925. *The Direct Measurement of the Absorption Spectra of Some Plant Phenols on Paper Strip Chromatograms.*

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A method is described for the measurement *in situ* of the ultra-violet absorption spectra of substances on paper chromatograms. A number of typical plant phenols have been examined and the effects of structural differences on light absorption are discussed.

The existence in various tissues of fruit trees of a complex mixture of phenols is readily demonstrated by paper strip chromatography of suitably prepared extracts, a variety of spots appearing either in visible or in ultra-violet light or becoming evident after treatment with various reagents. In a systematic investigation, it has become clear that R_F values, colour, fluorescence, and colour reactions sometimes fail to indicate reliably even the type of compound in question and it appeared likely that absorption spectra would be helpful, particularly if they could be combined directly with paper strip chromatography.

The usual technique involving elution of appropriate portions of the paper chromatogram was impossible in some cases as the substances were not eluted with boiling water or boiling alcohol. A similar difficulty has been reported by Gage and Wender (*Analyt. Chem.*, 1950, **22**, 708). Elution may be avoided and time saved by direct measurement of the absorption spectrum on the paper. This also has the advantage that, where the spots partly overlap, a small area of the desired spot may be utilised on the side remote from the overlap, and the effect of contamination reduced. The technique lacks somewhat in precision when compared with measurements on solutions and the data obtained are incomplete in that extinction coefficients cannot be calculated. The method has been tested by determination of the absorption spectra of a number of substances of various types known to occur in plants. Its application to the study of the phenols of fruit trees will be described elsewhere.

Experimental

Materials.—Some of the substances used were commercial materials, some were prepared in the laboratory, while specimens of others were provided by Dr. E. C. Bate-Smith and Mr. R. C. Seeley, to whom the authors express their warm thanks.

Paper Chromatography.—The spectra described, except where otherwise mentioned, were observed by utilizing the spots obtained by chromatography on Whatman No. 1 filter paper with butanol–acetic acid–water (Campbell, Work, and Mellanby, *Biochem. J.*, 1951, **48**, 109).

Light-absorption Measurements.—Measurements were made with a Unicam S.P. 500 quartz spectrophotometer. A strip of paper, about 1·2 cm. wide and 4—5 cm. long, carrying the spot to be examined, was cut from the paper chromatogram. Two of the spring spacers supplied with the instrument were inserted back to back in one of the compartments of the cell carrier in the two slots which are closest together, *i.e.*, the slots which would be used with 2-mm. and 5-mm. cells respectively, and the paper was inserted between them. A strip of paper cut from a blank chromatogram was similarly inserted in an adjacent compartment of the cell carrier. Because of the relative opacity of paper, two modifications of the normal procedure for taking readings were used. Method (*b*) gives rather more accurate readings, but the simpler procedure (*a*) is satisfactory in many cases. Neither method gives significant readings below 225 mμ.

Method (*a*). The hydrogen lamp is used and the slit width set at 1·5 mm. over the range 400—270 mμ; below 270 mμ the slit is increased to 2 mm. The instrument switch is set at 0·1 and the normal procedure for this setting followed. When strong absorption occurs between 320 and 400 mμ, it is sometimes necessary to use the tungsten lamp, but such readings may require adjustment (see below).

Method (*b*). The band width is kept at 5 mμ from 400 to 255 mμ. Below 255 mμ with the maximum slit width (2 mm.) the band width is less than 5 mμ. From 400 to 310 mμ, readings are taken with the tungsten lamp in the usual manner; below 310 mμ the hydrogen lamp and the switch setting of 0·1 are employed. Owing to the difference in cross-sectional area of the beams and the uneven optical density of the spots, readings at the same wave-length taken with the hydrogen and the tungsten lamp may differ. In such cases, from a few measurements with both lamps at several wave-lengths, a correction is readily calculated to bring the log D plots of the curves into coincidence.

The precision of the measurements of light absorption on cellulose (paper) is less than that obtainable with solutions, but for the present purpose, namely, recognition of the type of substance under examination, the " band envelopes " are adequately displayed. Values for $\lambda_{max.}$ are probably correct to within about 3 mμ. As the number of absorbent molecules in the path of the beam is, in general, unknown, it is particularly convenient to eliminate its effect on the shape of the absorption curve by plotting log D, rather than D, against wave-length. Further, the resulting curves may then be easily compared by suitable vertical transposition, as has been done in the accompanying Figures.

Discussion

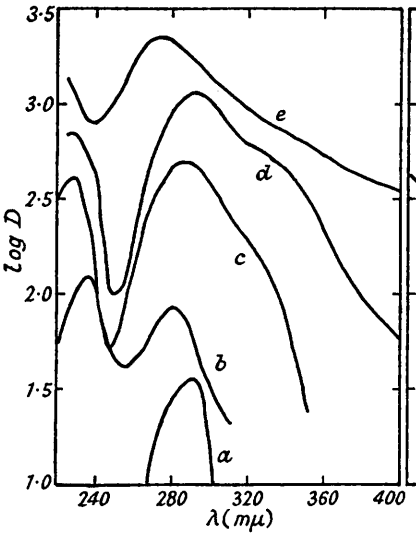
The physical condition of the substance constituting the " spot " on the paper chromatogram is somewhat uncertain, and it would be difficult to predict to what extent the absorption spectrum of a substance on paper would differ from its spectrum in water or alcohol. However, some experimental results may be given. The main peak for phloridzin on cellulose (λ 286 mμ) is the same as in water or alcohol and there is a general similarity in the curves. Again, with quercetin on cellulose and in alcohol the peaks of the curves correspond in position, but on cellulose there appears to be a considerable decrease in the intensity of absorption when compared with alcohol solution as the wave-length decreases. The latter effect, presumably due to adsorptive forces, appears to be fairly general in the series of compounds studied.

On the other hand, the 3-glycoside, quercitrin, has on cellulose a band with $\lambda_{max.}$ 385 mμ (see Fig. 1) but in alcohol (Gage and Wender, *loc. cit.*) this band has $\lambda_{max.}$ ~365 mμ. Here again decreased intensity of absorption at shorter wave-length is evident. Other examples of this effect are referred to below. Caution is required however, in comparing the relative intensities of two or more bands as measured on paper with an instrument of the type

employed. It has been pointed out by Braude, Fawcett, and Timmons (*J.*, 1950, 1019) that with certain instruments an apparent failure of Beer's law may result from fluorescent emission by the absorbing substance. Such an effect has been noted several times in the present studies and is illustrated by curves *a* and *b* in Fig. 2 which were determined with spots having maximum densities of 0·56 and 0·98 respectively at 385 mμ.

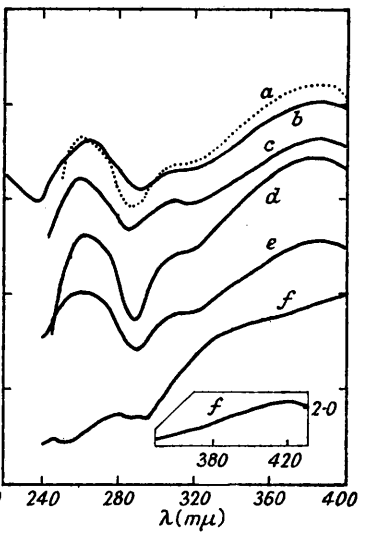
On cellulose, arbutin, a quinol glucoside, shows, rather weakly, a single band with $\lambda_{max.}$ 291 mμ (Fig. 1) which is without doubt the displaced *B*-band of quinol, for which Morton and Sawires record $\lambda_{max.}$ 294 mμ in alcohol (*J.*, 1940, 1052). As is to be expected, glucoside formation has little effect. *epi*Catechin (I), with two "insulated" phenolic rings, in alcohol has a *B*-band displaced by the auxochromic influence of the hydroxyl

FIG. 1.



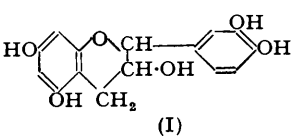
a, *Arbutin*.
b, epi*Catechin*.
c, *Phloridzin*.
d, *Hesperitin*.
e, *Irigenin*.

FIG. 2.

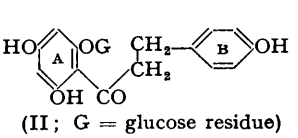


a, *Quercetin*, $D_{max.}$ 0·56 *at* 385 mμ.
b, ,, $D_{max.}$ 0·98 *at* 385 mμ.
c, *Quercitrin*.
d, iso*Rhamnetin*.
e, *Myricetin*.
f. *Leptosidin*.

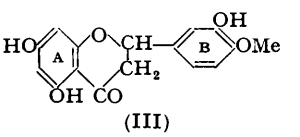
groups to 280 mμ, the effects of the phenolic rings on intensity being approximately additive (Bradfield, Penney, and Wright, *J.*, 1947, 32; Bradfield and Penney, *J.*, 1948, 2249). Below 240 mμ absorption is intense. On cellulose, two bands appear (Fig. 1)



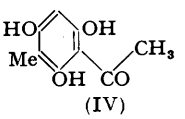
(I)



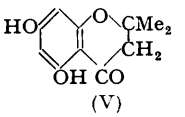
(II; G = glucose residue)



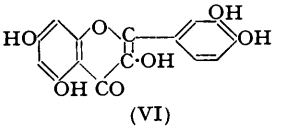
(III)



(IV)



(V)



(VI)

with $\lambda_{max.}$ 282 and 236 mμ respectively, the former being the *B*-band as in alcohol, and the latter presumably the *E*-band of benzene displaced by the cumulative effects of the hydroxyl groups and, as compared with alcoholic solution, diminished in intensity.

The spectrum of *epi*catechin may now be compared with those of phloridzin (II) and hesperitin (III) (Fig. 1), in which an additional chromophore, the carbonyl group, is attached to ring A. In alcoholic solution phloridzin shows much more intense absorption (*E* 20,260 at $\lambda_{max.}$ 284 mμ) than (—)-*epi*catechin (*E* 3300 at $\lambda_{max.}$ 280 mμ).

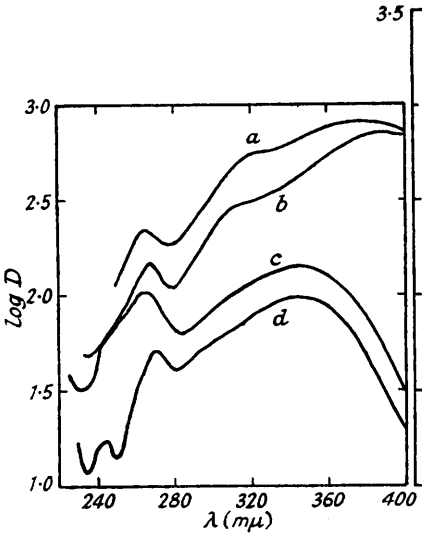
On cellulose, (II) and more particularly (III) resemble the acetophenone (IV) and the chromanone (V) in alcoholic solution, as shown in the Table. The main band results from

		$\lambda_{max.}$ (mμ)				$\lambda_{max.}$ (mμ)	
(II)	~320 *	284	228	(IV) †	~333 *	291	223
(III)	~325 *	293	(235)	(V) †	~345 *	292·5	—

* Inflexion. † Morton and Sawires, *J.*, 1940, 1052.

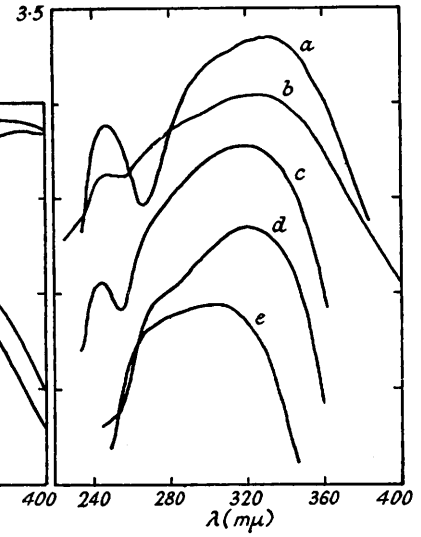
a displaced *B*-band showing only as an inflexion superposed on a *K*-band. Presumably another *B*-band originating in the unconjugated phenolic ring is submerged in this. The short-wave bands are again regarded as *E*-bands displaced to longer wave-lengths.

FIG. 3.



a, *Fisetin.*
b, *Butein.*
c, *Kaempferitrin.*
d, *Apigenin.*

FIG. 4.



a, *Chlorogenic acid.*
b, *Caffeic acid.*
c, *Ferulic acid.*
d, *Methylenedioxycinnamic acid.*
e, p-*Coumaric acid.*

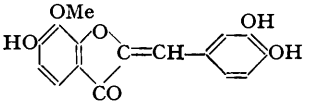
With the introduction of a double bond, converting a flavanone into a flavone, a more complex situation arises, as now ring B as well as ring A becomes conjugated with the carbonyl group. For the present purpose of empirical correlation of structure and light absorption it is convenient first to consider the flavonol quercetin (VI).

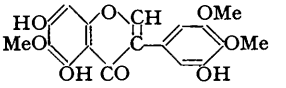
On cellulose this substance (Fig. 2) shows bands at 385 and 260 mμ (referred to as *K* and *B'* bands respectively) and an inflexion at 312 mμ (referred to as the *K'* band). The *K* and *K'* band-envelopes probably result from the partial resolution of a number of bands, some benzenoid in origin, some arising from the complex conjugated system. The effect on these three bands of changing the substituents may now be summarised.

(*a*) Methylation of the 4'-hydroxyl group or introduction of an additional hydroxyl group in the 5'-position has little effect on the position or relative intensity of the bands (see *iso*rhamnetin and myricetin, Fig. 2). The 3-rhamnoside of quercetin, quercitrin, gives the same spectrum as quercetin (Fig. 2). (*b*) Removal of the 3'-hydroxyl group from quercitrin, to give kaempferitrin, results in coalescing of the *K* and the *K'* bands to give one at 345 mμ, *i.e.*, less displaced to longer wave-lengths. As well as a *B'* band at 264 mμ there are indications of an inflection at ~250 mμ (Fig. 3). On the other hand,

removal of the 5-hydroxyl group from quercetin, to give fisetin, leaves the positions of the bands practically unchanged, but the *K'* band is relatively stronger and the *B'* band relatively weaker (Fig. 3). (*c*) The 3-hydroxyl group, whether bound as a glycoside or not, has little effect, as may be seen from a comparison of the spectra of the chalkone butein and the flavone apigenin (Fig. 3), from both of which it is absent, with those of quercetin and kaempferitrin respectively. The spectrum of the glycoside apiin is similar to that of its aglucone, apigenin. It may be noted however that in the spectra of apigenin and apiin a very weak additional band appears at 240—250 mμ.

The striking effects on light absorption of changes of structure resulting in increase or decrease of conjugation are shown by the spectra of leptosidin (VII) and irigenin (VIII).

(VII) 



(VIII)

In (VII), the phenolic nuclei, in addition to being conjugated with the carbonyl group are conjugated with each other by the –O·CH:CH– group. The ability of -O- to transmit conjugation (chromolatory effect) has been shown by Bowden, Braude, and Jones (*J.*, 1946, 948). For the *K* and the *K'* band of leptosidin, the values of $\lambda_{max.}$ 420 and 370 mμ may be compared with $\lambda_{max.}$ 390—320 mμ for butein. On the other hand, the absorption spectrum for the *iso*flavone, irigenin (VIII), resembles that for phloridzin or hesperitin rather than those of the flavones and flavonols with a peak at 270 mμ and an inflexion at 340 mμ. This is partly due to the fact that ring *B* is now no longer conjugated with the other phenolic ring *via* the –O·CH:CH– chain. The CO group attached to the ethylenic carbon atom, like an α-methyl group attached to a stilbene system, may however give rise to non-planarity of the ethylene link and the directly attached benzene ring, with the result that light absorption resembles that of a styrene rather than that of a stilbene system (cf. Braude, *J.*, 1949, 1902), *i.e.*, the bathochromic effect is diminished. Again, the conjugation of the ethylene link with the carbonyl group crosses its conjugation with the ring B. The net result is that conversion of a flavone into an *iso*flavone has a notable hypsochromic effect.

Some hydroxycinnamic acids as representatives of another group of well-known plant constituents have also been examined by the present technique. The absorption spectra on cellulose shown in Fig. 4 call for little comment. The hydroxyl group of *p*-coumaric acid gives rise, as expected, to a bathochromic displacement of the cinnamic acid band ($\lambda_{max.}$ 269 in alcohol; Morton and Sawires, *loc. cit.*) to 304 mμ, while caffeic acid, with two hydroxyl groups, has $\lambda_{max.}$ 325 mμ. With ferulic acid and methylenedioxycinnamic acid the maximum occurs at slightly lower wave-lengths. With chlorogenic acid, in which the carboxyl group of caffeic acid is esterified by one of the hydroxyl groups of quinic acid, displacement of the main band is rather greater than for caffeic acid ($\lambda_{max.}$ 330 mμ), while a peak at 240—250 mμ which in caffeic acid and its ethers is of very low intensity becomes more marked.

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