A CHROMATOGRAPHIC STUDY OF THE CURCUMINOIDS IN *CURCUMA LONGA*, L.

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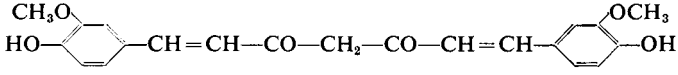
Received March 30, 1953

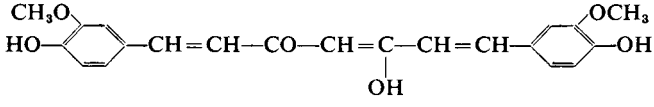
IN an attempt to evaluate samples of turmeric (the rhizome of *Curcuma longa*, L.) on the basis of their curcumin contents, using the familiar reaction with boric acid for colorimetric estimation, the author found that the colour developed with extracts was markedly different from that obtained with pure curcumin and this observation has led to the present investigation.

Curcumin has the molecular formula $C_{21}H_{20}O_6$ or $C_{19}H_{14}O_4(OCH_3)_2$, first suggested by Ciamician and Silber[1] from determinations of methoxyl. The work of Milobedzka, *et al.*[2] and the subsequent synthesis effected by Lampe[3] showed that curcumin was diferuloyl methane (I):



(I)

Attempting a synthesis of curcumin by condensing acetylacetone with vanillic aldehyde in presence of ethanolic hydrochloric acid, Heller[4] obtained two products which differed from curcumin in their failure to react with boric acid and ferric chloride, and were therefore called by him α- and β-*iso*curcumins. As 1:3-diketones form stable metallic compounds by virtue of their ability to enolise, the enolic H being acidic, Heller on the basis of the reaction with ferric chloride came to the conclusion that normal curcumin which gives a deep reddish-brown colour with ferric chloride has the keto-enol structure (II), while the two



(II)

*iso*curcumins which give only a faint yellowish-brown colour have the diketonic structure (I), the α- and β-forms being stereo-isomers of the same diketone[5]. Pavolini[6] obtained curcumin from acetylacetone and vanillin by using boric anhydride as condensing agent, and later in collaboration with others[7] has synthesised a number of "curcuminoids" or analogues of curcumin by condensing different aromatic aldehydes with β-diketones. As it is possible that natural curcumin is accompanied by minor amounts of its analogues and its structural and stereo-isomerides, a study of the constituents of the colouring matter in turmeric by a chromatographic procedure was undertaken. Preliminary experiments have indicated that the pigments could be resolved into three main constituents and a few minor fractions.

EXPERIMENTAL

Preparation of the Extract.

Curcumin is insoluble in water, light petroleum and hexane, moderately soluble in benzene, chloroform and ether, while ethanol and acetone are good solvents. Benzene however has the merit of taking up very little of the resinous impurities from turmeric[8] besides being nonpolar, and was therefore used throughout both for extraction of the pigments and for chromatography. About 25 g. of air-dried turmeric powder ground to pass 80 mesh is extracted with light petroleum (b.pt. 40° to 60° C.) to remove the fixed and volatile oils. The dried residue is again exhausted with about 200 ml. of boiling benzene in a percolator with a Wiley-Soxhlet extraction syphon cup. The syphon cup is constructed from a Pyrex boiling tube (3 cm. × 12 cm.) and 2 mm. bore Pyrex tubing. The extract is allowed to stand overnight and filtered free from insoluble materials.

Adsorbents.

A number of adsorbents were tried for their suitability for chromatographic procedure, with reference to their adsorptive power, rate of development, separation and visibility of the zones on the column. Alumina and magnesia were too active, the separation of the zones was poor and some of the adsorbed materials could not be stripped easily from the column with any of the ordinary solvents. Alumina (B.D.H. for chromatographic purposes) rendered neutral by washing with dilute hydrochloric acid and deactivated[9] was still found to be of no use. Precipitated calcium carbonate, magnesium carbonate, sodium bicarbonate, starch, Fuller's earth, etc., had very little adsorptive power for curcumin. Dried silica as an adsorbent was also not satisfactory; it becomes translucent in contact with the solvent, causing poor visibility and separation of the zones. But silica incorporated with about 50 per cent. of its weight of moisture proved satisfactory. The silica gel used in this study was prepared from sodium silicate dried (Merck) dissolved in water to form a 10 per cent. solution by precipitation with dilute hydrochloric acid as described by Gordon, Martin and Synge[10]. The precipitated silica gel was "aged" for 2 days in 4N hydrochloric acid, filtered and washed free from acid on a Buchner funnel and dried at 110° C. for 16 hours. The small lumps were crushed and the material passed through an 80-mesh screen and preserved in a stoppered bottle. For use, 100 g. of silica is treated with 53 ml. of water in a mortar and thoroughly mixed. With this material good visibility and separation of the zones with almost colourless interzones are secured, with a carefully packed column and a slow rate of percolation of developing solvent, if overloading of the column is avoided.

Apparatus and Procedure.

The chromatographic apparatus used consisted of a Pyrex tube (2·2 cm. × 120 cm.) fitted with a tap at the lower end. A plug of

cotton wool soaked in benzene and freed from air bubbles is pushed to the lower end of the tube and a thin slurry of the prepared silica gel in benzene is poured down the sides of the tube and allowed to settle.

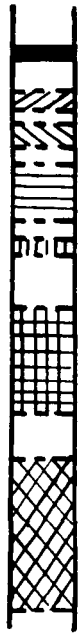


FIG. 1.
Chromatogram of benzene extract of turmeric on silica.

Application of positive pressure of about 20 to 30 cm. of mercury at the top of the column helps uniform and firm packing and the column is packed to a height of 45 cm. About 80 g. of the silica gel gives a column of this height. The length of the column holding 1 ml. of solvent is 4·4 mm. and 1 ml. of solvent occupies a tube length of 2·4 mm. giving a packing ratio (S)[11] for the column of about 1·8.

The filtered turmeric extract is then passed down the column and when the solution has just sunk into the column, fresh benzene equilibrated with water is added for the development of the chromatogram and pressure is applied at the top so that a rate of flow of solvent of about 3 ml./minute, which corresponds to a movement of the solvent front down the column at about 8 mm./minute, is maintained. There is a gradual separation of bands on the column and the completed chromatogram will show 3 distinct zones and a few minor zones represented in Figure 1.

R Values[12].

The rates of migration of the bands relative to the movement of the surface of the developing liquid are measured as follows. The initial position of the liquid level in the tube above the column and that of the estimated maximum concentration of each band are first marked when the solution has just entered the column. The distances through which these points move are measured again when the zones have completely separated on the column. The *R* values for the different fractions are given in Table I.

Separation of the Fractions.

Development is continued with benzene. Any fixed or volatile oil of turmeric not completely removed by light petroleum, passes down the column first without definite zoning and is collected as a pale yellow

TABLE I

PHYSICAL PROPERTIES AND ANALYTICAL CONSTANTS

Substance in zone number	M.pt. °C.	*R* values	Mol. wts.	Methoxyl found per cent.
1 (Curcumin)	182	0·27	371, 362, 369 [$C_{19}H_{12}O_2(OH)_2(OCH_3)_2$ = 368]	16·44 16·62 (calc. 16·88)
2	168	0·14	333, 341, 337 [$C_{19}H_{13}O_2(OH)_2(OCH_3)$ = 338]	9·05 9·10 (calc. 9·18)
3	80 to 130	0·10	364, 371	—
4	224	0·09	306, 304, 309, 308 [$C_{19}H_{11}O_2(OH)_3$ = 308]	0·0

fraction. Evaporation of the solvent leaves a yellow oily residue having the characteristic odour of turmeric. The different zones are collected as liquid chromatogram in separate containers as each washes down the column. It is found that the widths of the zones increase considerably as they move down the column. The eluates from the interzones are nearly colourless or only faintly coloured and are rejected. The different fractions collected are concentrated by distillation of the solvent under reduced pressure and further purified by re-chromatography on smaller columns (1·5 cm. × 20 cm.) of silica gel.

Fraction 1. This is the eluate containing the orange-yellow zone (adsorbed lowest on the column) consisting of curcumin. As the benzene solution is evaporated, curcumin is thrown out of solution in the form of bright cherry-red crystals which, however, soon change into a yellow form. The crystals were filtered off on a small sintered-glass funnel (No. 3 porosity), washed with benzene and dried (yield about 500 mg.). On recrystallisation from ethanol, curcumin is obtained in the form of reddish-orange prisms melting at 182° C.

Fraction 2. Evaporation of the eluate of the next zone above yields an amorphous orange-yellow powder (yield about 200 mg.). Under the microscope it appears as minute spheres of reddish-orange colour. The spheres break into irregular pieces when crushed under the cover slip. Attempts to obtain it in crystalline form were not successful. M.pt. 168° C.

Fraction 3. An amorphous reddish sticky residue (about 20 mg.) is obtained on evaporation of the benzene extract of the next zone. This material also could not be crystallised though its purity and homogeneity was assured by re-chromatography on silica gel several times. The substance was therefore precipitated from benzene solution by addition of light petroleum; the precipitate was washed with light petroleum and dried under reduced pressure. A pale yellow amorphous powder is obtained which softens at about 80° C. and melts at about 130° C. It reverts to the sticky form in contact with benzene.

Fraction 4. Yields a product (about 120 mg.) sparingly soluble in benzene and crystallising out in yellow plates. By precipitation from an ethanolic solution by cautious addition of water, the substance is obtained in the form of glistening golden-yellow spangles. M.pt. 224° C.

Fractions 5 *and* 6 are obtained in minor amounts which could not be crystallised. They had to be repeatedly passed through silica gel before chromatographic homogeneity could be secured. The dark brownish-black layer occupying a few cm. of the column at the top remains there, despite prolonged development with benzene. It probably consists of resinous impurities and other conversion products of the pigments.

Molecular Weights and Methoxyl Values.

Microdeterminations of molecular weights of the different fractions were carried out in replicate by Rast's method[13], with resublimed camphor (m.pt. 177·8° C. and mol. depression of freezing point 40° C.) as solvent. Microdeterminations of methoxyl by Zeisel's method using the Vieboch and Brecher modification[14] were made on fractions 1, 2 and 4, and these values are given in Table I.

Reactions.

The reactions, with the following reagents, of the substances isolated are summarised in Table II. The first 3 reagents are applied to the solutions of the substances in glacial acetic acid, reagents 4 and 5 are applied to a speck of the material on a white porcelain tile.

TABLE II

REACTIONS

Substance in zone number	$FeCl_3$	Boric acid	Boric-oxalic acid	Sodium hydroxide 1 per cent.	Concentrated sulphuric acid
1	Dark reddish brown	Orange	Pink	Red	Dark red
2	,,	,,	Orange red	,,	,,
3	,,	,,	Red	,,	,,
4	,,	Bright yellow fluorescence	Pink with orange fluorescence	Orange	Orange red
5	,,	Orange	Red	Orange red	,,
6	,,	Deep yellow	Orange	Orange	,,

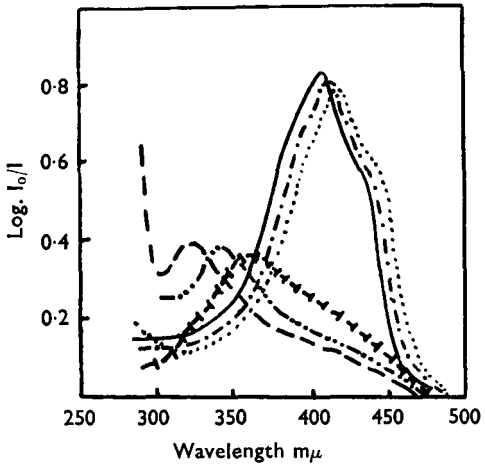


FIG. 2. Absorption spectra in benzene.

..... Zone 1 of chromatogram
·—·—· ,, 2 ,, ,,
+ + + + ,, 3 ,, ,,
——— ,, 4 ,, ,,
—···— ,, 5 ,, ,,
— — — ,, 6 ,, ,,

Reagents.

1. Ferric chloride—1 per cent. solution of anhydrous $FeCl_3$ in glacial acetic acid.
2. Boric acid—0·5 per cent. solution in glacial acetic acid.
3. Boric-oxalic acid—1 g. of oxalic acid, and 0·5 g. of boric acid dissolved in 100 ml. of glacial acetic acid.
4. Sodium hydroxide—1 per cent. aqueous solution.
5. Concentrated sulphuric acid.

Absorption Spectra.

The absorption spectra in the visible and ultra-violet region of the substances in benzene and ethanol were recorded with a Beckman Model DU Quartz Spectrophotometer, with a water-cooled hydrogen discharge lamp light source and 1-cm. silica cells. Solutions containing 0·5 mg. in 100 ml. of the solvents were prepared by weighing out the substances on a microchemical balance and their extinctions relative to the solvents were measured from 480 to 280 mμ in the case of benzene solutions and down to 220 mμ in the case of ethanol solutions, at intervals of 10 mμ, while, in the region of an inflexion, measurements at every 2 mμ were made. With the recommended setting of the sensitivity knob for

maximum accuracy the zero adjustments at each particular wavelength were made by adjustment of the slit width. Figures 2 and 3 show the typical curves obtained with benzene and ethanol solutions respectively, and the absorption data are given in Table III.

TABLE III

ABSORPTION DATA

Substance in zone number	Ethanol		Benzene	
	$\lambda_{max.}$	$E^{1\%}_{1\,cm.}$	$\lambda_{max.}$	$E^{1\%}_{1\,cm.}$
1	430	1560	420	1520
2	425	1580	415	1560
3	370	620	365	—
4	420	1640	410	1640
5	—	—	345	—
6	—	—	325	—

Thermal Isomerisations of Fractions 3, 5 *and* 6.

Benzene solutions of fractions 3, 5 and 6 are found to be unstable and they undergo a slow change, whereby each of them separates into two zones of re-chromatography after a few days. This change is accelerated by heat. A solution of chromatographically homogeneous fraction 3 in benzene was refluxed for 10 hours. Another portion of the same solution was evaporated to dryness and the residue was heated at 110° C. for 5 hours and then taken up in benzene. Both these solutions were passed through silica columns. There was a separation in both cases into two bands, the topmost one being due to the unchanged substance while the fast moving zone was identified as curcumin, from its absorption spectrum, reaction and *R* values (i.e., by mixed chromatogram with pure curcumin). Similar changes were noted in the case of fractions 5 and 6 also, and these yield respectively on isomerisation substances identical with fractions 2 and 4.

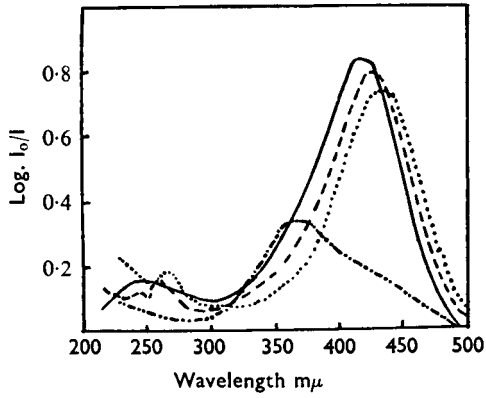


FIG. 3. Absorption spectra in ethanol.

····· Zone 1 of chromatogram
— — — „ 2 „ „
— · · — „ 3 „ „
——— „ 4 „ „

DISCUSSION

The Chromatographic Process.

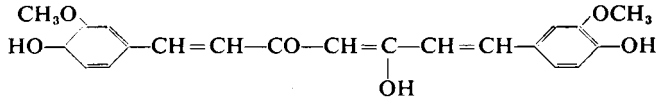
Silica gel is widely applied for the resolution of mixtures by partition between water held by the silica and the immiscible solvent phase. In the present case, however, since the pigments are completely insoluble

in water it is obvious that partition chromatography in the sense of a continuous counter-current liquid-liquid extraction process as used by Martin and Synge cannot be playing any significant role in the mechanism of the chromatographic process here, and the mechanism is most probably one of surface adsorption and desorption as in classical chromatography, the separation of the zones depending on differences in the adsorption affinities of the fractions for silica. The addition of water to the dried silica has served only to control its adsorbent activity by partial deactivation. Further, the widening of the zones as they move down the column, with a diffuse trailing boundary, may point to a variation of R with concentration and to a non-linear sorption isotherm which is more often the case with adsorption[15]. If dilute ammonia (say a 5 per cent. solution) in which the pigments are soluble is used in the place of water for the preparation of the adsorbent, the difference in partition coefficient may also be a contributory factor in their separations on the column, where they appear as bright red or orange zones. The use of ammonia as the immobile phase causes slower movement but better resolution of the zones, with sharp leading and trailing boundaries which do not spread out very much during development. However, as it was suspected that the zones on long contact with the column tend to fade in colour and undergo some irreversible change, the use of ammonia was not preferred.

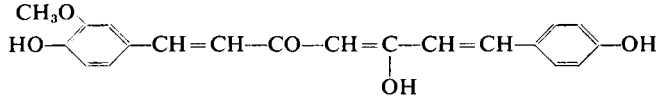
Successful chromatographic separation of the zones with silica and benzene depends on factors such as particle sizes of the adsorbent, dimensions of the column, degree of packing, and rate of flow of developing solvent. The smaller the size of the particle, the better the separation, but too fine a material renders the rate of permeation of the solvent inordinately slow. The 80 to 100-mesh powder used is found to be of suitable size. With the column dimensions used, viz., 2·2 cm. $\times$ 45 cm., complete separation of the 3 main zones takes place with benzene extracts from 25 g. of turmeric. Use of larger quantities of material will lead to overloading of the column and poorer separations. The minor zones higher up separate as development continues and not until the fast moving zones have completely left the column. Their movements can be speeded up by cautious addition of ether to the benzene. The rate of movement of the solvent front down the column of about 5 to 10 mm./minute is found to be the optimum for good separation.

Major Constituents.

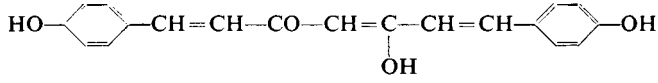
Fractions 1, 2 and 4 form the three major constituents of the colouring matter in turmeric. Curcumin, which is adsorbed lowest on the column (Fig. 1), forms by far the most important constituent. Fractions 2 and 4, which react in a similar way with ferric chloride and other reagents, are probably its analogues. Their molecular weight and methoxyl values (Tables I) indicate their relationship with curcumin. Thus, while curcumin has two OCH_3 groups, fraction 2 has only one, and fraction 4 has none; a reasonable guess as to the structure of these analogues can therefore be made as follows:

(II) 

Fraction 1. Diferuloyl methane

(III) 

Fraction 2. *p*-Hydroxy-cinnamoyl-feruloyl-methane

(IV) 

Fraction 4. *pp'*-Dihydroxy-dicinnamoyl-methane

(IV) has been synthesised by Lampe and Godlewska[16] and described by them as orange coloured needles melting at 218° to 220° C. and giving an orange colour with boric acid.

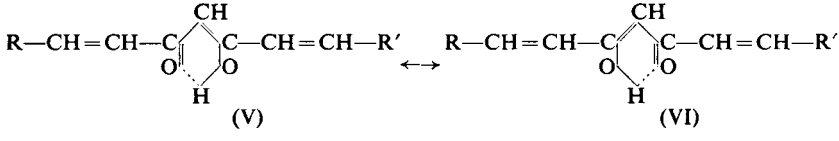
The close similarity in the structure of these 3 compounds is reflected in the similarity of the shapes of their absorption curves (Fig. 2). (IV) in benzene (fraction 4) has a maximum absorption at 410 mμ, while (III) and (II) (curcumin) have their peaks at 415 mμ and 420 mμ respectively, and have slightly lower extinction values. The minor bathochromic shifts of $\lambda_{max.}$ and decreasing absorption intensities can be accounted for on the basis of the weighting effect on the chromophore of successive *m*-substitution of the two aromatic nuclei by methoxy groups[17].

Minor Constituents.

Three minor fractions have so far been isolated and further work is in progress. Difficulty is experienced in their isolation in a state of purity owing to their small *R* values and consequent very slow rate of migration and development on the silica column, their instability, and other factors. Chromatographic homogeneity could be secured only by repeated re-chromatography on silica columns. Fraction 3 has been obtained in just sufficient amounts to enable a study of its behaviour and a guess as to its nature to be made. It has the same molecular weight as curcumin, gives the same reactions, and undergoes isomerisation into curcumin by heat. It is evidently a geometrical isomer of curcumin.

Stereo-isomerism of the Curcuminoids.

As 1:3-diketones, the curcuminoids could be represented as resonance hybrids between structures (V) and (VI) with a hydrogen bond between enolic H and carbonyl oxygen:



(V) (VI)

The two ethylenic double bonds on either side of the chelate ring structure would allow of *cis-trans* configurations of the substituent groups. For a symmetrical molecule with two double bonds, 3 isomers are possible, viz., *trans-trans*, *trans-cis* and *cis-cis*, and in the case of an unsymmetrical molecule (as III) 4 are possible[18], since the *trans-cis* and *cis-trans* configurations will not be equivalent. Those substances which occur in appreciable amounts on the column have obviously a *trans-trans* configuration which is the most stable form; the less stable forms will occur only in comparatively smaller amounts, while the least stable forms, as for instance the *cis-cis* isomers, may not be present at all or occur in such insignificant amounts as to be incapable of isolation. That probably explains why out of the 10 possible substances only 6 could be discerned on the column so far. Thus, while ordinary curcumin has possibly a *trans-trans* configuration, fraction 3 is to be assigned a *cis-trans* form, considering its lower m.pt. and lower stability. Its lower extinction coefficient and the shift of its absorption maximum to considerably lower wavelengths are also therefore to be interpreted in terms of a *cis-trans* isomerism. Representing the models of the structures of these

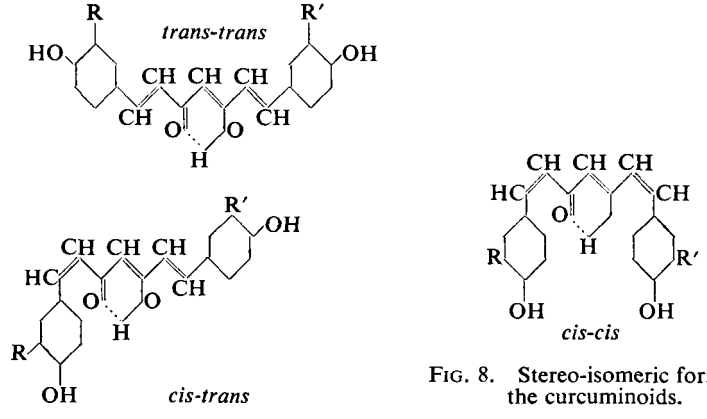


FIG. 8. Stereo-isomeric forms of the curcuminoids.

isomers, using the accepted values of bond distances and valence angles[19], it may be seen that a strainless coplanar *cis* configuration is not possible as the *ortho*-H of the benzene nucleus comes into steric conflict with the carbonyl O, necessitating a slight twist of the benzene nucleus about its 1:4 axis. But considerations of resonance among the different stable structures of the molecule would require a planar configuration and the resonance stabilisation energy will give rise to a potential energy gradient which would oppose any rotation of the benzene ring[20]. The molecule will thus be under a strain and this strain not only reduces its stability, but also causes a hypsochromic shift of the maximum of the principal absorption band[23].

Fractions 5 and 6 possibly bear similar relationships respectively to (III) and (IV) and in the case of fraction 5 there is at present no means of deciding its exact configuration.

iso*Curcumins*.

All the substances isolated give a strong colour reaction with ferric chloride and it therefore appears that the purely diketonic *iso*curmins described by Heller are not present in the natural product.

Summary

1. Chromatographic resolution of the colouring matter of *Curcuma longa*, L., on silica gel from benzene extracts of the powdered rhizome shows a separation into 3 main and 3 minor zones which are isolated by a liquid-chromatogram procedure, the details of which are given.

2. The chemical reactions, physical properties and absorption characteristics of the different fractions are recorded.

3. The three main constituents consist of curcumin, by far the most important, and its two analogues, viz., *p*-hydroxycinnamoyl feruloyl methane (III) and *pp'*-dihydroxydicinnamoyl methane (IV). The minor fractions appear to be the geometrical isomerides of the 3 main constituents.

4. The purely diketonic forms of *iso*curcumins described by Heller are apparently not present in the natural product.

The author is greatly indebted to Dr. T. R. Govindachari, Professor of Chemistry, the Presidency College, Madras, for the use of the Beckman spectrophotometer, microchemical balance, micro methoxyl apparatus and other facilities. The paper is published by kind permission of the Director of Medical Services, Government of Madras.

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