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A Method for Separating Lipid Components of Leaves

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In the separation by paper chromatography of tocopherols or carotenoids and other lipids it is customary to study only one group of compounds and to remove as many as possible of the interfering substances by such pretreatments as saponification or column chromatography. For the study of leaf extracts it was found that these preliminary stages are best omitted. The method is thereby simplified, with consequent reduction in loss of lipids; indeed, in this paper the 'interfering substances' are themselves studied.

The paper chromatograms were developed in the first dimension by adsorption and in the second by reversed-phase chromatography. The technique separated many of the lipids, including known substances such as pigments, vitamins and quinones, as well as fluorescing substances and other unknowns that accompanied chlorophyll.

METHODS

Small samples of fresh leaf were weighed and extracted repeatedly in the cold (Booth, 1959) with redistilled acetone and light petroleum (30–40°) that had been purified by passing it through aluminium oxide. To avoid enzymic destruction of lipids the extracts must be made quickly (Booth, 1960). The extraction of small samples by grinding with quartz in a beaker was quicker than the extraction of

large samples in a mechanical blender. Acetone was removed by washing with water in an automatic drip wash (Booth, 1957, p. 51), and the light-petroleum solution was concentrated to about 1 ml. in a stream of nitrogen gas on a water bath at about 70°.

Chromatographic procedure was based on that of Green, Marcinkiewicz & Watt (1955) as fully described by the Analytical Methods Committee (1959). For the present work many modifications were developed to shorten the method, or to increase a paper's capacity for lipids. The modifications are included in the following procedure. Whatman no. 31 (et) filter papers, size 22 cm. × 32 cm. with machine direction along the short dimension, were impregnated with zinc carbonate according to Analytical Methods Committee (1959) except that the sodium fluoresceinate was limited to 2 mg./l. of the ammoniacal zinc carbonate solution. The lipids were not saponified, but the whole green extract was chromatographed on two papers. Distortion of the spots on the chromatograms was minimized by applying the solution over an area of about 7 cm. × 3 cm. from a modified Trenner pipette (Anderson, 1952) having a curved polished tip and holding about 50 µl. The papers were mounted on a frame and the chromatograms were developed (ascending) with 1% acetone in light petroleum (40–60°) in a 35 cm. high tank lined with thick filter paper. After an hour the lipids had separated into bands in the 'corridor' (the left two-fifths of each chromatogram; Fig. 1). The papers were removed from the tank and the clear portions were coated with medicinal paraffin. In the second dimension the chromatograms were developed by the ascending method across the paraffin for about 1.5 hr. with methanol-water (23:2, v/v). Methanol is hygroscopic and volatile: it was maintained at 92% by daily addition of pure methanol to the tank.

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Papers were dried in air, then examined by daylight and with ultraviolet irradiation of 254 and 320 m μ . Substances absorbing ultraviolet irradiation were detected through quenching the fluorescence of fluorescein (Analytical Methods Committee, 1959). Other colourless substances were detected with spray reagents. Spots were cut out and eluted into ethanol, cyclohexane or *n*-hexane for assay or for spectrophotometry or other tests. Weak spots from several replicate papers were pooled when needed. Loca-

tions of spots are described with chromatograms in the aspect shown in Fig. 1.

Note on size of sample. To obtain strong spots it was necessary to apply a large amount of extract to each paper, yet too much led to crowding and distortion. About 1 g. of leaf (more or less according to percentage moisture) was suitable for two papers. Good results were obtained when enough extract was used to make the chlorophyll front ascend about $\frac{1}{4}$ the paper's length in the first dimension.

Fig. 1. Formal diagram of a two-dimensional chromatogram of lipids from leaves. In the following list, lipids marked * have been seen often, and may be present always in green leaves; those marked † are known to occur in all leaves; r, reduces FeCl₃; d, gives dianisidine reaction. c1, β -Carotene†; c2, luteol†; c3, violaxanthin†; c4, carotenoid*; c5, carotenoid*; c6, carotenoid; ch1, chlorophyll a†; ch2, chlorophyll b†; ch3, unknown chlorophyll*; ch4, derivatives of chlorophyll*; α t, α -tocopherol†, r; γ t, γ -tocopherol*, r, d; δ t, δ -tocopherol*, r, d; q1, plastoquinone*; q2, vitamin K₁*; q3, u.v.-absorbing, 257 m μ *; q4, u.v.-absorbing, 265 m μ ; q5, u.v.-absorbing, 271 m μ *; q6, u.v.-absorbing, 255 m μ *; q7, u.v.-absorbing, 255 m μ *; q8, u.v.-absorbing; f1, white fluorescent*; f2, blue fluorescent; f3, yellow fluorescent; f4, yellow fluorescent*; f5, yellow fluorescent*; f6, yellow fluorescent; f8, blue fluorescent; f9, yellow fluorescent; f10, yellow fluorescent*; u1, u.v.-absorbing*, r, d; u2, u.v.-absorbing*; u3, u.v.-absorbing*; u4, u.v.-absorbing, r, d; u5, u.v.-absorbing, 258 m μ ; u6, u.v.-absorbing*, r; u7, colourless, d; u8, unknown; u9, unknown. [] Pigment; [] u.v.-absorbing; [] fluorescent; [] other material.

RESULTS

Fig. 1 is a composite picture of a two-dimensional chromatogram of the light-petroleum-soluble lipids from green leaves of many species. Ubiquinone, plastoquinone, vitamin K₁, carotenoids 4, 5 and 6, chlorophyll ch3, fluorescent compounds f1, f2 and f4, substance q3 and several others were seen on chromatograms from many species, and are almost certainly universally distributed. The other lipids have been observed on chromatograms prepared from at least three species but were sometimes too weak for positive recognition. Some lipids fluctuated seasonally: for example, γ t, u1, u2, q6 and q7 almost disappeared in late winter. No leaf has yet been found that contained at one time all the lipids represented in Fig. 1. Spots were often distorted or displaced by the propinquity of other compounds: indeed distortion sometimes gave a clue to the presence of an invisible spot, as for example u8.

Carotenoids. β -Carotene, c1, ran near the solvent front in the first dimension and moved slowly across the corridor in the second dimension. β -Carotene was used as a reference substance in locating other materials.

Orange-yellow pigments c4 and c5, and lemon-yellow pigment c6, are probably carotenoids. Their $\lambda_{\max}$ values in *n*-hexane were at 418, 440, 469, at 421, 443, 472 and at 405, 427, 451 m μ respectively. Spectra, adsorption and partition data suggest that c4 and c5 may be the diepoxide of kryptoxanthol or monoepoxides of monohydroxy- α -carotene (two are possible). No. c6 resembles mutatochrome in some of its properties. I cannot find any published report on these compounds in leaves. No. c4 was present to the extent of about 0.5 p.p.m. fresh weight. [Compare β -carotene contents of >100 p.p.m. in clover, grass and nettle (Booth, 1957, p. 83).]

Chlorophylls. The green chlorophyll-like substance ch3 was less polar than other chlorophylls and had $\lambda_{\max}$ 409.5 and 662 m μ in ethanol. No reference has been found to a chlorophyll with this spectrum. The concentration of the new chlorophyll was about one-thousandth that of chl. There was occasional evidence of another chlorophyll between ch3 and u4, which may be chlorophyll α' (Strain, 1958). Many other compounds, including phospholipids, are obscured by the chlorophylls and carotenols; indeed the group of pigments etc. in the lower-right part of the chromatogram requires different conditions for its resolution. A start has been made by Jeffrey (1961), who separated from marine algae the pigments only, and by Holden (1962), who separated chlorophylls from coloured breakdown products. Kates (1959) has separated certain phospholipids from leaves by paper chromatography, and I have evidence for

the presence in the chlorophyll area of colourless substances.

Tocopherols. α -Tocopherol α t was always present (Booth & Hobson-Froehock, 1961), and the spot was used as reference for locating other spots. The identity of α -tocopherol was confirmed by spraying with ferric chloride-bipyridyl reagent or silver nitrate, both of which it reduced; by its $\lambda_{\max}$ 293 m μ in ethanol; and by mixed chromatography with authentic α -tocopherol. Substance α t had the same retention volume as authentic α -tocopherol on the gas-liquid chromatograph with 4% silicone polymer SE-30 (General Electric Co.).

On chromatograms from leaves of *Hedera helix* (ivy) and *Taxus baccata* (yew), spots in the γ -tocopherol and δ -tocopherol positions absorbed ultraviolet irradiation of both wavelengths, reduced ferric chloride and gave coloured spots when sprayed with diazotized *o*-dianisidine reagent (Analytical Methods Committee, 1959). With other species faint spots were often seen in the γ t and δ t positions that were usually too weak for certain recognition. Green (1958) found small amounts of non- α -tocopherols in young whole plants of maize, wheat, barley and pea.

Quinones. Substance q1, which was never completely separated from β -carotene, is presumed to be plastoquinone (Bishop, 1961; Crane, 1961; other references are given by Isler, Rüegg & Langemann, 1960), for this is where the authentic substance ran when applied to paper together with β -carotene as reference. The $\lambda_{\max}$ of q1 at 255 and 260 m μ in ethanol, and the shift to 290 m μ on reduction with potassium tetrahydroborate, support the identity.

Spot q2 was seen on chromatograms from most leaves. It showed $\lambda_{\max}$ in cyclohexane of 248, 260 and 270 m μ . Authentic vitamin K₁ (20) gave a spot of similar shape and position. The two substances had the same retention volume on a Pye gas-liquid chromatograph with Dow-Corning 5% silicone polymer QF1, and they are almost certainly identical. The faint spot q8 was seen only occasionally. Synthetic vitamin K₂ (20) ran to this position, but the identity is not proved.

The $\lambda_{\max}$ of q3 in ethanol was at 257 m μ . After addition of potassium tetrahydroborate the extinction fell to less than half, and a new maximum was observed at 294 m μ . The appearance under ultraviolet irradiation and the spectral properties were similar to those of plastoquinone, and the two substances migrated to about the same extent in the second chromatographic dimension. On the other hand they were well separated by adsorption chromatography.

Substance q4 was usually weak. Its $\lambda_{\max}$ was 265 m μ in cyclohexane and in ethanol. This agrees with Boyer's (1960) value for tocopherol quinone. After α -tocopherol was oxidized with ferric

chloride and chromatographed, several spots were seen, including a prominent one at about position q4. The identity is not proved.

Authentic ubiquinone-50 (Isler *et al.* 1960), when chromatographed with α -tocopherol as reference, gave a narrow spot at about position q5. The ubiquinone, after elution into cyclohexane, showed $\lambda_{\max}$ 272 m μ , the same as for the original material, and in agreement with Morton *et al.* (1958). Substance q5 was seen on chromatograms from many varieties of leaf. When obtained from leaf of carrot and of lucerne its $\lambda_{\max}$ was 271 m μ in cyclohexane and 272 m μ in ethanol. The substance q5 from *Narcissus* leaf had $\lambda_{\max}$ 272 m μ in ethanol, and the extinction was reduced to a quarter on the addition of potassium tetrahydroborate. When sprayed with leucomethylene blue, spot q5 gave a blue colour immediately. The most prominent difference between chromatograms from leaves and from chloroplasts was that q5 was weaker on the latter (see below). These results suggest that q5 is ubiquinone-50. Spot q5 was prominent on chromatograms from mushrooms, from tubers of *Helianthus tuberosus* and *Solanum tuberosum*, from the bulb of *Allium cepa* (onion) and from flowers of cauliflower, a cultivated *Brassica oleracea*.

Close to δt there commonly appeared another spot u6, that, in common with δ -tocopherol, reduced ferric chloride-bipyridyl reagent slowly, but was distinguished from δ -tocopherol by its failure to react with dianisidine reagent. The substance was unstable and had $\lambda_{\max}$ about 269 m μ in ethanol.

Spots q6 and q7 were very pale yellow. They had similar properties and were not always completely separated. When reduced with ascorbic acid and rechromatographed, each ran to position u6. Both had $\lambda_{\max}$ 255 m μ in *n*-hexane and in ethanol, and the extinction was reduced to half after addition of potassium tetrahydroborate. One or both of the compounds together with u6 may form a redox system.

Unknown lipids. Fluorescent spots f1, f2 and f4 were always seen. Spots f5 and f10 were nearly always observed, f3, f6, f8 and f9 occasionally. The $\lambda_{\max}$ values (m μ , all in cyclohexane) were: f1, 253; f2, 253 and 319; f5, 273; f9, 259. Some of these compounds may be polyenes. Sometimes β -carotene soon faded, exposing f5 prominently. This substance, and perhaps others, may be produced by breakdown during chromatography.

When chromatograms were sprayed with dianisidine reagent, some of the spots became purplish brown. Spot u7 was invisible even under ultraviolet irradiation, and has not been detected by other tests than that with dianisidine. Spots u1 and u4 absorbed ultraviolet light, reacted with dianisidine reagent and reduced ferric chloride-bipyridyl reagent, as also did γt and δt . The $\lambda_{\max}$ values of u1 were 262 and 294 m μ in ethanol.

Ultraviolet-absorbing spot u5 was very strong on some chromatograms but not seen on others. It may have been masked by the very strongly fluorescing f1 on some chromatograms. The spectral absorption maximum of u5 was at 258 m μ in ethanol.

Spots u2 and u3 were relatively weak, and their properties have not been studied. Spot u9 was hydrophobic and was detected only by spraying with water.

Substances with limited distribution. Certain conspicuous spots have regularly been observed in extracts from leaves of particular species. These lipids may not occur in all leaves and they are not shown in Fig. 1. For example, on chromatograms from the leaf of *Narcissus* a spot adjoining α -tocopherol between u8 and c5 was seen under ultraviolet irradiation of 254 m μ (but not under 320 m μ ; Booth & Hobson-Frohock, 1961). The substance with $\lambda_{\max}$ 244 m μ in ethanol was present in extracts of several cultivated varieties including *N. recurvus* and *N. pseudonarcissus*. A similar spot was seen on chromatograms from leaves of *Crocus flavus* and from *Fragaria vesca* (wild strawberry), but not from a cultivated variety of strawberry nor with certainty from any of 100 other species. α -Carotene from extracts of carrot leaf, in which it is known to be present in substantial amounts (Booth, 1956), formed a band above β -carotene. Carrot-leaf extracts produced below β -carotene a faint yellow band that was presumably γ -carotene. Near the upper-right corner of chromatograms from cultivated *Mentha* (mint) and *Tropaeolum majus* (nasturtium) areas containing aromatic compounds were located by smell. Lavender leaf produced several fluorescent spots, including one that fluoresced green. *H. helix* produced near u5 a strong spot ($\lambda_{\max}$ at 220 and 259 m μ) that became green when sprayed with antimony trichloride in chloroform.

Site of lipids. Chloroplasts were separated from leaves by a method based on that of Granick (1938), that is maceration in a blender for half a minute under ten times their weight of 0.35M-sodium chloride in aqueous 0.1M-phosphate buffer, pH 6.8, followed by differential centrifuging. Apparatus and solutions were kept in a refrigerated working space, separations were made in a refrigerated centrifuge and bright light was avoided throughout. Centrifuging requirements varied considerably, and had to be found experimentally for each variety. In general it was found that chloroplasts could be prepared more easily and quickly from leaves of ivy, bushes and trees than from softer leaves of spinach and nettle. The preparations were not washed but were used for extraction as quickly as possible. Chloroplasts were separated from *N. pseudonarcissus*, *H. helix*, *T. baccata*, *Rubus fruticosus*, *Crataegus monogyna*, *Urtica dioica* and other

plants. When chromatograms from extracts of chloroplasts were compared with chromatograms from leaves, all spots were common to both, although γ t, δ t and q5 were much weaker on the former.

DISCUSSION

The technique described allowed many phyllo-lipids to be separated in less than 5 hr. on one chromatogram. A chlorophyll and three carotenoids not before reported in leaves were partially characterized, and at least four compounds were tentatively identified as quinones. Over 20 substances remain unidentified and some may be 'new'. Other faint yellow spots or vague ultra-violet-absorbing areas were sometimes seen, especially in the area bounded by u3, α t and c2. As the technique improves, these and other spots may be concentrated and identified. The method is sensitive to low concentrations, which may explain why several substances have not been reported earlier.

The technique could be applied in such a way as to avoid certain ambiguities. For example, plastoquinone, vitamin K₁ and ubiquinone were widely separated. On the other hand, they are not easily separated by one-dimensional adsorption chromatography or by reversed-phase chromatography, when either is used alone. This may explain why Bishop (1961), Zill & Harmon (1962) and others were unable to find appreciable amounts of vitamin K₁ in chloroplasts, although it shows clearly on my chromatograms, and even though Dam, Glavind & Gabrielsen (1946) produced biological evidence for vitamin K in many leaves and suggested that it is present in all species. It may also explain why Page, Gale, Koniuszy & Folkers (1959) found no detectable amounts of coenzyme Q (ubiquinone) in the leaves of spinach, cabbage or lettuce, although Crane (1959) found it in all the leaves he examined.

The similarity of chromatograms from chloroplasts and from leaves is in accord with the accepted belief that most of the phyllo-lipids are in the chloroplasts but does not rule out their occurrence in other subcellular fractions. The observations that ubiquinone and γ - and δ -tocopherol were weaker on chromatograms from chloroplasts suggests that these lipids do occur elsewhere than in chloroplasts. Ubiquinone is concentrated in animal mitochondria, and may be similarly accumulated in plant cells. This suggestion is supported by the finding that ubiquinone was prominent on chromatograms from extracts of non-leaf tissues.

SUMMARY

1. Green leaves were extracted with acetone. The extracted lipids were transferred to light

petroleum and chromatographed on paper in two dimensions.

2. About 40 spots were observed on the chromatograms, many being found in extracts of all species examined. The spots included well-known chloroplast pigments, quinones and tocopherols.

3. A new chlorophyll was found, less polar than chlorophyll *a* and having spectral-absorption maxima at 409.5 and 662 m μ in ethanol. Three carotenoids have been observed whose properties do not coincide with those of carotenoids previously reported in leaves.

4. Over 20 other lipids await identification.

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