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SEASONAL CHANGES IN THE TANNIN CONTENT OF OAK LEAVES

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Abstract—Changes in the phenolic content of oak *Quercus robur* L. leaves were investigated by paper chromatography throughout the growing season. Condensed tannin was found to appear in late May, along with (+)-catechin, (+)-gallocatechin and other vanillin-positive components of low molecular weight. Hydrolysable tannins, comprising at least five components, are present throughout the season. The condensed and hydrolysable tannins from crude tannin, prepared by salting-out aqueous acetone extracts of mature leaves, were separated by chromatography on Sephadex columns. An alternative source of two of the hydrolysable tannins was found to be oak marble-galls. The mean molecular weight of these two hydrolysable tannins was found to be 1680; on hydrolysis with dilute acid, they yielded ellagic acid, with some gallic acid, and D-glucose, in addition to some unidentified phenolic products. The condensed tannin was found to have a molecular weight of 6750 and yielded phlobaphene and cyanidin on acid hydrolysis. Based on the salting-out procedure, a quantitative method was devised for the analysis of tannins in the aqueous acetone extracts. By this method it was found that the tannin content of oak leaves increased from 0·5 per cent of leaf dry weight in April to nearly 5·0 per cent of leaf dry weight in September.

INTRODUCTION

THE following work was carried out as part of a wider study to determine the possible ecological significance of seasonal changes in the phenolic content of the leaves of the Pedunculate or Common Oak *Quercus robur* L. This species is heavily infested by insects in the spring, occasionally resulting in complete defoliation, but is relatively unattacked after mid-June; Professor G. C. Varley suggested to us that changes in the content of leaf phenolics, especially of tannins, might be partially responsible for the relative lack of insect attack on mature leaves. It had already been reported[1] that condensed tannin was present in July leaves of *Q. robur*, although absent from May leaves, and there was casual evidence to suggest that the content of leaf tannins increased during the growing season. Plant polyphenols are known to have adverse effects on the growth and survival of other organisms: For example, tannins can inhibit the growth of fungi, probably by tanning the pectolytic enzymes,[2] and the transmission of viruses, probably by tanning the virus nucleoprotein.[3] Although the

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[1] B. R. BROWN, C. W. LOVE and W. R. C. HANDLEY, *Report on Forest Research*, pp. 90–93. H.M.S.O., London (1962).

[2] A. H. WILLIAMS, in *The Enzyme Chemistry of Phenolic Compounds* (edited by J. B. PRIDHAM), pp. 87–95. Pergamon, Oxford and MacMillan, New York (1963).

[3] C. H. CADMAN, in *Phenolics in Plants in Health and Disease* (edited by J. B. PRIDHAM), pp. 101–105. Pergamon Press, Oxford (1960).

effects of polyphenols on insect growth have been little studied, we considered that tannins might have an inhibitory effect on protein digestion, either by direct enzyme inhibition or by forming relatively indigestible complexes with leaf proteins, thus effectively reducing the nutritive value of the leaves.

Here we review an investigation into the qualitative changes in the phenolic components of oak leaves during the growing season and describe the isolation and investigation of the oak leaf tannins and their quantitative change during the season.

RESULTS AND DISCUSSION

Phenolic Constituents of Oak Leaves

Two-way paper chromatography of acetone/water extracts of fresh oak leaves, collected monthly during 1965, revealed more than twenty components which gave a positive reaction with ferric chloride/potassium ferricyanide reagent. Five of these components were vanillin-positive (Table 1). The R_f values and behaviour of certain of the components under u.v.

TABLE 1. PHENOLIC COMPOUNDS IN OAK LEAVES.

Symbol	R_f* in HAc	R_f* in BAW	u.v.	u.v./NH_3	Vanillin	Diazotized *p*-nitraniline	Tetrazotized benzidine	Identity (if known)
A	0·10	0·54	abs.	YG	—	—	—	
B	0·09	0·10	abs.	abs.	—	—	—	? Hydr. tannin
C	0–0·25	0	abs.	abs.	R	Y	YBr	Cond. tannin
D_1	0·45	0·02	abs.	abs.	—	Br	YBr	Hydr. tannin
D_2	0·33	0·04	abs.	abs.	—	Br	YBr	Hydr. tannin
E	0·61	0·29	—	—	—			
F_1	0·33	0·57	—	—	—			Gallic acid
F_2	0·33	0·57	—	—	R	Y	R	(+)-Catechin
G	0	0–0·30	—	—	—			? Hydr. tannin
H	0·44	0·13	abs.	abs.	—	—	—	? Hydr. tannin
I†	0·42	0·08						
J	0·31	0·30	—	—	R			
K†	0·16	0·19						
L	0·18	0·22	—	—	—			
M	0·14	0·35	—	abs.	—			
N	0·02	0·47	—	—	—			
O	0·43	0·27	—	—	R	Y	R	
P†	0·31	0·22						
Q	0·28	0·40	—	—	R			(+)-Gallocatechin
S	0·02	0·66	Y	Y	—			Quercetin
T	0·02	0·80	Y	Y	—			
U	0·01	0·33	B	Y	—			Ellagic acid
V	0·15	0·07	—	B	—			
W	0·08	0·17	—	—	—			

* R_f values in 2% acetic acid ("HAc") and n-butanol/acetic acid/water ("BAW") of compounds detected by potassium ferricyanide/ferric chloride reagent on chromatograms of aqueous acetone extracts of oak leaves, together with behaviour with other developing reagents and in u.v. light (360 nm).

abs. = dark spot due to absorption of light; R = red; Y = yellow; B = blue; Br = Brown; YG = yellow-green; YBr = yellow-brown.

† In extracts of stored leaves only.

light and with various developing reagents corresponded with the properties of pure samples of gallic acid, ellagic acid, (+)-catechin, (+)-gallocatechin and quercetin, all of which have been reported previously from oak leaf extracts.[4] Representations of chromatograms from extracts of April buds and June leaves are shown in Fig. 1.

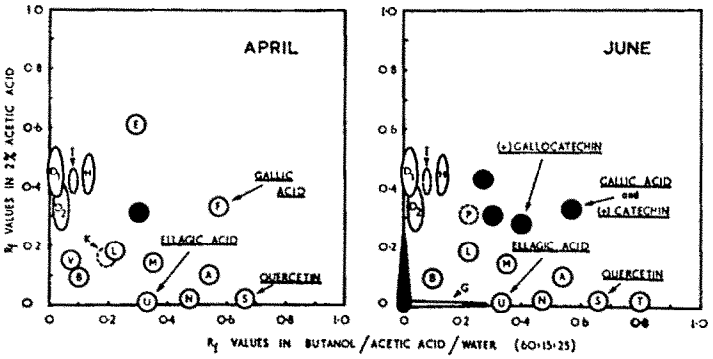

FIG. 1. REPRESENTATIONS OF TWO-WAY PAPER CHROMATOGRAMS IN HAc AND BAW SOLVENTS OF AQUEOUS ACETONE EXTRACTS OF APRIL OAK BUDS AND JUNE OAK LEAVES.

The positions are shown of all compounds located with the ferric chloride/potassium ferricyanide reagent. Compounds indicated by shading were, in addition, vanillin-positive. Compounds indicated by broken lines were found in stored extracts only.

Tannins were isolated from July oak leaves by a modification of the ether-precipitation method developed by Brown and Love.[5] After dialysis and freeze-drying, the resulting pale-brown tannin powder was dissolved in acetone/water (70:30) and run on two-way chromatograms in HAc and BAW solvent systems. C, D and H were revealed on the developed chromatograms as heavy dark-blue streaks (D usually being resolved into two closely-spaced components, D_1 and D_2), G as a faint pale-blue streak and B as a faint blue spot or streak. C was the only vanillin-positive component and clearly represented the oak leaf condensed tannin.[1] All other substances found in the aqueous acetone leaf extracts were absent and presumably represent compounds of low molecular weight. All the components of the crude tannin (C, D, B, H and G) were absorbed from an aqueous extract of fresh September oak leaves by hide powder.

We have no direct proof that tannins exist as such in intact oak leaves and it is conceivable that they are formed from precursors as soon as the tissue is damaged. However, stringent precautions were taken to minimize any potential chemical changes during extraction (see Experimental Section) and we consider that the information obtained from the leaf extracts accurately represents the phenolic composition of the intact leaves.

Seasonal Variation of Oak Leaf Phenolics

The variation in the occurrence of the oak leaf phenolics from April to September is shown in Table 2. The pattern remains fairly constant during the growing season, though there are some interesting changes, notably among the vanillin-positive compounds: condensed tannin, (+)-catechin, (+)-gallocatechin, J and O (which may be leucodelphinidins[4]).

[4] D. E. HATHWAY, *Biochem. J.* **70**, 34–42 (1958).
[5] B. R. BROWN and C. W. LOVE, *Report on Forest Research*, pp. 90–92. H.M.S.O., London (1961).

TABLE 2. SEASONAL VARIATION OF PHENOLIC COMPOUNDS IN OAK LEAVES.

Symbol*	Identity (if known)	April†	May	June	July	August	September
A		++	+	+	+	+	+
B	? Hydr. tannin	+	++	+++	+++	+++	+++
C	Cond. tannin		+	++	+++	+++	+++
D_1	Hydr. tannin	+++	+++	+++	+++	+++	+++
D_2	Hydr. tannin	+++	+++	+++	+++	+++	+++
E		+	+				
F_1	Gallic acid‡	++	++	?	?	?	?
F_2	(+)-Catechin		+	+++	+++	+++	+++
G	? Hydr. tannin		+	++	+	+	+
H	? Hydr. tannin	+++	+++	+++	+++	+++	+++
J		+	+	++	++	++	++
L		++	++	++	++	++	+
M		+++	+++	+++	+++	+++	+++
N		+++	++	+	+	+	+
O				+	+	+	+
Q	(+)-Gallocatechin			+	+	+	+
S	Quercetin	+	+	++	++	++	++
T			+	+	+	+	+
U	Ellagic acid	+	+	+	+	+	+
V		+++	+				
W			+				

* Compounds detected as in Table 1.
+++ = strong; ++ = medium; + = weak.
† Descaled buds.
‡ Shielded by (+)-catechin after May; (+)-catechin judged by intensity of reaction with vanillin.

With the exception of J, present in low concentration, these compounds were undetected in April and were first detected in the leaf extracts in May. Thus condensed tannin, and its probable flavonoid precursors,[4] are absent from oak leaves during the early spring, in accordance with the findings of Brown, Love and Handley.[1] It is of interest that the suspected hydrolysable tannins, especially D and H, are present throughout the growing season. G, the previously reported hydrolysable tannin,[1] was never more than weakly shown on chromatograms.

Properties of Oak Leaf Tannins

Attention was focused on the tannins C and D (by far the strongest streaks on chromatograms), since these were considered to be the phenolics most likely to influence the growth of insect larvae. The remaining components showed little seasonal change, apart from the disappearance of E and V after May, and their tanning effect, as judged by hide powder absorption, is likely to be slight. No further attention was therefore given to them.

Tannin was extracted in bulk from acetone/water (70:30) extracts of fresh oak leaves, using the ether-precipitation method.[5] During the removal of salt by dialysis, some of the tannin came out of solution as a brown precipitate; this water-insoluble fraction (P) was sedimented and subsequently freeze-dried from aqueous suspension, separately from the soluble fraction (S). After freeze-drying, both tannin fractions were obtained as light-brown powders, soluble in aqueous acetone. Several analyses were carried out of September oak leaf tannins extracted by the above method (Table 3).

TABLE 3. ELEMENTARY ANALYSES OF TANNIN EXTRACTED FROM SEPTEMBER OAK LEAVES

Sample	% C	% H	% N	% Ash
September oak leaf tannin—(S) and (P) fractions unseparated	48·8	4·8		
September oak leaf tannin—fraction (S) only*	48·7	4·7	nil	nil
September oak leaf tannin—fraction (S) only, after drying for 1 day†	49·4	4·7		
September oak leaf tannin—fraction (S) only, after drying for 3 days†	49·7	4·6		
September oak leaf tannin—fraction (S) only, after drying for 9 days†	49·8	4·4		
September oak leaf tannin—fraction (P) only*	51·8	5·4	nil	nil

* Average of duplicate determinations.
† Drying was carried out over P_2O_5 at 30–35°.

Two-way paper chromatography of the soluble tannin fraction (S) in HAc and BAW showed that it was made up of the components C (the vanillin-positive condensed tannin), D and H. Other spots visible on the chromatograms, though much weaker, were B, I and E′. Two-way cellulose thin-layer chromatography of fraction (S), using the same solvent systems, revealed the same pattern but, in addition, a component Y, hidden on the paper chromatograms owing to the poorer resolution (Fig. 2). The thin-layer chromatograms also

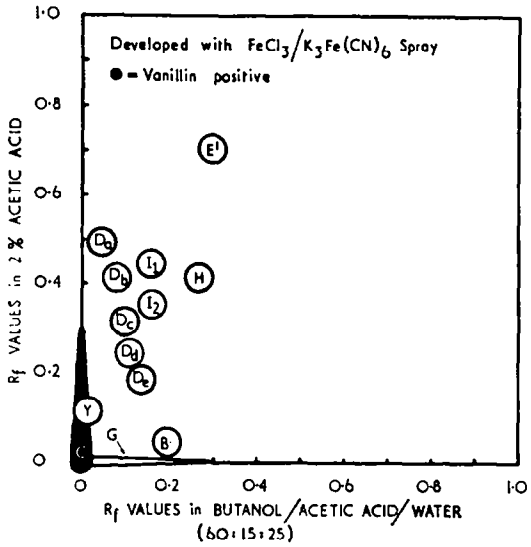

FIG. 2. TWO-WAY THIN-LAYER CHROMATOGRAMS ON CELLULOSE IN HAc AND BAW SOLVENTS OF OAK LEAF TANNINS, EXTRACTED BY THE METHOD OF BROWN AND LOVE.

showed that D could be resolved into five components (D_a to D_e; see Fig. 2) and I into two components (I_1 and I_2). The R_f values in BAW on thin-layer plates were found to be higher than those on the corresponding paper chromatograms.

Chromatograms of the water-insoluble fraction (P) differed from those of (S) only in the occurrence of the streak G along the BAW axis and in the greater concentration at the origin of the reaction of C with vanillin, suggesting that (P) contains components of higher molecular weight than (S).

All the components of the crude oak tannin fraction (S) were absorbed from aqueous solution by hide powder. The complexes were stable to water, but were dissociated at least partially by 70% acetone. An aqueous solution of fraction (S) gave a white precipitate with gelatin solution at pH 4·0.

After boiling samples of both (S) and (P) fractions of crude July oak leaf tannin with concentrated HCl, cyanidin chloride was detected in the hydrolysates, confirming the presence in the crude tannin of condensed flavonoid tannin.[1] No cyanidin was produced after similar treatment of tannin from April oak buds and the reaction with May leaf tannin was extremely weak, so confirming the virtual absence of condensed tannin from oak leaves in the early spring.

Prolonged hydrolysis of July oak leaf tannin with dilute sulphuric acid gave rise to gallic and ellagic acids, in addition to D-glucose and some cyanidin. The presence of a sugar and of the simple phenols gallic and ellagic acid in the hydrolysate indicated for the first time the occurrence of at least one hydrolysable tannin in the leaves of *Quercus robur*, the presence of which had previously been suspected solely on the basis of chromatographic behaviour.[1] However, the crude tannin extracted from the leaves of another species of oak, *Q. sessilis* Ehrh., has previously been shown to contain both condensed tannin and components which yield ellagic and gallic acids together with D-glucose on hydrolysis with dilute acid.[6]

Separation of the Constituents of the Leaf Tannin Fractions

Crude oak leaf tannin was separated into its components by elution from Sephadex G-25 columns with 60% ethanol, containing 0·1% HCl, as described by Somers[7] for the separation of wine tannins. C was eluted in 0·5 bed volumes, B in 3·5 bed volumes and D and H in 4 bed volumes. Somers[7] gives a value of about 2000 for the molecular exclusion limit of G-25 Sephadex in 60% ethanol, so that molecular weights somewhat lower than this would be expected for D and H and a much higher value for C. Although condensed tannin could be obtained chromatographically pure from a single separation on Sephadex, D and H were still contaminated with small amounts of C and B and not separated from each other. It was found that D and H could be freed from B and the remaining traces of C by running them through a second Sephadex G-25 column, using 50% acetic acid as eluent. Under these conditions, C appears to be irreversibly absorbed onto the Sephadex. Subsequent separation of D and H was achieved by two-way chromatography on thick paper.

Properties of the Separated Tannin Components

Tannin C was obtained as a reddish brown powder, partially soluble in water, but completely soluble in acetone/water (20:80). Its u.v. spectrum showed a distinct maximum at 277 nm. Elemental analyses were carried out (*a*) on the freeze-dried powder (Found: C, 53·1%; H, 4·9%; average of two determinations) and (*b*) after the freeze-dried powder had been dried for 24 hr at 150° *in vacuo* (0·2 mm) (Found C, 55·6%; H, 4·9%; average of two determinations). A value of 6750 was obtained for the average molecular weight of C, using an analytical ultracentrifuge. Aqueous solutions gave copious precipitates with gelatin solution and the tannin was completely removed from aqueous solution by hide powder. The flavonoid nature of C was confirmed by the liberation of cyanidin and the concomitant formation of insoluble phlobaphene on boiling with concentrated HCl.

[6] A. BOUDET and P. GADAL, *Compt. Rend.* **260**, 4057–60 (1965).
[7] T. C. SOMERS, *Nature* **209**, 368–370 (1966).

It was found that marble galls from *Q. robur*, caused by the gall wasp *Adleria* (= *Cynips*) *kollari* Hartig., provided a rich source of tannins D_b and D_c, which were purified by chromatography on Sephadex columns and thick paper. Hydrolysis of these two tannins with dilute acid gave rise to ellagic acid, with a trace of gallic acid, and D-glucose, confirming their identity as hydrolysable tannins and suggesting that they might be further classified as ellagitannins. However, the occurrence in the hydrolysates of several unidentified phenolic components, none of which could be hydrolysed further, indicated a complex constitution.

Whether obtained from oak leaves or galls, D was a dull white amorphous powder, completely soluble in water. Elemental analysis was carried out (*a*) on the freeze-dried powder (Found: C, 45·5%; H, 4·0%) and (*b*) after heating for 24 hr at 150° *in vacuo* (0·2 mm) (Found: C, 48·4%; H, 4·1%). Its u.v. spectrum was featureless, showing gradually increasing absorption towards lower wavelengths, with no maxima. Aqueous solutions gave precipitates with gelatin and the tannins were absorbed by hide powder. A value of 1680 was found for the average molecular weight of D_b and D_c.

Quantitative Estimation of Tannins

No suitable method for the quantitative analysis of tannins could be found in the literature (for a discussion of the inadequacies of various published methods see, for example, the

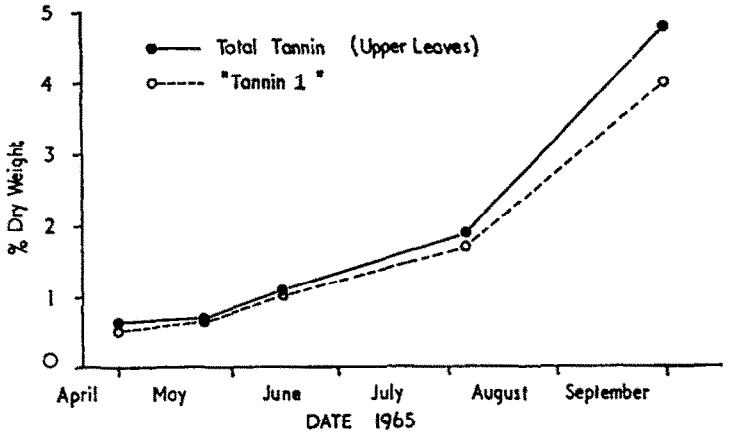

FIG. 3. SEASONAL VARIATION IN TANNIN CONTENT OF THE LEAVES OF A SINGLE OAK TREE *Quercus robur* L., DETERMINED BY THE GRAVIMETRIC METHOD.

reviews by Hathway[8] and Roberts[9]). We found, however, that the Brown and Love[5] salting-out procedure could be made quantitative, although the method is tedious and involves considerable quantities of ether. Details of the method are given in the Experimental Section.

Quantitative analysis of tannins was carried out monthly during 1965 by the modified Brown and Love method, using acetone/water extracts of fresh oak leaves from the study tree. Total tannin content was estimated as the sum of the fractions "Tannin 1", obtained from the acetone layer after addition of salt, and "Tannin 2", obtained from the aqueous layer (see Experimental Section). The results are shown in Fig. 3, where it can be seen that tannin

[8] D. E. HATHWAY, in *Chromatographic and Electrophoretic Techniques* (edited by I. SMITH), Vol. 1, *Chromatography*, pp. 104–109. Heinemann, London, and Interscience, New York (1960).

[9] E. A. H. ROBERTS, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), pp. 468–512. Pergamon, Oxford (1962).

content does increase during the growing season, as previously suspected, though slowly at first. The most rapid increase occurs in late summer, when the tannin content rises to as much as 5% of the dry weight of the leaves. The condensed tannin, C, and hydrolysable tannins (chiefly D and H), making up the "Tannin 1" fraction, account for about 80% of the total tannin throughout the season. Judged from the intensities of the chromatogram streaks, it would seem that the increase in tannin content during the summer is largely due to increase in the level of condensed tannin, the level of the hydrolysable tannins D and H remaining fairly constant.

Conclusion

The most obvious changes in the phenolic content of the leaves of *Q. robur* during the growing season are the appearance in late May of condensed tannin, together with vanillin-positive components of low molecular weight, and the progressive increase in total tannin content. Thus the period of highest insect attack on oak leaves (early spring) corresponds to the time when total tannin content is at a minimum and condensed tannin is absent or almost so. Whether or not this correlation has any causative basis will be discussed in a separate paper examining the effects of oak leaf tannins on insect growth.

EXPERIMENTAL

Analyses

Analyses were carried out by Messrs. Weiler and Strauss, Oxford.

Leaf Collection and Extraction

Leaf collections for analysis were restricted to the upper sun leaves (south side) of a single mature oak tree *Quercus robur* L. situated in mixed deciduous woodland in Wytham Wood, near Oxford. All leaf collections were made between 1400 hr and 1700 hr B.S.T. on days of fine weather. Leaves were also collected occasionally from five other oak trees in Wytham Wood for qualitative comparison of leaf phenolics; chromatograms from the resulting extracts were indistinguishable from those derived from leaves of the main study tree.

Intact leaf clusters from several branches in the appropriate part of the tree were transferred to the laboratory in a refrigeration box and randomized by hand. 50 g of leaves (fresh wt.) were then promptly cut from the twigs and the leaf sample immediately homogenized in 250 ml acetone/water (70:30) in an "Atomix" blender (Measuring and Scientific Equipment Ltd.) in the cold room (4°). To reduce the risk of chemical change due to enzyme action during extraction, 0·5 g of L-ascorbic acid and 0·1 g KCN[9] were added to the solvent prior to extraction. The extract was made up with washings to 500 ml and stored under N_2 at 4°. A further 50 g of the original randomized leaf sample was dried at 80° for dry weight equivalent.

For bulk extraction of leaf tannins, leaves were collected as above and either extracted immediately or stored in the deep-freeze (−20°) until needed. Leaves were extracted into acetone–water (70:30), under N_2. After 7–10 days' storage in the dark, the suspension of leaves was homogenized in an "Atomix" blender and the dark-green extract filtered. Tannins were then isolated by the method of Brown and Love[5]: excess solid NaCl was added to the extract, resulting in a separation into two layers. The dark-green upper, acetone, layer was separated and twice its volume of ethanol added, followed by 5–10 vol. of anhydrous diethyl ether to precipitate a mixture of NaCl and tannin. The precipitate was sedimented (2500 rev/min for 10 min) and, after washing with fresh ether and drying under N_2, taken up in distilled water and dialysed ("Visking" tubing, 1 in. dia.) against distilled water under N_2. The dialysate was replaced at intervals of a few hours until it ceased to give a positive reaction with $AgNO_3$ and $FeCl_3$ solutions. The dialysed solution was then freeze-dried to yield the brown tannin powder.

Effects of Storage of Leaves and Extracts on their Phenolic Constituents

Aliquots from an acetone/water extract of fresh September oak leaves were run on two-way chromatograms in HAc and BAW (*a*) immediately after extraction and (*b*) after storage for 4 months under nitrogen in the dark. The patterns of spots on the two chromatograms were indistinguishable and there was no obvious difference in the relative spot intensities, suggesting that extracts, prepared as described above, can be stored for considerable periods without risk of significant change to the phenolic constituents.

The effects of storing leaves in the deep-freeze before extraction of phenolic constituents were also investigated. Fresh acetone/water extracts of April buds and June oak leaves were compared chromatographically with extracts from the same samples of leaves after deep-freeze storage at −20° for a year. Almost no difference was detectable between the two sets of chromatograms, except that substances I and K were present in the stored April bud extract and substances I and P in the extract from stored June leaves (Fig. 1 and Table 1). I, P and K were not found in extracts from fresh leaves. Thus slight changes do apparently occur during storage, but the tannins are seemingly not affected.

Investigation of Oak Phenolics by Chromatography

Immediately prior to analysis, the acetone/water extracts from oak leaves were centrifuged to remove cellular debris and 0·01 ml aliquots run on two-way paper chromatograms (Whatman No. 1 paper) in standard "Smith" chromatanks (Shandon Ltd.) at 16–18° using ascending solvent. The solvent systems used were 2% acetic acid ("HAc"), followed by n-butanol/acetic acid/water (60:15:25) ("BAW"). Developing reagents used were ferric chloride/potassium ferricyanide, vanillin-HCl, diazotized *p*-nitroaniline and tetrazotized benzidine. Before spraying with a developing reagent, the chromatograms were examined in u.v. (360 nm) with and without the presence of NH_3 vapour. Commercial samples of gallic acid, ellagic acid, (+)-catechin and quercetin and a sample of (+)-gallocatechin, kindly supplied by Dr. D. E. Hathway, were used for reference.

Two-way TLC was carried out at 24°, using 20 × 20 cm cellulose (MN-300) plates.

Hide Powder

Aqueous solutions of suspected tannins were shaken with wetted hide powder (BDH Ltd., standard grade) or run through short columns, packed with wetted hide powder. The presence or absence of phenolic components in the supernatants or eluates was determined by two-way paper chromatography.

Gelatin

To aqueous solutions containing suspected tannins was added slowly a 1% aqueous solution of gelatin, containing 10% (w/v) NaCl, at pH 4·0.

Hydrolysis of Oak Leaf Tannins

(*a*) *Concentrated hydrochloric acid.* 0·2 g each of the (S) and (P) fractions of July oak leaf tannin, extracted by the method of Brown and Love,[5] were boiled briefly in separate tubes with 2 ml of conc. HCl and 1 ml n-butanol. In both tubes, a cherry-red colour developed and was largely extracted into the butanol layer. 0·01 ml samples of the butanol layers from the two hydrolysates were run one-way on a paper chromatogram, together with a butanolic solution of pure cyanidin chloride, using the "Forestal" solvent (water/acetic acid/hydrochloric acid, 10:30:3 v/v).[10] The R_f of the cerise spot obtained from both hydrolysates was found to be 0·40—identical to that of the cyanidin reference spot. The absorption spectra of the butanol layers from the two hydrolysates were obtained in a Beckman DB spectrophotometer, using pure n-butanol as a reference blank. The spectra (λ_{max} 465 and 560 nm) were indistinguishable from that of a pure butanolic solution of cyanidin chloride. Cyanidin was also found to be present in the solution obtained by boiling 0·2 g pure oak leaf condensed tannin with 2 ml concentrated hydrochloric acid.

(*b*) *Dilute sulphuric acid.* 0·05 g July oak leaf tannin (S and P fractions unseparated) was heated at 90° in a sealed glass test-tube with 3 ml of dil. (1% v/v) H_2SO_4 for 24 hr. The resulting pink hydrolysate was decanted from insoluble residue and 0·01 ml aliquots run on two-way paper chromatograms in HAc and BAW solvents. Cyanidin, gallic and ellagic acids were identified on the developed chromatograms by their R_f values and behaviour in u.v. light and with developing reagents. The pure compounds were run on separate chromatograms for comparison. In addition, three other unidentified phenolic hydrolysis products were noted on the chromatograms (R_f HAc/BAW: 0·62/0·10, 0·12/0·54, 0·02/0·55). 0·01 ml samples of the hydrolysate were also run one-way on paper chromatograms in iso-propanol/water (4:1) and the chromatograms sprayed with aniline/diphenylamine, aniline hydrogen phthalate or phthalic acid reagents. A single spot was revealed, identical in R_f value (0·45) and in coloration with that obtained on a reference chromatogram using a solution of pure D-glucose.

A solution of the oak gall hydrolysable tannins D_b and D_c (50 mg) in 1% H_2SO_4 (50 ml) was saturated with N_2 and refluxed in a water bath under a stream of N_2 for 2 days. After extraction of the hydrolysate with ether, TLC on cellulose revealed the presence of ellagic acid and a trace of gallic acid in the ether layer and several unknown phenolic components (R_f HAc/BAW: 0·69/0·13, 0·60/0·17, 0·06/0·27, 0·10/0·28, 0·10/0·32) in the aqueous layer. Cellulose TLC in isopropanol/water (4:1) revealed a single spot, opposite the glucose marker, on development with aniline hydrogen phthalate.

[10] C. W. Love and B. R. Brown, *Report on Forest Research*, pp. 104–109. H.M.S.O., London (1959).

Determination of Molecular Weights of Tannins

Solutions of condensed tannin C, in acetone/water (1:2, v/v), and hydrolysable tannin D (D_b and D_c from oak galls), in water, each containing 2·5 mg/0·5 ml, were supplied to Mr. C. J. Teal, at the Department of Biochemistry, Oxford, together with samples of the solvents. The molecular weights were determined on a Beckman "Spinco" Model E analytical ultracentrifuge, using Schlieren optics, by the short column method of Yphantis.[11] Partial specific volumes of the tannins (Tannin C: 0·64; Tannin D: 0·63) were obtained using a 10 ml density bottle and 50-mg tannin samples.

Quantitative Analysis of Oak Leaf Tannins

A quantitative adaptation of the Brown and Love[5] method of tannin extraction was tested as follows: 50 g (fresh wt.) of fresh September oak leaves were extracted into 500 ml acetone/water (70:30) and, after addition of NaCl, the acetone and aqueous layers were separated. To a 50 ml aliquot from the acetone layer were added 2 vol. (100 ml) of ethanol and 5 vol. (750 ml) of anhydrous diethyl ether; to a second 50 ml aliquot were added 4 vol. of ethanol and 5 of ether and, to a third aliquot, 2 vol. of ethanol and 10 of ether. The precipitates of tannin and NaCl were sedimented (2500 rev/min/10 min), dialysed, freeze-dried and weighed, yielding 0·163 g, 0·134 g and 0·165 g tannin, respectively. The ethereal supernatants in each case were concentrated by vacuum distillation to 25 ml, of which 0·01 ml aliquots were applied to two-way chromatograms. Some tannin was detected in the ethereal layers from aliquots 1 and 2, but complete precipitation had been achieved from aliquot 3. As judged by the yield of tannin, however, addition of 10 vol. of ether is only marginally more effective than addition of 5 vol.

The yield of tannin from the 50-ml acetone aliquots was used to calculate the total tannin content of the acetone layer and hence the percentage of tannin in the original leaves. The tannin fraction obtained from the acetone layer was designated "Tannin 1"; it was chromatographically free from the non-tannin phenolic material present in the original extract and consisted largely of condensed tannin, C, and the hydrolysable tannins, D.

After addition of salt to the 500 ml extract from September leaves, it was noticed that the lower, aqueous, layer was golden brown in colour. This layer was vacuum distilled (30°) to remove traces of acetone, dialysed and freeze-dried, when it yielded a light-brown powder (0·211 g). A sample of this powder yielded some cyanidin on boiling with conc. HCl, indicating that it contained some condensed tannin. Two-way chromatography of an ethanolic solution of the powder, subsequently referred to as "Tannin 2", revealed only a streak along the HAc axis (R_f 0–0·9) when sprayed with $FeCl_3$/potassium ferricyanide reagent, with a concentration near the origin, which was weakly vanillin-positive. Tannins D, H, B and G were absent. Elemental analysis revealed a small amount of nitrogen and a large amount of inorganic material (Found: C, 51·65, 51·76; H, 4·81, 4·48; N, 1·45; ash, 6·76%). "Tannin 2" was absorbed from aqueous solution by hide powder. Although it clearly contains some non-tannin material, probably including some complexed protein, the above observations suggest that it consists largely of tannin, partly condensed and partly of unknown nature, and we therefore decided to include "Tannin 2" in the routine leaf analyses, but to tabulate the results separately from those of "Tannin 1".

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[11] D. A. YPHANTIS, *Ann. N.Y. Acad. Sci.* **88**, 586–601 (1960).