Positive identification of flavanone aglycones by paper chromatography of their alkaline degradation products

Studies of flavanones found in citrus fruits revealed the need for an improved method of identifying very small quantities of closely related flavanone aglycones, such as hesperetin, homoeriodictyol, and isosakuranetin, which are difficult to identify unequivocally by paper chromatography because of very similar R_F values and color reactions. Such a method has, therefore, been developed in our laboratory. In this method, paper chromatography is utilized for identification of the degradation products (phloroglucinol and substituted cinnamic acids) which are obtained by adapting to semi-micro scale the classical aqueous potassium hydroxide degradation of flavanone aglycones. Chromatographic data for phloroglucinol and several substituted cinnamic acids which are, or might be, produced on degradation of flavanone aglycones are presented in Table I. The data show that these compounds can be differentiated and positively identified on the basis of their R_F values in different solvent systems, of their fluorescence under ultraviolet light in air and when exposed to ammonia vapor and of their color reactions with chromogenic spray reagents. Hence, if a small quantity of a flavanone aglycone is properly degraded, the degradation products can be positively identified by paper chromatography, and the identity of the original molecule can thus be established with certainty.

Degradation of flavanones may be satisfactorily accomplished by mixing the aglycone with a small quantity of 30% aqueous potassium hydroxide (usually about 2–3 ml of alkali per mg of aglycone) and refluxing for 2.5 h. The degradation mixture is then made acidic (pH 4) with dilute sulfuric acid, and extracted 3 times with ethyl ether. The ether extract is washed twice with small quantities of water, reduced in volume and chromatographed in comparison with standard substituted cinnamic acids and phloroglucinol.

FEWSTER AND HALL'S[1] *n*-butyl alcohol: ethyl alcohol: ammonia–ammonium carbonate buffer (40:11:19 v/v/v) solvent system, and a modification of this system prepared by mixing 1 part nitromethane with 2 parts of *n*-butyl alcohol saturated with ammonia–ammonium carbonate buffer (1.5 *N* with respect to both ammonia and ammonium carbonate) are very useful solvent systems for identification of the cinnamic acids produced on degradation. These systems give small, concentrated spots with no tailing, and therefore permit easier location of weak fluorescing spots and give more clearly defined spots with indicator spray reagents, particularly for very low concentrations of acids. They are especially suitable for chromatography of cinnamic acids containing only methoxy substituents. Table I indicates that observation of R_F values in these solvents, plus fluorescence, permits differentiation between such closely related compounds as *o*-, *m*-, and *p*-methoxycinnamic acids.

Diazotized sulfanilic acid[2] and *p*-nitroaniline[3] spray reagents are profitably employed with chromatograms of the cinnamic acids containing hydroxyl groups, and phloroglucinol, not only to assist in locating the spots, but also to produce colors which are often characteristic and useful in identification. For chromatograms of the

TABLE I

R_F VALUES, FLUORESCENCE AND COLOR REACTIONS OF SUBSTITUTED CINNAMIC ACIDS AND PHLOROGLUCINOL

Compound	R_F values in given solvent[a]				Fluorescence and color reactions with reagent given			
	n-Butyl alcohol–acetic acid–water (6:1:2)	*n*-Butyl alcohol: ethyl alcohol: NH_3–$(NH_4)_2CO_3$ buffer	*n*-Butyl alcohol: nitromethane: NH_3–$(NH_4)_2CO_3$ buffer	Chloroform–acetic acid–water (2:1:1)	Ultraviolet light	Ultraviolet light in NH_3 fumes	Diazotized sulfanilic acid spray reagent	Diazotized *p*-nitroaniline spray reagent
o-Coumaric acid	0.88	0.47	0.34	0.85	blue-white	yellow-green	orange	wine
p-Coumaric acid	0.86	0.44	0.26	0.79	negative	bright blue	red-pink	gray-purple
Ferulic acid	0.81	0.31	0.25	0.92	blue	sky blue	purple	gray-purple
Isoferulic acid	0.83	0.40	0.29	0.90	blue	tan or cream	orange-pink	dark purple
Phloroglucinol	0.70	0.79	c	c	negative	blue	yellow-brown	brown
o-Methoxycinnamic acid	b	0.61	0.38	d	blue	fades	e	e
m-Methoxycinnamic acid	b	0.59	0.34	d	blue	fades	e	e
p-Methoxycinnamic acid	b	0.54	0.27	d	deep purple (faint)	fades	e	e
3,4 Dimethoxycinnamic acid	b	0.44	0.28	d	blue	fades	e	e

a. Whatman No. 1 paper. Ascending chromatography with all solvent systems except *n*-butyl alcohol–acetic acid–water.
b. Spots produced are too diffuse for accurate location by fluorescence or reaction with indicator spray reagents.
c. Compound streaks or tails badly in these solvents.
d. Compounds move with solvent front.
e. Compounds do not couple with these diazotized reagents.

cinnamic acids possessing only methoxy substituents, and which, therefore, will not couple with the diazotized reagents mentioned, indicator solutions such as buffered methyl red (0.1% alcoholic methyl red–0.167 *M* phosphate buffer, 1:2) and 2,6-dichlorophenol-indophenol (0.4% in 95% ethyl alcohol) are useful as spray reagents for locating the acid spots, particularly those with weak fluorescence.

Employing the procedures outlined here, we have successfully degraded 1 mg samples of the flavanone aglycones hesperetin, homoeriodictyol, isosakuranetin, and naringenin, and identified their degradation products by paper chromatography. We have not obtained satisfactory results with eriodictyol, apparently because the caffeic acid which should be produced on degradation of this compound is destroyed by the alkaline degradation conditions. This method has been employed successfully in our laboratory for positive identification of very small quantities of unknown flavanone aglycones obtained by hydrolysis of glycosides from citrus fruits.

This research was supported in part by Sunkist Growers, Inc.

Chemistry Department, University of Oklahoma, Norman, Okla. (U.S.A.)

WILLIAM J. DUNLAP
SIMON H. WENDER

1 M. E. FEWSTER AND D. A. HALL, *Nature*, 168 (1951) 78.
2 E. C. ALBRIGHT, F. C. LARSON AND W. P. DEISS, *Proc. Soc. Exptl. Biol. Med.*, 84 (1953) 240.
3 H. G. BRAY, W. V. THORPE AND K. WHITE, *Biochem. J.*, 46 (1950) 271.

Received December 21st, 1959

J. Chromatog., 3 (1960) 505–507

BOOK REVIEW

Chromatographie en chimie organique et biologique, Vol. I, edited by E. LEDERER (Collection de monographies de chimie organique, compléments au *Traité de Chimie organique*), Masson et Cie, Paris, 1959, 671 pages.

This is the first book on chromatography to be written in French since 1949. Volume I is divided into two parts: *Généralités* (358 pages) and *Applications de la chromatographie en chimie organique* (276 pages), while Volume II will deal with the applications of chromatography to biochemistry.

Part I consists of an excellent account by CHOVIN of the theory and methods of adsorption chromatography (110 pages), which is well illustrated, and gives an adequate and clear account of the theory. The chapter on ion exchange (by BUC, 34 pages) is rather superficial and in places not quite clear. A table on the properties of resins, for example (page 120), has a column entitled "temperature" without specifying that the maximum operating temperature is meant. It could also be the optimum temperature.

An excellent treatise on partition chromatography was written by BOULANGER AND BISERTE (92 pages), who give a wealth of practical information, for example on