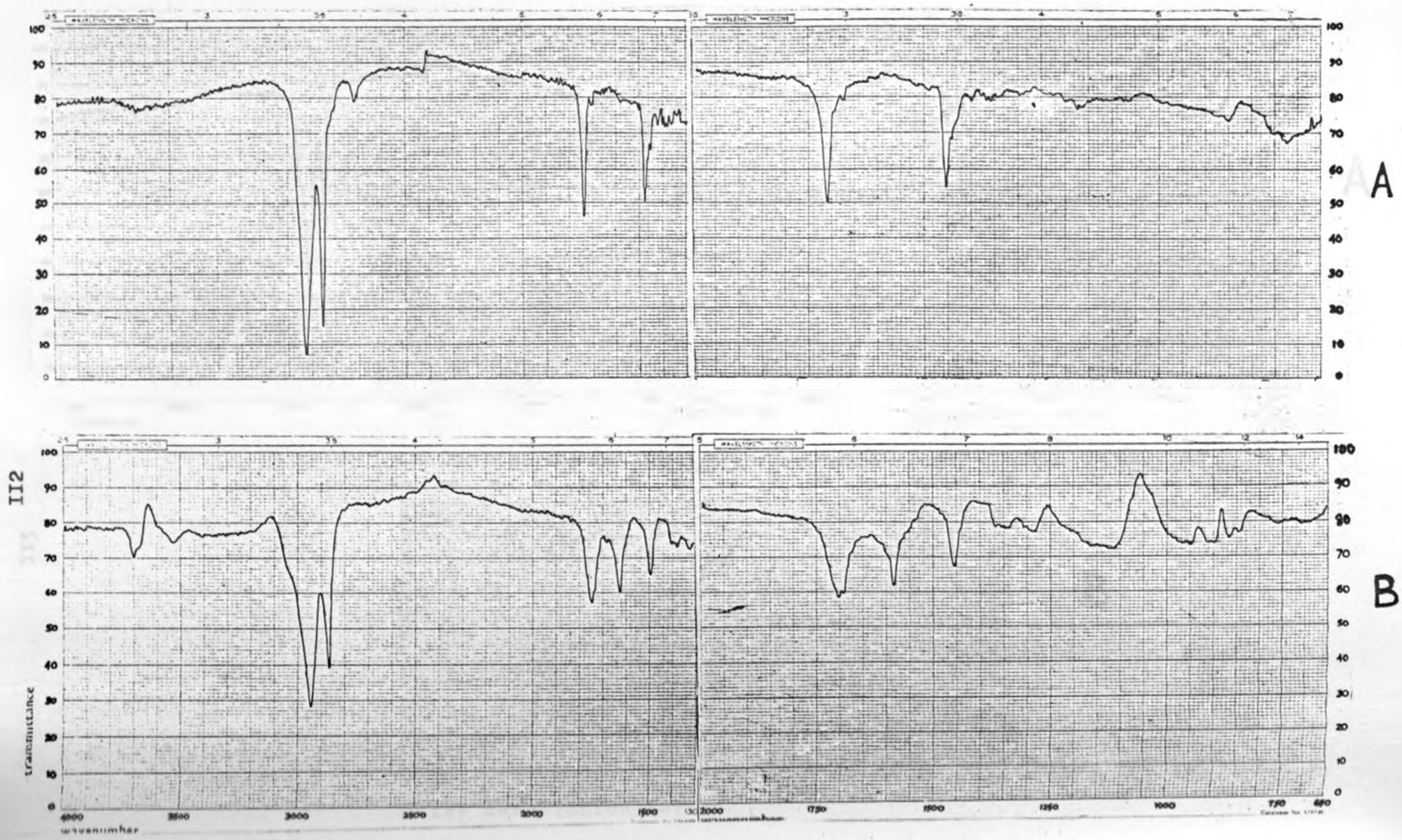
Appendix 1 Infra-red spectra of hydrocarbons (A) and aldehydes (B) in CCl_4.



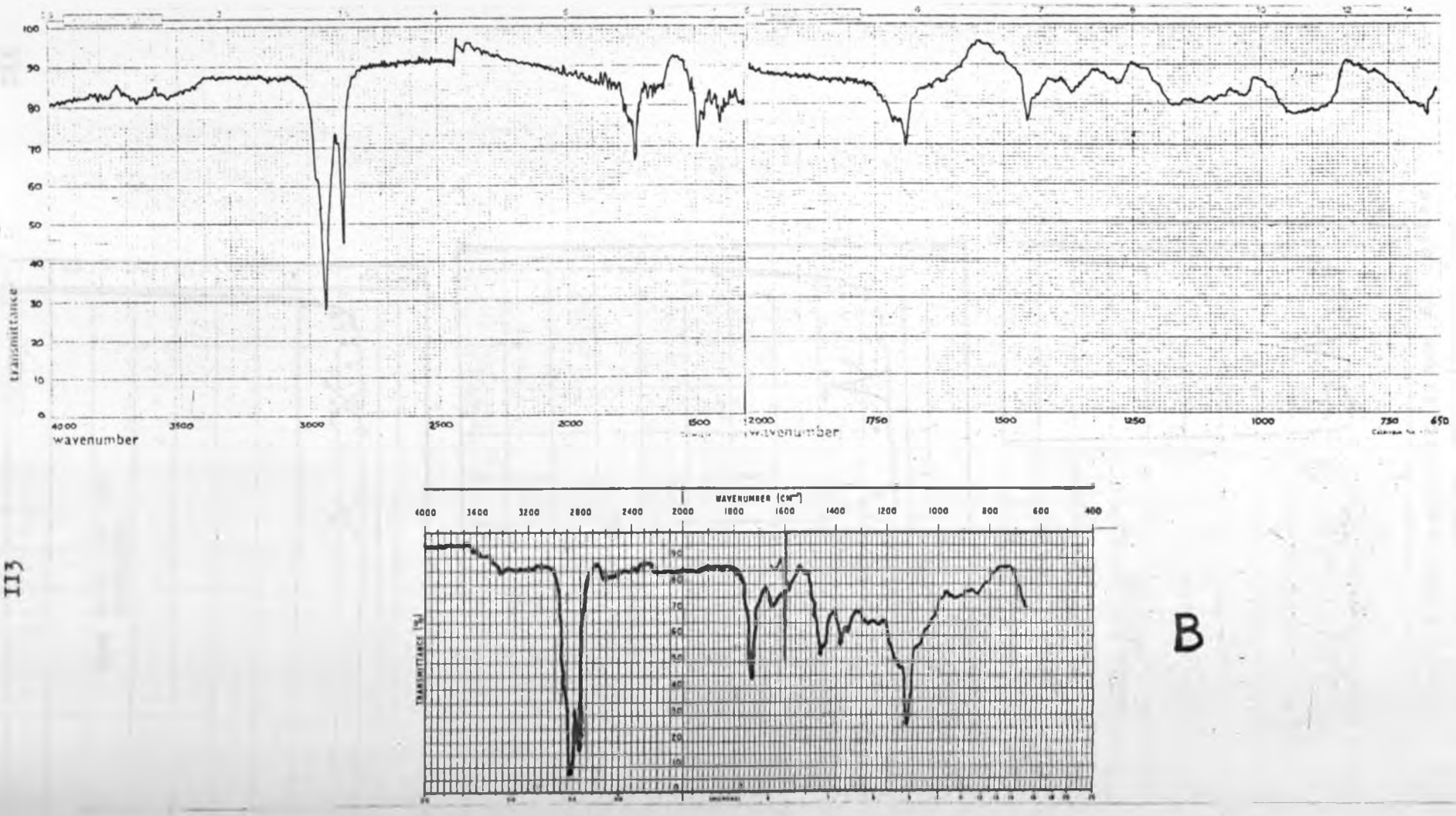
Appendix 2A and B Infra-red spectra of primary alcohols in CCl_4 before (A) and after acetylation (B).



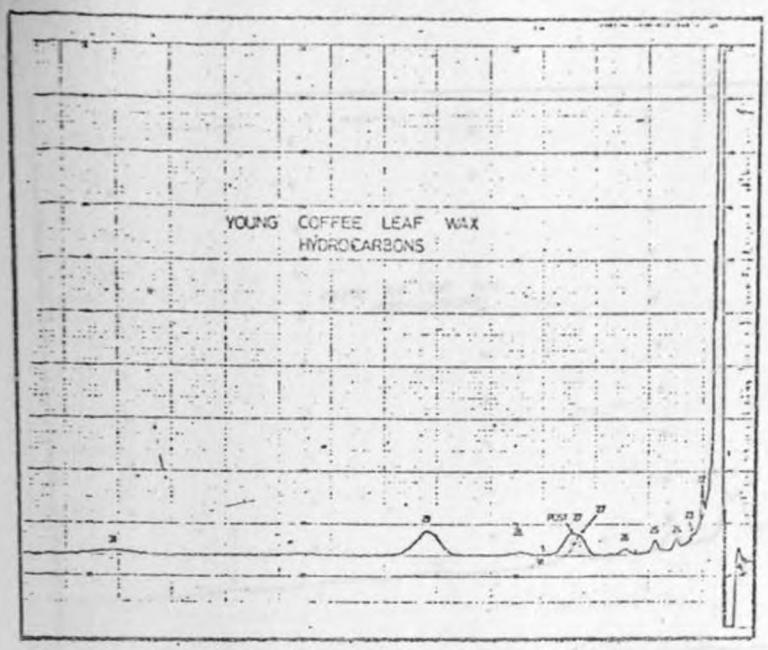
Appendix 2C and D Infra-red spectra of authentic C_{20} primary alcohol in CCl_4 before (C) and after acetylation (D).

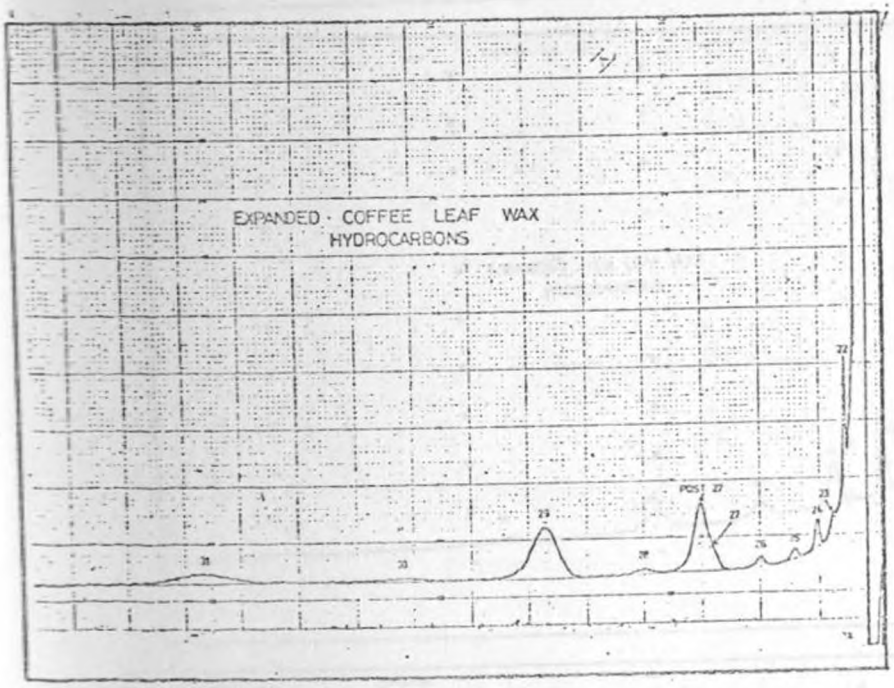


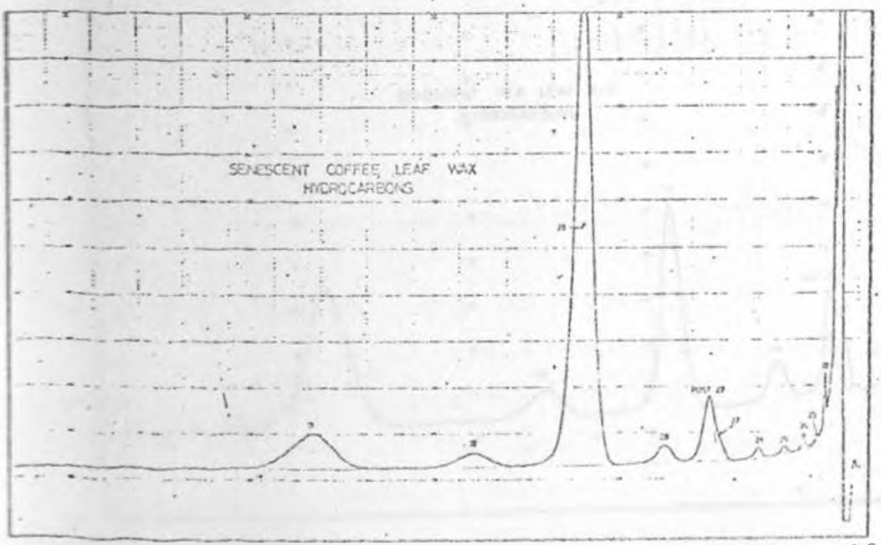
Appendix 3: Infra-red spectra of sugar-cane wax-aldehydes (A) in CCl_4 and free fatty acids from coffee leaf wax (B) in $CHCl_3$.



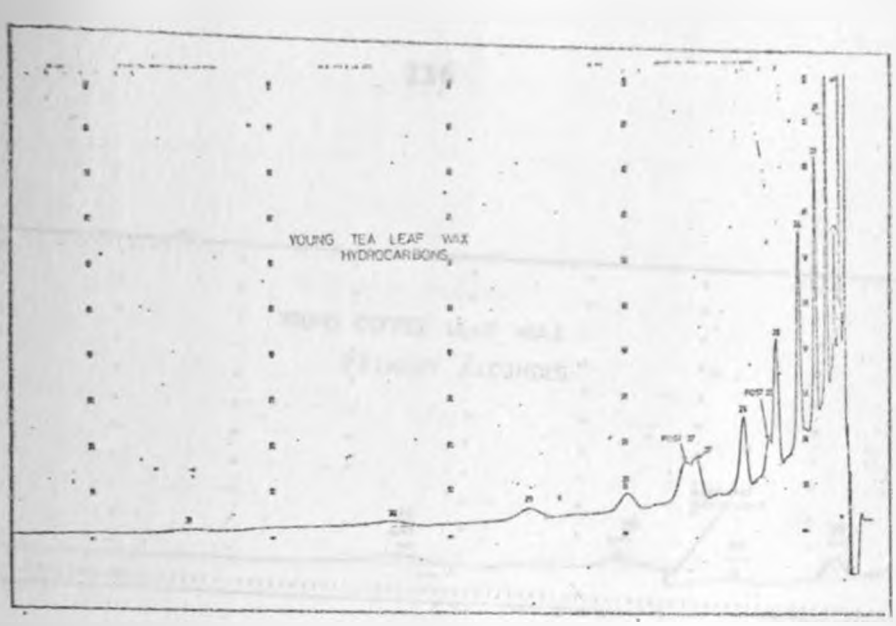
Appendix 4 Infra-red spectra of free fatty acids in CCl_4 before (A) and after methylation (B).

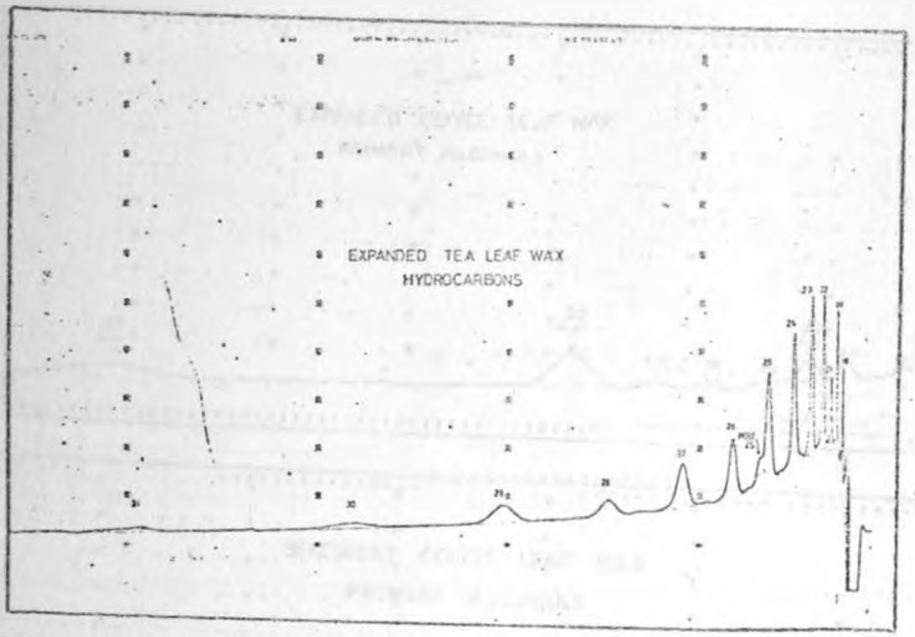


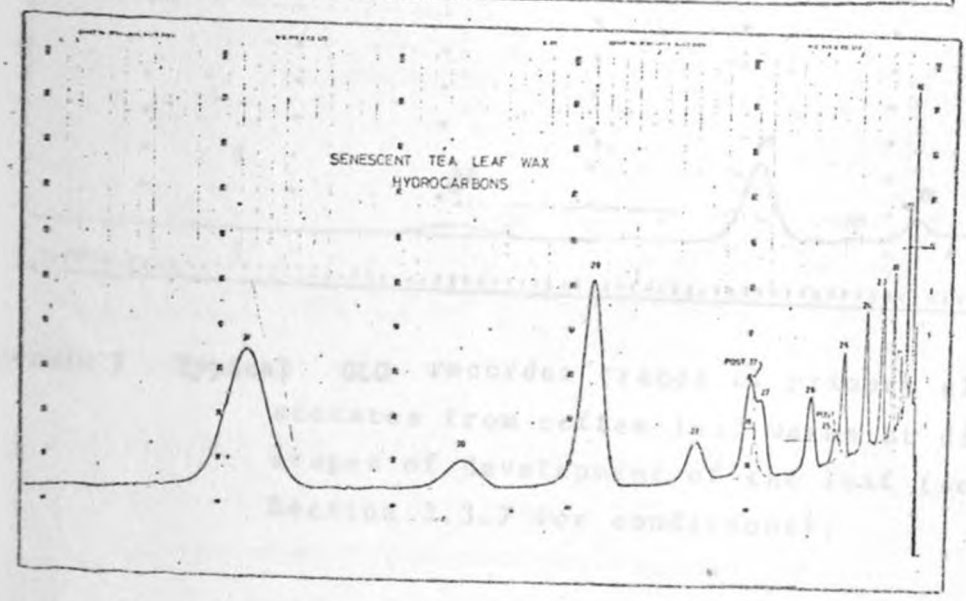




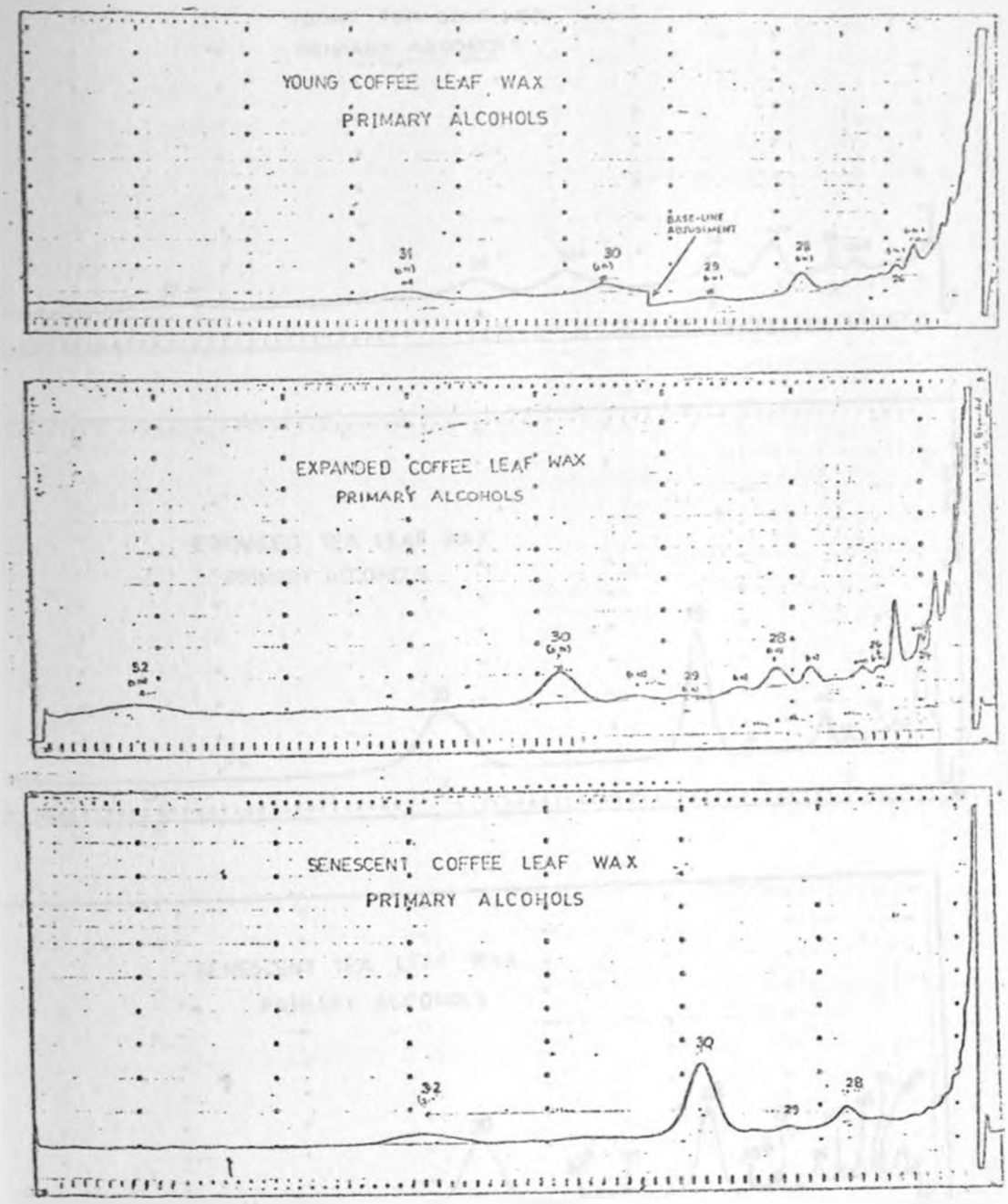
Appendix 5 Typical GLC recorder trace of coffee leaf epicuticular wax alkanes (see Section 3.3.7 for conditions).



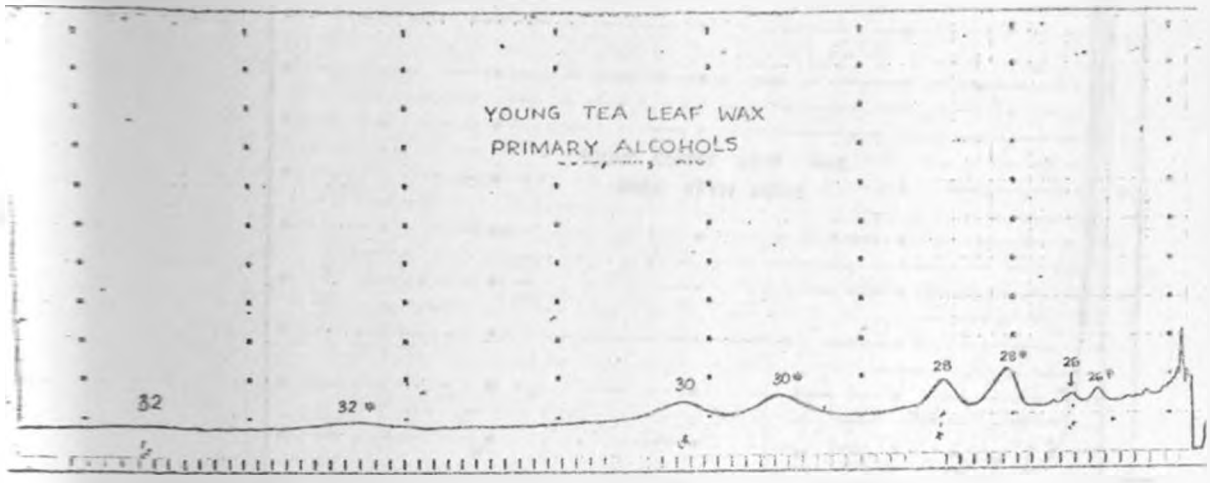


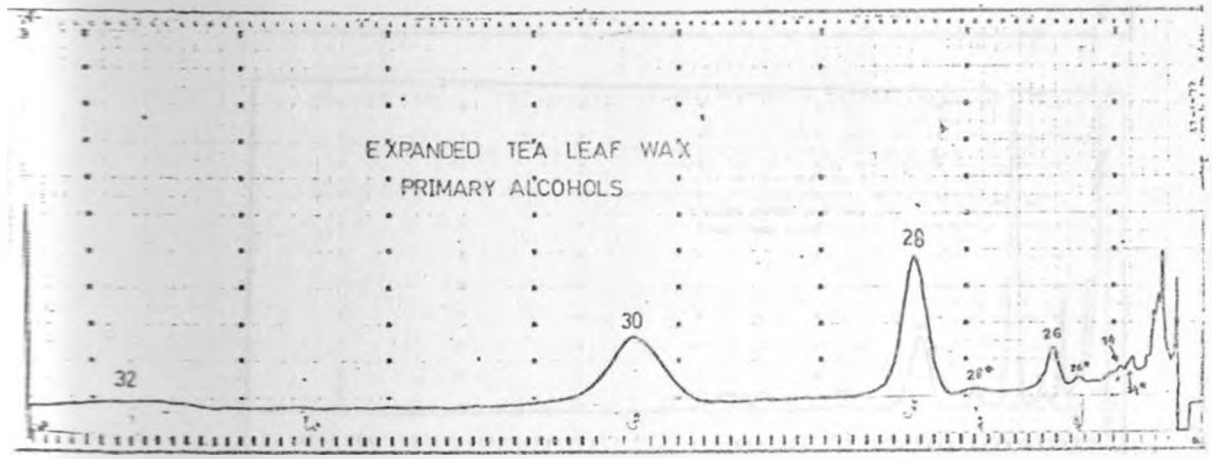


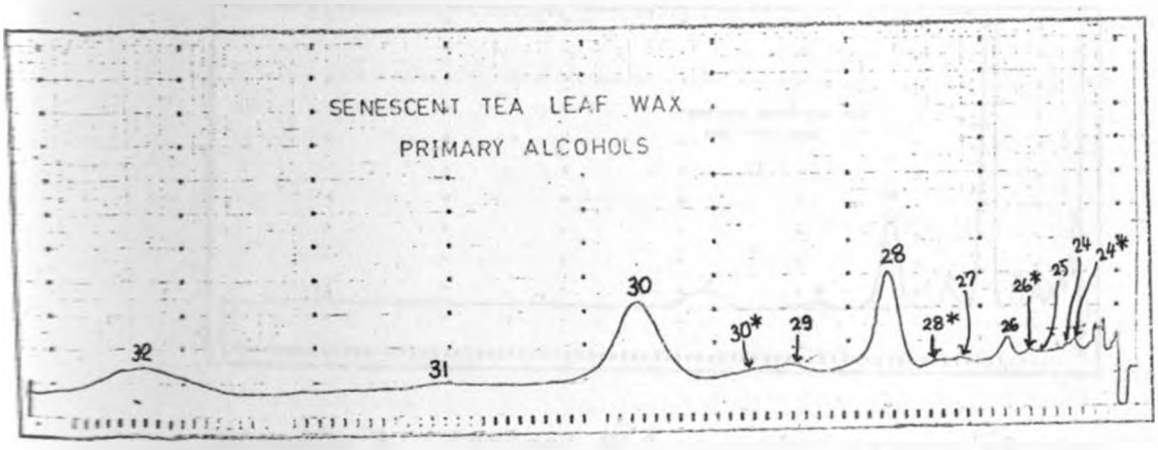
Appendix 6 Typical GLC trace of Tea Leaf epicuticular wax alkanes (see Section 3.3.7 for conditions).



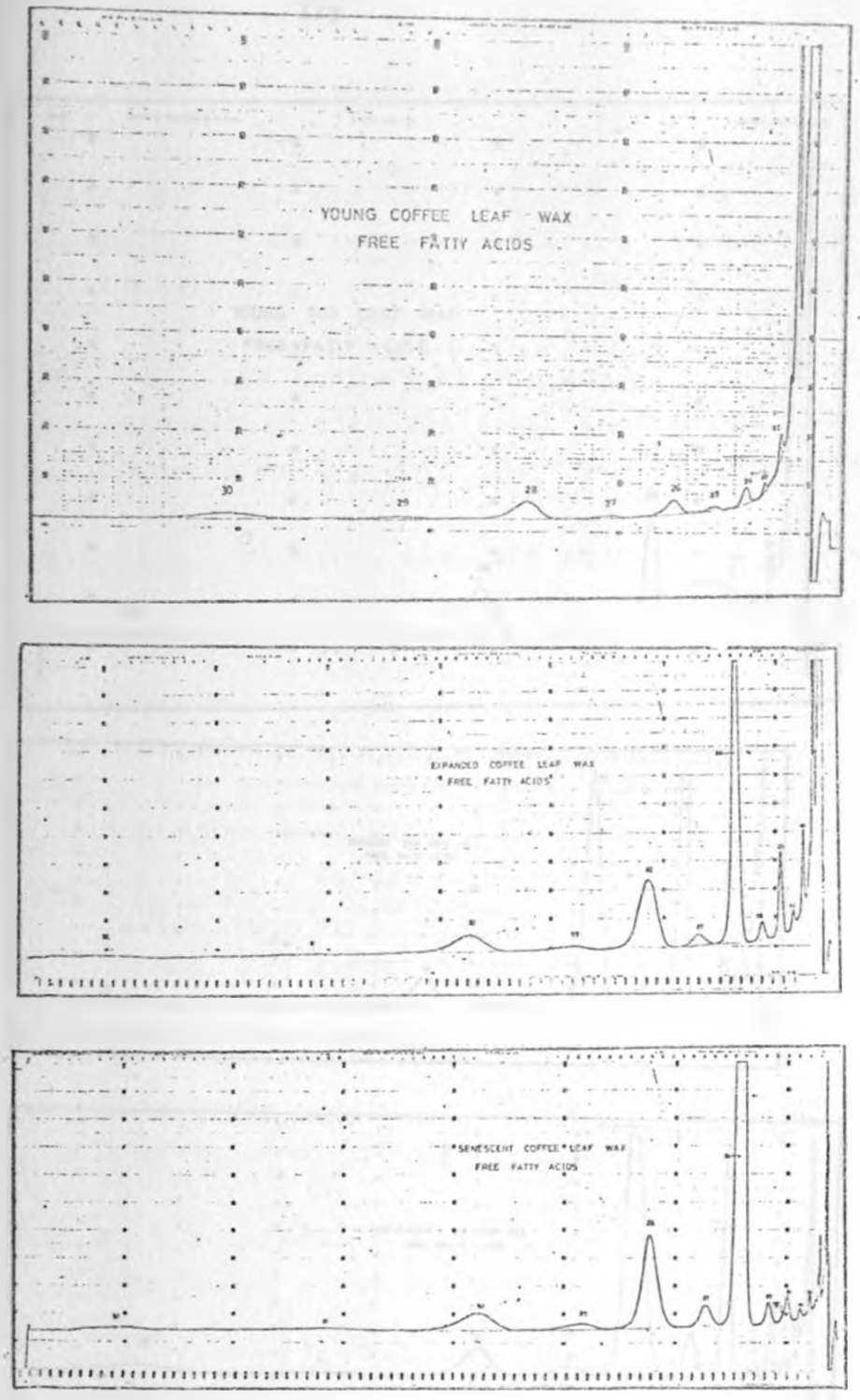
Appendix 7 Typical GLC recorder traces of primary alcohol acetates from coffee leaf waxes at different stages of development of the leaf (see Section 3.3.7 for conditions).



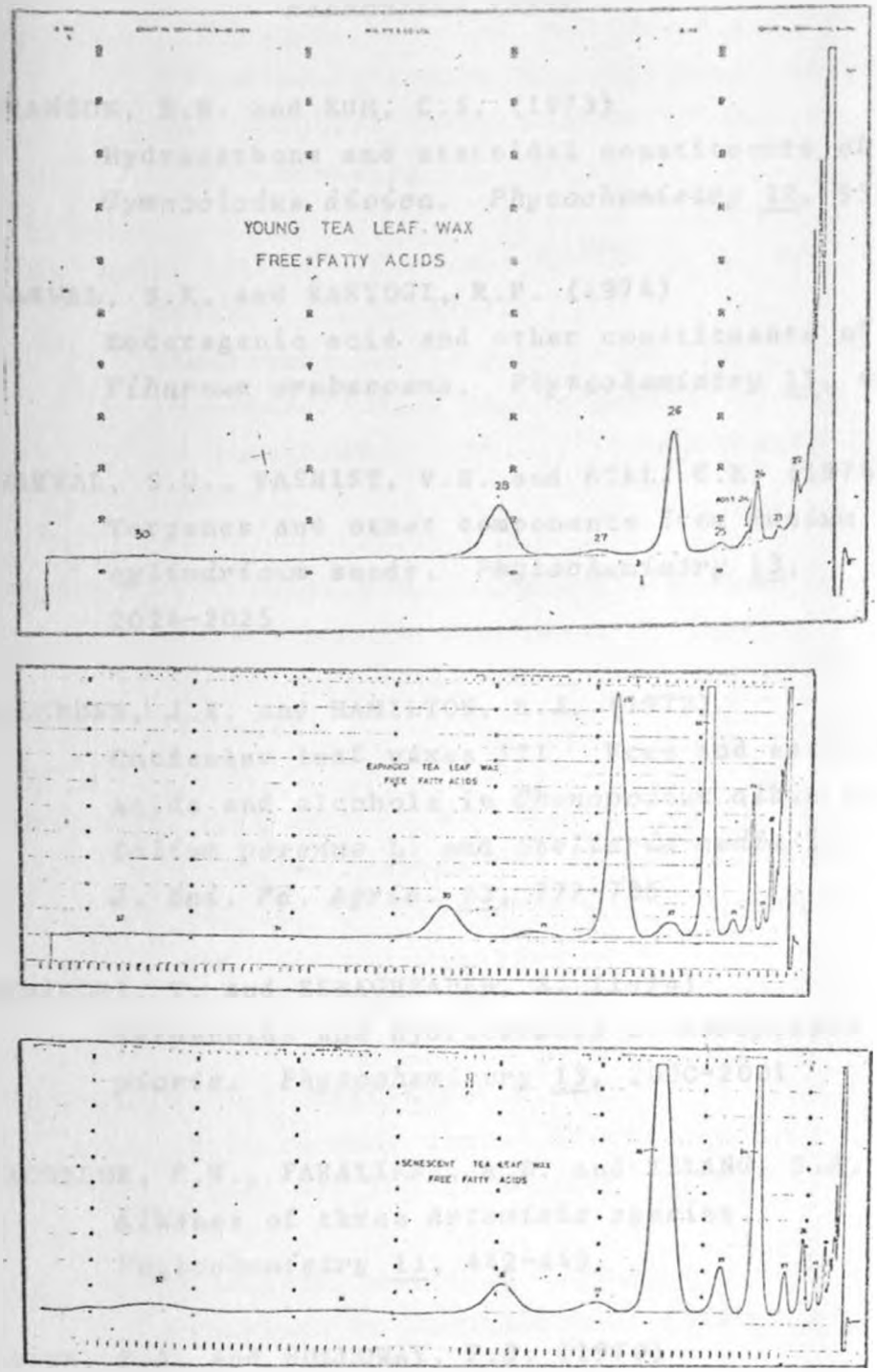




Appendix 8 Typical GLC recorder traces of Tea Leaf epicuticular wax alcohols (as acetates). See Section 3.3.7 for conditions.



Appendix 9 Typical GLC recorder traces of the methyl esters of free fatty acids from the epicuticular waxes of coffee leaves at three stages of maturity (see Section 3.3.7 for conditions).



Appendix 10 Typical GLC traces of the methyl esters of free fatty acids in the waxes of tea leaves at different stages of development (see Section 3.3.7 for conditions).

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