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SOLUBLE BROWNING REACTION PIGMENTS OF AGED BURLEY TOBACCO—II.
THE DIALYSABLE FRACTION*

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Abstract—The major phenolics of air-cured tobacco exert a pronounced influence on the color of cured leaf, the maximum effect of which is revealed in the soluble and insoluble brown pigments produced by enzymic oxidation of chlorogenic acid and rutin. The soluble brown pigments may be classified into two fractions: the dialysable and the nondialysable.

Brown pigments contained in concentrated dialysate fractions of aqueous extracts were separated by gel filtration on a column of Sephadex G-25. Constituent pigments so obtained were, in the order of decreasing molecular weight, a major dark brown pigment and two minor reddish brown pigments. The major pigment was characterized as a polymeric iron–protein–chlorogenic acid complex with a molecular weight estimated to be in the order of 4000. The minor pigments were each a polymeric protein–chlorogenic acid complex. Neither of the minor pigments contained iron. There was no evidence of the involvement of rutin in the compositions of the dialysable pigments, in distinction to that of a soluble nondialysable pigment previously isolated and characterized as an iron–protein–chlorogenic acid–rutin complex with a molecular weight of the order of 20,000–30,000. The amount of dialysable pigments so isolated corresponded to 0·2% of dry leaf weight. Methods of isolation and characterization are described, and some of the more significant biochemical aspects of the pigments are considered.

INTRODUCTION

THE phenolics of tobacco [1] are unique among other leaf components because of their relation to both aroma and color, factors which are recognized as extremely important criteria of leaf quality. The contribution of the phenolics to leaf aroma, for the most part, is restricted to that of the low concentrations of highly aromatic simpler phenols associated with the essential oils of the leaf. More significantly, the phenolics exert a pronounced influence on the color of cured leaf, particularly in the case of air-cured types. The intensity of chromogenic properties, in general, is in direct correlation with molecular complexity. The maximum effect is revealed in the brown pigments produced by enzymic oxidation of phenolic substrates, notably chlorogenic acid and rutin, the predominant phenolics of tobacco.

The brown pigments of air-cured Burley tobacco consist of both water-soluble and, in greater proportion, water-insoluble types. The soluble pigments may be further classified into two fractions: the dialysable and the nondialysable. Previous work on the nondialysable fraction [2] led to the isolation of a dark brown pigment which was characterized as a polymeric

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[1] H. E. WRIGHT, JR., in *Plant Phenolics and Their Industrial Significance* (Ed. V. C. RUNECKLES), p. 39, Symposium, Plant Phenolics Group of North America, Corvallis, Oregon (1962).

[2] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *Arch. Biochem. Biophys.* **86**, 94 (1960).

iron–protein–chlorogenic acid–rutin complex with a molecular weight of the order of 20,000–30,000, which was insoluble in dilute acetic acid. A second brown pigment of similar qualitative composition, but soluble in dilute acetic acid, was also isolated. However the second pigment was contaminated by an accompanying polysaccharide which was later identified as the calcium-magnesium salt of a complex acid composed of glucuronic acid, galactose, arabinose and rhamnose.[3] During a recent re-examination of the two nondialysable pigments evidence was obtained by gel filtration on a column of Sephadex G-75 that the pigments, although possessing similar qualitative chemical compositions, differed in molecular weight. This in itself could account for the difference in solubility in dilute acetic acid. Accumulated experimental evidence indicates there is an inverse relation between solubility of the pigments in acetic acid and molecular weight. Jacobson,[4] in an investigation on the brown pigments of air-cured cigar tobacco, has reported the occurrence of complex high molecular weight pigments composed of protein, chlorogenic acid and rutin, chemically similar to the pigments of Burley tobacco.

The formation of brown pigments is much more extensive in air-cured types than in flue-cured tobacco. A limited production of brown pigments does occur in flue-cured tobacco during the initial stages of curing. However the high temperatures attained during the final stages of curing inactivate the polyphenolase complex with concomitant curtailment of pigment formation. This fact is interpreted as positive evidence that the formation of brown pigments in the leaf is basically an enzymic oxidative process. The presence in flue-cured tobacco of high molecular weight brown pigments composed of proteinaceous and phenolic moieties, similar to the pigments of air-cured tobacco, was noted by Runeckles[5] in a recent report describing the isolation of a low molecular weight phenolic complex. The low-order polymer, pale brown in color, was composed of chlorogenic acid, rutin and scopolin, but did not contain a proteinaceous component.

Investigation of the soluble brown pigments of aged Burley tobacco has now been extended to include the dialysable fraction. Data on the composition of the pigments and methods of isolation and characterization are reported in this communication.

RESULTS

The organic solvents used in the preliminary extraction of the Burley tobacco removed about 25% of the dry weight of the leaf, and the subsequent aqueous extraction removed an equivalent quantity. This latter extract was dialysed and the mixture of solids comprising the major dialysable fraction was more complex in composition than that of the minor non-dialysable fraction. The dialysable material, in addition to the pigments, contained considerable quantities of other substances, predominantly calcium and potassium salts of citric, malic and glycolic acids. An araban-like polysaccharide and small amounts of free thymine and tryptophan were also detected. The initial dialysate fractions which contained minor amounts of the pigments were therefore discarded, and subsequent fractions containing the pigments were essentially devoid of tryptophan and the nonvolatile organic acids, as determined by paper chromatography, selective precipitation methods and ultraviolet spectroscopy. Brown pigments contained in concentrated dialysates were resolved into three well-separated fractions by gel filtration on a chromatographic column of Sephadex

[3] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *Phytochemistry*, **1**, 125 (1962).
[4] J. S. JACOBSON, *Arch. Biochem. Biophys.* **93**, 580 (1961).
[5] V. C. RUNECKLES, *Arch. Biochem. Biophys.* **102**, 354 (1963).

G-25. These fractions represented, in the order of elution and hence in the order of decreasing molecular weight, a major dark brown pigment and two minor reddish brown pigments. The major pigment, by precipitation in 50% ethanol containing a drop of glacial acetic acid, was isolated free of the araban-like polysaccharide which persistently accompanied the pigment during gel filtration. The minor pigments, isolated by precipitation with acetone, were not completely free of polysaccharide contamination.

In dialysate fractions containing residual amounts of thymine and tryptophan as contaminants of the pigments, it was observed that the monomeric nitrogenous components were strongly adsorbed on the Sephadex and were thus removed from the more mobile pigment bands. The adsorption of these components was consistent with previous observations of Gelotte[6] and Porath[7] regarding the behavior of amino acids and heterocyclic substances on Sephadex.

Phenolic Components

Caffeic acid and quinic acid were identified in the ether-soluble and ether-insoluble fractions, respectively, of the alkaline hydrolysates of the pigments. Initial hydrolyses carried out with hydrochloric acid led to substantial losses of caffeic acid and, in addition, esculetin was produced in quantity as an artifact.[8,9] The alkaline hydrolysis minimized both

TABLE 1. R_f VALUES OF ALKALI FUSION PRODUCTS OF CHLOROGENIC ACID

Compound	Solvent*				*p*-Nitroaniline color
	HA	NaF	IPrAm	NaCl	
m-Hydroxybenzoic acid	0·59	0·74	0·35	0·61	Rose red
p-Hydroxybenzoic acid	0·56	0·65	0·24	0·56	Rose red
Protocatechuic acid	0·48	0·55	0·02	0·45	Violet
3,5-Dihydroxybenzoic acid	0·47	0·61	0·21	0·45	Yellow

* See Experimental section for composition of solvents.

the degradative losses of caffeic acid and the formation of esculetin. In addition, the lactonization of quinic acid, which occurs during acid hydrolysis or simply by heating,[2,4,10,11] was inhibited. Both caffeic and quinic acids co-chromatographed with authentic compounds in four solvent systems and the identification of these acids suggested that chlorogenic acid was a component of the pigments.

Alkali fusion of the pigments provided additional evidence of the presence of quinic acid. Alkali fusion of caffeic acid normally yields protocatechuic acid, and quinic acid yields protocatechuic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid (Table 1). Fusion of the minor reddish brown pigments, which did not contain iron, yielded the normal mixture of fusion products for chlorogenic acid. However, fusion of the major dark brown pigment, which contained iron, yielded 3,5-dihydroxybenzoic acid, no protocatechuic acid, and above-normal amounts of *m*- and *p*-hydroxybenzoic acids.

[6] B. GELOTTE, *J. Chromatog.* **3**, 330 (1960).
[7] J. PORATH, *Biochim. Biophys. Acta*, **39**, 193 (1960).
[8] C. F. VAN SUMERE, F. PARMENTIER and M. VAN POUCKE, *Naturwissenschaften*, **46**, 668 (1959).
[9] W. L. BUTLER and H. W. SIEGELMAN, *Nature*, **183**, 1813 (1959).
[10] R. A. CARTWRIGHT and E. A. H. ROBERTS, *Chem. & Ind. (London)*, 230 (1955).
[11] Y. NAKAGAWA, C. H. YANG and S. H. WENDER, *J. Org. Chem.* **26**, 3017 (1961).

Fusion of the previously reported nondialysable brown pigment,[2] which contained iron, likewise yielded a mixture of products devoid of protocatechuic acid, but fusion of the partially hydrolysed pigment residue, which was devoid of iron, yielded protocatechuic acid. The results of the fusion experiments on the dialysable and the nondialysable pigments would seem to indicate that mode of linkage of iron to chlorogenic acid, in the polymeric pigment complex, may preclude the formation of protocatechuic acid.

Protein Component

The qualitative amino acid compositions of the protein components were the same for each of the pigments. Hydrolysis of the pigments with 6 N hydrochloric acid yielded a mixture of sixteen amino acids: cystine, lysine, histidine, arginine, serine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, tyrosine, valine, phenylalanine, leucine and isoleucine. The amino acids co-chromatographed with authentic compounds in four solvent systems. Tryptophan was not found after hydrolysis either with acid or with 14% barium hydroxide solution, although it was a prominent constituent of the proteinaceous component of the nondialysable pigment.[2] No ninhydrin-positive spots were obtained when the pigments were chromatographed prior to hydrolysis.

The Pigment Complexes

The nitrogen content of the major dark brown pigment was 3·9%, equivalent to a protein content of 24·4%. Quantitative nitrogen determinations were not performed on the minor

TABLE 2. COMPARATIVE PROPERTIES OF MAJOR SOLUBLE BROWN PIGMENTS FROM AGED BURLEY TOBACCO

Pigment factor	Pigment	
	Dialysable	Nondialysable*
Molecular weight	*ca.* 4000	20,000–30,000
Phenolic moiety	Chlorogenic acid	Chlorogenic acid and rutin
Protein content	24·4%	33·2%
Number of amino acids	16	18
Iron	0·9%	0·3%
Content in tobacco	0·2%	0·6%

* Source of data: Ref. 2.

reddish brown pigments due to the extremely small amounts which were isolated. Qualitative tests on the ash of the pigments, were negative for copper, calcium and potassium, and iron was only found in the major dark brown pigment (0·9%). The molecular weight of the major pigment was presumed to be in the order of 4000 based on the degree of cross-linkage of the G-25 type Sephadex (crushed form) which excludes compounds with higher molecular weights. The pigment was just within this limit. The molecular weights of the two minor pigments were, of course, less than 4000.

In accordance with the evidence it was concluded that the major dark brown pigment was an iron–protein–chlorogenic acid complex, and that the two minor reddish brown pigments were each a protein–chlorogenic acid complex. The major pigment was isolated in an amount corresponding to about 0·2% of dry leaf weight. The minor pigments were isolated in

considerably smaller amounts. The pigments are amorphous substances and melt with decomposition over a wide temperature range. They are without characteristic aroma. The brown pigments possess strong chromogenic properties, and are directly responsible for the color of air-cured Burley tobacco leaf. In this respect they differ from other tobacco pigments of various shades of yellow, e.g., the carotenoids.[12] The contribution of the latter pigments to the color of air-cured leaf is limited, at most, to the imparting of certain tones and hues.

The mode of linkage of the apparently tightly bound phenolic and proteinaceous components of the brown pigments remains obscure. Infrared spectroscopy has been of no help in this matter. There was no evidence of quercetin or rutin in the dialysable pigments. Phloroglucinol, a prominent alkali fusion product of quercetin, was not found among the fusion products. The absence of rutin constitutes the major distinguishing factor of the dialysable and nondialysable soluble pigments. The protein content of the major dialysable pigment was four-fifths that of the nondialysable pigment, but tryptophan and methionine were not constituent amino acids. Comparative data on the major dialysable and nondialysable soluble pigments are shown in Table 2.

DISCUSSION

The absence of iron in the minor pigments and its presence in the major dialysable and the nondialysable soluble pigments suggests that the participation of iron in pigment formation may be of greater significance than has heretofore been considered. The amount of iron in the soluble pigments, dialysable and nondialysable, accounts for about 10% of the iron content (0·034%) of the Burley tobacco. While it would seem probable that iron is a responsible factor for the dark brown color of the pigments in which it is a constituent element, in contrast to the lighter reddish brown color of the minor pigments devoid of iron, the difference in color, at least in part, may also be a matter of degree of polymerization and linkage. The presence of protein in the minor pigments may indicate that, during the early stages of pigment formation, chlorogenic acid or its quinone were linked to a protein component prior to the participation of iron in the sequence of reactions leading ultimately to the formation of the dark brown highly polymerized pigments. The hypothesis is therefore suggested that the minor pigments may represent basic units which, during subsequent stages of polymerization, are linked by iron through a system of chelation. The results of recent studies on iron chelates in stalk exudate from Wisconsin tobacco,[13] an air-cured type, and in soybean exudate[14] strongly suggest that iron in chelated form is the general mode of absorption from soil by plants, and, in the solubilized form, is transported throughout plant systems at a pH which otherwise would cause precipitation of the iron as insoluble salts. The present view is that there is a relation between iron and the soluble nature of these dialysable and nondialysable brown pigments. It is hoped that research presently in progress on the more prominent insoluble brown pigments will provide answers to this and other questions regarding the chemistry of the pigments.

The foregoing concept of a stage in pigment formation in air-cured tobacco is in conformity with current knowledge that the reactions involved in the formation of brown pigments by enzymic oxidation of phenolic substrates take place in sequence rather than simul-

[12] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR. *Arch. Biochem. Biophys.* **82**, 107 (1959).
[13] W. E. SCHMID and G. C. GERLOFF, *Plant Physiol.* **36**, 226 (1961).
[14] L. O. TIFFEN and J. C. BROWN, *Science*, **135**, 311 (1962).

taneously.[15,16] Weaving[17] has postulated that chlorogenic acid is first oxidized by phenolase to the corresponding quinone which can then oxidize rutin, and is itself converted back into chlorogenic acid. The regenerated chlorogenic acid may participate in further oxidations while the rutin quinone is capable of undergoing coupling reactions. The process is repeated until, in the case of flue-cured tobacco, the enzyme is inactivated by the high temperatures of the curing process, while in the case of air-cured types, in the absence of heat during curing, rutin and other regenerating compounds are oxidized. The quinones then polymerize to produce the brown pigments which characterize air-cured tobaccos. This mechanism explains why relatively large concentrations of chlorogenic acid, rutin and other minor phenolics are found in flue-cured leaf, while in the leaf of air-cured tobaccos large amounts of brown pigments accumulate at the expense of the corresponding phenolic precursors. The isolation of brown pigments from the aged Burley tobacco with phenolic moieties composed solely of chlorogenic acid indicates that in the absence of regenerating compounds, such as rutin, the quinone of chlorogenic acid can undergo autopolymerization. This is a reflection of the higher concentrations of chlorogenic acid in the tobacco with respect to rutin.[17,18,19] It appears that the quinone of chlorogenic acid preferentially oxidizes rutin as long as the regenerating flavonoid is present.

No flavonoid constituent was detected in the dialysable material contained in the aqueous extracts of the tobacco. Of particular interest is the fact that no brown pigment has been found of which the phenolic moiety is composed solely of rutin. This would appear to be *in-vivo* evidence in support of biochemical studies by Dawson *et al.*,[20] Shiroya *et al.*[21] and Reid[19,22] who found that, in model experiments, rutin alone was not oxidized by preparations of tobacco polyphenol oxidase. Reid however observed that rutin was oxidized by the enzyme when in the presence of caffeic acid or chlorogenic acid, and described the reaction as an oxidative coupling. There is no evidence that free caffeic acid or esculetin participates in the formation of soluble brown pigments in the Burley tobacco, although both of the *o*-dihydric compounds react with polyphenol oxidase in model experiments.[17] These phenolics have been found only in extremely small amounts in air-cured tobacco. Neither is there evidence of a pigment representing a condensation product of chlorogenic acid or rutin with a single amino acid, unless such a product was removed during the preliminary extraction of the leaf with the series of organic solvents.

Chlorogenic acid and rutin are unquestionably the most important of the known tobacco phenolics. Zucker and Stinson,[23] in a study of the role of chlorogenic acid in the browning of variegated cigar leaf, observed that the concentration of chlorogenic acid in the fresh tobacco was in direct correlation with the concentration of brown pigment formed during curing. Isolation and characterization of the brown pigments from aged Burley tobacco and from cigar[4] tobacco provides conclusive *in-vivo* evidence that the ultimate fate of chlorogenic

[15] M. A. JOSLYN and J. D. PONTING, in *Advances in Food Research* (Eds. E. M. MRAK and G. F. STEWART), Vol. 3, p. 1, Academic Press, New York (1951).
[16] H. S. MASON, in *Advances in Enzymology* (Ed. F. F. NORD), Vol. 16, p. 105, Interscience, New York (1955).
[17] A. S. WEAVING, *Tobacco Sci.* 2, 1 (1958).
[18] R. F. DAWSON and E. WADA, *Tobacco Sci.* 1, 47 (1957).
[19] W. W. REID, in *The Chemistry of Vegetable Tannins*, p. 75, Symposium, Society of Leather Trades' Chemists, Croydon (1956).
[20] R. F. DAWSON, M. SOLT and E. WADA, *Progress Report on Cigar Manufacturers Association Research Grant*, Columbia University, New York (1955).
[21] M. SHIROYA, T. SHIROYA and S. HATTORI, *Physiol. Plantarum*, 8, 594 (1955).
[22] W. W. REID, *Tobacco Sci.* 3, 103 (1959).
[23] M. ZUCKER and H. T. STINSON, JR., *Tobacco Sci.* 4, 229 (1960).

acid and rutin in the fresh leaf of air-cured types, and to some extent in flue-cured tobacco,[5] is participation in browning reactions involving protein which, activated during curing by enzymic oxidation, result in the formation of complex brown pigments. The activity of chlorogenic acid in pigment formation is more extensive than that of rutin due to the higher concentrations of chlorogenic acid in the fresh tobacco leaf.

EXPERIMENTAL

Materials

The tobacco (*Nicotiana tabacum*) used in this investigation was the lamina portion of leaf (middle stalk position) of field-grown Burley, U.S. type 31, 1958 crop, aged. In preparation for extraction, the tobacco, 5·6% moisture, was ground to a particle size of 1 mm. Sephadex, types G-25 and G-75, medium grade, crushed form, was obtained from Pharmacia Fine Chemicals, Inc., Rochester, Minnesota. All reagents used were of the highest purity obtainable.

Extraction

The tobacco was initially extracted successively with pentane, ether, chloroform, acetone and ethanol in a Soxhlet apparatus for periods of 20 hr per solvent. A sample (15 g) of the pre-extracted tobacco, usually in duplicate, was next extracted twice with 175 ml of deionized water at room temperature in a Waring Blendor. The combined aqueous extracts and washings were filtered by suction through a pad of cellulose powder (Whatman, standard grade), and concentrated *in vacuo* at 37° to a volume of 100 ml.

Dialysis

The concentrated extract was placed into two lengths of dialysis tubing (Visking, $\frac{24}{32}$ in.) which were then attached to a glass rod of appropriate length and suspended, from an air stirrer, in a 1-l. glass cylinder. Sufficient deionized water was placed into the cylinder to cover the casings which were rotated slowly during the total dialysis period of 140 hr. Dialysate fractions collected during the first 20 hr, representing six changes of water, were discarded. Thereafter two 12-hr and four 24-hr fractions were collected successively, after which dialysis was discontinued.

Gel Filtration

Dialysate fractions were concentrated *in vacuo* at 37° and each fraction was then run several times through a chromatographic column (40 × 600 mm) containing a packed bed of 150 g of type G-25 Sephadex.[24] The eluant, deionized water, was passed through at a flow rate of 120 ml/hr. Appropriate pigment fractions were combined. By this procedure three pigment bands were separated which, in the order of elution, represented a major dark brown pigment and two minor reddish brown pigments. The major pigment was precipitated from a concentrated solution upon the addition of 1 vol. of ethanol and a drop of glacial acetic acid. The pigment was centrifuged, washed several times, first with the acidified 50% ethanol and finally with ethanol, and then dried *in vacuo* at room temperature. The minor pigments were each precipitated from concentrated solutions upon the addition of 5 vol. of

[24] P. FLODIN, *J. Chromatog.* **5**, 103 (1961).

acetone. The pigments were centrifuged, washed several times, first with 80% acetone and finally with acetone, and then dried *in vacuo* at room temperature.

Hydrolysis

Phenolic component: Chlorogenic acid. A sample (10 mg) of pigment in 10 ml of 1 N KOH solution under N_2 was allowed to stand at room temperature for 4 hr. The solution was then acidified with 1 NHCl to pH 2 and extracted with five 20-ml portions of ether. The combined ether extracts were evaporated to dryness *in vacuo*, and the residue was taken up with 1 ml of ether and centrifuged. The ether-insoluble fraction of the acidified hydrolysate was evaporated to dryness on the steam bath. Excess hydrochloric acid was removed by adding a small quantity of water to the residue and evaporating it to dryness three successive times. The residue was finally taken up with 1 ml of absolute ethanol and centrifuged. The ether-soluble fraction, containing caffeic acid, and the ether-insoluble fraction in ethanol, containing quinic acid, were reserved for subsequent examination by paper chromatography.

Protein component. A sample (5 mg) of pigment in 15 ml of 6 N HCl was refluxed for 20 hr. The hydrolysate was evaporated to dryness on the steam bath, and the residue was taken up with 2 ml of a solution of acetone containing 5% by vol. of 2 N HCl and centrifuged. The supernatant solution containing amino acids was reserved for subsequent examination by paper chromatography. To determine the presence of tryptophan a sample (5 mg) of pigment in 15 ml of 14% $Ba(OH)_2$ was refluxed for 20 hr. The alkaline hydrolysis and preparation of sample for subsequent paper chromatographic examination has been described in detail elsewhere.[2]

Alkali Fusion

A sample (5 mg) of pigment was added to 3 g of molten potassium hydroxide (200°) in a Pyrex test-tube (13 × 128 mm). The temperature was raised during 2 min to 300° and maintained at this temperature for an additional 3 min. The cooled mass was dissolved in water and diluted to 50 ml, acidified to pH 2 with 6 N HCl, and extracted with four 20-ml portions of ether. The combined ether extracts were evaporated to dryness *in vacuo*, taken up with 1 ml of ether and centrifuged. The supernatant solution containing the phenolic fusion products was reserved for subsequent examination by paper chromatography.

Chromatography

Chromatograms were run on Whatman No. 1 paper by the ascending technique for a period of 18–20 hr, except for runs of 4 hr in the aqueous solvents. Samples were examined by co-chromatography with authentic compounds in all solvent systems. Solvent systems for two-dimensional chromatography are described as (*a*) first direction and (*b*) second direction. R_f values are given in brackets.

Alkali fusion products. (*a*) HA (2% acetic acid) and (*b*) NaF (5% sodium formate–formic acid, 200:1); (*a*) IPrAm (isopropanol–ammonium hydroxide–water, 20:1:2) and (*b*) NaCl (8% sodium chloride–acetic acid, 100:1). The phenolic compounds were detected on chromatograms by diazotized *p*-nitroaniline spray reagent.[25]

Caffeic acid. (*a*) HA (0·27) and (*b*) NaF (0·28); (*a*) *n*-butanol–pyridine–water, 14:3:3 (0·41) and (*b*) HA; (*a*) BuA (*n*-butanol–acetic acid–water, 12:3:5) (0·79) and (*b*) NaF.

[25] I. SMITH, *Chromatographic Techniques*, p. 195, Interscience, New York (1958).

Caffeic acid was detected on chromatograms by its blue fluorescence in u.v. light. The fluorescence was enhanced after spraying the chromatograms with 1% sodium borohydride,[26] prepared by dissolving 0·1 g of reagent in a minimum vol. of water and diluting to 10 ml with isopropanol.

Quinic acid. (*a*) BuA (0·30) and (*b*) ethanol–ammonium hydroxide–water, 20:1:4 (0·56); (*a*) isopropanol–pyridine–acetic acid–water, 8:8:1:4 (0·34) and (*b*) isopropanol–*n*-butanol–water, 7:1:2 (0·17). Quinic acid was detected on chromatograms by the periodate–nitroprusside–piperazine spray reagent.[10]

Amino acids. (*a*) BuA and (*b*) *n*-butanol–methyl ethyl ketone–ammonium hydroxide–water, 5:3:1:1 run twice; (*a*) sec-butanol–formic acid–water, 15:3:2 and (*b*) *n*-butanol–pyridine–water, 1:1:1. Amino acids were detected on chromatograms by a ninhydrin spray reagent.[27]

Acknowledgement—The authors are grateful to Mrs. Ellen Campbell for the quantitative analyses.

[26] E. Eigen, M. Blitz and E. Gunsberg, *Arch. Biochem. Biophys.* **68**, 501 (1957).
[27] J. B. Jepson and B. J. Stevens, *Nature*, **172**, 772 (1953).