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OPTIMUM CONDITIONS FOR BONDING OF PLANT PHENOLS TO INSOLUBLE POLYVINYLPYRROLIDONE*

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Abstract—Maximum bonding of the principal plant phenols of *Nicotiana tabacum* to insoluble polyvinylpyrrolidone occurred at pH 3·5 in 1–10 per cent methanol. Three successive additions of 0·5 g each of purified polyvinylpyrrolidone quantitatively bound 100 μg amounts of each phenol. The bonding was reversible; 8 M urea, 5 M guanidine hydrochloride and *N*-methyl-2-pyrrolidone released undegraded caffeic acid from the polyvinylpyrrolidone–caffeic acid insoluble complex. The analytical and spectral data presented in this paper indicate that the phenols were attached to the polyvinylpyrrolidone principally by hydrogen bonds.

INTRODUCTION

IT IS often desirable to separate the phenol fraction from plant tissue extracts. This is necessary during the course of analytical determinations of plant phenols[1–4] and carbohydrates,[5] and in the isolation of certain enzymes.[6–7] Lead acetate has been widely used as a precipitant for *ortho* hydroxy phenols, but a disadvantage is that it does not precipitate some plant phenols and may coprecipitate other compounds.[8] More recently, the insoluble polymer polyvinylpyrrolidone (PVP) has been used to separate phenols, supposedly by hydrogen bond formation (Fig. 1). It has been fairly specific in this regard.[6] The purpose of this paper

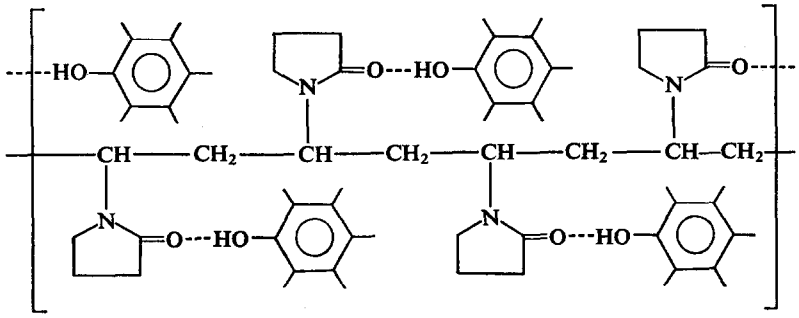

FIG. 1. POSTULATED HYDROGEN BONDING OF PLANT PHENOL TO POLYVINYLPYRROLIDONE.

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is to report the results of work to establish optimum conditions for the formation of the PVP-plant phenol complex and to provide evidence for the nature of the postulated hydrogen bonding. The study is a prerequisite for the development of an analytical method for total phenols in tobacco leaf. The principal plant phenols of *Nicotiana tabacum* (namely caffeic acid, quercetin, scopoletin and their naturally occurring derivatives) were investigated.

RESULTS

Optimum Conditions for Bonding

Solutions of plant phenols were prepared by dissolving the compounds in 10 per cent methanol and adjusting to the desired pH by addition of acetic acid or sodium carbonate. The effects of pH differences upon the formation of the plant phenol–PVP insoluble complex were determined by the addition of 0·250 g purified PVP (see Experimental) to 10·0 ml of the 18 μg/ml standard plant phenol solution contained in a glass-stoppered Erlenmeyer flask. The flask contents were gently shaken with the aid of a mechanical shaker for 1 hr. Next the mixture was transferred to a conical centrifuge tube and centrifuged at 3000 rev/min for 20 min. The absorption spectrum of the supernatant was compared with that of the original solution from 220 to 420 nm on a Beckman DB recording spectrophotometer. The effect of pH on the bonding of rutin to PVP is represented in Fig. 2. From this spectral data it can be seen

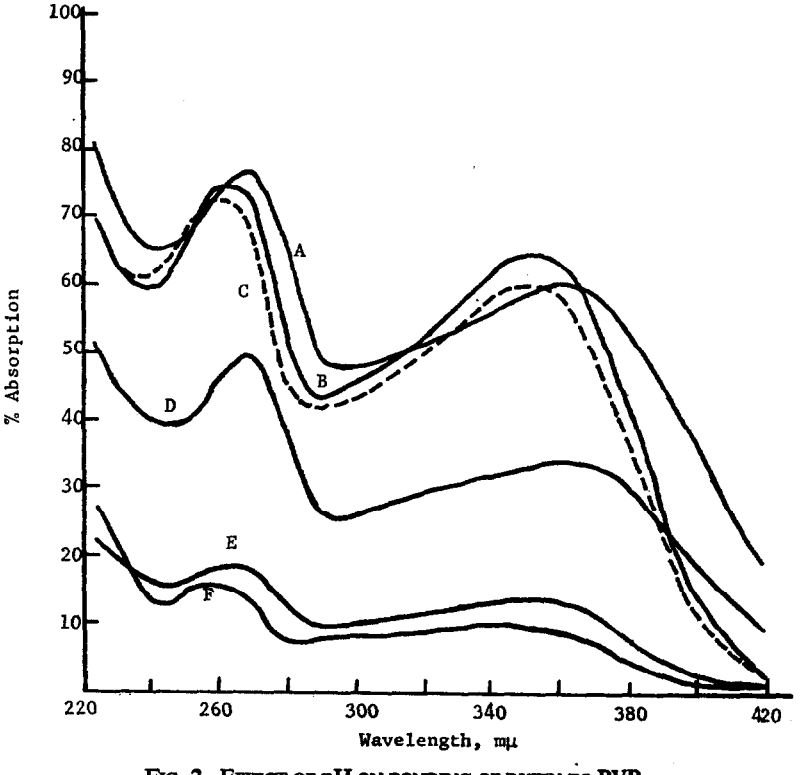

FIG. 2. EFFECT OF pH ON BONDING OF RUTIN TO PVP.

Original rutin concentration: 18 μg/ml, 10 per cent methanol. Reference curves A, B, C=pH 8·5, 6·0 and 3·5, respectively, without PVP; curves D, E, F=pH 8·5, 6·0, and 3·5, respectively, with PVP, 25·0 mg/ml.

that at pH 3·5 more rutin is removed from solution than at either pH 6·0 or 8·5. Bonding was least in the alkaline solution in which phenolic hydroxyl groups are largely converted to the sodium salt. Similar results were obtained for solutions of chlorogenic acid, quercetin and scopoletin. Furthermore, when the pH effect was studied in the same manner with caffeic acid at 0·5 unit pH differences in the range pH 2·5 to 5·5, it was found that pH 3·5 provided the optimum conditions for formation of the PVP–caffeic acid insoluble complex. These results are given in Table 1. Similar results were obtained for the other plant phenols studied, and this provided evidence that the bonding was of the hydrogen type.

TABLE 1. EFFECT OF pH ON BONDING OF CAFFEIC ACID TO PVP

pH	Absorbance at 322 nm		Caffeic acid removed from solution* %
	Before PVP	After PVP	
2·5	0·545	0·258	52·7
3·0	0·481	0·101	79·0
3·5	0·530	0·069	87·0
4·0	0·481	0·072	85·0
4·5	0·423	0·079	81·3
5·5	0·403	0·098	75·7

* Calculated on the basis of the change in absorbance at 322 nm after PVP additions.

Because hydrogen bonding was more likely to occur in aqueous than in organic solvent systems, experiments were performed to determine the effect of methanol upon the amount of phenol removed from solution. Relatively small losses in the efficiency of PVP with respect to caffeic acid and scopoletin bonding occurred at increasing methanol cencentrations of 1–10 per cent (cf. Table 2). However, at a 25 per cent methanol concentration, there was much less formation of bound caffeic acid.

TABLE 2. EFFECT OF INCREASED METHANOL CONCENTRATION ON BONDING OF PHENOLS TO PVP

Phenol	Methanol %	Phenol removed from solution* %
Caffeic acid	1	92·2
	5	91·3
	10	91·2
	25	65·3
Scopoletin	1	56·3
	5	52·3
	10	48·2

* Calculated on the basis of the change in absorbance at wavelength maxima for compound as given in Table 3.

The effect of shaking time with PVP upon the amount of caffeic acid removed from solution was studied. From the results it appears that an equilibrium between bound and

unbound caffeic acid is reached within a 10-min shaking period. Longer periods of shaking up to 2 hr had no effect upon the amount of caffeic acid bound.

Three weights of PVP were used with an equal concentration of chlorogenic acid, rutin or scopoletin in an experiment designed to determine whether the amount of PVP–plant phenol insoluble complex was dependent upon the weight of PVP used or the number of successive PVP treatments. Figure 3 indicates that more chlorogenic acid, rutin and scopoletin were bound at the 1·000 g PVP level than at the lower 0·250 g level. However, because of the high ratio of solid phase to liquid phase, the higher weight was not conveniently used. When three successive treatments with 0·500 g were tested, there was nearly quantitative bonding of the three phenols to PVP.

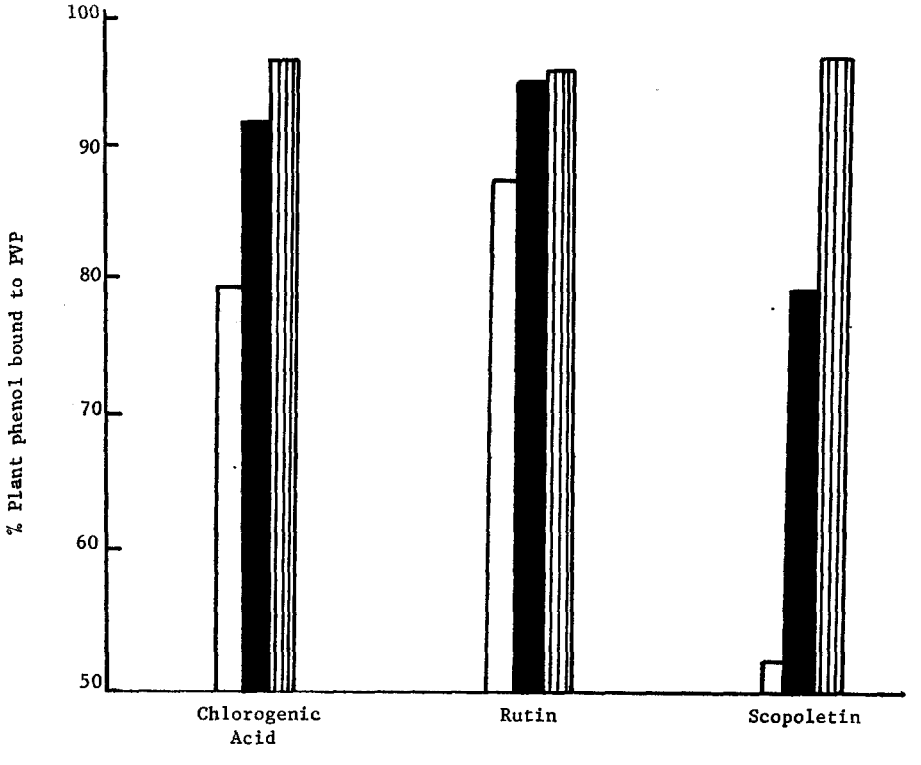

FIG. 3. EFFECT OF WEIGHT OF PVP AND NUMBER OF TREATMENTS UPON AMOUNT OF BOUND PHENOL.
100 μg standard plant phenol in 10 ml 10 per cent methanol at pH 3·5.
□ One treatment, 0·250 g PVP
■ One treatment, 1·000 g PVP
|||| Three successive treatments. 0·500 g PVP each.

Precipitation of Plant Phenols with Polyvinylpyrrolidone at pH 3·5

Solutions of standard tobacco plant phenols at a final concentration of 18 μg/ml in 10 per cent methanol were prepared and adjusted to pH 3·5 as described in the previous section. A 10·0-ml aliquot of each solution was treated with 0·250 g PVP for 30 min and subsequently separated from the PVP-insoluble complex as previously indicated. Spectral curves before and after the addition of PVP were compared, and the absorbance change at an appropriate

wavelength maximum was used to calculate the amount of plant phenol removed from solution. A representative curve upon which the calculations were based for chlorogenic acid is given in Fig. 4. The data are summarized in Table 3.

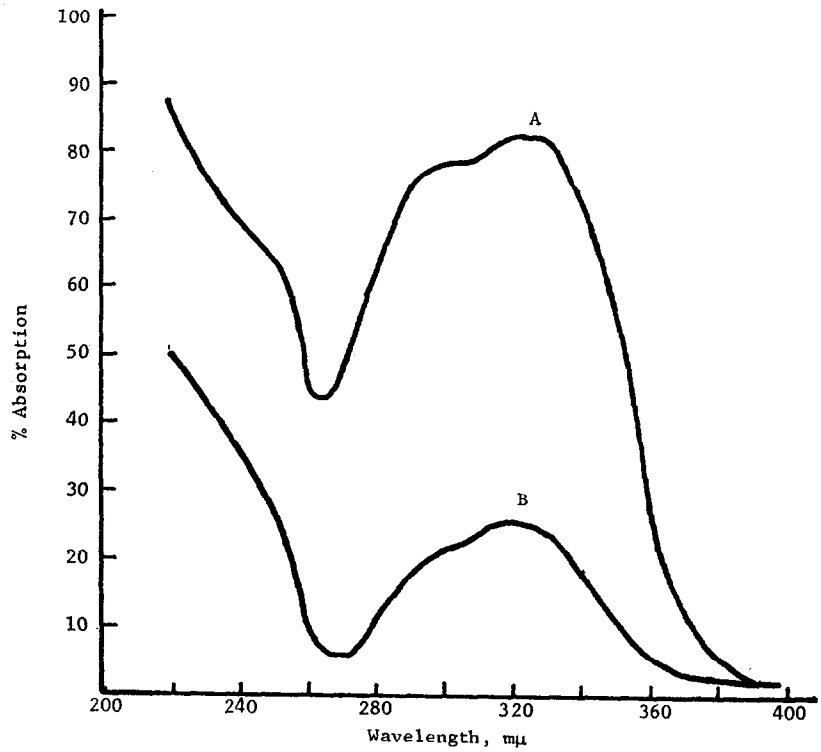

FIG. 4. BONDING OF CHLOROGENIC ACID TO PVP.
18 μg chlorogenic acid/ml, in 10 per cent methanol, pH 3·5. Curve A, reference, no PVP; curve B, PVP treated.

TABLE 3. REMOVAL OF PLANT PHENOLS BY POLYVINYLPYRROLIDONE AT pH 3·5

Compound	Absorbancy maxima,* nm	Percentage removed by PVP†
Caffeic acid	322,‡ 300	91·2
Chlorogenic acid (3-caffeoyl quinic acid)	323,‡ 300	79·5
Scopoletin	343,‡ 295	48·2
Scopolin (scopoletin-7 glucoside)	338,‡ 288	0
Quercetin	291‡	95·2
Rutin (quercetin-3-rhamnoglucoside)	352,‡ 258	88·3

* In 10 per cent methanol at pH 3·5.
† Calculated in terms of absorbancy change at indicated absorbancy maximum following 30 min treatment with PVP.
‡ Absorbancy maximum used in calculation of per cent removal.

All compounds with free aromatic hydroxyl groups were bound to PVP. In each case (cf. Table 3) the percentage of bound phenol was higher for the aglycone than it was for the corresponding derivative. Further, there was no reaction with PVP in the case of the glucoside of scopoletin, scopolin, which has no free aromatic hydroxyl group. The results also suggest that increased numbers of aromatic hydroxyl groups per molecule increase the amount of PVP bond formation. This is evidenced by an increased order of the amount of removal from solution of scopoletin, caffeic acid and quercetin which possess 1, 2 and 4 free phenolic groups respectively. It is probable that carboxyl groups present in caffeic acid and chlorogenic acid also hydrogen bond with PVP. In the opposite sense, it is likely that there is competition to PVP–phenol bond formation from intramolecular hydrogen bond formation presumed to occur in *ortho* hydroxy phenols such as caffeic acid, chlorogenic acid, quercetin and rutin.

Dissociation of Phenols Bound to PVP

Experiments were performed to determine whether plant phenols bound to PVP could be dissociated without degradation. In addition, the nature of reagents which dissociate or elute bound compounds could provide further evidence for the nature of the PVP–phenol insoluble complex.

A standard solution of 18 μg/ml caffeic acid in 10 per cent methanol at pH 3·5 was prepared, and 20·0 ml was treated with 0·500 g PVP as previously described. The PVP-complex was reserved. An absorbance spectrum of the supernatant was taken. The amount of caffeic acid bound to PVP was calculated by the absorbance difference between the original solution and the supernatant after PVP treatment. The PVP-insoluble complex in the tube was washed by three successive 1-min shakings with 20-ml portions each of distilled water acidified with acetic acid to pH 3·5, followed by centrifugation and removal of the supernatant each time. The PVP-complex was then vacuum dried at 60°. Several equivalent portions of PVP–caffeic acid complexes were prepared in this manner.

Next a 20·0-ml portion each of acetone, 95 per cent ethanol, pyridine, 20 per cent sodium carbonate, *N*-methyl-2-pyrrolidone (Eastman Kodak Co., Rochester, N.Y.), 5 M guanidine hydrochloride (reagent grade, Nutritional Biochemicals Corp., Cleveland, Ohio) or 8 M urea was added to a prepared portion of the PVP–caffeic acid complex contained in a 50-ml, glass-stoppered Erlenmeyer flask. The flask contents were gently shaken with the aid of a mechanical shaker for 2 hr. Next, the mixture was transferred to a conical centrifuge tube, centrifuged as previously described, and the supernatant was reserved for spectral analysis. In the case of the use of acetone, 95 per cent ethanol and pyridine, the solvents were removed *in vacuo* and the residue was taken up in 20·0 ml of 10 per cent methanol at pH 3·5. Spectral curves of this solution were then examined to determine the amount of recovered caffeic acid or the presence of degradation or other dissolved products of the PVP–caffeic acid complex. The sodium carbonate solution was acidified to pH 3·5 and adjusted to 10 per cent with respect to methanol prior to spectral analysis. Solutions of 8 M urea, 5 M guanidine hydrochloride and *N*-methyl-2-pyrrolidone were directly analyzed spectrophotometrically.

Some of the results of these experiments are presented in Fig. 5 in which spectra of the eluted caffeic acid are compared with a hypothetical reference curve which would be obtained if there was 100 per cent recovery. Complete solution of bound caffeic acid was achieved with 8 M urea, while partial recoveries were obtained with 5 M guanidine hydrochloride and *N*-methyl-2-pyrrolidone. The other solutions (namely, acetone, 95 per cent ethanol, pyridine and 20 per cent sodium carbonate) yielded spectral data that indicated they were ineffective

for the elution of PVP-bound caffeic acid. Both 8 M urea and 5 M guanidine hydrochloride are solutions commonly employed experimentally to dissociate hydrogen bonds. Their ability to elute caffeic acid from its PVP-bound state, and the fact that other solvents which ordinarily dissolve caffeic acid failed in this regard, provide further evidence for the presence of hydrogen bonding between PVP and phenols.

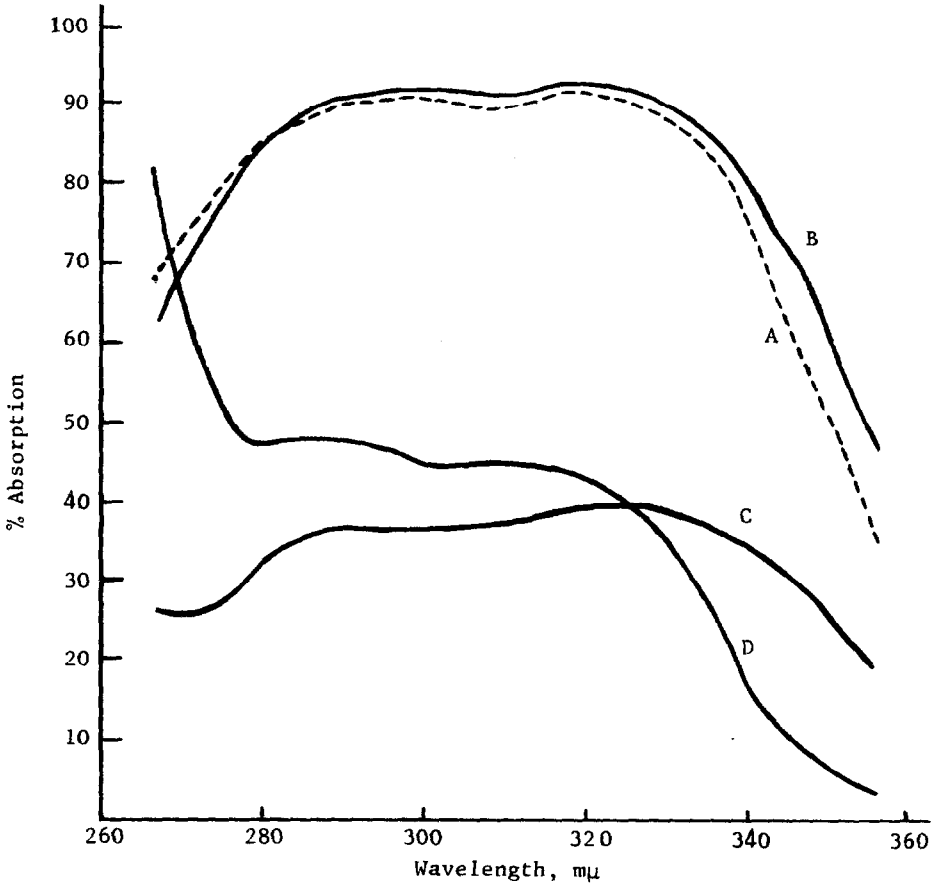

FIG. 5. RELEASE OF PVP-BOUND CAFFEIC ACID.

Curve A, reference, hypothetical 100 per cent release of caffeic acid. Curve B, caffeic acid released with 8 M urea. Curve C, caffeic acid released with *N*-methyl-2-pyrrolidone. Curve D, caffeic acid released with 5 M guanidine hydrochloride.

We selected a flue-cured sample of tobacco which was characteristic of tobaccos known to contain fairly high amounts of the plant phenols caffeic acid, quercetin and scopoletin, either in the free state or as sugar or quinic acid derivatives.[9] This sample was treated by the scheme given in Fig. 6 and analyzed for phenols by paper chromatographic procedures as described by Runeckles.[10] Identification of caffeic acid, quercetin and scopoletin were made by the comparison of R_f values obtained for the sample with R_f values for standard solutions

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[10] V. C. RUNECKLES, *Arch. Biochem. Biophys.* **102**, 354 (1963).

of these compounds. In addition, the zones corresponding to the R_f values of these compounds from a chromatographic run were cut out and refluxed with 50 per cent methanol in a Soxhlet extractor. Absorbance spectra of these extracts from 220–450 nm were then compared with standards. The absorbancy maxima of the extracts from the samples were identical to those of the reference compounds. These results provided evidence for non-degradative bonding to PVP of naturally occurring plant phenols and non-degradative release of the bound phenols by 8 M urea.

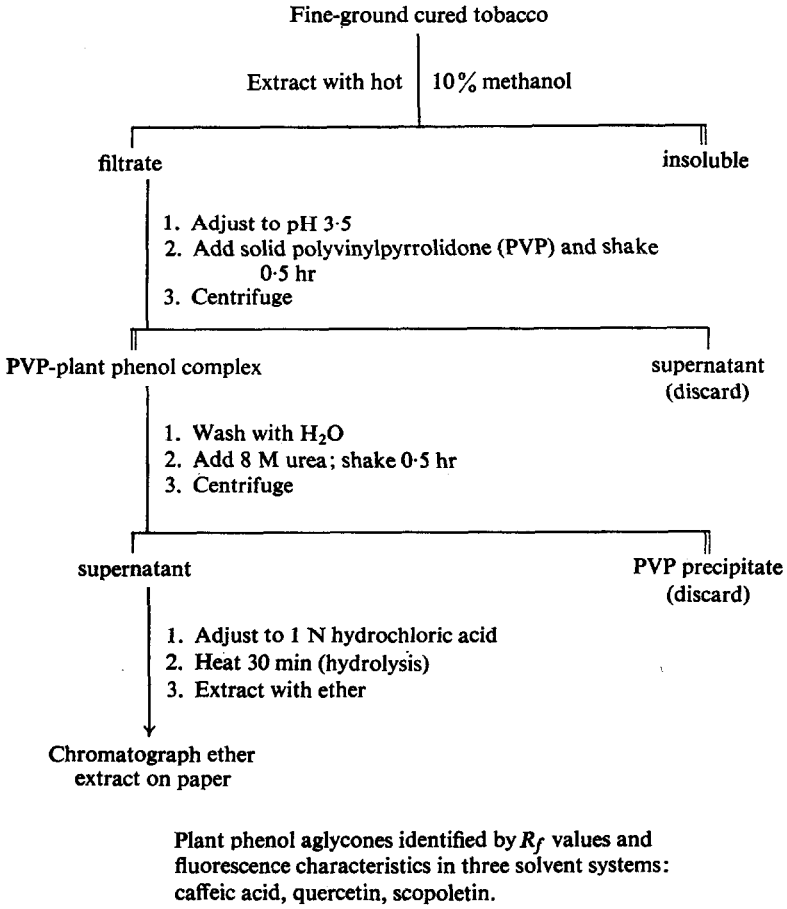

FIG. 6. FLOW SHEET FOR SEPARATION AND IDENTIFICATION OF PLANT PHENOLS IN TOBACCO.

DISCUSSION

We have demonstrated the use of specified amounts of insoluble polyvinylpyrrolidone as a precipitant for plant phenols under controlled pH and solvent conditions. The phenols tested could be quantitatively removed from solution provided enough PVP and successive treatments were used. Therefore, the use of PVP for quantitative analysis of total plant phenols should be possible, provided that a suitable method for determining the amount of bound phenols is developed and appropriate interference studies are performed.

The experimental results of pH effects, the loss of PVP-bonding capacity with increased methanol concentration, and the release of bound phenols by 8 M urea strongly indicated that the phenols were bound to PVP by hydrogen bonds. The specificity of PVP for removal of the phenol fraction from plant extracts then needs to be considered from the standpoint of functional groups other than aromatic hydroxyls on phenols or interfering substances which are likely to form hydrogen bonds under the conditions used. Thus, under mildly acidic conditions, compounds possessing carboxyl, sulfhydryl, sulfonic acid, imino, hydroxamic acid or enolic groupings might bond.

The use of polyvinylpyrrolidone should find further application to biological studies in which a preliminary separation of phenolics from other substances is desirable. In some cases where conventional techniques (such as the removal of *ortho* hydroxy phenols by precipitation with lead acetate) are not feasible, PVP may function to advantage.

EXPERIMENTAL

Purification of Polyvinylpyrrolidone

High molecular weight, insoluble, cross-linked polyvinylpyrrolidone was obtained as Polyclar AT from the General Aniline and Film Corp.* The powder as received contained substances which reduced alkaline potassium ferricyanide solution. To remove these reducing substances and any alkali or acid reactive materials, the following procedure was used:

Polyclar AT was stirred with successive volumes (solid phase: liquid phase = *ca.* 1:4) of a solution which contained 9·0 g sodium chloride, 0·25 g potassium ferricyanide and 20·0 g sodium carbonate diluted to 1000 ml with distilled water. After each stirring period of sufficient length to decolorize the yellow color of the ferricyanide solution, the mixture was filtered through Whatman No. 1 paper with suction. When the polymer no longer decolorized freshly added ferricyanide, and the lack of reduction persisted during a stirring period of at least 3 hr, the mixture was filtered as before and the PVP was washed with distilled water. Next, the polymer was stirred for 1 hr periods prior to filtrations with successive volumes of 10 per cent sodium carbonate, 10 per cent hydrochloric acid, distilled water, and acetone. The PVP was then air-dried at 60°. Finally, the polymer was passed through a 40-mesh sieve and stored in a desiccator.

Standard Plant Phenols

Caffeic acid—obtained from Aldrich Chemical Co., 2371 North 30th St., Milwaukee, Wisconsin 53210.

Chlorogenic acid, quercetin, and *rutin*—obtained from Nutritional Biochemical Corp., 21010 Miles Ave., Cleveland, Ohio 44128.

Scopoletin—synthesized according to procedure of Braymer *et al.*[11]

Scopolin—synthesized from scopoletin[11] and *O*-tetracetyl-α-glucosidyl bromide[12] according to the procedure used by Chaudhury *et al.*[13]

Acknowledgements—We thank Mr. Jack Todd and Dr. T. C. Tso for valuable suggestions related to this work. In addition, we are indebted to Mrs. Charlotte Coffman for valuable technical assistance.

* Trade names are given as part of the exact experimental conditions and not as an endorsement of products.

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