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Anomalous Chromatographic Behavior of Chlorogenic Acid in Extracts of Tobacco

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Paper chromatographic procedures are widely employed in studies on the isolation and identification of polyphenolic compounds occurring in plants. In fact, it has been stated that the appearance of single, well-defined spots on paper chromatograms having Rf values identical with authentic reference standards and characteristic color reactions may be used as a basis for absolute identification of polyphenols (Geissman, 1955). This criterion, of course, presupposes that each polyphenol in a complex plant extract will partition on paper in an independent, uniform manner giving characteristic Rf values. The present report describes an instance in which such a polyphenol, chlorogenic acid, partitioned in an abnormal manner due to the presence of inorganic substances in the extract.

The phenomenon was observed during the chromatographic separation of concentrated polyphenol extracts obtained from flue-cured tobacco by a modification of the isolation procedure of Reid (1956). Two hundred and fifty grams of U. S. Type 12 tobacco (mixed grades) were extracted with 12 liters of 80 percent ethanol, the polyphenols were precipitated by adding basic lead acetate at pH 8.0, and the lead-polyphenol complex was separated and suspended in 400 ml. water. The suspension was adjusted to pH 1.0 with sulfuric acid, the lead sulfate was filtered off and the filtrate was adjusted to pH 5.0 with sodium hydroxide. The filtrate was then successively extracted with chloroform, diethyl ether, ethyl acetate and *n*-butanol to remove most of the polyphenols from the aqueous layer.

On checking the completeness of extraction the aqueous layer ("aqueous concentrate") was chromatographed on small (5″ x 5½″) two dimensional Whatman No. 1 papers using butanol-acetic acid-water (56:14:30 $^{v}/_{v}$) (BAW) followed by 2 percent acetic acid. The papers were found to contain a large spot having Rf values different from any previously reported polyphenol in tobacco (Reid, 1956; Roberts and Wood, 1951; Stedman, 1957). Actually, the spot consisted of two almost completely overlapping substances. One was blue under ultraviolet light and gave typical color reactions for chlorogenic acid with ammonia vapor, diazotized p-nitroaniline (Swain, 1953) and the Hoepfner reaction (Roberts and Wood, 1951). The second substance was brown in visible and ultraviolet light but valid diagnostic color tests could not be applied since the blue substance was intimately intermingled with the entire area of the brown. The Rf's of the dual spot were 0.20 in the direction of the organic solvent and 0.80 in the aqueous. On the basis of chromatographic behavior the blue material did not appear to be chlorogenic acid or its isomers, isochlorogenic acid and neochlorogenic acid, which have reported Rf values in the organic solvent of 0.64, 0.76 and 0.55, respectively[2] (Roberts, 1956).

Separation of the blue and brown substances could not be achieved on chromatographing in five different solvents on small papers. By increasing the size of the papers (18¼″ to 22½″), however, the two overlapping substances separated in a surprising manner when the same amount of concentrate was chromatographed. The blue spot migrated to the position taken by authentic chlorogenic acid in the BAW and 2 per cent acetic acid solvents: its Rf in the organic solvent increased from 0.20 to 0.76, but its Rf in acetic acid remained essentially unchanged. The brown spot increased slightly in Rf in both directions. From these findings, it was concluded that the blue spot was chlorogenic acid which, on small papers, partitioned in an abnormal manner. To confirm this, the blue areas of overlapping spots on small chromatograms were cut out, eluted in water and the eluate chromatographed again on small papers. This time the blue spot migrated to the correct position for chlorogenic acid. The ultraviolet spectrum also confirmed the identity of the blue spot as chlorogenic acid.

Ultimately, it was found that the abnormal behavior of the polyphenol on small papergrams was due to a "salt effect." By precipitation with 80% ethanol, the inorganic material was removed from the aqueous concentrate and on chromatographing of the latter after removal of the ethanol, migration of the chlorogenic acid to its normal position was observed on small papergrams. On adding this inorganic matter (as ash) to the de-salted aqueous concentrate, a reversal was obtained, the blue and brown material once more partitioning together at low Rf. On reconstituting the de-salted concentrate in a similar matter with progressively less ash, gradual separation of chlorogenic acid and migration to its normal position could be observed. Apparently the increased distance of migration of BAW solvent on large papers tended to minimize the water-logging effect of the salt and permit more normal resolution.

Analysis of the aqueous concentrate showed 11% ash (44 gm. in

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[2] *In the present study, chlorogenic acid has given an Rf value of 0.76 in the butanol-acetic acid-water solvent.*

total volume of 400 ml.) Since the inorganic matter of tobacco is relatively insoluble in the 80 per cent ethanol used for primary extraction (1.8 gm. ash recovered from 12 liters of extract), the bulk of the inorganic substances must have been contributed by the isolation procedure. The most probable source was the acid and alkali used for pH adjustments. From personal observation it is felt that addition of sulfuric acid in quantities sufficient to precipitate the lead completely is preferable to addition to a specific pH; this should reduce the inorganic matter by eliminating excess sulfate and sodium ions in the final solution. However, not all of the "salt effect" can be attributed to sodium sulfate since addition of the pure salt to de-salted aqueous concentrates has shown that quantities of sodium sulfate two to three times the amount of ash removed are required to produce the abnormal effect.

Although the addition of inorganic salts to developing solvents used in paper chromatographing procedures is commonly employed for buffering action, the effect of salts present in extracts or mixtures to be chromatographed has not been studied extensively, except in the case of amino acids and sugars. Consden and coworkers (1944, 1948) have demonstrated the dehydrating and waterlogging effect of sodium chloride and sodium sulfate on the chromatographic behavior of amino acids, and a number of workers have employed de-salting procedures in separations of amino acids on paper (Zweig and Hood, 1957; Lederer and Lederer, 1957). Smith has discussed the "salt effects" encountered during the chromatographic separation of sugars (Smith, 1952). In general, the migration of salts in organic solvent mixtures used in paper chromatography is complex, and it is difficult to predict their effect (Pollard, 1954). At any rate, it appears advisable to be aware of the possibility of salt effects when interpreting paper chromatograms of concentrated plant extracts containing chlorogenic acid and possibly other polyphenols prepared by methods requiring extensive adjustments in pH.

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