THE CHEMISTRY OF THE WATTLE TANNINS

(Acacia mollissima Willd., A. decurrens Willd., A. pycnantha Benth. and A. dealbata Link)

by

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INDEX.

CHAPTER VII

THE ANALYSIS OF BLACK WATTLE TANNINS

Much analytical work preceded the chromatographic approach in the present investigation. As the degradative study has shown that only three fundamental nuclei are present in the tannins, it is considered likely that the majority of areas of high concentration shown in the fresh-bark chromatogram, are very closely related if not consisting of different states of aggregation of one or two fundamental units. These tannins will be separated with difficulty. Analyses of the tannin fraction as a whole, which supply much useful information regarding the composite nature and chemical behaviour of these tannins, are thus presented in this chapter.

The previous work of Stephen (149) showed little or no relationship between analytical figures obtained from the original tannins and their derivatives. The same discrepancies are evident when comparing the work of nearly all the earlier investigators. Kirby (163) and Lowitt (159) based their work and speculation entirely on methoxyl and acetyl derivatives respectively.

Stephen not only used tannins still contaminated with an admixture of carbohydrates as starting-material, but also analysed and compared derivatives which were incompletely substituted. Opposed to this, the present work on the whole tannin fraction showed for the first time that a constant proportion of carbon,

hydrogen and hydroxyl groups exist throughout, and also established that the large proportion of non-reactive oxygen present in the tannins, could possibly be ether linkages.

(1) Elementary Analyses of the Tannins

Stephen previously analysed the acetone-salt purified material on which all his work was based. He recorded the values :

C = 57.4 - 59.1%, H = 4.8 - 5.0%

C = 60.0% H = 4.92% (dried at 110°C. for 3 - 10 days)

C = 60.0% H = 5.06% (electrodialysed sample)

Nunn's (234) values were :

C = 58.6 - 59.3%, H = 4.69 - 4.92%

Tannins "purified" by Russel's (136) acetone-salt method were also analysed by the author for comparison (dried at 110°C. for 2 hours) :

C = 57.75% H = 4.54% Ash = 0.0%

C = 57.61% H = 4.66% Ash = 0.2%

C = 57.48% H = 4.54% Ash = 2.3%

As Williams (158) claimed that methanol extraction was more effective than Russel's method for removing sugary non-tannins, tannins leached from fresh-bark with absolute methanol were, therefore, also analysed.

Found :

C = 58.15, 57.89% H = 4.92, 4.55%

Tannins = 80.00% Non-tannins = 17.6% % Solids = 2.4%.

In the previous section on purification (Chapter IV), samples separated by the various methods were subjected to combustion analyses.

Found : C = 60.90 - 61.04%

H = 4.83 - 4.94%

Due to the high resistance of the tannins to combustion, it was considered that slight pressure in the tube might assist more complete combustion. Potash absorption tubes were thus substituted for soda-lime and further analyses (dried at 110°C. for 2 hours in an Abderhalden gun) gave :

C = 61.38, 61.80%

H = 4.69, 4.84%

Corbett (146), from previous work on tannins purified by ultrafiltration and dried for 24 hours at 95 - 100°C., recorded the values :

C = 61.6, 61.7%

H = 4.53, 4.61%

Calculated for

$C_{15}H_{14}O_6$: C = 62.07% H = 4.83%

$C_{15}H_{13}O_6$: C = 62.29% H = 4.50%

$C_{15}H_{12}O_6$: C = 62.40% H = 4.16%

Compared with tannins obtained by methanol extractions and acetone-salt purifications, the above combustion values show an increase in carbon content consistent with some elimination of sugary non-tannins.

For the interpretation of the above results the following must be taken into account :

(a) The tannins, even taken directly from the fresh bark cannot be "purified" without causing a certain amount of darkening in colour. As the darkening of black wattle tannins is known to be due to atmospheric oxidation (See Chapter XI) all samples analysed were in a slightly oxidised condition.

(b) The tannins show a very high resistance to combustion. After initial charring the samples set into a hard carbonaceous mass which burns away only at high temperature and invariably leaves a trace of greyish residue.

(c) The tannins show a great tendency for complex formation through the presence of orthohydroxy-groups, and thus retain small traces of salts with avidity.

(d) The highly hygroscopic nature of the completely dried tannins always introduces a small error into this estimation. In equilibrium with air they usually contain 8 - 12% moisture, part of which they rapidly reabsorb after complete drying.

From this discussion it is obvious that slightly low carbon and possibly high hydrogen values may be expected, and the results obtained are thus in agreement with the formula

$$C_{15}H_{12-13}O_6$$

The Functional Groups in Black Wattle Tannins

(ii) Hydroxy-groups via Acetylation

5 grm. amounts of purified and dried tannins were dissolved in 20 ml. dry pyridine. The solution was cooled in an ice-salt freezing mixture to 0°C. and fresh acetyl chloride (10 grms.)

added in small quantities. A fairly vigorous reaction was evident, and the temperature rose to about 30°C. accompanied by the formation of a solid white amorphous mass which settled from the pyridine. The reaction-mixture was allowed to stand in contact with ice for 30 minutes and then poured into iced water with vigorous stirring. The insoluble acetylated product was thoroughly washed with a large quantity of cold water and air-dried. The white product was dried, dissolved in chloroform and poured through an alumina (100 - 200 mesh) column. The acetylated compound was completely adsorbed and the solvent passing on yielded a trace of oiliness of terpinoid odour. The column was washed with further additions of chloroform and the acetylated tannins finally eluted off with excess of the same solvent.

The acetyl determinations were carried out by the trans-esterification method of Matchett and Levine (150) standardised against recrystallised triacetylpyrogallol of melting point 163°C. This method is a modification of the technique used by Perkin (244) for catechins. In all cases the material was dried at 110°C. for 2 hours under vacuum.

Found : % C = 59.64, 59.48, 59.77

% H = 4.81, 4.85, 4.78

% Acetyl = 38.32, 38.21, 37.92

The above material was reacetylated as before, repurified by chromatography and dried as before :

Found : % C = 60.08, 60.01, 59.87

% H = 4.74, 4.73, 4.73

% Acetyl = 37.55, 38.17, 37.87, 37.48

Calculated for $C_{15}H_9C_2(OAc)_4$: % C = 60.94 % H = 4.59

% Acetyl = 36.8

Previous investigators have obtained the following results :

Investigator	Tannins purified by	Method of Estimation	% Acetyl	% C	% H
Lauffmann (235)	impure	gravimetric	41.3	-	-
Corbett (146)	impure	gravimetric	41.0	-	-
Stephen (149)	acetone - salt	trans-est.	39.0	-	-
	"	"	39.3	-	-
Lowitt (159)	ethyl acetate extract	"	40.8	58.4	4.7
	acetone extract	"	40.1	58.7	4.7
Williams (157)	ethyl acetate extract	"	41.5	58.94	4.93
Author	acetone - salt		37.94	59.64	4.72
			38.16	59.50	4.73
Author	methanol fresh-bark extract		39.14	58.79	4.56
			39.04	58.61	4.66

The elimination of sugary non-tannins has thus caused an expected reduction in the acetyl value and an increase in carbon content.

Reductive Acetylation

Reductive acetylation has been used (236) for the simultaneous reduction and acetylation of oxidised quinonoid structures.

It is possible that such reaction, corresponding to a Clemmensen reduction followed by acetylation, might split pyrane ring structures and cause an increase in acetyl content.

Lead-salt purified black wattle tannins (8 grms.) were refluxed with sodium acetate (10 grms.), acetic anhydride (100 ml.) and zinc dust (10 grms.) over a 3 hour period, and the product poured into iced water. After standing and stirring to hydrolise the acetic anhydride the white precipitate was sucked off, washed and dried as before.

Found : % Acetyl = 38.64, 38.52.

This product was reacetylated as before for a further 3 hour period:

Found : % Acetyl = 38.95, 39.58.

Reductive acetylation, therefore, does not increase the acetyl content significantly and no easily reducible groups appear to be present.

Drastic Double Acetylation

Very recently Putnam and Gensler (131) twice acetylated a phenolic fraction of quebracho extract with drastic reagents and thus obtained an acetyl value which accounted for all the oxygens in the tannins as hydroxy groups. They also intimated that black wattle tannin behaves similarly.

Putnam and Gensler's acetylation technique, which consists of (a) a short (15 mins.) reductive acetylation in an acetic anhydride, tetraethylammonium bromide, zinc dust and triethylamine mixture, followed by (b) a prolonged (46 hr.) drastic acetylation

with an acetyl chloride/dimethylamine mixture, was accordingly used on the lead-salt purified black wattle tannins.

Dimethylamine was removed from the acetylated product after completing the second stage of the reaction by freezing the latter from ethanol instead of methanol.

Found : % Acetyl = 39.10, 39.48.

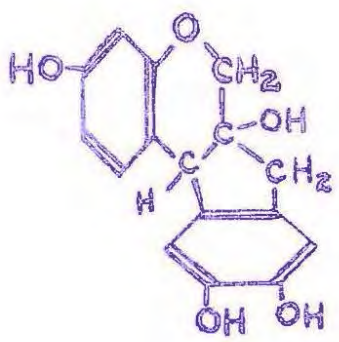
No significant increase of acetyl content is reflected in this analysis, which disagrees with Putnam and Gensler's findings.

d-Catechin from cube gambier was similarly treated to establish whether this drastic acetylation is capable of opening the pyrane ring. After the first stage of the dual acetylation pentaacetyl d-catechin mpt. 130°C. was already isolated. The second reaction did not affect the pentaacetyl derivative (Mpt. and mixed mpt. 130°C) and no ring opening, therefore, results from either or both of these reactions.

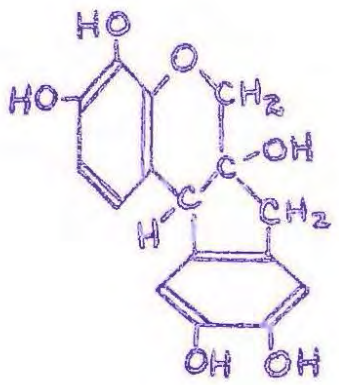
Discussion

Compared with previous work on impure or partly purified acetylated tannins, the new figures reflect a lower acetyl and higher carbon value. The elimination of carbohydrates which have a low carbon content and high hydroxyl content, is almost certainly responsible for these differences. The use of freshly purified ethanol is essential for the trans-esterification method of acetyl estimation. Commercial absolute ethanol causes high results (40 - 41%) and is also considered responsible for some of the high values obtained previously by other investigators.

Acetylation with acetyl chloride/pyridine and acetic anhydride/sodium acetate mixtures easily substitutes secondary aliphatic hydroxyl groups of the type present in catechin (237), XXXI and gallocatechin (238) LXV and tertiary aliphatic hydroxyls of the type present in brazilin (239) XCVI and haematoxylin (240) XCVII.

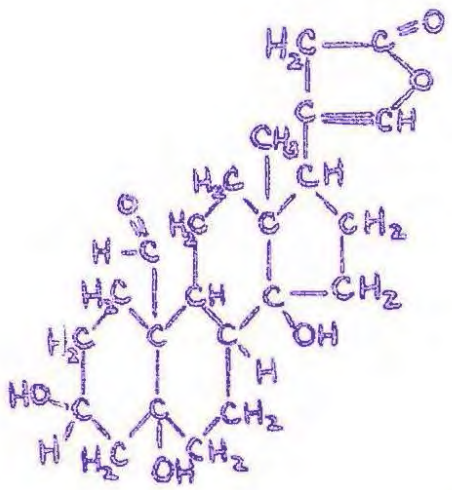


XCVI



XCVII

Tertiary aliphatic hydroxyls such as those present in the cardiac glycosides e.g. strophanthidin (241) XCVIII do not acetylate under such conditions, and only the secondary aliphatic hydroxyl reacts in this nucleus. Tertiary aliphatic hydroxyl groups are generally known not to react quantitatively with acetyl chloride/pyridine mixtures (245)(246) or with acetyl chloride only (247). Nevertheless the close proximity of phenyl nuclei, as in the case of brazilin and haematoxylin, appears to confer added reactivity to tertiary aliphatic hydroxyls, and it appears unlikely that possible tertiary aliphatic hydroxyl groups present in black wattle tannins will be non-reactive.



(suggested structure)

XCVIII

(iii) Hydroxy Groups via Methylation

Dimethyl sulphate has been used for the methylation of black wattle tannins by Corbett, Stephen and Kirby, and is certainly the easiest, although a drastic method, for obtaining the fully methylated product. The reaction has the disadvantage of occurring in strongly alkaline methanolic solution which causes a small amount of unavoidable oxidation and possible cleavage of labile linkages as in the case of lignins (242). Tannins containing unreacted phenolic groups may be removed from the fully-substituted product by chromatography on an alumina column (164). The quantity of dimethyl sulphate required has always been regarded as excessive but ortho-hydroxy groups, e.g. gallic acid (243) apparently require a large excess of this reagent for complete methylation.

Analysis of Methylated Tannins

Dried tannins (55 grms.) purified by the lead-salt method, were dissolved in methanol and methylated with dimethylsulphate

(120 ml.) and methanolic KOH first gently and finally vigorously at the boil. After complete reaction the mixture was poured into slightly acidified water; the light pink insoluble methyl ether sucked off, washed with water and air-dried (70 grms.). After drying in an Abderhalden gun under vacuum at 110°C. for 2 hours it was analysed.

Found : % C = 65.36, 65.31 % H = 5.88, 5.91

and % $-OCH_3$ = 32.50, 32.55

The product was finely powdered, shaken with cold absolute ethanol (300 ml.) and allowed to stand for 8 hours. About 15 grams dissolved. The ethanol-soluble portion was adsorbed on to an alumina column from benzene and eluted with a benzene-ethanol (100 : 2) mixture according to Kirby's method.

Found : % C = 66.19, 66.45 % H = 6.07, 6.04

and % $-OCH_3$ = 35.19, 35.36.

The ethanol-insoluble portion was similarly chromatographed and eluted with a benzene-ethanol (100 : 3) mixture.

Found : % C = 66.53, 66.37 % H = 6.09, 6.16

and % $-OCH_3$ = 34.49, 34.57, 34.60

Both fractions gave a mixture of veratric and O-trimethylgallic acids on oxidation, and no fractionation was thus achieved by this method.

Maximum Methoxyl Values

No proved "maximum" methoxyl value has yet been achieved. Stephen showed that dimethyl sulphate was the most effective methy-

lating reagent for the tannins, and obtained an apparent maximum value of 35.5% on repeated action. Purdie and Irvine (151) have shown that methyl iodide and silver oxide effectively methylate sugars. This method would be unsuitable for tannins because of the oxidising action of the silver oxide. Stephen accordingly used it on the above 35.5% methoxyl material, and increased the methoxyl content to 36.5%. Kirby, to prove that his fractions were fully substituted, remethylated these with the weaker reagent, diazomethane and recorded one value of 36.3%.

To check the above, dimethyl sulphate was used repeatedly followed by the purification of the product on an alumina column after each reaction. 20 grms. of methylated tannins of 34.5% methoxyl content was used as starting material. Subsequent reactions were carried out in methanol at the boil using 40 ml. quantities of dimethyl sulphate for each reaction. Very small but decreasing traces of an aromatic oil were separated during each purification.

Found :

No. of Methylations	Methoxyl Value %	
1	34.5	
2	36.21	35.99
3	36.35	36.81
4	36.49	36.51
5	36.47	

Combustion analyses were performed on the final product.

Found : % C = 65.85 65.79 65.83 66.01

% H = 6.14 6.26 6.17 6.06

Calculated for $C_{15}H_9O_2(OCH_3)_4$:

% C = 66.09 % H = 6.10 % $-OCH_3$ = 35.9

Discussion

The "maximum" methoxyl value is in slight excess of 4 methoxyl groups per hypothetical C_{15} unit. Under drastic conditions methylation in its final stages proceeds with greater difficulty than acetylation. The maximum value attained agrees with Stephen's and is in slight excess of Kirby's. Previous combustion analyses were performed on incompletely substituted materials :

Investigator	How Purified	% $-OCH_3$	% C	% H
Stephen	Acetone-salt	32.0	63.30	5.61
Kirby	Acetone extraction	34.8	66.45	6.54
	Acetone extraction	35.9	66.03	6.08

Kirby claimed that two fractions with the above analyses, although not differing much analytically gave veratric and O-trimethyl gallic acids respectively on oxidation.

(iv) Hydroxyl groups by Combined Acetylation and Methylation Procedures

Both the previous derivatives show agreement in the number of hydroxyl groups per C_{15} unit when fully substituted. Such agreement could, however, result from the opening of pyrane rings during drastic methylation to free phenolic hydroxyls, and

the simultaneous non-reactivity of secondary aliphatic hydroxyls, which, however, acetylate easily. Kirby acetylated the almost completely methylated tannin, and obtained evidence of some substitution. He thus concluded that a proportion of unsubstituted aliphatic hydroxyl groups still exist in the methylated product.

In order to establish whether any such free hydroxyl groups still exist in the fully methylated (36.5%) compound, it was acetylated with acetyl chloride and pyridine as previously described. As solid pyridine hydrochloride is known to be a demethylating agent (248) the mixture was allowed to stand for only 15 - 20 minutes after the addition of acetyl chloride. The acetylation of the methylated tannins was also performed by Stephen using acetic anhydride and sodium acetate.

Found :

Investigator	% $-OCH_3$ of starting-material	% $-OCH_3$ in product.	% -Ac
Author	36.5	34.55	3.56
Stephen	36.5	34.3	4.0
Stephen	34.0	30.1	5.4
Stephen	34.5	32.5	3.0

Corbett also noticed a reduction in methoxyl value after the acetylation of the methylated derivatives. Assuming that 4.13 is the correct number of hydroxyl groups per C_{15} unit the above result and the first of Stephen's are in perfect agreement. (Fig. XXXIII.)

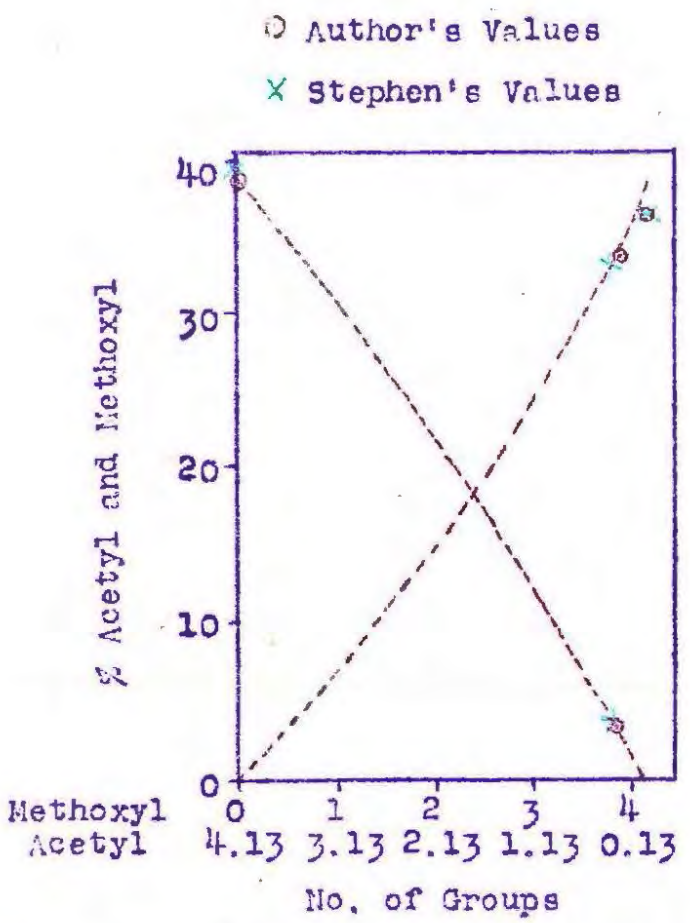


Fig. XXXIII. The Variation of Acetyl and Methoxyl Content in fully Substituted Black Wattle Tannins and their Mixed Derivatives.

A small amount of replacement of methoxyl by acetyl radicals appears to occur and thus no "free" hydroxyl groups exist in the methylated tannin. Kirby's findings are thus incorrect.

The above evidence establishes that, with the exception of possible tertiary aliphatic hydroxyls in abnormally sterically hindered positions, just over 4 hydroxyl groups per C_{15} unit exist in black wattle tannins.

(v) The Nature of the Hydroxy-Groups

It is to be expected that the majority of the hydroxy-groups in the tannins are phenolic in character, as the basic building-stones are di- and tri- hydroxy phenols. Nevertheless the presence of at least one aliphatic hydroxyl per C_{15} unit in tannins has previously been assumed purely by analogy to the catechins, which have hitherto been regarded as "precursors" of tannins due to their close association in nature.

The assumption for black wattle tannin was partly supported by Stephen's (149) emphasis on the extreme difficulty of the reaction between tannins and diazomethane in methanol-ether solution, and by evidence of the abnormally weak acidic nature of the tannins from conductimetric titrations (249).

No thorough comparative study to confirm or disprove these trends has yet been attempted, and a synthetic study involving the use of diazomethane has prompted a repetition of various methylation procedures, with the aim of establishing the proportions of aliphatic and phenolic hydroxyl groups in the fundamental unit.

Previous Methylation Studies

Stephen (loc. cit.) performed the bulk of the earlier work and employed a variety of methlating agents. With dimethyl sulphate he obtained methoxyl values varying from 32% to 35.5% on repeated treatment, and regarded this as the most satisfactory reagent. The majority of investigators have subsequently obtained

a value of 32% methoxyl with ease, but further increase to a maximum of 36.5% was slow and only effected by repeated reaction.

Stephen treated the above material (34% methoxyl) with methyl iodide and silver oxide, and caused an increase to 36.5% after two treatments

He also methylated black wattle tannins with excess diazomethane in alkaline methanol-ether solution, but the reaction proceeded with extreme difficulty and an apparent maximum of 18% was obtained after four treatments. In the absence of alkali the action of diazomethane was equally difficult, but gave a product of 32% methoxyl after several treatments. The presence of alkali, therefore, apparently exerts an inhibiting effect.

Discussion on Previous Work

With black wattle tannins a value of 36.5% methoxyl appears to be the maximum attainable. This represents both primary and secondary aliphatic and phenolic hydroxyls, as it agrees closely with the maximum acetylation value, and as both dimethyl sulphate and methyl iodide/silver oxide reagents are capable of methylating the more difficultly reactive secondary aliphatic groups (250 - 253). Other investigators (254) also consider that the complete action of dimethyl sulphate and sodium hydroxide on tannins methylates both types of hydroxy groups.

The slow and difficult increase above 32% methoxyl using dimethyl sulphate, was taken as indicative of the presence of aliphatic hydroxyl groups in black wattle tannins. Perkin (237)

used this reagent for the methylation of catechin, and continued using it until no colour change occurred between acid and alkaline conditions. All the phenolic groups were thus satisfied and the products consisted mainly of the tetramethylether (all phenolic hydroxyls methylated), and a possible low proportion of the penta-methylether (the aliphatic hydroxyl also methylated). With black wattle tannins, the colour-change between alkaline and acid conditions continues till well above 32% methoxyl, indicating free phenolic groups still at this stage of the reaction. The solid product of 32 - 34% methoxyl is always light pink in colour and Kirby (163) showed this to be probably due to unreacted phenolic or quinonoid groups. When subjected to chromatography on an alumina column the pink colour was eliminated and material remained on the column which was extremely difficult to elute.

From an oxidation study, Heugh (161) concluded that higher yields of O-trimethylgallic and veratric acids were obtained from methylated tannins of 34.7% methoxyl than from material of 32% methoxyl and that the former product was also more resistant to permanganate oxidation.

This indirect evidence indicates that phenolic groups, probably the ortho-hydroxy-phenolics, and not hypothetical aliphatic hydroxyls, are the last to methylate when using dimethyl sulphate.

Three factors, however, possibly contribute to the final slow methylation-reaction of black wattle tannins :

(a) the fission of labile bonds

(b) the presence of secondary aliphatic hydroxyl groups, and (c) the steric effects of ortho-hydroxy-phenolic groups. The effect of one or more of these factors might easily be intensified when occurring in an amorphous polymer.

Methylations with Diazomethane

The reaction of diazomethane with tannin must be regarded with caution as the methylating agent combines with many functional groups. Ketones (255), aldehydes (256)(257), as well as ethylenic linkages and labile bonds (258) are known to react under special experimental conditions, or where these groups have specially high reactivities.

Diazomethane has been used with lignins (259)(260)(261)(262) as a means of evaluating the number of acidic hydroxyl groups, and also with tannins (254) for distinguishing between phenolic and alcoholic hydroxyls. It is well known that such distinctions do not rest on a sound basis since the reagent is also capable of reaction with certain aliphatic hydroxyl groups. The simple aliphatic alcohols and glycols (263) are not methylated, but cellulose (264) and soluble starches (265) react to a slight degree. According to Meerwein (266) the reactivity of an aliphatic hydroxyl group towards diazomethane may be increased by the introduction of polar groups to the α-carbon. Thus allyl alcohol reacts very slowly; benzyl alcohol forms 13% of benzyl methyl ether, and d-tartaric acid with excess diazomethane forms 95% of the dimethyl ester of dimethoxysuccinic acid (267). Even

when further removed, polar groups still have a strong influence and monoacetyl-ethyleneglycol easily forms β-methoxy ethyl acetate (263).

In general the reactivity of hydroxyl groups, whether acidic, phenolic or alcoholic depends on their "acidic strength". Acidic hydroxyls react most rapidly and esterify easily with diazomethane; the reaction with phenols is also rapid but decreases with decreasing acidity (268), whilst that with alcoholic groups is very slow. The aliphatic alcohols form the lower end of this scale, and there appears no absolute dividing line between phenols and aliphatic alcohols. Comparison must therefore be made with compounds of known structure to facilitate the interpretation of the results obtained with black wattle tannins.

The unfractionated reaction-products were isolated by identical methods where possible, and treated as amorphous substances. In this way only, could legitimate direct comparison be made with the amorphous tannins.

(a) Black Wattle Tannins

Diazomethane was prepared from nitroso-methylurea (269) by the method of Arndt (270). 42 grms. of nitrosomethylurea was employed in each instance giving 350 ml. ethereal solution containing about 10 grms. diazomethane. This amount of reagent ensured sufficient excess for the conversion of 5 grms. of pyrogallol-4-carboxylic acid into the methyl ester of 3,4-dimethoxy-2-hydroxy-benzoic acid, with the additional formation of a small amount of

2,3,4-trimethoxybenzoic acid methyl ester, e.g. sufficient excess for the esterification of one acidic and two phenolic hydroxyl groups per 170 molecular weight unit.

3 grms. lead-salt purified black wattle tannins, dissolved in 100 ml. absolute methanol, were cooled to 0°C., and added to the above ethereal solution of diazomethane kept below 0°C. in an ice-salt mixture. A vigorous evolution of nitrogen, comparable to that usually associated with acidic groups, took place. The reaction slowed down after 30 minutes, and the mixture kept at 0°C. for 24 hours when the reaction appeared complete. The ether was removed, the remaining methanol solution filtered, concentrated and poured into water. A milky emulsion resulted which was broken by the addition of a small amount of sodium chloride. The pure white product was sucked off and washed with water and freed from salt. Yield : 3.5 grms. Analysis : (Dried at 110°C. for 2 hours) : 32.45% and 32.35% methoxyl. This was repeated on a different sample of tannins, the reaction proceeding for 30 hours and giving a slightly higher value of 33.07% methoxyl. The above products were mixed and again subjected to a further 24 hours reaction with diazomethane increasing the methoxyl content to 34.17%.

The latter product was acetylated with acetyl chloride in pyridine. The product contained 30.75% methoxyl and 5.61% acetyl groupings corresponding closely to the previously found total of 4.1 hydroxyl groups per C_{15} unit. This duplication of previous findings, as well as the absence of nitrogen in the methylated product, points to the absence of serious side reactions.

The ease and rapidity of the reaction of excess diazomethane with black wattle tannins is quite remarkable and, therefore, contrary to Stephen's findings. Of the total of 4.1 hydroxyl groups per C_{15} unit, 3.5 react with ease over 24 hours, and 3.75 after 48 hours' reaction. The product is white, as compared to the flesh colour of methylated tannins from the dimethyl sulphate/alkali reaction, and gives a negative nitrogen test by the Lassaigne sodium fusion method.

(b) Catechin XXXI

Catechin was isolated from cube gambier by the method of Perkin (89). MPt. 175°C. The methylation with diazomethane, repeated under precisely the above conditions, appeared slow. The whole reaction product was isolated after 24 hours' reaction, dried over P_2O_5 in vacuum, and analysed. Found : % Methoxyl groups = 32.30. More vigorous drying in an Abderhalden gun increased this value to 33.13%.

Catechin contains five hydroxyl groups : one secondary alcoholic and four phenolics, two of which are in the ortho-position on a catechol nucleus. The above value represents just over 3.6 methoxyl groups per C_{15} unit.

(c) Brazilin XCVI

Judged by the rate of evolution of nitrogen, the reaction of brazilin with diazomethane appeared very slow. The whole product was isolated as above and treated as an amorphous precipi-

tate. After drying over P_2O_5 it contained 23.65% methoxyl groups, and after more drastic drying in an Abderhalden gun, 23.97% methoxyl.

Brazilin contains a total of 4 hydroxyl groups per C_{15} unit; one tertiary alcoholic and three phenolic, two of which are in the ortho position on a catechol nucleus. The above methoxyl value corresponds to just under $2\frac{1}{2}$ hydroxyl groups methylated.

(d) Haematoxylin XCVIII

Haematoxylin reacts slowly but more rapidly than brazilin. After 24 hours' reaction the white product was isolated as before and contained 22.30% methoxyl groups.

Haematoxylin has five hydroxyls per C_{15} unit; one tertiary alcoholic, and four phenolic, all of which are in ortho positions. Two are present in a catechol nucleus, and two in a 3-ether-pyrogallol unit. The above methoxyl value corresponds to 2.4 hydroxyl groups methylated per C_{16} unit.

(e) Catechol

The reaction of catechol with diazomethane appears slow. After the usual reaction for 24 hours, the ether was removed, the remaining methanol solution filtered, and the product taken to dryness without pouring into water. The whole product was analysed. Found : % Methoxyl = 39.10 and 38.85. Theoretical for veratrole = 41.89%. The reaction with catechol, therefore, goes almost to completion.

(f) Pyrogallol

(Mpt. 131°C. Recrystallised from a benzene/ethanol mixture). The reaction of pyrogallol in methanol-ether solution appeared more vigorous than any of the above compounds, with the exception of the tannin itself. The product was treated in the same way as catechol, and after a thorough heating to remove all the methanol, it set to a crystalline mass. Found : % Methoxyl = 47.08.

This product was treated with dilute alkali solution and ether-extracted. The alkaline solution contained a fair proportion of partly methylated or unmethylated phenolic material. The neutral ether extract was taken to dryness, and recrystallised once from an ethanol-water mixture. Found : MPt. 41°C. and % Methoxyl groups = 52.90. Theoretical for O-trimethylpyrogallol : MPt. 47°C. and % Methoxyl groups = 55.37.

The reaction was repeated using less pyrogallol (1.5 grms) to the same amount of diazomethane. After 72 hours' reaction at 0°C. a trace of phenolic material still remained which gave a red $FeCl_3$ reaction.

The value of 47% methoxyl represents 2.4 - 2.5 out of a possible 3 hydroxyls methylated. This value is probably low since the neutral fraction contains a small proportion of impurity. Nevertheless the reaction does not go to completion in 24 hours, due possibly to steric hindrance. This is supported by Herzig and Pollak's (271) finding that the methylester of O-4-methylgallic acid

requires repeated treatment with diazomethane for its conversion into O-trimethylgallic ester. The latter substance (243) and O-trimethyl pyrogallol (272) are thus usually prepared by drastic action with dimethyl sulphate.

Discussion

(1) Diazomethane is known to react with ethylenic, aldehyde, keto, alcoholic and acidic hydroxyl groups besides phenolic hydroxyls. Ethylenic and aldehyde groups are unlikely to be present in the tannins, whilst acidic groups are known to be absent from potentiometric curves (273). The carbonyl group of a ketone is not very reactive and in general ketones do not react with diazomethane except in the presence of a catalyst such as water (255). With the exception of special cases the reaction between diazomethane and aliphatic hydroxyl groups is very slow, and comparison has thus been made with compounds thought to be closely allied to the tannin and containing both phenolic and aliphatic hydroxyl groups.

(2) In the reaction of diazomethane with brazilin and catechin under the above conditions, 1½ groups less than the total number available remain unmethylated. In each case, one of the unmethylated groups was almost certainly the tertiary or secondary aliphatic hydroxyl group respectively, as, except in special cases, primary aliphatic hydroxyls react with extreme difficulty in large excess of diazomethane. Catechin, for example, when vigorously treated with dimethyl sulphate, still retains a proportion of the

aliphatic hydroxyl group unmethylated (89).

(3) It was thought that the incomplete reactivity of the remaining phenolic groups in catechin and brazilin was possibly due to chelation or steric hindrance of ortho-hydroxy groups. Schonberg and Mustafa (274) have shown that diazomethane in methanol solution is capable of methylating chelated hydroxyl groups which are otherwise non-reactive. As all the above reactions were performed in the presence of methanol, it is unlikely that chelation effects would have much influence.

Steric hindrance thus appears a more likely factor, and catechol and pyrogallol were therefore treated with diazomethane in methanol-ether solutions to assess the reactivity of ortho-hydroxy groupings. Catechol gave virtually complete, but pyrogallol incomplete methylation. The incomplete reactivity of the hydroxy groups in the tannins towards diazomethane might thus be partly due to steric hindrance, but some other factor associated with large molecular size seems to be involved. This is supported by the finding that haematoxylin, structurally identical to brazilin but containing an extra phenolic hydroxyl group in ortho-position to both an ether linkage and a second phenolic hydroxyl, gives a methoxyl value just below that of brazilin after methylation with diazomethane.

(4) Black wattle tannins contain 4.1 reactive hydroxyl groups, of which 3.6 methylate with ease in the presence of diazomethane over 24 hours. The methoxyl value attained is the same as

with catechin containing 4 phenolic groups, and the equivalent of one methoxyl more than brazilin with 3 phenolic groups.

Assuming the absence of strongly polar groups capable of conferring abnormal reactivity on aliphatic hydroxyls, it appears that all the reactive hydroxyl groups in black wattle tannins are phenolic in character. The known presence of ortho-hydroxy groups in the tannins is probably responsible for the incomplete methylation with diazomethane over a 24 hour period. This finding is in conflict with previously-held notions regarding the constitution of the tannins, and appears to cast some doubt on the presence of catechin bodies in the phenolic fraction of the extract.

Alternatively, it might be possible that aliphatic hydroxyl groups, capable of some reactivity with diazomethane due to the proximity of polar groups, are present in the tannins. This is considered unlikely because of the observations listed below.

(5) The apparent completely phenolic character of all active hydroxy groupings is supported by the observations :

(a) that tannins in methanol solution during the reaction with dimethyl sulphate and alkali, still exhibit colour changes between acidity and alkalinity when nearly fully methylated.

(b) that part of the nearly fully methylated product is irreversibly absorbed on an alumina column, as are other large phenolic bodies and tannins. This is accompanied by a reduction in colour as well as an increase in methoxyl value, and

(c) that full methylation of the tannins is essential

for obtaining high yields of veratric and O-trimethylgallic acids, while a slight decrease in methoxyl value causes low yields of these acids on oxidation.

All these observations emphasise that in the final stages of methylation with dimethyl sulphate, phenolic hydroxyls are still being satisfied.

The Action of Diazomethane on Tannins in Acetone Solution.

Black wattle tannins are insoluble in completely anhydrous acetone, but dissolve in the presence of a trace of water. The tannins, in equilibrium with atmospheric moisture, were thus dissolved in dry acetone instead of methanol, and the reaction performed as above. The course of the reaction and the product, appeared similar to that previously observed. % Methoxyl = 31.20 and 31.02. Acetone is known to react with diazomethane but requires the presence of a fair amount of water as catalyst (255). The presence of metharol, known to have a pronounced effect in some instances (274), appears to have no special influence on the reaction between diazomethane and black wattle tannins.

The Action of Diazomethane on Acetylated Tannin.

Diazomethane in the presence of water is said to be capable of "displacing" the acetyl group of an acetylated phenol (268), but the direct reaction with the phenol itself is more complete than with the acetylated derivative (275). Nierenstein (276) used diazomethane with piperidine as base to convert the

acetyl derivatives of protocatechuic acid and β-resorcylic acid to their respective methyl ethers. He proposed extending this method to acetylated catechin, but such work was not reported in the literature. Lowitt (159) claimed that the acetyl derivatives of alcohols were not methylated by diazomethane/piperidine in ethereal solution, and it was thought that this reaction would, therefore, be excellent for distinguishing between aliphatic and phenolic hydroxyls in black wattle tannins.

2 grms. fully acetylated tannins (37.5% acetyl) were thus treated with excess diazomethane in methanol-ether solution as before, but also with the addition of 8 grms. piperidine. The reaction was vigorous but slowed down rapidly. After standing for 64 hours at 0°C., the product contained 26.73% methoxyl and 2.32% acetyl groupings. A further 48 hours' reaction with fresh diazomethane and piperidine altered these values to 27.85% methoxyl and 0.58% acetyl. All the hydroxyl groups in the tannins are not satisfied here, as the product could be methylated to 29.6% with diazomethane only, and Lowitt in previous work, substituted the full total of 4.1 hydroxyls only by reacetylating this methylated product.

Recrystallised pyrogallol triacetate (51% acetyl) was also treated over a 3 day period with diazomethane and piperidine. Removal of the piperidine was difficult and required fairly drastic heating. The product contained 19.83% methoxyl and 0.4% acetyl groupings. Herzig (277) recorded that the direct reaction between triacetyl-pyrogallol and diazomethane over 48 hours gave a

product of 10.4% methoxyl content.

Hydrolysis of the acetyl group by piperidine therefore seems to be the primary, and methylation with diazomethane the secondary step in this reaction. There is thus apparently no "replacement" of acetyl by methoxyl groupings. The presence of piperidine appears to have an inhibiting effect, as in its absence methylation with diazomethane proceeds further. Such inhibition is not surprising as piperidine easily forms labile compounds with catechol, pyrogallol and tannins (278). The methylation of acetylated derivatives with diazomethane under these conditions seems to have no advantage over the direct reaction, and no differentiation between phenolic and aliphatic hydroxyl groups could be achieved by this method.

Methylations with Methyl Iodide and Sodium Methoxide.

The phenolic groups in brazilin (279) were methylated with sodium methoxide and methyl iodide without affecting the alcoholic hydroxyl group. This reaction did not, however, go to completion, since a proportion of partly methylated material was removed before isolating the trimethoxy-brazilin. These reagents were also used with myricetin (280), and were applied here as a possible method for studying the acidic nature of the hydroxy-groupings in the tannin.

The reaction-conditions for brazilin were repeated with black wattle, using slightly more than the required molar quantities. The product was poured into acidified water with stirring,

when a yellowish amorphous precipitate formed. Analysis : 22.01% methoxyl. The process was repeated twice on the above product, increasing the methoxyl value to 29.22%. 30.40% methoxyl was attained after a fourth methylation process. The latter product was adsorbed on to an alumina column from benzol and eluted with benzol containing 5% ethanol. A small amount of material remained on the column and the methoxyl content was raised to 32.40%.

The initially rapid, but overall slow methylation of the tannin with CH_3I/MeONa reagent is possibly the result of (a) the polymerised nature of the material and (b) the relatively high acidity of one, but abnormally low acidity of the majority, of the hydroxyl groups, as evidenced by potentiometric and conductimetric titrations. The presence of aliphatic hydroxyl groups could not be established in this way, and the slow but continuous increase of methoxy groups might point to their absence.

Conclusion

The reaction of diazomethane with black wattle tannins has been shown to be an easy, rapid reaction, yielding a white unoxidised product free from nitrogen. It is superior to all other methylating agents with the exception of dimethyl sulphate, and has many advantages over the latter.

The extent of this reaction when compared with a variety of compounds, has established the predominance of phenolic and virtual absence of aliphatic hydroxyl groups of the type present in catechin and brazilin. The presence of ortho-hydroxy phenolic

groups in the tannins appears responsible for the difficulty of the last stages of methylation, both with dimethyl sulphate and diazomethane.

No differentiation between aliphatic and phenolic hydroxyl groups is obtained by Nierenstein's "replacement" reaction of acetyl with methoxyl groups using diazomethane, as well as by methylations with methyl iodide and sodium methoxide.

(vi) A Study of the Ortho-Hydroxy Groups and the Colorimetric Behaviour of Black Wattle Tannins

An assumed relationship has always existed between the phlobatannins and true catechins. This assumption is based chiefly on their close association in many plants; on their similarity in degradative products and qualitative colour reactions; on the facile conversion of crystalline catechins into amorphous products possessing tannins properties; and on the ability of both to form insoluble red phlobaphenes in the presence of mineral acids.

Various structures and many condensation mechanisms have been proposed for the phlobatannins, but in all cases, apart from ring opening, the intact C_{15} unit has always been visualised as the building stone for these amorphous substances.

Black wattle tannins appear to contain no such free catechin "precursors", but yield degradation products typical of the 2-phenylbenzpyrane class of compounds on alkali fusion and permanganate oxidation, and resemble catechins in the formation of insoluble red phlobaphenes. Combustion analysis has also shown

the tannins to be a multiple or submultiple of the formula $C_{15}H_{12}O_6$, and Stephen, Williams, Kirby and the author all calculated their respective analytical figures to a C_{15} basis.

In the present investigation the application of a physical method demonstrated for the first time that some justification exists for the assumed relationship of black wattle tannins with the $C_6-C_3-C_6$ class of compounds. In addition it sheds further light on the nature of this important tanning material and provides the basis for a rapid method of tannin estimation. (See Chapter VIII).

Some Colour Reactions of Black Wattle Tannins

Pyrogallol, catechol and resorcinol nuclei are present in black wattle tannins. The colour reactions of the parent material should thus furnish important evidence as to the state of combination of these basic units, and Russel (136) has already listed many of these. The ferric chloride reactions, which supply much information, have been repeated on authentic commercial and fresh bark extracts, and others added to the already long list. (Table XLV).

Alcoholic ferric chloride (0.1 grm. phenol in 20 ml. absolute ethanol - 1 drop aqueous ferric chloride) seems to be the most discerning of these reagents, since it gives no colour with resorcinol, orcinol or phloroglucinol, and the colour developed with the tannins is characteristic of pyrogallol, but different from catechol. These solutions were examined spectrophotometrically

TABLE XIV

Reagent	Colour Produced by			
	Black Wattle Tannin	Pyrogallol	Catechol	Resorcinol
Aqueous $FeCl_3$	Blue-black followed by ppt.	Blue-black	Green	Violet
Alcoholic $FeCl_3$ (136)	Clear blue-black	Blue-black	Green	No colour
$FeCl_3$ - $NaHCO_3$ (small amount)	Ultra-marine	Ultra-marine	Ultra-marine	No colour
$FeCl_3$ - $NaHCO_3$ (excess)	Red	Red	Red	No colour
$FeCl_3$ - Na_2CO_3	Red	Red	Red	No colour
Ferrous Tartrate (281)	Violet-blue	Violet-blue	Green	No colour
Ferrous Tartrate - NH_4Acetate(281)	Violet-blue	Violet-blue	Violet blue	No colour
Phthalic Anhydride - $ZnCl_2$(121)	weak fluorescence	weak fluorescence	None	Very strong fluorescence
Osmic Acid (206)	Blue-black	Blue-black	Blue-Black	No colour
Glyoxalic Acid (282)	Brown ppt.	Light-blue	Violet	Blue-black

(Fig. XXXIII). The tannin and pyrogallol complexes exhibited similar maxima at 600 mμ, whilst that of catechol occurred at 700 mμ.

Although this difference exists in acid solution, ferrous and ferric complexes with pyrogallol, catechol, catechin and gallic

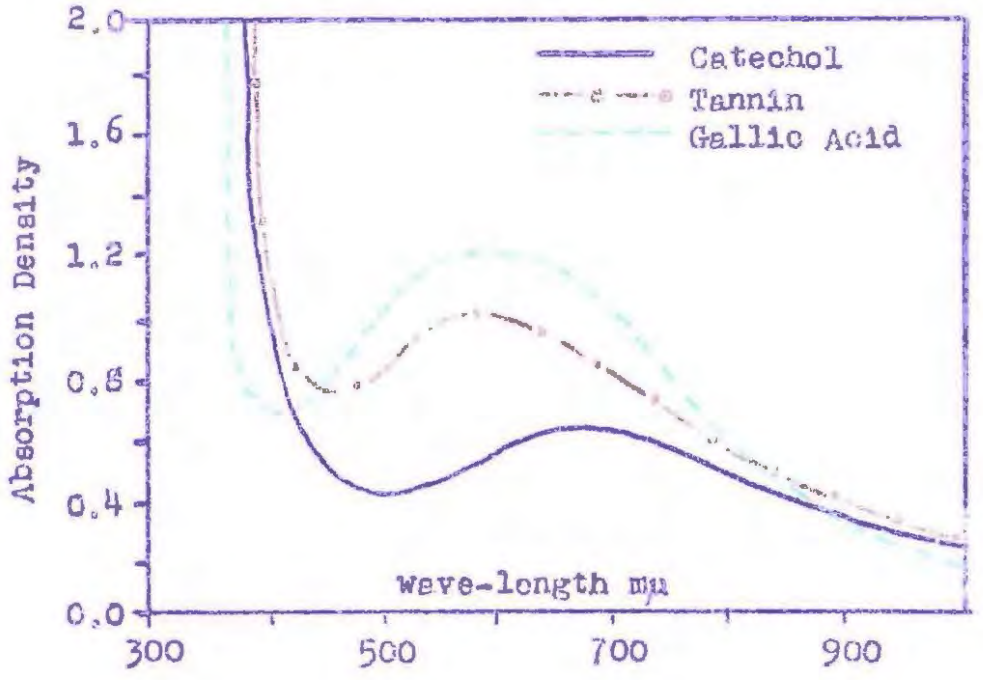


Fig. XXXIII. Alcoholic Ferric Chloride-Phenolic Complexes

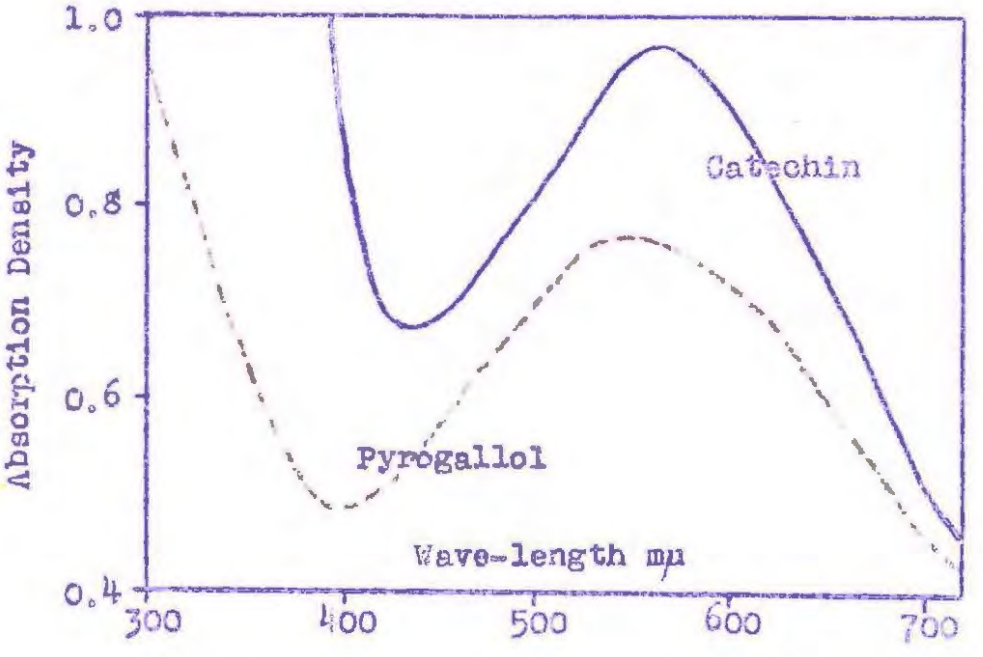


Fig. XXXIV. Ferric Complex in Alkaline Solution.

acid (e.g. orthodihydroxy- and orthotrihydroxy-phenols) and also black wattle tannin, in neutral and alkaline solutions give similar colorations (Table XIV), and maxima at the same wavelength (Figs. XXXIV and XXXV).

This indicates that only two hydroxy groups in ortho position are essential for complex formation with ferrous and ferric salts, and spatial considerations make it very improbable for all three vicinal hydroxyls of the pyrogallol type to be involved simultaneously. Osmic acid (206) also forms the same blue colorations having identical absorption maxima (545 mμ) both with pyrogallol and catechol. Silbermann and Ozorovitz (283) considered only two hydroxyls to be involved in coordinate links in ferrigallic links both in acid and alkaline solutions. Zetzche and Loosli (284) showed that one part of ferric iron combines with one part of both orthodihydroxy- and orthotrihydroxy-phenols, although they erroneously believed all three hydroxyl groups to be involved in coordination in the latter case. With protocatechuic and gallic acids they even included the carboxylic group in complex formation (285) although these compounds produce similar colorations to catechol and pyrogallol.

Complexes formed with orthodihydroxybenzenes are similar to the anionic "ato" complexes formed by dibasic oxy-acids (286). The orthohydroxy chelate groups, joined through two atoms to the central ferrous or ferric atom and so forming a ring, give complexes of exceptional stability. Thus catechol is capable of forming complex anions with 27 metals. Mono-, di- and

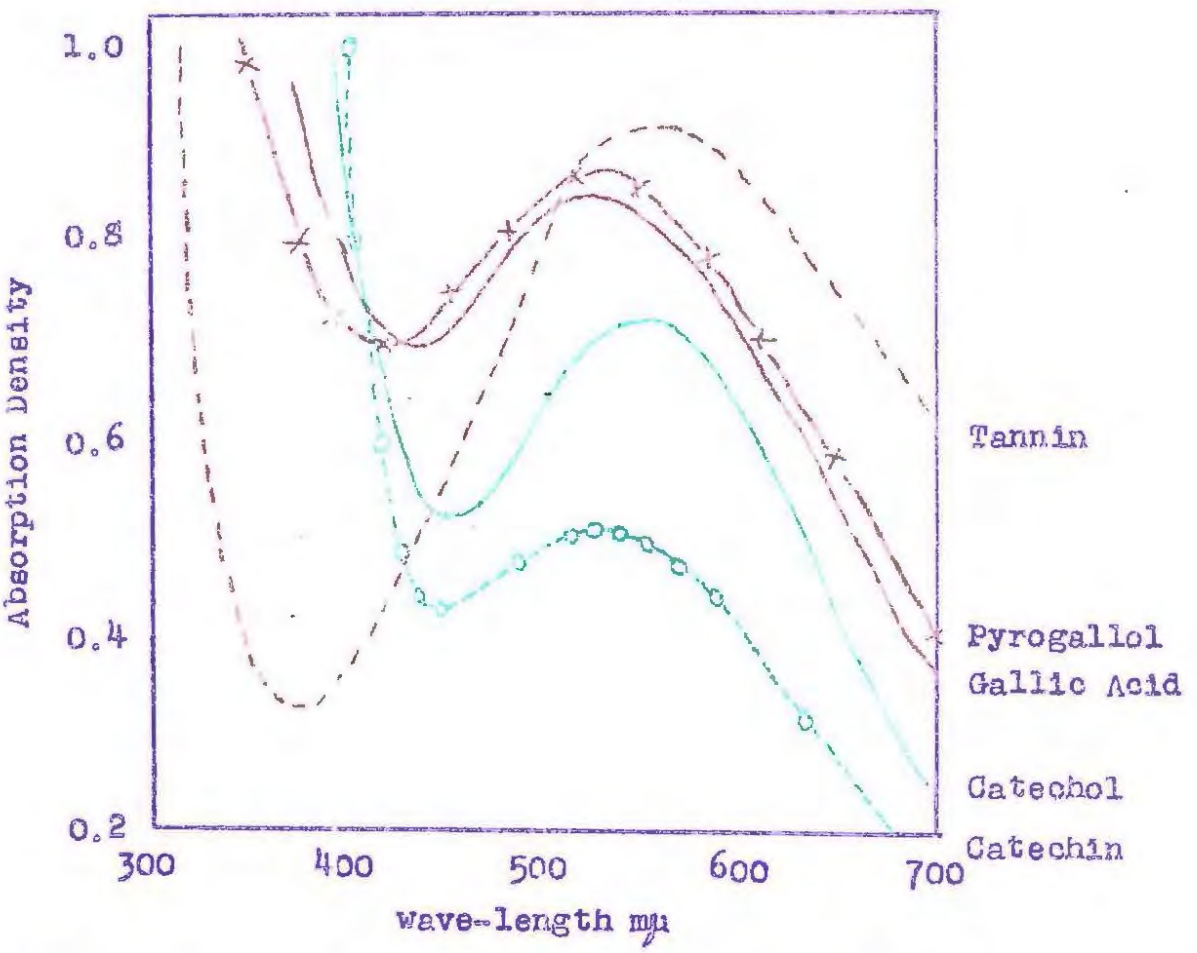


Fig. XXXV. Ferrous Tartrate-Phenolic Complexes.

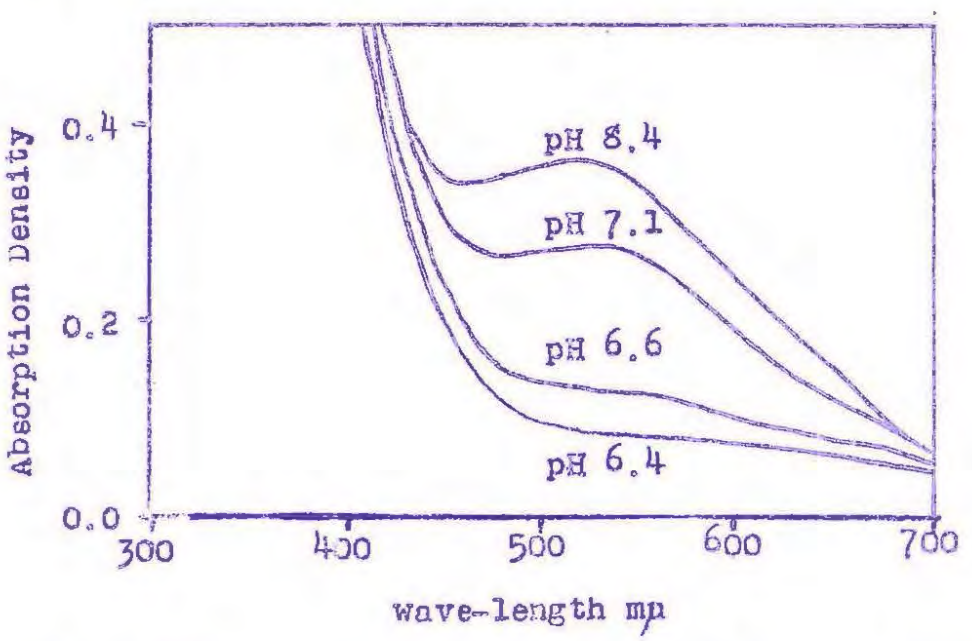


Fig. XXXVI. Catechol-Ferrous Tartrate Complex.

tri-hydroxyphenols containing no orthohydroxy groups, e.g. phenol, resorcinol and phloroglucinol, also form colorations with ferric chloride in acid solution. As they are weakly proton-donating with only one hydroxyl coordinating, the complex is easily broken in the presence of alcohol or weak alkali (Table XIV). A parallel is found with oxalic and other dibasic acids which form more numerous and more stable complexes than the mono-carboxylic acids such as acetic acid.

The Jablonski test (121) for quebracho and mimosa gives a very weak fluorescence with pyrogallol but not with its derivatives, e.g. gallic acid. The fluorescence developed by black wattle tannin is also very weak, comparable to that developed by morin, and it is possible that some resorcinol nuclei linked by carbon chain only, exist in the tannins.

These qualitative colour reactions, therefore, only indicate the certain existence of "free" pyrogallol nuclei. The ferrous tartrate reagent used below gives no colour with resorcinol, orcinol or morin, and the colour developed with the tannins can only result from the coordination of orthodihydroxy or ortho-trihydroxy groups attached to the tannin residues.

Development of the Colorimetric Method.

Mitchell (281) devised a colorimetric method for the quantitative comparison of pyrogallol and gallic acid, which depends on the formation of a blue-violet solution on addition of a ferrous tartrate reagent to a dilute solution of these phenols.

The chromogenic group appeared to be the three vicinal hydroxyls, and the intensity of colour developed was apparently the same for one equivalent of the pyrogallol nucleus independent of its state of combination. Mitchell attempted its application to gallotannin, but obtained variable results due to the non-homogeneity of the tanning material. He claimed that the colour developed was specific for the pyrogallic grouping, but Price (287) showed that two hydroxyls in ortho-position were sufficient for the formation of the same blue-violet coloration. Accordingly, Price attempted the extension of Mitchell's colorimetric method to catechol derivatives, but found that although it was satisfactory for the comparison of catechol with catechol, protocatechuic acid with protocatechuic acid and catechin with catechin, the colorimetric ratios obtained when comparing these substances with each other were unsatisfactory.

The main criticism of this work was that the hydrogen-ion concentration was not taken into account, especially as the violet colour of Mitchell's reagent was discharged to a light green on reducing, and converted to a pronounced orange on raising the pH. Glasstone (288) showed the vital importance of pH control in this colorimetric method. By varying the pH of various pyrogallol and catechol derivatives in the presence of ferrous tartrate reagent he found that in all cases the violet colour developed over a variable and limited range, and that the maximum colour development occurred over the very narrow range pH 7.5 - 8.0. With this strict pH control a molecular colori-

metric relation is completely valid, and it has been so repeatedly confirmed that it was regarded as incontrovertible (289). From Price's work Glasstone showed that even without pH adjustment the colour developed by 0.1% protocatechuic acid and catechin solutions was inversely proportional to their molecular weights.

Preliminary Spectrophotometric Examination of Ferrous Complexes

In afore-mentioned work Nessler tubes were employed, but in this investigation a Beckman Model DU spectrophotometer was used to examine the complexes and to measure the colour-densities developed. To establish whether orthodihydroxy- and orthotrihydroxy-phenols form similar complexes with ferrous tartrate, dilute solutions of these compounds were treated with Mitchell's reagent and ammonium acetate buffer, and the similar blue-violet colorations obtained examined over the range 300 - 1000 mμ. The resulting curves all show absorption maxima in the region 545 mμ irrespective of concentration, and go to complete extinction just below 300 mμ (Fig. XXXV). Catechin and black wattle tannins behave similarly.

The above solutions were all of pH = 6.6 - 6.8 and subsequent adjustment to pH 8.0 - 8.3 with dilute ammonia showed no effect on the maxima or shapes of the curves of tannins, pyrogallol and gallic acid, but caused a striking difference in the case of catechol (Fig. XXVI) and catechin. (See Figs. XXXVIII - XL).

In the above curves (Fig. XXXV) the absorption maxima at 545 mμ are most sensitive to alterations in concentration, and

all subsequent absorption density measurements were thus taken at this wavelength. As the previous work of Glasstone had shown that the colour-density varied with pH, and as the catechol complex was obviously less stable than that of pyrogallol, measurements were taken over a wide pH range. The complexes obey Beer's law over a limited concentration-range. (See Chapter VIII).

The ferrous tartrate reagent used contained 1 grm. A.R. ferrous sulphate and 5 grms. C.P. Rochelle salt per litre. This was always freshly made up and used immediately. The lead-salt purified tannins were dried at 110°C. for 2 hours in an Abderhalden, and the phenols over P_2O_5 in vacuum for at least 48 hours.

5 ml. of an exactly 0.1% phenol or tannin solution was treated with 25 ml. fresh ferrous tartrate reagent and diluted to 100 ml. The pH of this solution was usually about 5.6, showing a light green colour with catechol derivatives, and a blue colour with black wattle tannin and all pyrogallol derivatives. This procedure was now repeated, diluting with water first to 80 ml. then adding 1, 2, 3, 5 and 10 ml. amounts of 10% ammonium acetate buffer, and finally diluting to 100 ml. The pH values of these solutions corresponded approximately to 5.9, 6.0, 6.1, 6.2 and 6.4 respectively and showed an increase in absorption density with pH at 545 mμ. Similarly the pH was raised to 8.4 by addition of dilute ammonia to the ammonium acetate buffered (10 ml. of 10% solution) solution before finally diluting to 100 ml., and the absorption densities determined in each case. Beyond this point a reddish colour developed resulting in a slow reduction of the

absorption density.

The resulting curves (Fig. XXXVII) re-emphasise the similarity between black wattle tannins and pyrogallol previously found qualitatively with alcoholic ferric chloride. Assuming therefore, a molecular weight of 288 for black wattle tannin from the empirical formula, the molar extinction coefficients may be calculated from the formula

$$\epsilon = \frac{D}{c \times d}$$

Where D = absorption density

C = concentration in moles/litre

and d = thickness of the cell in cms.

TABLE XV

	ϵ at 545 mμ.	
	pH = 6.5	pH = 8.3
Black Wattle Tannins	2,370	2,428
Pyrogallol	2,370	2,342
Catechol	602	1,661
3-Ethoxypyrogallol	708	1,638

In the ferrous-catechol complex the coordination between the orthohydroxyls and the ferrous atom is weaker than in those of the pyrogallol and tannins, and a relatively high pH is required

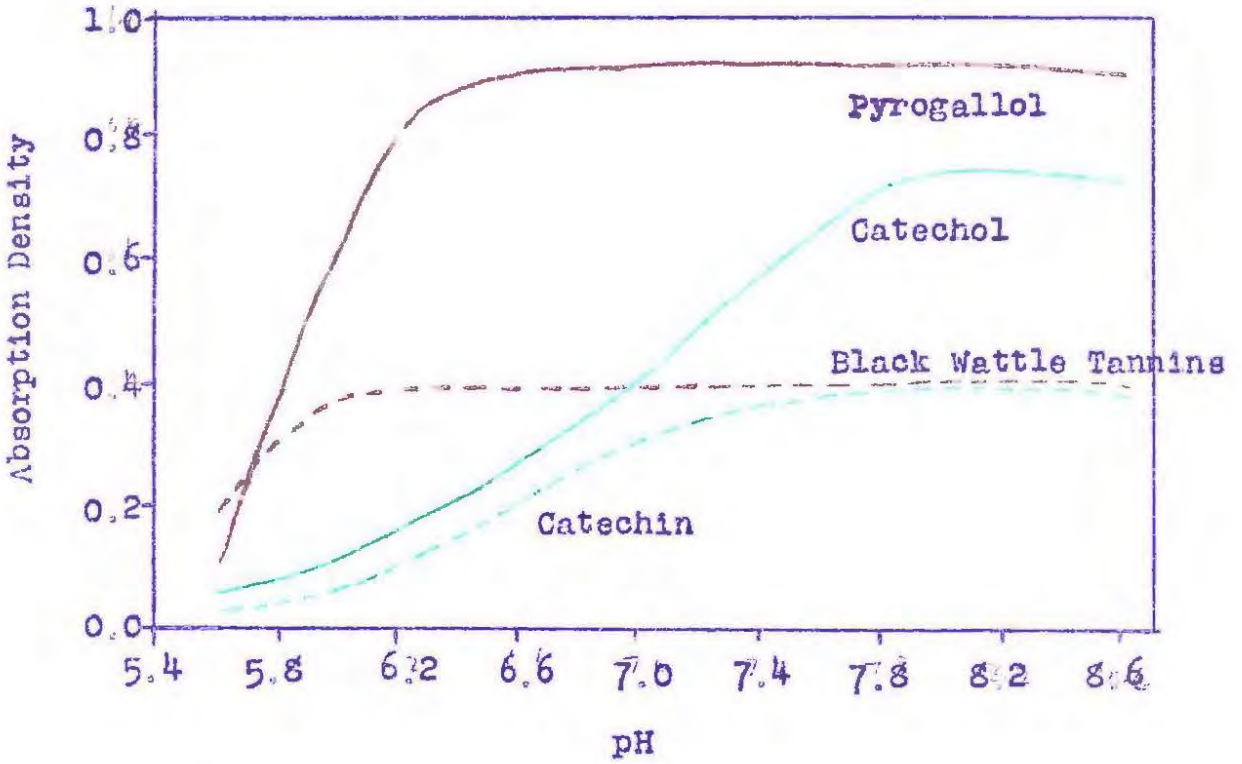


Fig. XXXVII. Variation of Absorption Density with pH at 545 mμ.

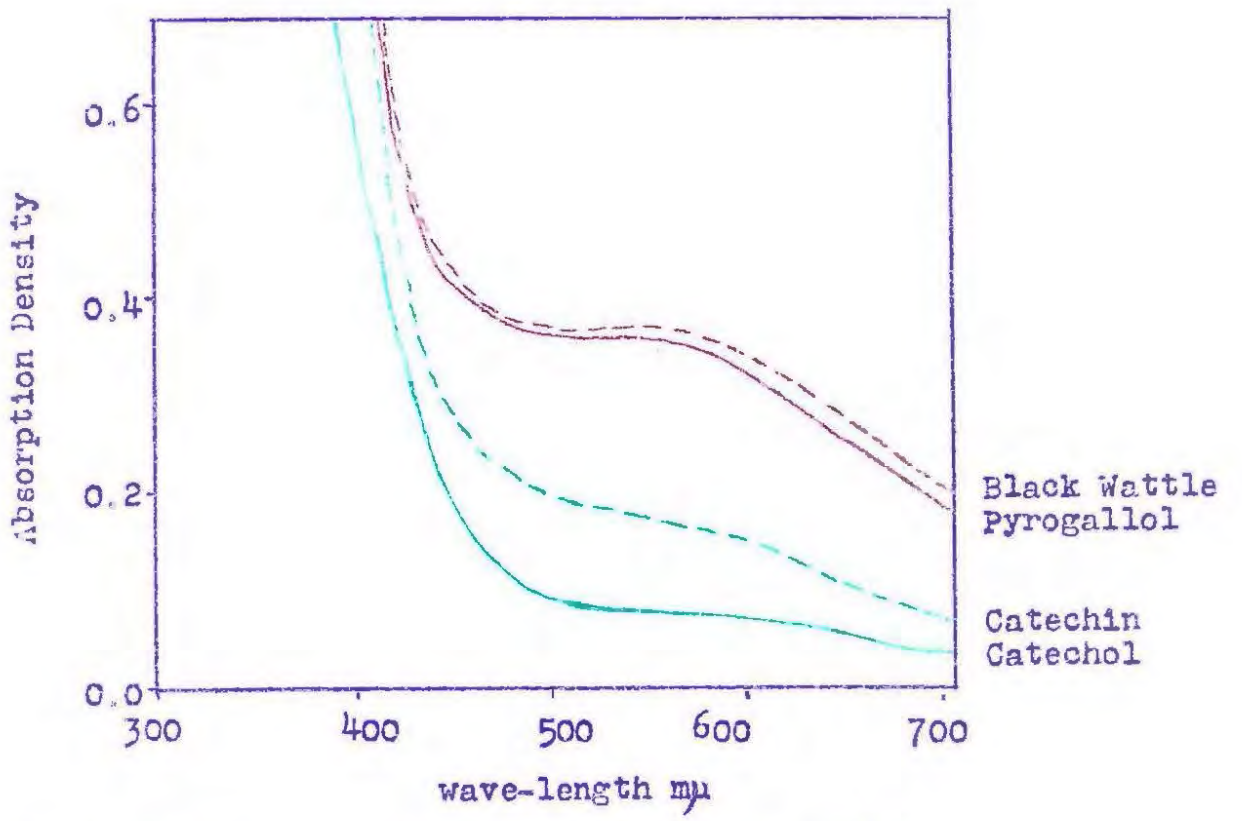


Fig. XXXVIII. Ferrous Complexes at pH 6.4.

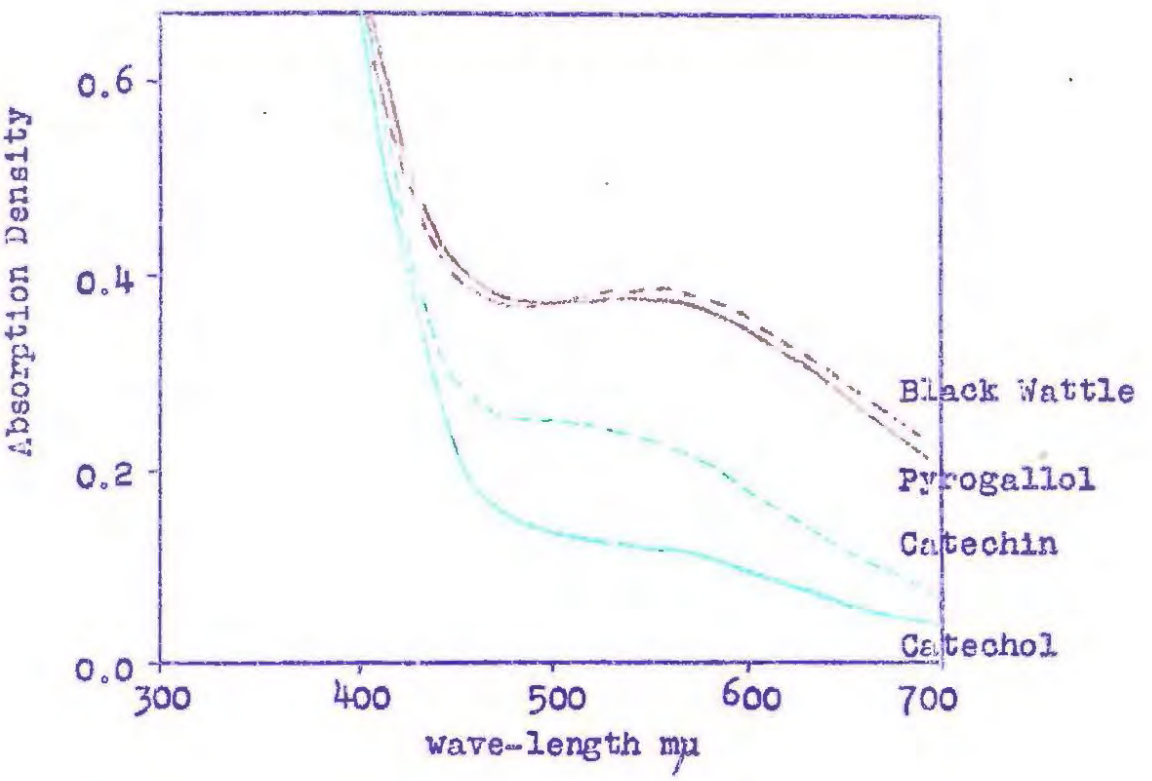


Fig. XXXIX. Ferrous Complexes at pH 6.6.

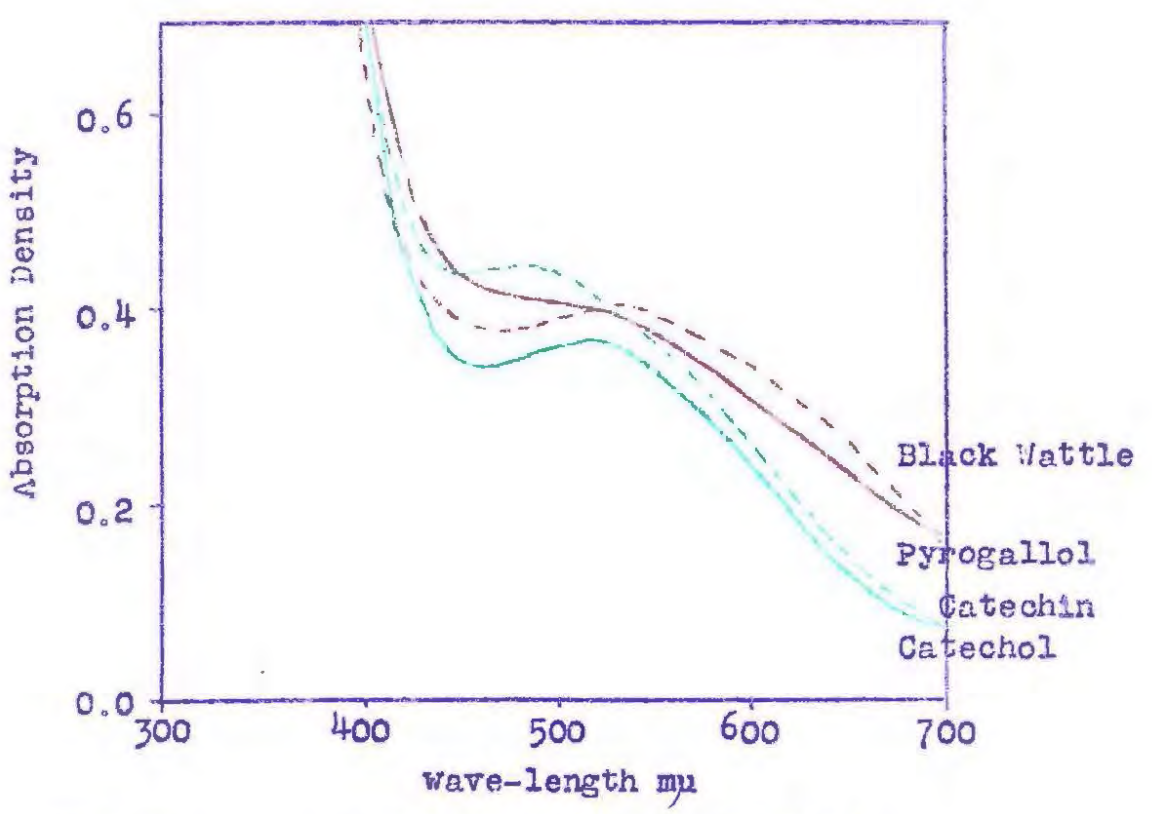


Fig. XL. Ferrous Complexes at pH 8.4.

for complete coordination. Catechin, 3-methoxypyrogallol and 3-ethoxypyrogallol all behave in the same way as catechol, whilst o-trihydroxyphenols behave as pyrogallol. Not only do black wattle tannins and pyrogallol behave similarly over the whole range pH = 5.6 - 8.4 but their molar extinction coefficients correspond remarkably well when assuming the empirical formula $C_{15}H_{12}O_6$ for the tannin. These results are reproducible and show little variation. The stability of the ferrous tartrate complex is discussed in Chapter VIII.

Comparative Spectrophotometric Examination of Pyrogallol, Tannin, Catechol and Catechin.

In the above work the colour-densities for pyrogallol and catechol were much higher than with catechin and the tannins, and although the complexes were found to obey Beer's law over a limited range, the concentrations of the former solutions were now reduced so as to furnish equivalent proportions of orthohydroxy groups. These compounds could thus be compared on a similar basis and many anomalous effects eliminated. 0.0379 grms. catechol, 0.0434 gms. pyrogallol and 0.10 grms. catechin and tannins were made up to 100 ml. as before. To 5 ml. phenol was added 50 ml. water, 5 ml. of 10% ammonium acetate buffer, 5 ml. ferrous tartrate reagent (0.5 grm. ferrous sulphate and 2.5 grms. Rochelle salt per 10 ml.) and the whole made up to 100 ml. This gave a solution of pH = 6.4, which was examined spectrophotometrically over the range 300 - 700 mμ. The above was repeated on solutions of pH = 6.6 (10 ml. buffer) and pH = 8.4 (10 ml.

buffer + dil. ammonia) as before.

The resulting curves (Figs. XXXVII, XXXIX and XL) again emphasise the similarity between the tannin and pyrogallol and the different colorimetric behaviour of catechin and catechol. Catechol and catechin show lower absorption densities at pH = 6.4 and 6.6 but attain similar values at pH = 8.4 and 545 mμ.

Confirmation of the colorimetric ratio between black wattle tannins and pyrogallol by comparison with some other o-trihydroxyphenolic bodies, is desirable. Suitable polyphenols should contain no strongly polar group which could influence the stability of the complex or the degree of coordination.

Casuarin, identified by Osima (200) as a d-gallocatechin was isolated from the bark of Casuarina equisetifolia for this purpose, but was found to contain an admixture of catechin by one- and two-dimensional chromatography. (See Chapter V). Due to the low yields of casuarin the separation of the two constituents on a silica gel by Bradfield and Penney's method (112)(113) was not feasible. The arrival of green tea is awaited for the separation of gallocatechins for this purpose.

Examination of Ferric Chloride as Chromogenic Reagent

The Rochelle salt in the above ferrous tartrate reagent probably acts as an efficient "masking agent" since it is used as such where ferric sulphate is employed as a tanning material under basic conditions (290). Ferric chloride normally causes the precipitation of tannins although it forms stable blue complexes

with catechin, pyrogallol and other orthodihydroxy compounds in alkaline solutions. An attempt was thus made to use it in the presence of a "masking agent". Although stable colourations were produced with catechin and pyrogallol with ferric tartrate reagent in the presence of the ammonium acetate buffer, a colloidal effect developed slowly in the presence of the tannin, and the method was discarded.

Discussion and Summary

(a) Pyrogallol, catechol and their derivatives form complexes with ferrous tartrate in neutral solution which are identical in colour and have absorption maxima at 545 mμ. Only two hydroxyls are therefore necessary for the formation of the complex. This is to be expected as the spatial arrangement of the hydroxyls on a pyrogallol unit would make it impossible for all three to be involved in complex formation. These complexes involving five-membered rings are similar to the anionic type formed by dibasic oxy-acids and are characterised by their exceptional stability. Other hydroxybenzenes are only weakly proton-donating and unstable and do not form complexes under the conditions of the experiment.

(b) The molar extinction coefficient at 545 mμ. for catechol is lower than that of pyrogallol over the pH range 5.6 - 8.4. This fact was also observed by Price (287) and Glasstone (288). The coordination in the catechol complex is thus weaker than in the

pyrogallol complex. Potentiometric (273) and conductimetric evidence (249) shows that although the stronger hydroxyls in pyrogallol and catechol titrate over nearly the same pH range, the second hydroxyl group of catechol is much weaker than hydroxyls of secondary strength in pyrogallol. The stronger coordination in the latter unit is thus probably due to the enhanced acidity conferred on two orthohydroxyls by the presence of a third in juxtaposition in the pyrogallol nucleus. These ideas were confirmed by a study of the 3-alkyl ethers of pyrogallol.

3-methoxypyrogallol was synthesised by the conversion of guaiacol to o-vanillin (291) and oxidising the latter with alkaline peroxide (292), as methyl iodide was not available for direct synthesis. The product was not quite pure even after lead-salt purification (293) and vacuum distillation. It gave a pH-absorption density curve similar to catechol, and at pH = 8.1 and 545 mμ ϵ = 1,554.

3-ethoxypyrogallol was easily synthesised from ethyl iodide and pyrogallol (293). The pH-absorption density curve was similar to that of catechol and 3-methoxypyrogallol. At pH = 8.2 and 545 mμ ϵ = 1,638. (For catechol ϵ = 1,661.)

The 3-alkyl ethers of pyrogallol thus behave similarly to catechol and give the same molar extinction coefficients at pH = 8.1 and 545 mμ.

(c) The molar extinction coefficients for pyrogallol and its derivatives at 545 mμ maintain an even maximum from pH = 6.4

to pH = 8.4. Those for catechol and its derivatives increase rapidly with increasing pH and only attain a maximum at pH = 7.9. This is probably due to the greater stability conferred by the strong coordination in the pyrogallol-ferrous complex as compared with the relatively weak coordination in the catechol complex. True pyrogallol and catechol nuclei cannot therefore be compared with each other at relatively low pH values. At higher pH values the absorption densities of pyrogallol and catechol derivatives are nearly identical (Fig. XL).

(d) **Black wattle tannins** and pyrogallol form ferrous complexes which behave identically over the range pH = 6.0 - 8.4 (Figs. XXXVIII - XL). No decline in absorption density over the range pH = 7.8-6.6 typical of catechol derivatives could be detected, although a low proportion of catechol nuclei are known to be present in the tannins from alkaline degradations, and the permanganate oxidations of the methylated tannins. The molar extinction coefficients of pyrogallol and black wattle tannins show agreement assuming a basic unit of $C_{15}H_{12}O_6$ based on the empirical formula. This may be a chance agreement, and further confirmation must be sought from gallocatechins.

(e) This colorimetric study shows that black wattle tannin contains a very high proportion of "free" pyrogallol nuclei, which are attached to the rest of the tannin by carbon chain only. It thus supports the evidence from the degradation study where the isolations of gallic acid (from alkaline fusion) and O-trimethyl-

gallic acid (from permanganate oxidation) in high yields, were obtained. The pyrogallol nuclei, therefore, appear to "free" not only after methylation but also in the tannin itself.

(f) This study provides an excellent basis for a colorimetric method of tannin analysis (See Chapter VIII).

(vii) Terminal Methyl Groups

For determining the presence of carbon-linked methyl groups the method of Barthel and Laforge (294) was applied. Although not a completely quantitative estimation, the result obtained depending on the position of the group, it gives a good qualitative indication of the presence or absence of such groups. The method was standardised against a variety of compounds.

Found :

Compound	No. of C-CH_3 groups per molecule	
Crotonic Acid	0.97	0.95
Orcinol	0.80	0.83
o-Cresol	0.88	0.84
Lead-salt purified tannin	0.08	
Chromatographed methylated tannin	0.08	
Morin	0.08	

For the tannin and its methylated derivative the empirical formulae corresponding to the molecular weights 290 and 346 were used for calculating the number of terminal methyl groups.

The above values are below those obtained by Kirby, who

found 2.1% terminal methyl groups in methylated tannins derived from the acetone extracted fraction of the commercial extract. As morin contains no terminal methyl groups but nevertheless gives a low value comparable to that obtained from the tannins, carbon-linked methyl groups may be assumed to be entirely absent.

(viii) Easily Reducible Ethylenic and Carbonyl Groups : a Hydrogenation Study

Useful evidence has been obtained from the hydrogenation of lignins by the isolation of propylcyclohexanol derivatives (295 - 299) from amongst the reaction-products resulting from drastic hydrogenation and hydrogenolysis. Stephen (149), therefore, attempted a similar degradation of acetone-salt "purified" black wattle tannins using water as solvent, and Raney nickel and copper chromite as catalysts, at high pressures and temperatures for prolonged periods. From large-scale hydrogenations he obtained much blackened and pyrogenetically decomposed material as well as good yields of clear oil. This oil obviously consisted of a mixture of constituents which could not be separated.

Silk (152) continued this work and showed that water was an unsuitable solvent as a large proportion of the hydrogenation and hydrogenolysis products were water-insoluble, and tended to char on the sides of the reaction-liner. The tannin appeared to lose its water-solubility after only slight hydrogenation. The selection of a solvent capable of dissolving both the tannin and its hydrogenation-products was obviously desirable. Silk used less drastic (lower temperature) conditions and showed from

molecular weight studies that Stephen was unjustified in using a C_{15} basis for the comparative analysis of the hydrogenation products, as the oils obtained fell into an average molecular range 114 to 216. Silk also found a variable average number of hydroxyl groups in the various fractions of the oil obtained from the fractional distillation of the whole product. No pure compound could be isolated from these fractions.

Both Stephen and Silk failed to separate identifiable compounds from the oils produced on drastic hydrogenation and hydrogenolysis. This was possibly largely due to the fact that neither investigator fractionated the oil through an efficient column (e.g. Widmer type (300), on which the successful separation of the propyl-cyclohexanol derivatives in the case of the lignins depended). The tannins, however, are known to consist of catechol, pyrogallol and resorcinol nuclei as compared with a single "basic" molecular type (e.g. phenylpropane) in most lignins. The dihydric and trihydric phenols have been shown by Fujita (301) to be reduced, during high temperature hydrogenations in the presence of a nickel catalyst, to a variety of mixtures consisting mainly of cyclohexane or cyclohexanol derivatives. Thus 100 moles of resorcinol forms a mixture of 47 moles of cyclohexanediol-1,3, 22 moles of cyclohexanol, 4 moles of m-hydroxy-cyclohexyl-phenylether, and leaving about 5% unreduced resorcinol. Catechol is converted to 74% cyclohexandiol-1,2 while 12% remains unchanged, and pyrogallol changes to a mixture of 55% cyclohexantriol-1,2,3, 21% cyclohexandiol and 5% unchanged pyrogallol. The hydrogenation

of a mixture of these three phenols under controlled conditions could, therefore, possibly result in a product containing at least nine compounds. The nature of the products obtained would depend on the solvents used (302); on variations in the mixture hydrogenated (303); on the catalysts employed; on the temperature and length of the reaction (304) as well as the rate of shaking (305). With phenols no stepwise reactions occur but a series of parallel independent reactions result, producing a wide variety of hydrogenation products (306). Hydrogenolysis of phenols also occurs easily and is difficult to control.

Because of the multiplicity of hydrogenation-products which are formed from single phenols (301)(307 - 309), coupled with the different hydrogenation rates of various compounds (310); their possible influence on each other (303) and the fact that hydrogenations will split carbon links (311)(312), it is obvious that the products formed from the tannins, even under controlled conditions, would be extremely complex. This appears to be confirmed by Silk's work. The degradative approach was abandoned on these grounds, and the behaviour of the tannins was rather observed at lower temperatures and pressures in order to determine whether easily hydrogenated carbonyl and ethylenic linkages were present.

From a study of chromones, flavones and anthocyanidins Mozingo and Adkins (313) concluded that with compounds containing several functional groups, rapid hydrogenation gives better yields of the chief product and fewer by-products due to side-reactions.

The reaction should be carried out at the lowest temperature at which rapid hydrogenation can be secured. That temperature at which the drop of pressure due to the reaction is just greater than the rise in pressure due to heating was considered most favourable. The hydrogenation of the tannins was carried out under these conditions. The increase of pressure with temperature was calculated from simple gas laws and deviations from predicted pressures were easily established by a plotting the course of the reaction on a graph. This approach was thus essentially different from that used by Silk and by Stephen.

Apparatus

The hydrogenation bomb used has already been described by Silk and by Stephen and was based on that of Adkins (314). The rocker and thermostatic control apparatus were found to be unsatisfactory and were reconstructed (Fig. XLI). The wooden rocker support was replaced by a rigid all-metal frame and the wiring of the heating-element was improved by passing the resistance wire through fire-clay beads held in one inch thick rings of Sindanyo boarding. A bimetallic spiral and a Sunvic relay system were used for accurate temperature control.

The bomb has an internal volume of 1.15 litres and is lined by a copper container with a screw-down lid. Absolute ethanol was chosen as solvent for all hydrogenations. These were carried out under conditions where no hydrogenolysis of the solvent was likely to occur. The solute and solvent occupied 0.15 litre,

Fig. XLI. The Assembled Hydrogenation Apparatus.

leaving an internal volume of 1.00 litre. During the hydrogenations of the methylated tannin the internal volume was reduced in order to obtain a more sensitive index of the absorption of hydrogen. This was brought about by reducing the copper liner to half its length and filling the remaining space with a steel cylinder which fitted accurately into the body of the bomb in order to avoid looseness during the rocking movement. (Fig. XLII). The total internal volume was thus reduced to 0.65 litres, leaving an effective volume of 0.50 litres after charging the bomb with the

Fig XLII. Reaction-Liner and Metal Cylinder Used for Reducing the Internal Volume of the Hydrogenation Bomb.

150 ml of solution. Initially much difficulty was experienced with hydrogen leaks past the copper gasket in the body of the bomb. This was overcome by substituting bolts of larger size and finer thread in the bomb-head. These could be tightened sufficiently without stripping the thread.

Selection of Method and Catalysts

The high-pressure hydrogenation with relatively inert catalysts was preferred to the use of active catalysts at one or

two atmospheres pressure, as more selective hydrogenation was possible with the former method.

(a) Copper Chromite Catalysts

Copper chromite is a more selective catalyst than Raney nickel, which was also used in this investigation. It reduces aldehydes and ketones quantitatively also in the proximity of phenolic nuclei, and does not attack phenolic nuclei at low temperatures (315). The presence of hydroxyl groups in benzene nuclei, however, aids hydrogenation (316). Aromatic ethylenic groups hydrogenate with greater difficulty than aliphatic ethylenics. Polyphenols, again, are more difficult to hydrogenate than phenol itself. Copper-chromium oxide catalysts are stabilised and also have their reactivity enhanced by the presence of barium, calcium and magnesium oxides (317).

Standardisation of Apparatus

0.15 litre of acetone was placed in the reaction-liner and the bomb-head screwed down securely. Hydrogen was admitted to a pressure of 1625 pounds per square inch at 15°C., and all the valves tightly closed. After slow heating over 8 hours with rocking, the temperature and pressure were raised to 190°C., and 2730 p.s.i., and the bomb allowed to cool to room temperature overnight. The pressure returned to 1615 p.s.i. at 14°C. The slight loss of pressure (10 p.s.i. at 15°C.) might be partly due to a thin film of rust formed on the inside of the bomb (318), and

was observed in almost all cases. The bomb thus retains the hydrogen satisfactorily up to a high temperature and pressure.

One-quarter mole acetone (14.5 grm.) was made up to 150 ml. with absolute ethanol, and 3.5 grms. copper chromite catalyst with BaO as promotor (315) was added. Hydrogen was admitted to a pressure of 1620 p.s.i. at 15°C. The increase of pressure with temperature was calculated from the simple gas equation, in order to obtain a rough indication of the predicted course of the reaction. Deviations from this straight line will occur mainly due to the fact that ethanol tends to vapourise from 180 - 200°C. upwards, even at high pressures. There is also a lag in the transfer of heat from the element to the thermometer, which is situated in a central well in the bomb.

Fig. XLIII shows the variation of pressure with temperature as well as the period elapsed during the reaction.

Hydrogenation of acetone thus occurs at 120°C. and 2175 p.s.i. The initial pressure was 1620 p.s.i. (15°C.), and the final pressure 1500 p.s.i. The absorption of $\frac{1}{4}$ mole of hydrogen corresponds to a drop of 120 p.s.i. at 15°C.

The Hydrogenation of Benzaldehyde

In order to test the activity of the catalyst and to determine the conditions under which aromatic aldehydes reduce, $\frac{1}{4}$ mole (26.5 grm.) benzaldehyde was hydrogenated as before (Fig. XLIV).

The initial pressure was 1650 p.s.i. at 18°C., and the

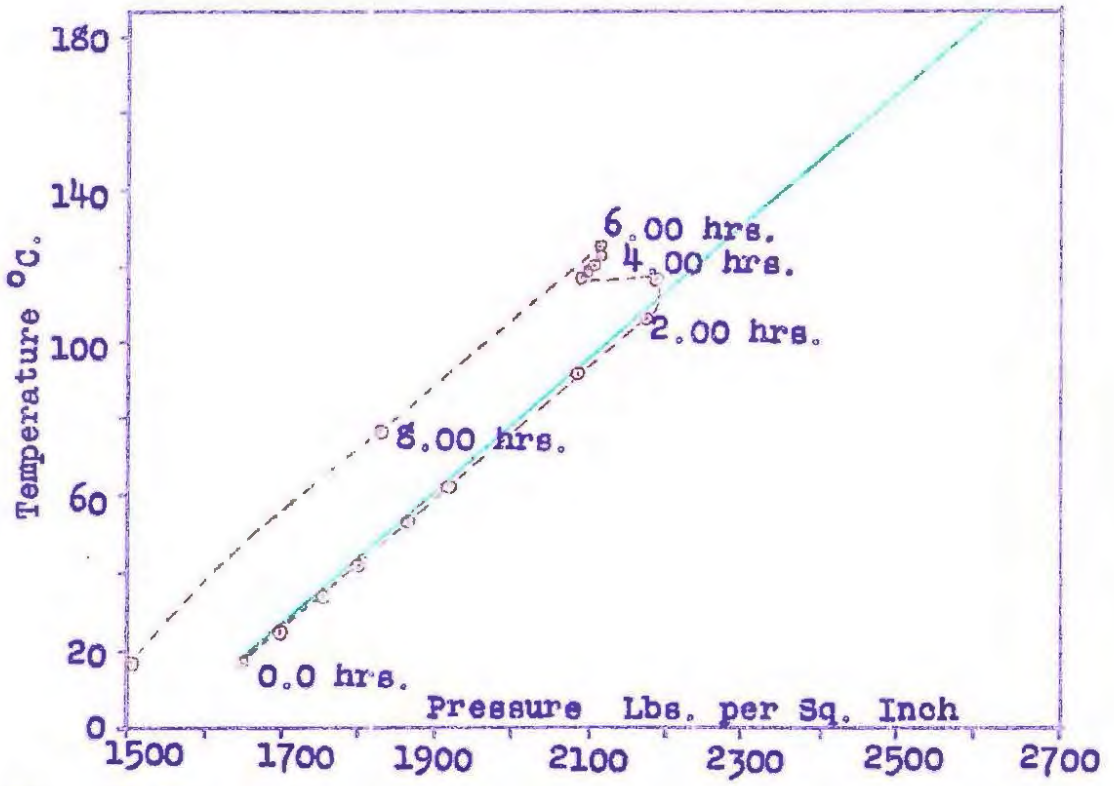


Fig. XLIII. The Hydrogenation of Acetone. Copper Chromite Catalyst.

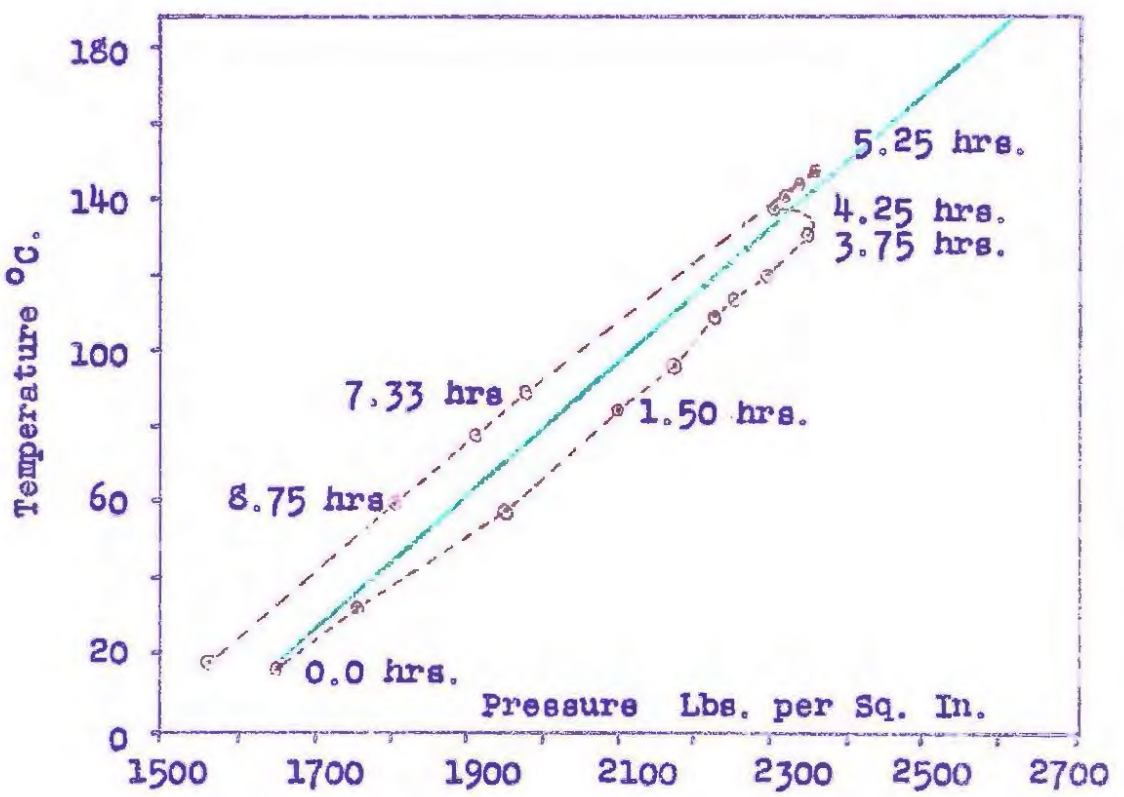


Fig. XLIV. The Hydrogenation of Benzaldehyde. Copper Chromite Catalyst (3.5 grm.)

final pressure 1560 p.s.i. at 18°C. Hydrogenation occurs easily at 133° - 138°C., and 2350 p.s.i. The product consisted of benzyl alcohol only.

The Hydrogenation of Resorcinol

In order to establish under what conditions phenolic nuclei, containing hydroxy groups, are stable in the presence of copper chromite catalysts, 1/8 mole (13.75 grm.) resorcinol was hydrogenated under the same conditions. The phenol was hydrogenated for an eight hour period with increase in temperature to 165°C., and 2350 p.s.i., and then cooled. The initial pressure was 1510 p.s.i. at 16°C., and the final pressure 1490 p.s.i. at 17°C. Assuming no leakage, a drop of 20 p.s.i. represents slight hydrogenation, possibly at the higher temperatures. This could not be gauged from a graphic plot.

As higher pressures would necessitate the use of a different gauge, the starting pressure was decreased to 1225 p.s.i. at 17°C., and the temperature slowly increased to a maximum of 215°C., at 2275 p.s.i. Hydrogenation appeared to commence at 160 - 165°C. (Fig. XLV). The final pressure was 1110 p.s.i. at 17°C. This corresponds to a drop of 115 p.s.i. at 17°C., or the absorption of 2½ equivalents of hydrogen. The product smelt strongly of cyclohexanol.

Hydrogenation of O-Dimethyl Resorcinol

The effect of methylation on the stability of resorcinol

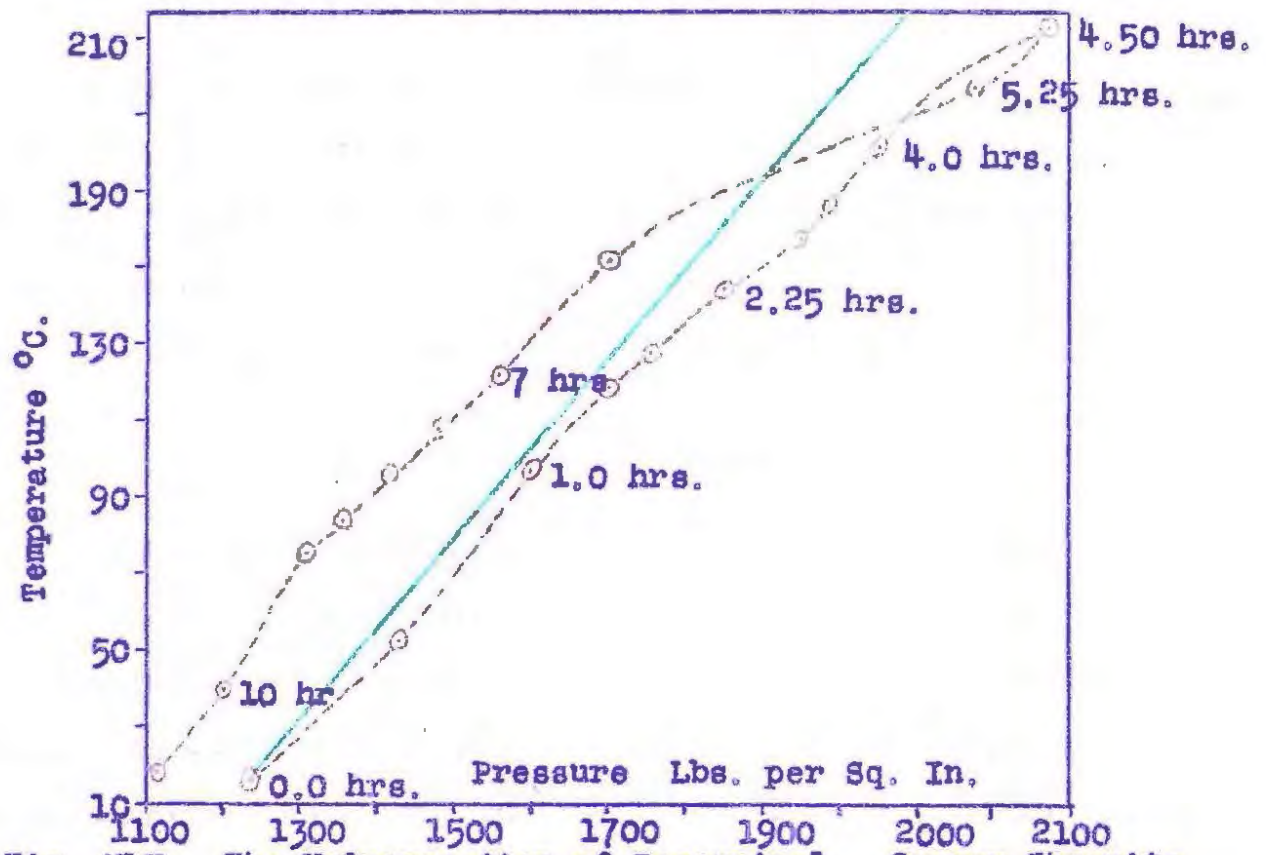


Fig. XLV. The Hydrogenation of Resorcinol. Copper Chromite Catalyst.

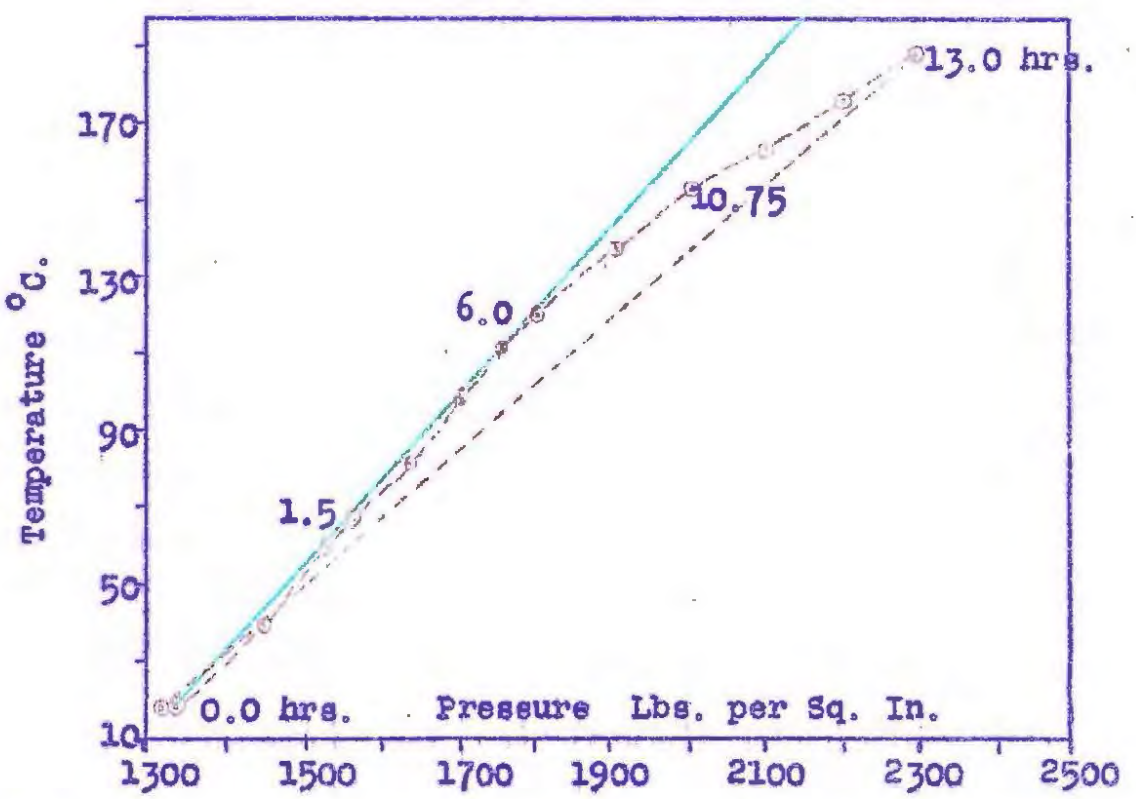


Fig. XLVI. The Hydrogenation of O-Dimethyl Resorcinol. Copper Chromite Catalyst.

was demonstrated by an identical hydrogenation on 1/8 mole O-dimethyl resorcinol (Fig. XLVI). The initial pressure was 1325 p.s.i., and the final pressure 1315 p.s.i. Methylation thus confers far greater stability on the resorcinol nucleus. Dimethyl resorcinol is stable up to 200°C.

The Hydrogenation of Methylated Tannin

Due to the relative instability of phenolic nuclei e.g. resorcinol, the tannin was methylated before hydrogenation. The hydrogenation curve of 1/10 th mole (35 grms.) methylated tannin followed the same course as that of dimethyl resorcinol. The temperature was raised to 185°C. at 2565 p.s.i. The initial pressure was 1540 p.s.i. and final pressure 1530 p.s.i. at 15°C. The product freed of catalyst was poured into water and sucked off. It was white compared with the pink colour of the starting-material.

Found :

% Methoxyl Before Hydrogenation = 31.08%

% Methoxyl After Hydrogenation = 30.77%

The methylated tannin, therefore, appears to be quite stable under conditions at which aldehyde and keto groups, and also alkene groups (320), are easily reduced in the presence of a copper chromite catalyst.

(b) Raney Nickel Catalyst

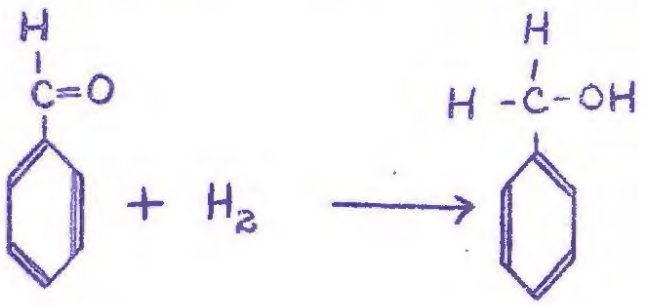
Confirmation of these results were sought using Raney

nickel catalyst which is far more active towards keto, aldehyde and ethylene groups, but less selective towards phenyl nuclei.

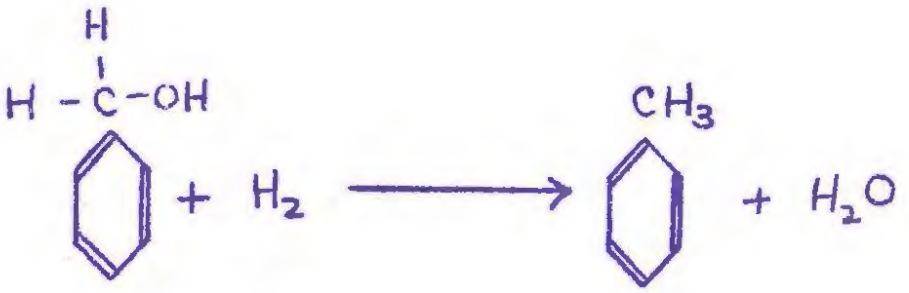
The catalyst was prepared according to the method of Mozingo (319), and stored under anhydrous ethanol. Hydrogenations were performed as before with ethanol as solvent, and fresh Raney nickel (3 grm.) substituted for copper chromite.

The Hydrogenation of Benzaldehyde

Hydrogenation commenced immediately with rocking, and increase of temperature. The reaction ran to completion at 88°C. and 1900 p.s.i. The bomb was allowed to cool to room temperature. The initial pressure was 1650 p.s.i. at 18°C., and the final pressure 1550 p.s.i. at 20°C. ¼ mole benzaldehyde thus absorbed one equivalent hydrogen in the reaction :



Heating was recommenced without opening the bomb, and above 130°C. and 2150 p.s.i. hydrogen absorption again occurred. The final pressure after cooling was 1380 p.s.i. at 16°C. Slightly more than one equivalent hydrogen was absorbed in the second stage. Only toluene from the hydrogenolysis-reaction :



was obtained in the reaction-product.

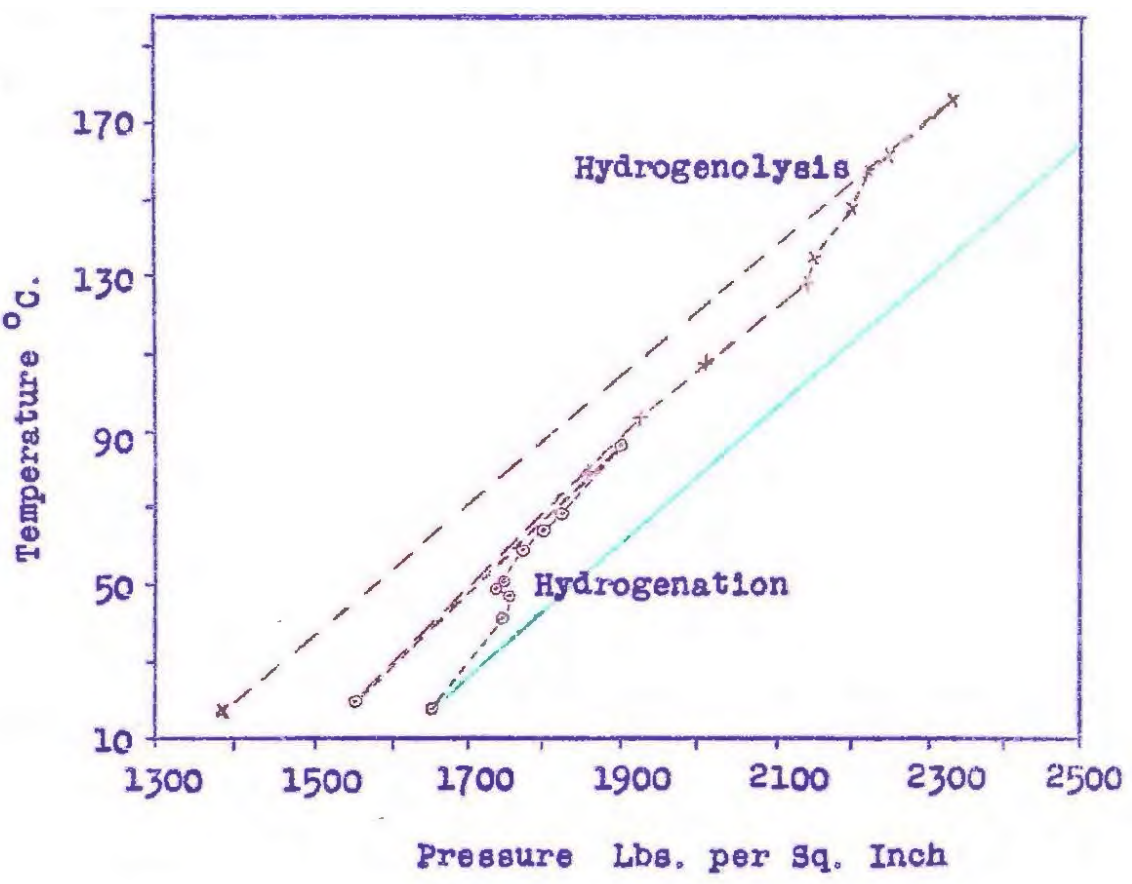


Fig. XLVII. The Hydrogenation and Hydrogenolysis of Benzaldehyde Raney Nickel Catalyst

Hydrogenation of Resorcinol

¼ mole (27.5 grm.) resorcinol was hydrogenated in the presence of Raney nickel catalyst. Hydrogenation commenced immediately and absorption continued over the nine-hour heating period. Three equivalents of hydrogen were absorbed during the reaction. After complete removal of the ethanol in vacuum the product set to a semi-crystalline solid.

Found : C = 61.48% H = 10.28%

Calc. for $C_6H_{12}O_2$: C = 62.05% H = 10.35%

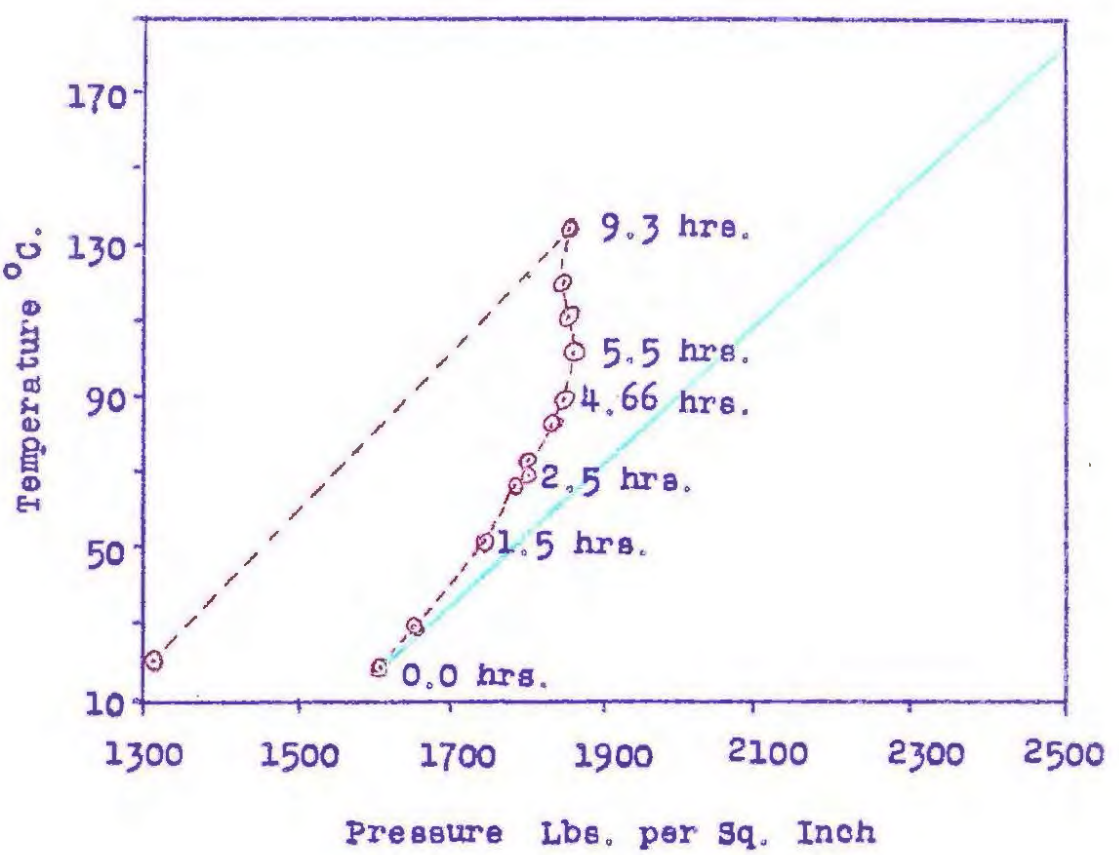


Fig. XLVIII. Hydrogenation of Resorcinol. Raney Nickel Catalyst.

The Hydrogenation of Acetone

By reducing the size of the liner and using a steel cylinder as filler the volume of the bomb was reduced from 1.15 litres to 0.65 litres. The bomb was then restandardised using Raney nickel catalyst. Hydrogenation commenced immediately (Fig. XLIX), and the absorption of $\frac{1}{4}$ mole hydrogen caused a drop in pressure of 225 p.s.i. at 18°C., or roughly about double the drop previously obtained before reducing the internal volume.

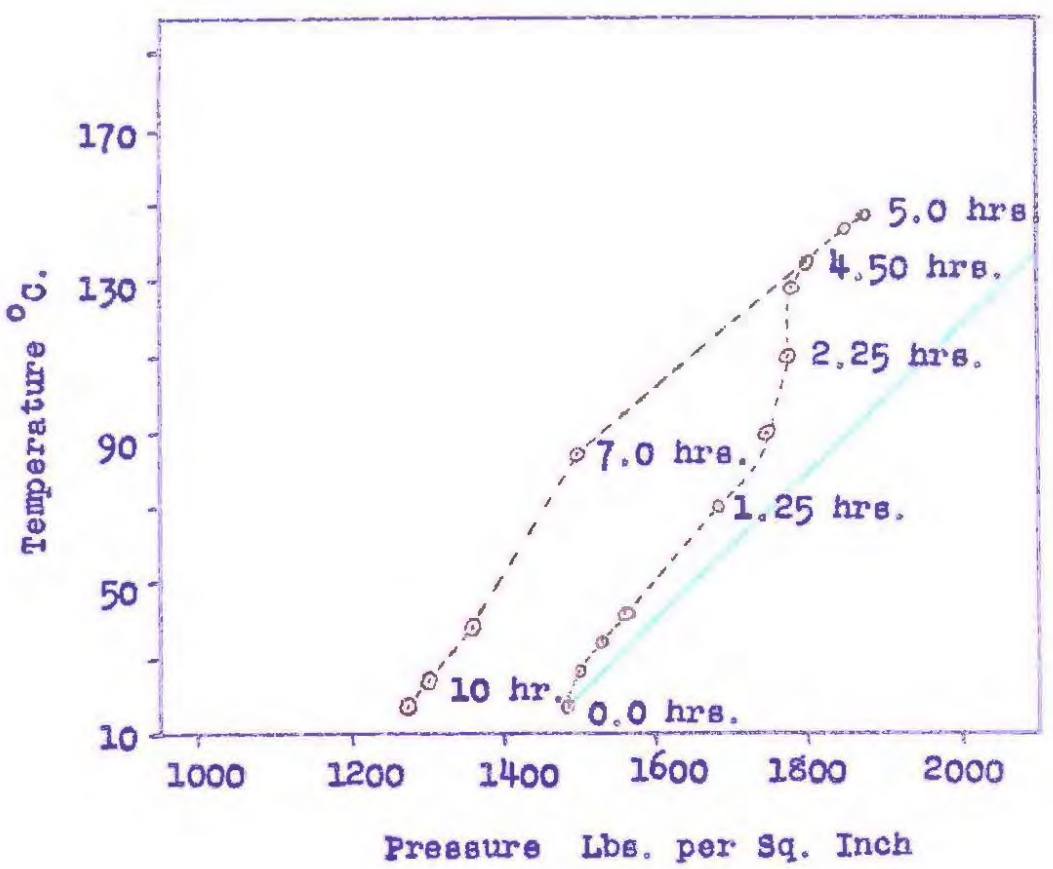


Fig. XLIX. The Hydrogenation of Acetone. Raney Nickel Catalyst.

The Hydrogenation of Methylated Tannin

35 grms. of methylated tannin (33% methoxyl) was treated as above. No immediate absorption of hydrogen took place but some slight reaction could have occurred above 120°C., and 2100 p.s.i. The initial pressure was 1560 p.s.i. at 20°C., and final pressure 1540 p.s.i. at 19°C.

The initial pressure was reduced to 1410 p.s.i. and hydrogenation repeated by increasing the temperature slowly to 181°C. and 2180 p.s.i. Final pressure 1390 p.s.i. at 20°C.(Fig.L).

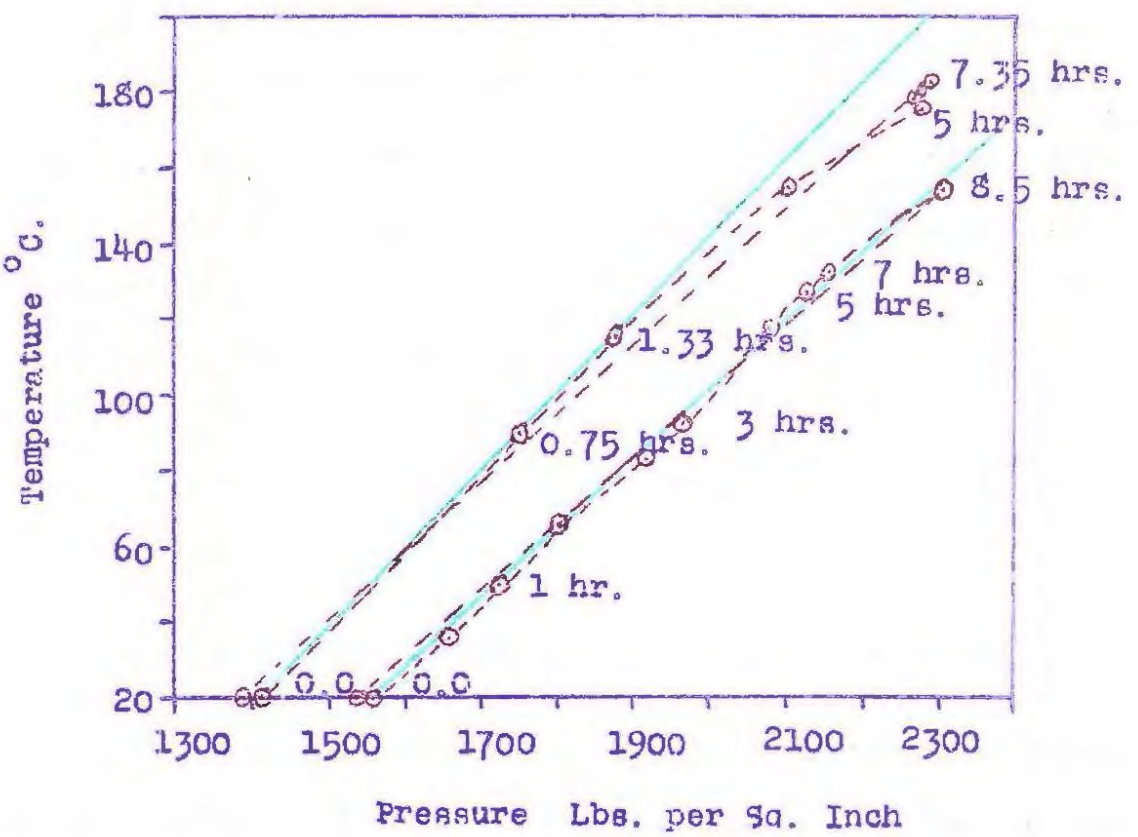


Fig. L. The Hydrogenation of Methylated Tannins. Raney Nickel Catalyst.

Found : Before hydrogenation :

% $-OCH_3$	= 33.07;	33.05
% C	= 64.22;	64.61
% H	= 5.79;	5.78

After hydrogenation :

% $-OCH_3$	= 31.47;	31.49	
% C	= 65.00;	64.22;	64.89
% H	= 6.54;	6.25;	6.31

Some reduction, accompanied by slight demethylation appears to have resulted at higher temperatures. The product was pure white in colour and a slight odour of cyclohexanol was evident.

A different sample of methylated tannin was kept at 200°C. (2300 p.s.i.) for 5 hours and the product subsequently remethylated. Some hydrogenation and hydrogenolysis appears to occur under these conditions. A far more pronounced cyclohexanol odour was evident.

Found : (after remethylation) :

% $-OCH_3$	= 32.45
% C	= 66.72
% H	= 7.19

Summary

A comparative hydrogenation study of various compounds using copper chromite and Raney nickel catalysts, and making use of a graphic method of following the course of the reaction, has shown that :

(a) Keto groups, e.g. acetone, are hydrogenated at 120°C. by copper chromite and at lower temperatures (15° - 120°C.) by Raney nickel catalysts.

(b) Aldehyde groups, e.g. benzaldehyde, are hydrogenated at 135°C. by copper chromite and at low temperatures, 18° - 88°C. by nickel catalysts.

(c) Ethylene groups are known to be reduced with ease by copper chromite (125° - 150°C. at 125 - 133 atm) and by nickel (20° - 100°C. at 25 - 110 atm) (320).

(d) Phenolic nuclei e.g. resorcinol, are relatively stable to hydrogenation in the presence of copper chromite. This selective action of copper chromite, which normally easily reduces ethylenic- and conjugated double-bonds, is well known (321)(322). Nickel catalysts, by contrast, easily hydrogenate resorcinol at low temperatures (20° - 135°C.) Methylation appears to confer additional stability on such phenolic nuclei.

(e) Although it is known that the degree of substitution alters the rate of hydrogenation (320), and that the nature of substituent groups may also modify hydrogenation reactions, methylated black wattle tannins were found to be non-reactive over the range in which keto, aldehyde and ethylenic groups are normally easily reduced in the presence of copper chromite and Raney nickel catalysts. Such easily-reducible groups, therefore, appear to be absent in the tannins.

(ix) Carbonyl Groups

The presence of carbonyl groups in amorphous polymers is difficult to establish. In black wattle tannins they must be present at least in very low proportion due to the presence of a trace of fisetin; but the hydrogenation study has shown that no easily reducible groups are likely to be present in major proportion in the methylated tannins. This examination was an attempt to establish more definitely the presence or absence of carbonyl groups.

(a) Infra-red Spectroscopy

No very satisfactory results could be obtained from the solid tannins or their methylated derivatives by mulling them in nujol. Methylated black wattle tannins (lead-salt purified) in chloroform solution gave two peaks at 1505 and 1595 cm^{-1}. (Fig. LI). These peaks are almost certainly associated with aromatic groups. The shoulder at 1620 cm^{-1} may be spurious (due to water absorption). Carbonyl groups in this molecular environment would be expected to give intense absorption in the range 1650 - 1720 cms^{-1} (366)(367). Hydrogen-bonded carbonyl groups again, show an absorption-band at 1635 cms^{-1} (367). The absence of such absorption sets an upper limit of one such group in a molecular fragment of several thousand.

(b) Attempted Formation of Hydrazones, Oximes and Semi-carbazones

Due to the phlobaphene-reaction which occurs under

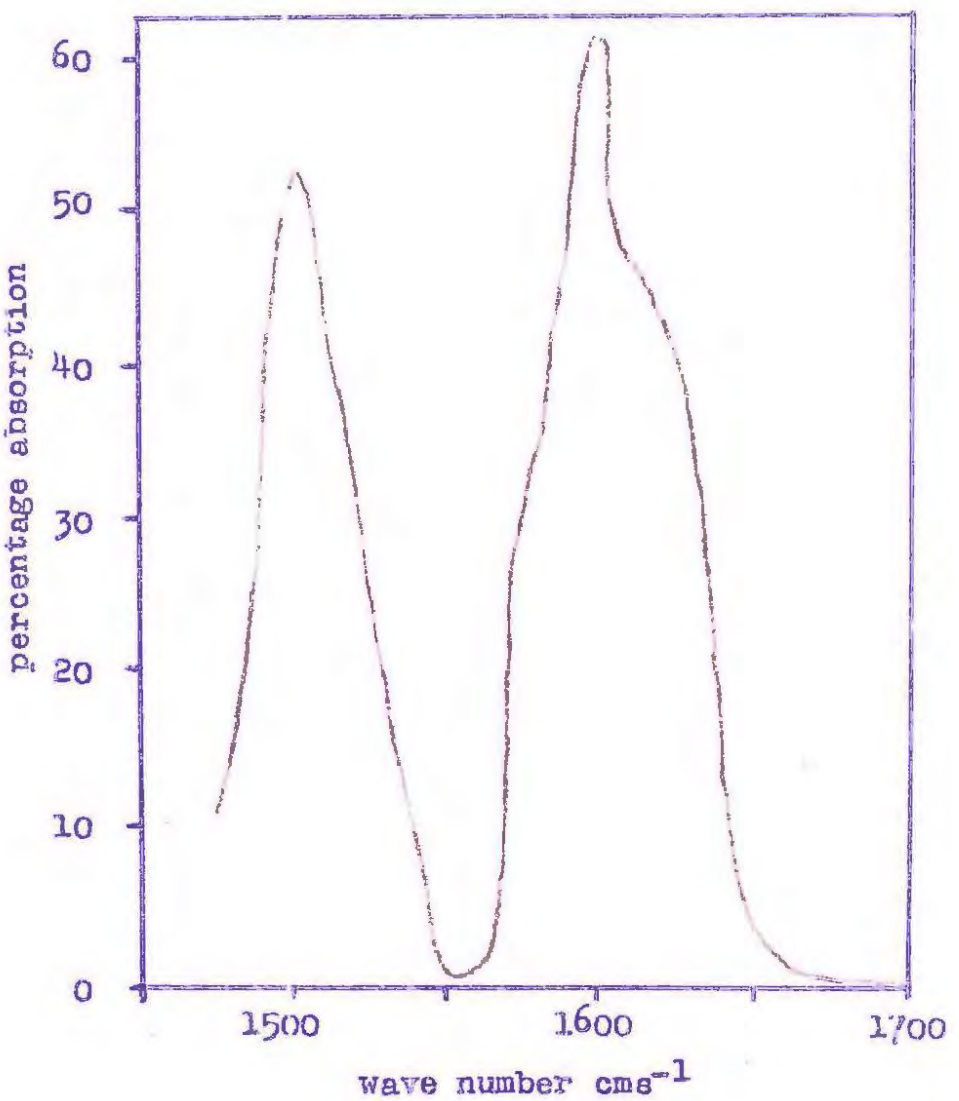


Fig. LI. Infra-red Absorption Curve of Methylated Black Wattle Tannin

acidic conditions necessary for the formation of the above derivatives, the tannins themselves could not be used. The fully methylated product (36.5% methoxyl) was, therefore, treated with the various reagents under standard conditions.

(1) Phenylhydrazones

2,4-dinitrophenylhydrazine was prepared by Allen's method (323). The formation of a hydrazone was attempted accord-

ing to Brady's technique (324).

1 grm. 2,4-dinitrophenylhydrazine was dissolved in 15 ml. absolute ethanol and 2 ml. conc. sulphuric acid added. 1.73 grms. fully methylated tannins in 15 ml. warm absolute ethanol was added to the warmed freshly-prepared reagent and the mixture kept under anhydrous conditions for 3 - 4 days. Free dinitrophenylhydrazine was removed from the reacted material by the repeated precipitation of the latter from benzol solutions with petrol ether. Kjeldahl analyses are known to give satisfactory results (323).

Found : % N = 1.33

This corresponds to one carbonyl group per 8.3 C_{15} units in the tannin residue. 2,4-dinitrophenylhydrazine is an excellent reagent, but is known to react with difficulty and is not satisfactory for α-hydroxy aldehydes and ketones (323)(324).

(2) Oximes

1 grm. methylated tannins, 1.2 grm. hydroxylamine hydrochloride, 4.4 ml. absolute ethanol and 4.4 ml. dry pyridine were refluxed for 3 hours. The solvents were removed in vacuum. The product dissolved in benzene, and was precipitated from such a solution with petrol ether. It was then redissolved in ethanol and poured into water.

Found : % N = 0.19

This corresponds to one carbonyl group per 20 C_{15} units.

(3) Semicarbazones

2.0 grm of semicarbazide hydrochloride and 2.5 grm. of

cryst. sodium acetate were added to 10 ml. water and the mixture warmed until a clear solution was obtained. 2 grms. of methylated tannins in 10 ml. absolute ethanol was added and kept warm on a waterbath for 3 days. No semicarbazone separated. The methylated tannin was recovered by two precipitations into water from alcoholic solutions.

Found : % N = 0.13

In all the above instances, owing to the amorphous nature of the tannin, the reagents could only be removed with difficulty. The low nitrogen content of the product indicated one carbonyl group per 2500 - 5800 molecular fragment.

With both these methods the absence of carbonyl groups in the methylated tannins are clearly established. Kirby (164) also found that the methylated tannin failed to form hydrazones even after Oppenaur's oxidation of hypothetical secondary hydroxy groups.

(x) <u>The Ultra-Violet Absorption Spectrum of Black Wattle Tannins and their Derivatives</u>

The ultra-violet absorption curve of commercial "mimosa" extract was previously recorded by Russel (138) and compared with that of his synthetic "flavpinacols". The tannin and its methylated derivative showed two close absorption maxima at 286 mμ and 270 mμ. The curve was similar in shape and agreed closely not only to that of the various synthetic "flavpinacols", but also resembled that of hemlock tannin and its methylated derivative. The dimeric structure of Russel's "flavpinacols"

was severely criticised by Freudenberg et Al (139)(See Chapter II). Freudenberg found that two phenolic nuclei joined by a carbon chain were sufficient to produce an absorption-curve characteristic of these tannins.

Buchanan, Lewis and Kurth (254) found that the ultra-violet absorption-curve for "mimosa" tannin show only one peak at 280 mμ and not two as obtained by Russel. Tannic acid and redwood tannins show absorption-maxima at the same wave-length, but their molar extinction-coefficients differ. Lignin preparations similarly show an absorption maximum at 280 mμ (325). The methylation of redwood tannins with diazomethane produce a reduction in the absorption density consistent with the increase of molecular weight.

Sohn (326) found that commercial quebracho, valonea, mimosa and pine-bark extracts as well as lignin-sulphonic acid preparations all show a single absorption-peak in the ultra-violet region at 280 mμ. As a linear relationship was found to exist between absorption density and concentration (i.e. Beer's law was applicable), Sohn recommended the development of an ultra-violet method of tannin analysis.

Bradfield and Penney (113) showed that all catechins have an adsorption-band in the range 270 - 280 mμ. The chromophoric nuclei constituting such catechins act almost independently Thus ϵ_{max} values for epicatechin and for gallocatechin correspond approximately to the sums of the ϵ_{max} values of 5,7-dihydroxy-2,2-dimethyl chromane and catechol and pyrogallol respec-

tively. Stereoisomerism has a small effect. l-gallocatechin and r-gallocatechin, for example, have ϵ_{max} values of 1340 and 1290 respectively at 271 mμ. With the exception of l-gallocatechin gallate which has twin peaks at 275 mμ and 279.5 mμ, all other catechin bodies show a single absorption-maximum in the ultra-violet region.

From the above it is obvious that the phenolic nuclei are the chromophoric groups responsible for the absorption observed with catechins and tannins in the ultra-violet region. Putnam and Gensler (131) recently entertained the idea that carbonyl groups or ether linkages alone, when attached to phenolic nuclei, might cause sharp absorption peaks in the ultra-violet region 271 - 283 mμ. Such speculation is unfounded.

Black Wattle Tannins

A Beckman Model DU spectrophotometer was used throughout the present investigation. With this instrument the tannin gave a single absorption-peak at 280 mμ (Fig. LII), and the twin peaks found by Russel could not be reproduced on a Hilger instrument of even greater resolving power. It is remarkable that the majority of tannins, constituted of different phenolic bodies each with a different ϵ_{max}, should almost all show absorption peaks at the same wave-length (279 - 281 mμ).

ϵ_{max} of black wattle tannin in ethanol assuming a molecular weight of 288 = 3,356. Gambier catechin consisting predominantly of d-catechin but containing also dl-catechin (327)

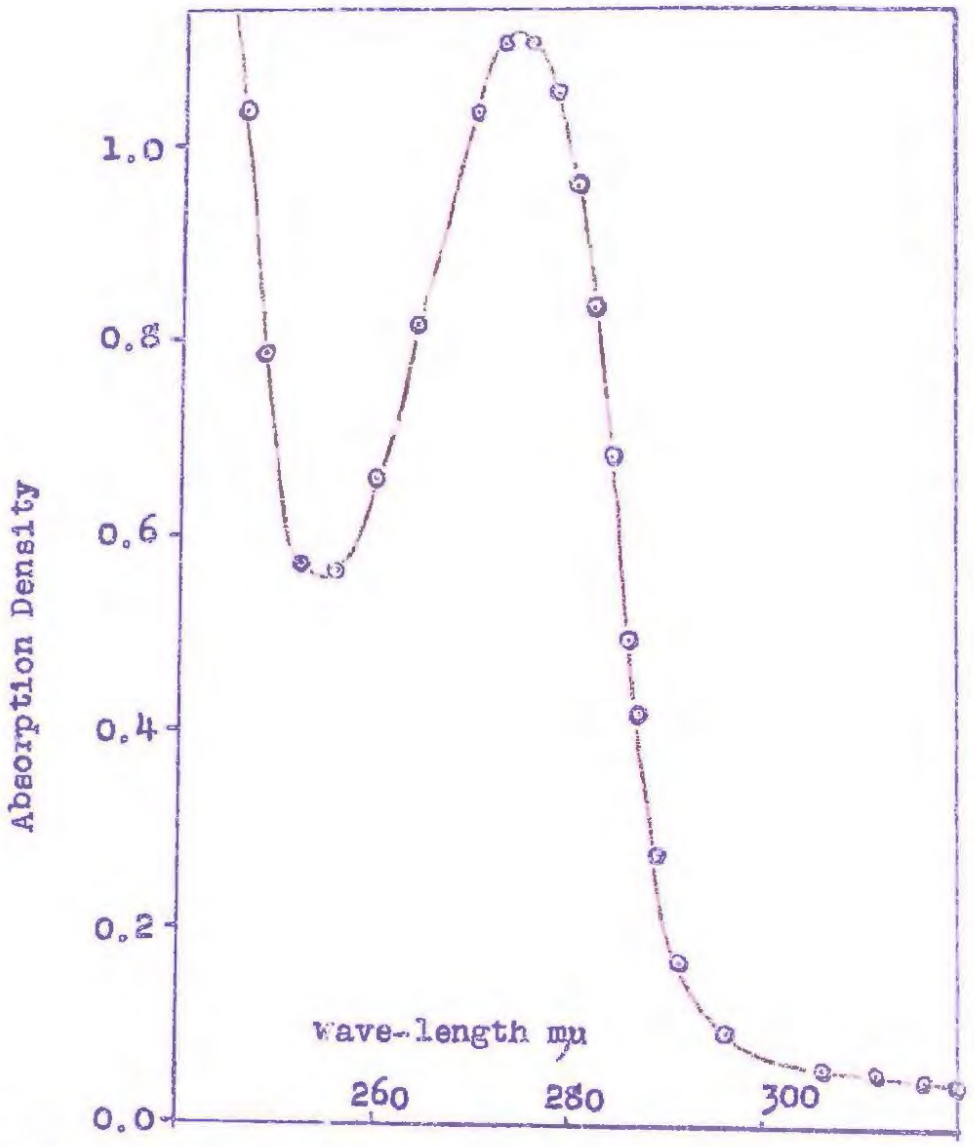


Fig. LII. Ultra-Violet Absorption Curve of Black Wattle Tannin.

was found to have a similar absorption-peak at 280 mμ. ϵ_{max} = 3647 in ethanol. Bradfield and Penney recorded ϵ_{max} = 3300 for l-epicatechin in ethanol at 280 mμ. The following are the relative values for the phenols which constitute both black wattle tannins and catechins ;

Compound	λ_{max} mμ	ϵ_{max}
Catechol (328)	278	2600
Pyrogallol (330)	266	800
Resorcinol (328)	276	2000
Phloroglucinol (329)	266	380

Bradfield's and Penney's findings, previously discussed, will prove an extremely useful check once the various tannins have been isolated and if they should prove to contain only two phenolic nuclei in definite proportion. From the afore-mentioned figures it is obvious that the sum of ϵ_{max} of resorcinol (2000) and ϵ_{max} of pyrogallol (800), is less than ϵ_{max} of the tannins (3,356). This is most likely due to the fact that a minor proportion of catechol nuclei (ϵ_{max} 2600) is also present.

Methylated and Acetylated Tannins

Lead-salt purified tannins methylated with dimethyl sulphate (36.5% methoxyl) have the same λ_{max} as the tannins themselves (279 - 280 mμ) (Fig. LIII). ϵ_{max} of the methylated tannins in methanol = 3599 assuming a molecular weight of 346. The molar extinction coefficient of the methylated tannins is thus higher than, but of the same order as that of the original tannins.

Acetylation causes a shift to slightly lower wave-lengths, (λ_{max} = 276 mμ with an inflection at 278 mμ) and a large decrease in the molar extinction coefficient ϵ = 2000 (assumed molecular

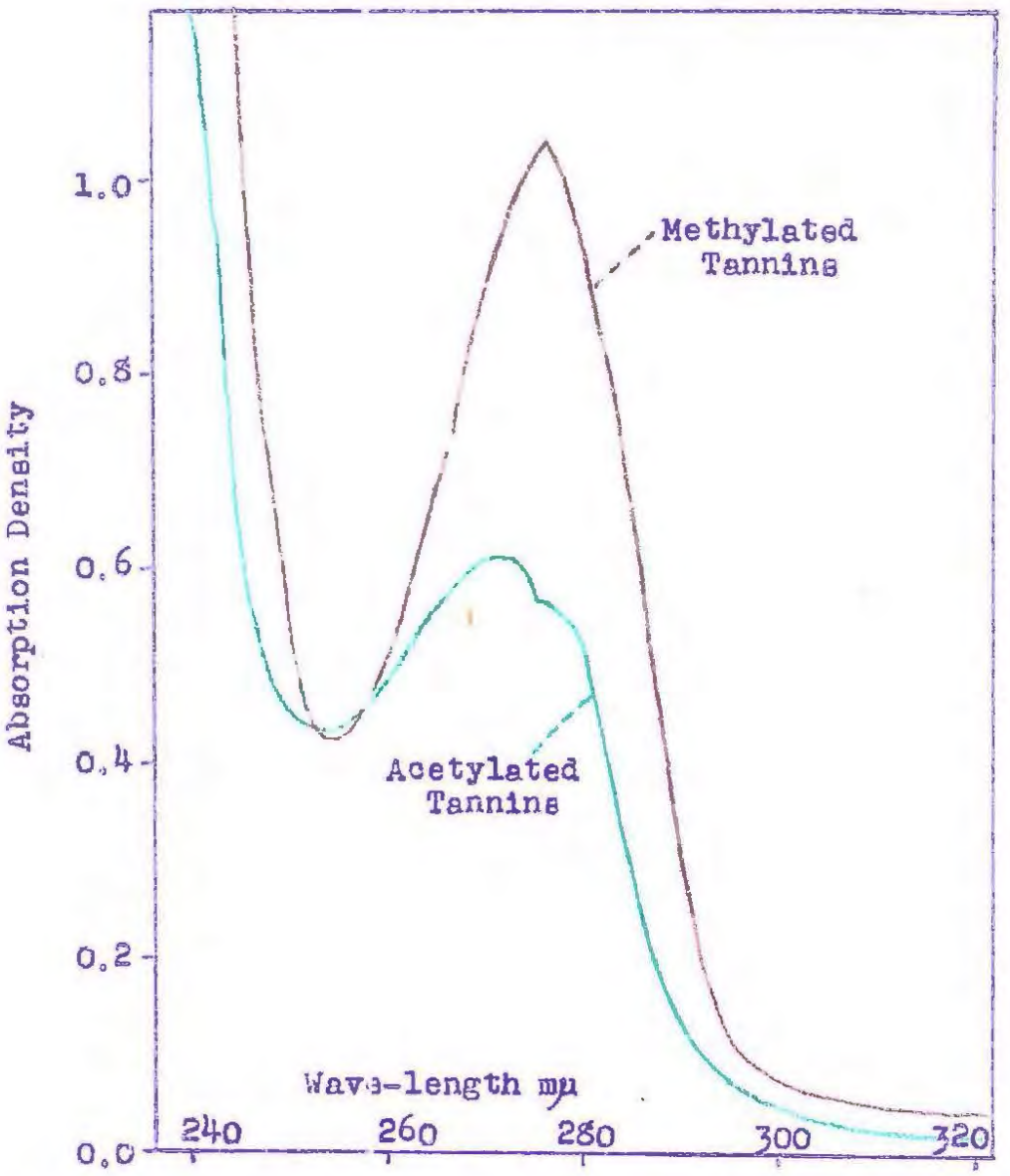


Fig. LIII. Ultra-Violet Absorption Curve of Methylated and Acetylated Tannins

weight = 450). This inflection appears more prominent at lower concentrations, and is also evident in the ultra-violet adsorption curves of 1,2-diacetyl-3-methoxypyrogallol, pentaacetyl-d-catechin, diacetylcatechol and triacetylpyrogallol, all of which were synthesised. Bradfield and Penney also recorded decreases in ϵ_{max} values of catechins on acetylation.

Discussion

It is obvious that a comparison of the molar extinction coefficients of the tannins with that of the sum of its constituents is premature at this stage. Such work awaits the isolation of the various individual tannin constituents. The use of ultra-violet spectroscopy for tannin analysis is, however, feasible and has been successfully applied (Chapter VIII).

(xi) Condensations of Black Wattle Tannins

Black wattle tannins are normally easily soluble in water, but form typical insoluble red phlobaphenes or "tanners' reds" in strongly acidic aqueous solutions. This behaviour classes it amongst the "phlobatannins" or condensed tannins. The phlobaphene-formation is considered to be a further condensation of the amorphous polyphenolic fraction of the extract, and a proportion of some condensed tannins e.g. quebracho and redwood exist naturally in the wood or bark in this highly condensed form. Solubilisation of these fractions is effected by bisulphite treatment.

This work on black wattle tannins was completed before the lead-salt separation and other "purification" methods were devised, but the results are nevertheless interesting when compared with the starting-material and with later work.

Formation of the Phlobaphenes

10 grms. of the acetone-salt "purified" (88% tannin by

the Official Method) commercial extract dissolved in 50 ml. water, was treated with 30 ml. conc. HCl and heated on a water-bath for 1 hour. A deep reddish-brown precipitate resulted. The phlobaphene was insoluble in cold water, and sparingly in hot water. It was also sparingly soluble in methanol and ethanol and fairly soluble in pyridine. All these solvents dissolve black wattle tannins. The phlobaphene was washed with water until free of chloride, and dried at 110°C. in a drying pistol under vacuum.

Found : % C = 62.56, 62.19, 62.19, 62.10, 62.38
% H = 4.34, 4.24, 4.37, 4.37, 4.22
% Ash = 0.00

Average Values : % C = 62.28, % H = 4.31

Acetone-salt purified tannins used as starting-material contained :

% C = 57.75, 57.61, 57.48
% H = 4.55, 4.66, 4.54
% Ash = 0.20, 0.23

For comparison, the lead-salt "purified" tannins were found to contain :

% C = 61.38, 61.80
% H = 4.69, 4.84

It thus appears that during the process of phlobaphene formation all the soluble sugars are eliminated from the acetone-salt "purified" tannins, resulting in an insoluble product of similar composition as the lead-salt "purified" tannins. A certain amount of oxidation appears to accompany this conversion

as the phlobaphene has a lower hydrogen content.

Derivatives of the Phlobaphenes

The phlobaphenes were acetylated and methylated by the previously-described techniques until "maximum" values were reached.

Methylated Phlobaphenes were obtained by the repeated action of dimethyl sulphate in boiling methanol.

Found :	No. of Methylations	% $-OCH_3$
	1	30.22
	2	32.47
	3	34.02, 34.43
	4	34.69, 34.77

Analysis of the latter product :

% C = 66.37, 66.22, 66.45, 66.31

% H = 5.84, 5.88, 5.68, 5.79

Both the initial and final methoxyl values are below that which is normally obtained (32.5% and 36.5% respectively) with the tannins by the same method. The hydrogen content of the methylated phlobaphene is also lower than that in the methylated tannin itself (6.04 - 6.16%).

Acetylated Phlobaphenes. The phlobaphenes were acetylated first with acetic anhydride/sodium acetate and finally with acetyl chloride/pyridine mixtures.

Found :	No. of Acetylations	% Acetyl
	1	31.66
	2	35.05
	3	34.47
	4	35.30, 35.51

Analysis of the fully acetylated phlobaphene :

% C = 60.42, 60.99, 60.78, 60.16, 60.26

% H = 4.75, 4.60, 4.64, 4.61, 4.66

As with the methylated products the initial and final acetyl values are well below that obtained with the tannins, (38.15% and 37.77% respectively). The percentage hydrogen is also slightly lower (4.73 - 4.87%) than that of the acetylated tannins.

Discussion

The analyses of the phlobaphenes and their derivatives show little difference when compared with those of the tannins from which they were derived. The slight reduction in hydroxyl groups could be due to :

(a) the elimination of a low proportion of such groups by ether formation due to condensation; or quinone formation resulting from oxidation; or by the elimination of water from a hydroxyl group or groups on carbon atoms,

or (b) increased steric hindrance in the more highly condensed units,

or (c) due to the only partial dispersion of the phlobaphene in the solvent media in which reaction occurs.

A slight reduction in hydrogen content is perceptible in both the phlobaphene and its derivatives, and it is probable that the slight analytical differences are due to a combination of some of the above possibilities. The phlobaphene thus appears to be a more highly condensed and also more highly oxidised product than the soluble tannins themselves.

(xii) Summary

(1) Previous analytical work had shown little or no relationship between the tannins and their derivatives. Tannins, from which the carbohydrate non-tannins were completely removed, were used for the first time in this analytical study.

(2) Combustion analyses of the "purified" tannins corresponded to an empirical formula of $C_{15}H_{13}O_6$.

(3) 4.1 hydroxyl groups per hypothetical C_{15} unit appear to be present from the analyses of the fully substituted acetyl, methoxyl and mixed derivatives.

(4) Ester linkages and carbonyl groups appear to be absent, and a hydrogenation study has also shown the absence of easily reducible groups, e.g. ethylenic groups.

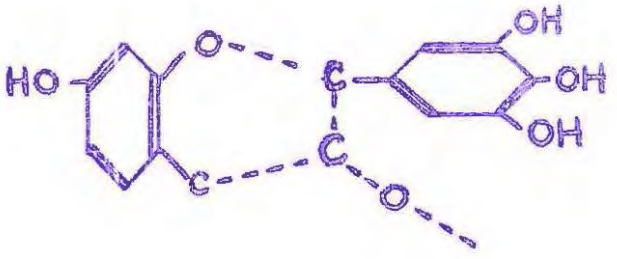
(5) Two oxygens are present per hypothetical C_{15} unit which refuse to acetylate even under exceptionally drastic conditions. They are considered to be ether links, although the possibility of the presence of non-reactive tertiary aliphatic

hydroxyls is not entirely eliminated.

(6) The four active hydroxyl groups all appear to be phenolic. This was proved by the fact that the tannins, contrary to Stephen's findings, react easily and rapidly with diazomethane to give a pure white product of high methoxyl value. Other phenolic bodies give the same degree of substitution.

(7) A colorimetric study has shown the presence of a very high proportion of orthohydroxy phenolic groupings, corresponding to one pyrogallol nucleus per C_{15} unit. This study forms a useful basis for a rapid method of tannin estimation.

(8) From the degradative study the tannins were shown to consist predominantly of pyrogallol and resorcinol nuclei. The resorcinol nuclei were destroyed during permanganate oxidation. It would thus appear that the predominant structures may be represented by the formula :



in which the mode of linkage is as yet unknown. Exceptionally high yields of degradation-products have been obtained for the first time.

(9) The tannins give an absorption peak at 280 mμ in the ultra-violet region, caused by the presence of phenolic chromophoric groups.

(10) The black wattle phlobaphenes, formed under conditions of high acidity, differ only slightly from the tannins on analysis. A slightly lower proportion of hydroxyl groups and percentage hydrogen appeared to be present in the phlobaphenes.

(11) Many of the above techniques represent useful methods for the investigation of the various amorphous tannin constituents, once isolated.

CHAPTER VIII

PHOTOMETRIC METHODS OF TANNIN ANALYSIS FOR BLACK WATTLE TANNINS

New rapid and reliable methods of analysis are urgently required for large-scale tannin estimations, as the recognised procedure, particularly the determination of non-tannins, is both laborious and time-consuming. Analytical differences evident in the existing method when the same sample is analysed by different chemists, may be attributed largely to environmental variations (331) and partly to the personal factor associated with any empirical method involving a large number of operations. Such differences are obviously undesirable.

Jamet (332) and Chambard and Jamet (333) successfully simplified the non tannin analysis by using dried pre-chromed hide powder, and obtained results in excellent agreement with the Official Method. Further work based on the above and aimed at the elimination of many unnecessary steps is receiving the attention of various supervisory committees (334), as the ultimate assessment of the tannin content of a vegetable extract depends on its percentage combination with animal proteins.

It is unlikely, however, that the hide-powder method will ever be reduced to a simple, rapid and very exact technique and the search for other methods more suited for mass determinations is thus a natural result. Vorsatz (335), for example,

developed a rapid colorimetric method for pine-bark extract, which is also applicable to quebracho and other tannins containing only catechol groupings. This was based on the previous work of Hoepfner (336) on chlorogenic and caffeic acids. The red colour developed by the action of nitrous acid on the catechol group, intensified by alkali and stabilised by urea, was standardised against a sample of known tannin content as determined by the hide-powder method. Comparative results of 38 samples analysed by the Official and colorimetric methods, show a maximum difference of 0.8% between methods on any sample analysed, and an average difference, neglecting the sign, of 0.3%. Adams and Merrill (337) extended Vorsatz's method to pyrogallol tannins and Batzer (338) recently contributed a thorough study of the reaction mechanism and stability of the coloration produced, since the method gave appreciable differences with different analysts. Bayak and Hirth (339) similarly perfected two methods for tannic acid; (a) a photometric technique using the blue colour produced with iron ammonium alum and (b) a volumetric method based on the decoloration of the blue colour used in the photometric technique.

Woodhead (340) previously devised a refractive index method for the rapid and comparative assessment of the tannin content of black wattle barks. More recent work of the author has, however, shown that sucrose is an important non-tannin constituent. As sucrose solutions have a high refractive index and the refractometer is actually used for sugar estimations, the Woodhead method would only be effective where the tan/non-tan ratio

is constant. Sherry (341) showed that this ratio increases gradually from 2.7 to 3.5 over a period of 1 to 9 years of growth. The refractive index method is thus unsuitable when barks representing different stages of growth are being investigated, but still applicable to commercial extracts with relatively constant tan/non-tan ratios.

Bryant and Martin (363) recently found that a linear regression exists between hot-water solubles and tannin content of black wattle barks, and derived an equation from which tannin contents may be calculated from known values of hot water solubles. Their samples on which statistical data were based were, however, collected from the lower portions of relatively mature trees (mainly 25 ft. high and also 5½ years old) which would show little variation in tan/non-tan ratios. The main criticism of Woodhead's method is, therefore, also applicable here.

From the previous constitutional investigation on black wattle tannins, two possible methods of analysis were evident and these have now been more thoroughly investigated. Due to its high solubility black wattle extract is eminently suited to photometric methods, and the recent advent of differential methods (342)(343)(344) through which accuracy equal to that of volumetric and gravimetric procedures is attainable (345), has rendered possible the use of easy, direct and accurate techniques.

Spectrophotometric Method

Organic materials containing resonating structures, e.g.

phenyl, naphthyl, anthryl, carbonyl, azo, nitro and hydrosulphido-groups absorb radiant energy in the ultra-violet and visible regions of the spectrum. These are known as chromophore groups. Thus aromatic compounds which possess the well-known resonance characteristics of the benzene ring show strong absorption in the ultra-violet region 230 - 280 mμ, whereas non-resonating structures such as paraffins and some sugars are completely transparent within this range. Furthermore, in a homologous series, e.g. the alkyl-benzenes, the spectra resemble each other very closely in location, shape and intensity as the resonance absorption is due to the unsaturation and resonance effects inherent in the benzene ring and is essentially independent of alkyl substitution (346).

Since absorption in the ultra-violet is governed by the chromophoric constitution of the compounds under investigation, the spectra of related materials will have the distinct general properties of that class of compounds. Infra-red and Raman spectra by comparison are unique for each compound. The similarity of the ultra-violet spectra holds the disadvantage that similar compounds are usually indistinguishable from each other and its use for the analysis of multicomponent systems is not feasible. On the other hand for quantitative analytical purposes, deviations from Beer's Law are less frequently encountered in the ultra-violet than in the more characteristic infra-red region (346), although exceptions do occur. One reason for this is that the energies involved in electronic transitions are much greater than those involved in vibrational transitions and are less affected by low

energy inter-molecular interactions.

Black wattle tannins contain chromophores in the form of phenolic nuclei, and previous work in these laboratories showed that dilute aqueous, methanolic and ethanolic solutions give a broad absorption band with a single peak on a Beckman Model DU Spectrophotometer. (Fig. I). Russel (138) previously obtained a double peak at 286 mμ (major) and 270 mμ (minor peak) using an instrument of higher resolving power. From the above discussion it is obvious that the peak is not characteristic of black wattle tannins, but is also found with catechin (Fig. LIV), hemlock, ellagic acid (138), Douglas fir bark tannin (347), lignins (348)(349), redwood tannin and tannic acid (254) although the molar extinction coefficients differ in each instance. The curve obtained by plotting the absorption density at 280 mμ against concentration, using solutions of varying dilution, was a straight line and it thus offered a rapid method of tannin analysis. This was confirmed recently by Sohn (326) who showed that in addition to mimosa, lignin sulphonic acid, quebracho and pine-bark extracts behave similarly.

Gums and sugars are known to constitute the non-tans in black wattle extracts from the present investigation (Chapter IX). The sugars, e.g. sucrose, do not absorb radiant energy in the visible and ultra-violet regions of the spectrum (351)(352).

The gums which are formed from galactose, arabinose, rhamnose and uronic acid residues would be expected to behave similarly. A solution containing 0.316 grms. of the natural

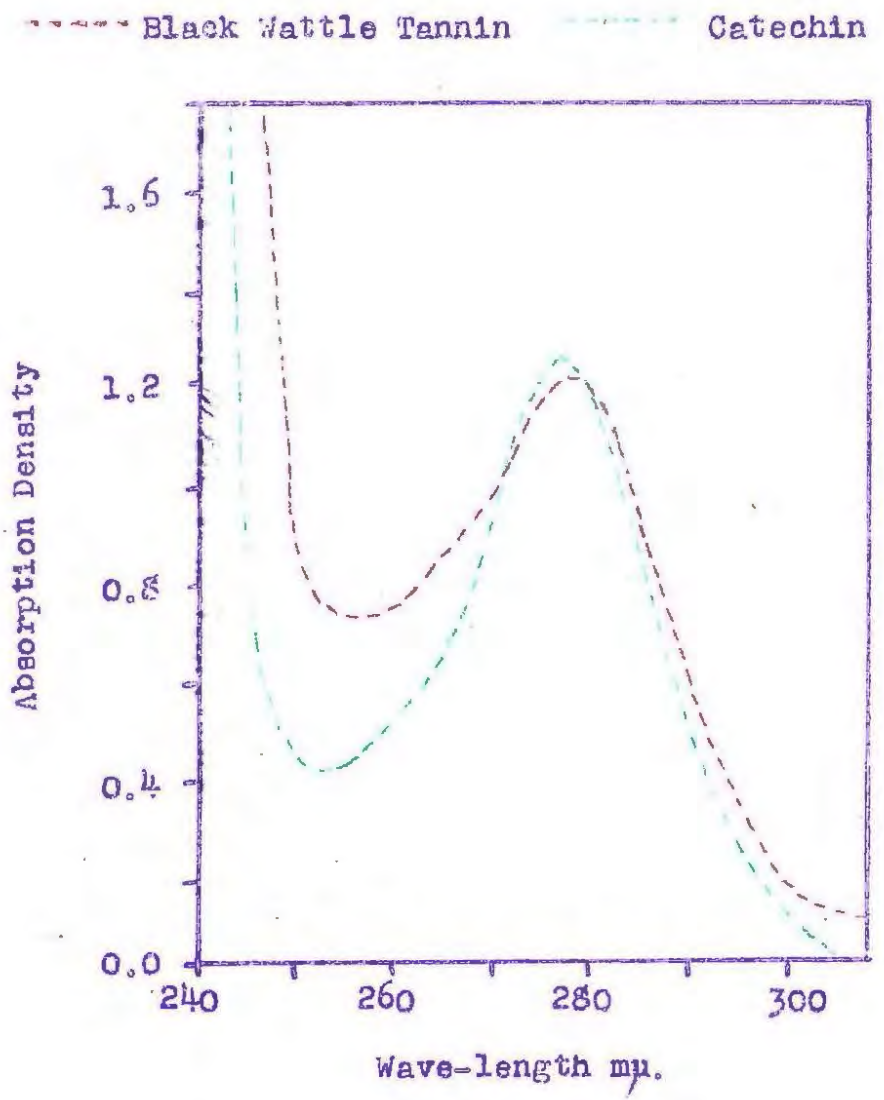


Fig. LIV. Ultra-violet Absorption Curve of Black Wattle Tannins

exudate of the bark per litre was found to show slight absorption at 280 mμ. (Absorption density = 0.031). This gum was unpurified and known to contain salts, but was chosen for examination in preference to the gum in the extract which was almost impossible to free of the last traces of tannins (See Chapter IX). The most concentrated solution used in the present photometric investigation contained 2.5 grms. extract per litre and gave an absorp-

tion density of 0.500 at 280 mμ when diluted 40 times (25 ml/litre). This extract solution before dilution corresponds to 0.175 grms gum per litre. (See Chapter IX). The aforementioned solution of the natural exudate was diluted about 72 times (14 ml./litre) to obtain a gum solution of equivalent strength for measurement. The small absorption found (δ = 0.002 at 280 mμ) corresponded to 0.4% of the total absorption density of the extract as a whole.

The absorption is a function of the phenolic tannin content only, as a very low proportion of "half tans" (small phenolic bodies which do not tan easily) are present in wattle extracts, and the sugars and gums do not absorb in the visible and ultra-violet regions.

Ultra-violet absorption spectroscopy has recently been employed for the accurate determination of Vitamin A (353), phenolic aldehydes (354) and nicotine (355).

Stability of Analytical Solutions

One difficulty with black wattle tannins is the ease with which oxidation occurs in the highly dispersed state necessary for analysis. This results in slight darkening of the water-white solution and a perceptible increase in absorption density even when freshly distilled water of low oxygen-content is used. (Table XV).

The height of the whole curve is raised and no lateral shift of the peak occurs (Fig. LV).

TABLE XV.

Increase of Absorption Density due to Atmospheric Oxidation

(2.5 grm. extract of 61% tannin content made to 1L. Diluted 25ml./Ltr)

Time after 2nd Dilution	Absorption Density at 280 mμ and 1.9 mm.S.W.	% Increase
23 mins.	0.498	0.0
80	0.501	0.6
160	0.503	1.0
380	0.508	2.0
1140	0.517	3.8

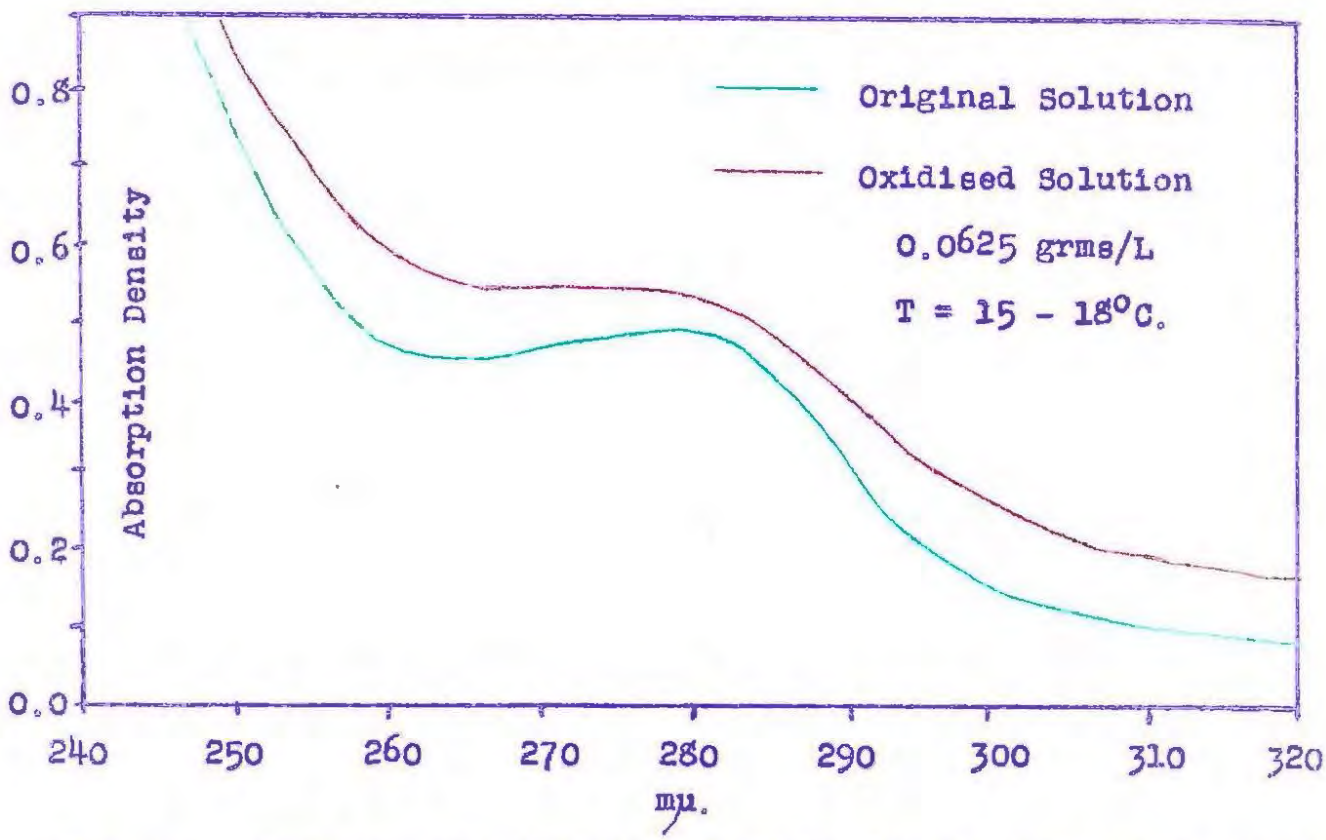


Fig. LV. Increase of Absorption Density due to Atmospheric Oxidation

Absorption increases due to oxidation may be avoided by two methods :

(a) When the tannins are kept in well-corked solutions of the usual analytical strength (6.5 grm/litre) or of approximately one-third analytical strength (2 - 2.5 grm/litre), darkening due to atmospheric oxidation is very limited. This is due to the slight oxidation of a much larger concentration of tannins by the relatively constant but limited amount of oxygen in solution. Rapid measurement was made immediately the tannin/oxygen ratio was decreased by dilution to that analytical strength which falls within the range of the instrument. The stability of a solution of high tannin/oxygen ratio is shown in Table XVI.

TABLE XVI

Stability of Solution of High Concentration (2.5 grm/litre) to Atmospheric Oxidation

Age of Concentrated Solution	Period before measurement after 25/1000 ml dilution	Absorption Density at 280 mμ & 1.9 mm. SW
60 minutes	23 minutes	0.498
1140 "	15 "	0.499
1980 "	20 "	0.503

19 hours standing of the original solution results in a 0.2% increase and 33 hours in a 1.0% increase in the absorption density of the diluted solution.

(b) The relatively stable concentrated solution (2.5 grm. extract/litre) was diluted (25/1000 ml.) as before, but the 25 ml. pipetted was run into a litre flask already containing 500 ml. of exactly 0.2% sodium bisulphite. When made up to the mark the resultant 0.1% bisulphite solution was capable of stabilising tannin solutions of much higher concentration (See Chapter X). 0.1% bisulphite solutions showed very low absorption at 280 mμ (0.014, 0.017, 0.014) and in the presence of tannins raised the absorption density of the dilute solutions used by 2% (from 0.499 to 0.509). The stability imparted to the latter is remarkable (Table XVII).

TABLE XVII

The Stabilising Effect of 0.1% Bisulphite on Tannin Solutions of High Dilutions (0.0625% extract)

Period after Dilution	Absorption Density at 280 mμ & 1.9 mm. S.W.	% Difference
15 minutes	0.509	-
160 "	0.508	-0.2
464 "	0.506	-0.6
1440 "	0.507	-0.4

By employing bisulphite, therefore, or by taking photometric measurement shortly after dilution to a suitable concentration range for the instrument, close agreement and high accuracy is obtained.

The high order of reproducibility attainable in the visible spectrum with the Beckman Model DU Spectrophotometer for inorganic solutions, even between different observers, has been recorded by Bastian, Weberling and Pallila (344). Allowing for differences due to oxidation, the above results show that similar reproducibility may be attained in the ultra-violet region.

Preparation and Analysis of Sample for Standardisation Curves

A large sample (1 kg.) of air-dried commercial black wattle extract, crumbling easily to the touch and free from hessian fibres, was selected so that it could be powdered easily without developing the stickiness of the fresh extract. Such a sample was in addition more closely equilibrated with atmospheric moisture and less likely to alter with time.

The whole sample was powdered, thoroughly mixed, and kept in a tightly corked bottle. Samples drawn from different portions of the whole were analysed by the Official hide-powder method, each analysis representing a duplicate determination :

% Tannin	= 66.43,	66.64,	65.83,	65.12,	65.67
% Non-tannin	= 18.74,	18.32,	18.97,	19.68,	20.20
% Insolubles	= 1.37,	1.52,	1.41,	1.82,	1.69

Average % Tannins = 65.98.

This sample was used in standardisation curves for both methods of analysis.

Analytical Method

In the conventional colorimetric procedure, the absorption

density or transmittancy of the light-absorbing or coloured substance is compared with that of water. This has certain disadvantages which seriously limit the accuracy of the method (343). Very recently, however, the ordinary procedure was replaced by "differential" method which gives results of high accuracy with inorganic solutions, and excellent accounts of which exist elsewhere (343)(344). Differential colorimetry in the ultra-violet region is also feasible (344). It consists briefly of setting the optical density scale zero (or 100% transmittancy with a solution of a light-absorbing subatance in place of the reagent blank (water in this instance) usually employed. This requires that the concentration of the latter zero-point solution be known accurately, and be absolutely stable for the precise reproduction of the standard. In inorganic solutions previously studied (343)(344), the aqueous solution of the compound under measurement was quite stable and reproducible.

Black wattle tannin solutions of high dilution are unstable (Table XV), but in the presence of 0.1% bisulphite high stability is attainable (Table XVII). The use of benzoic acid, obtainable in high purity and exhibiting two rounded absorption peaks at 233 mμ and 273 mμ (356), is recommended as a replacement for the zero-point tannin solution. 280 mμ on the latter rounded peak still corresponds closely to the absorption maximum. Benzoic acid (0.8400 grms/2 litres at 20°C. diluted 200/1000 ml.) proved to be an excellent and reliable standard (Tables XVIII and XIX).

TABLE XVIII

Stability of Benzoic Acid Zero-Point Standard

Period after Prepn.	Absorption Density at 280 mμ & 1.9 mm. S.A.	% Change
0 hours	0.434	-
16 "	0.434	-
31 "	0.433	-

TABLE XIX

Reproducibility of Benzoic Acid Standard

Wt./2 litres	Absorption Density after 200/1000 diln.
0.8400	0.434
0.8397	0.434
0.8389	0.433
0.8401	0.434

Preparation of the Standard Curve

In all measurements distilled water of low oxygen content was used, and fresh supplies continually checked against that previously employed. All large dilutions were performed with water at 20°C. to avoid volumetric errors due to temperature effects, as all volumetric equipment was standardised at this temperature.

(The specific gravity of water varies about 2 parts per 10,000 per °C. (344). Grade A NPL pipettes and grade B volumetric flasks were used throughout.

To prepare the standard curve, 2.05 grm. of the analysed sample was weighed into a litre measuring flask, washed in with hot water, completely dissolved, and colder water (20°C.) added with shaking until near the mark. The whole was slowly cooled to 20°C. and made up to volume. 25 ml. of this solution was accurately pipetted into a second litre flask containing 500 ml. of exactly 0.2% sodium bisulphite, and made to the mark with distilled water at 20°C. At 280 mμ and a slit-width of 1.9 mm., this solution had an absorption density of 0.434, similar to that of the benzoic zero-point standard previously prepared.

The benzoic acid standard was placed in adjacent cells, the slit-width set to 1.9 mm. and wave-length to 280 mμ. The dark current was adjusted and the galvanometer brought to the zero position using the sensitivity knob alone. The companion cell was now slid into position and if any difference was observed a corresponding correction was made on the solutions measured in that cell. This check was repeated at intervals, as tannin solutions tend to affect the cell-blank.

At 280 mμ and the same slit-width, the instrument was set against the benzoic acid solution and gave an absorption reading 0.000 with the tannin solution. Similar tannin solutions increasing by 0.10 grm. to 2.5 grm./litre were now made up, diluted as before into bisulphite solution and their absorption measured

against the benzoic acid standard. The resultant curve (Fig. LVI), was a straight line and the slight deviations obtained are most likely due to the sampling errors. Deviations from the line in parts per thousand is shown on the graph. The value of all these solutions are closely reproducible.

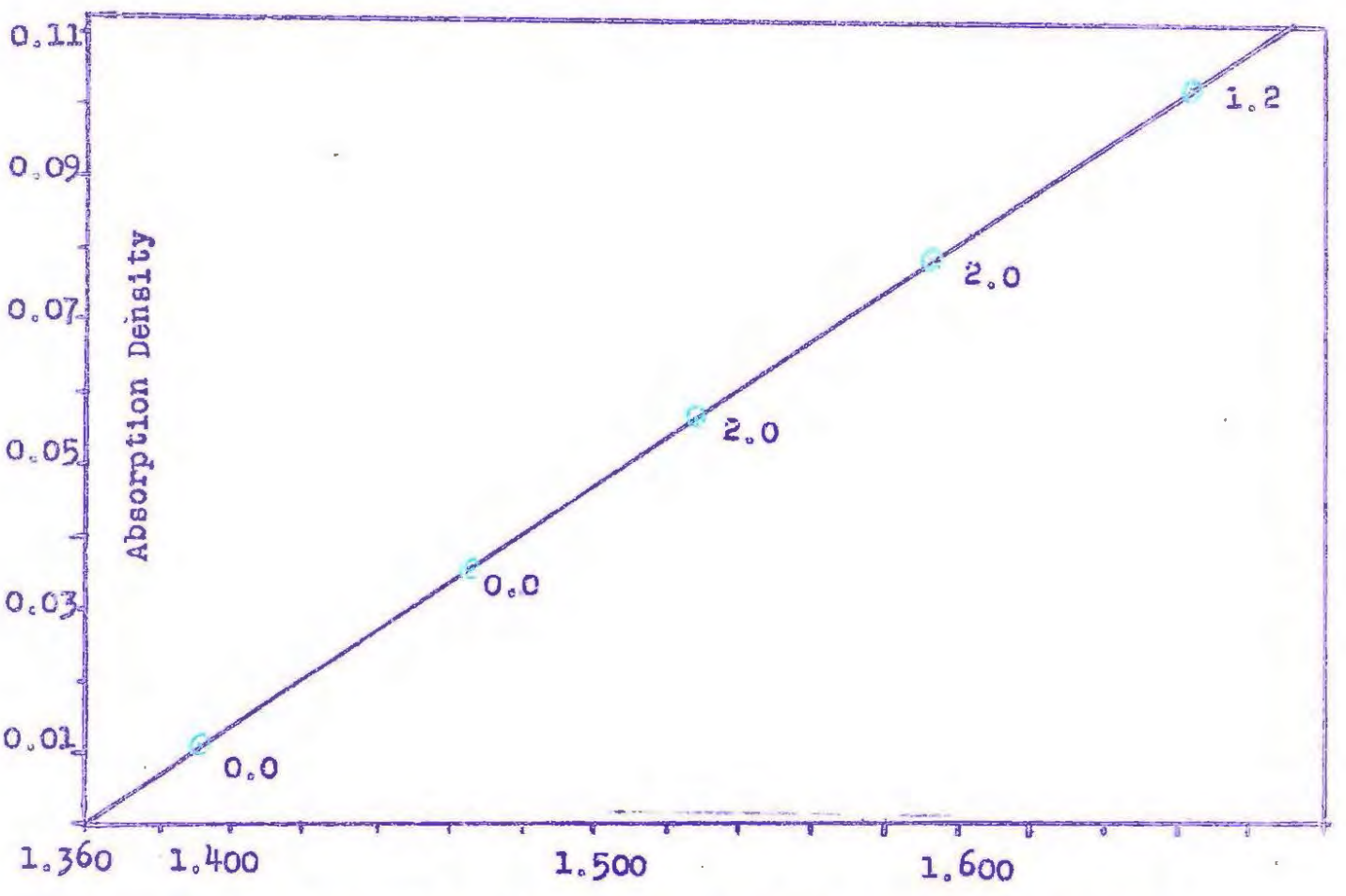


Fig. LVI. Standard Curve for Bisulphite-protected Tannin Solution.

The curve obtained when rapid readings were taken soon after dilution, instead of using the stabilising bisulphite solution, had a similar slope and agreed closely with the above.

Unknown solutions were treated according to their origin :

(a) Those from commercial extracts were prepared as described above, cooled to 20°C., and after dilution 40 times gave optical clarity.

(b) Bark extracts obtained by hot leaching in the Procter extractor according to the Official Method, showed turbidity due to a low proportion of sediment on cooling to 20°C. The sediment did not redissolve on further dilution and would affect absorption measurements. Solutions of the usual analytical strength (for the hide powder method) were thus centrifuged for 4 minutes at 3,500 r.p.m. This eliminated all insolubles. For accurate comparison with the hide-powder method the reproduction of the standard extraction method was considered essential.

Results :

(a) Bark Samples. The following weights extracted to 2 litres were diluted 10 ml./litre before use.

TABLE XX

Code No.	Weight	Observed Values (Benzoic Acid Zero-point Standard)		% Tannin Found	Hide-pdr.
W/15	23gm.	0.064	0.063	33.8	34.9
S/33	23gm.	0.080	0.080	35.0	35.3
S/34	24gm.	0.061	0.060	32.2	32.7
D.F.C.	22gm.	0.076	0.076	36.2 †	35.2
16/deW.	23gm.	0.053	0.053	33.0	33.3
17/deW.	22gm.	0.070	0.070	35.8 †	34.3
18/deW.	24gm.	0.072	0.072	33.0	33.2

† Similar high value (compared with the hide-powder method) were also recorded in the colorimetric method. (Table XXV).

(b) Tannin Extracts. 2.5 grm. extract per litre diluted 25 ml./litre before use.

TABLE XXI

Code No.	Observed Values		% Tannin U.V. Method.	Av.	Hide-Pdr.	Av.
409	0.062	0.062	61.84		61.8	
410	0.055	0.055	61.45	60.94	61.5	61.7
411	0.040	0.040	59.17		61.8	
412	0.0575	0.0575	61.30		61.8	
413	0.069	0.069	62.72		61.7	
414	0.065	0.065	62.24	61.40	61.1	61.2
415	0.052	0.052	60.63		61.1	
416	0.047	0.047	60.01		60.8	
417	0.051		60.60		61.9	
418	0.062		61.84	61.30	62.3	62.2
419	0.061		61.70		62.3	
420	0.055		61.30		62.3	

(c) Purified Black Wattle Extract. Previous work of the author has shown that the phenolic fraction of black wattle extracts could be almost completely "purified" by the removal of gums and sugary non-tannins, using a variety of methods. This was confirmed by the later work of Buchanan, Lewis and Weber (357). During the isolation of small quantities of such purified extracts a fair amount of colour-darkening due to oxidation was unavoidable. The use of purified extracts for the preparation of the standard

curve was thus thought to be inadvisable, but the following analysis is of interest.

0.3966 grm. of lead-salt purified tannin made to 250 ml. and diluted 25 ml./litre gave the absorption densities = 0.0505, 0.0495, 0.050.

Found : % Tannin = 95.2 (ultra-violet)

% Tannin = 94.4 and 93.7 (hide-powder).

Colorimetric Method of Analysis

Chromatographic studies by the author have shown that the bulk of the polyhydroxyphenolic fraction of black wattle extracts contains orthodihydroxy and orthotrihydroxy phenolic bodies. Only one meta-hydroxy phenol of high RF value (0.92), possibly a phloroglucinol derivative present in exceedingly low concentration, could be detected on paper chromatograms. The estimation of orthohydroxy groups would thus serve as an index of the total tannin content. From the discussion preceding the ultra-violet method it is obvious that the non-tannins will not interfere.

Colorimetric estimation of such groups has previously been studied by Mitchell (206)(281), Price (287) and Glasstone (288) using ferrous tartrate in the presence of a buffer. The complex formed produces a blue-violet coloration, and is an exceedingly sensitive test for phenols of this class. Complexes formed with orthodihydroxybenzenes are similar to the anionic "ato" complexes formed by dibasic oxy-acids (286). The orthohydroxy

chelate groups joined through two atoms to the central ferrous atom and so forming a ring, give complexes of exceptional stability.

The colour-density of the above complex varies with pH (288) at a given concentration, but maintains an even maximum over the wide range pH = 6.5 to 8.5 with black wattle tannin. The use of a buffer to keep the pH value within this range is, therefore, essential. In this photometric study a Beckman Model DU Spectrophotometer and the same principles employed with the ultra-violet method were used.

Materials

The materials described by Mitchell (281) and Glasstone (288) and the volumetric apparatus previously mentioned, were used.

Ferrous tartrate reagent : 1 grm. C.P. ferrous sulphate and 5 grms. C.P. Rochelle salt per litre. The solution was unstable and freshly prepared every 3 - 4 hours.

Ammonium acetate buffer. 10% solution filtered before use.

Water. The optical density of each new batch of distilled water was matched with that previously used.

Conditions of Complex Formation

10 ml. of the tannin solution containing about 2 grms. of extract per litre was carefully pipetted into a clean dry flask. To this was added, using pipettes, 120 ml. water, 50 ml. ferrous tartrate reagent and lastly 20 ml. ammonium acetate buffer. Addi-

tion of the ferrous tartrate reagent must precede the latter as the colloidal phenomena arise when the process is reversed. The ratio of ferrous tartrate reagent to the total amount of phenol present is of importance, as this influences the stability of the coloration. The accuracy of the method ultimately depends on the care with which the pipetting is carried out.

Spectrophotometric Examination and Selection of Wave-Length

The ferrous tartrate-orthodihydroxyphenolic complexes are visually identical for pyrogallol, catechol and their derivatives. The colour density developed, however, differs for pyrogallol and catechol derivatives (287) but both show the same broad absorption bands with peaks at about 545 mμ (Fig. LVII). This peak is most sensitive to concentration changes and all measurement was thus carried out at the above wave-length.

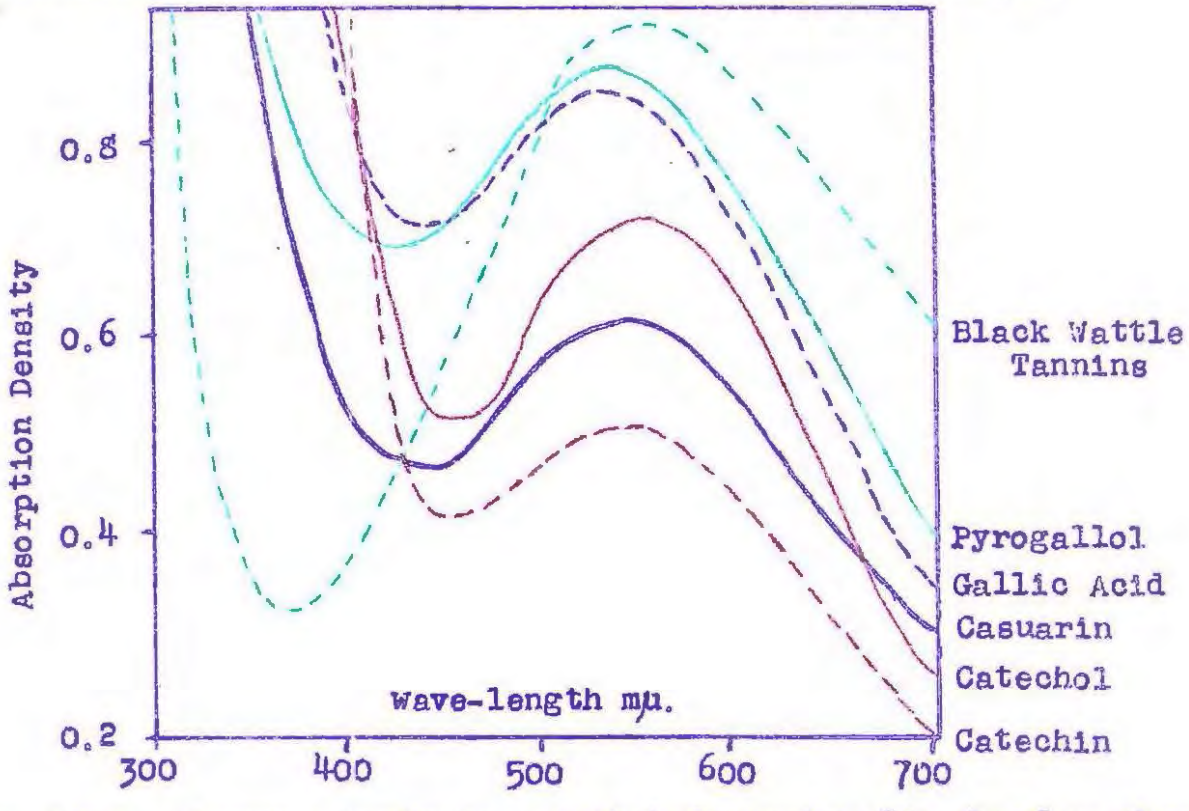


Fig. LVII. Ferrous Tartrate - Orthohydroxyphenolic Complex Absorption Curves.

The casuarin was isolated from Casuarina equisetifolia bark according to Osima (200) and catechin from gambier extracts by Perkin's (89) method.

Stability of the Coloration at 545 mμ

The absorption-density of the tannin-ferrous tartrate complex, prepared as above using a solution containing 2.5 grm. extract of 60-61% tannin content per litre (analytical strength subsequently employed), measured at 545 mμ and a slit-width of 0.02 mm., increases slowly with time (Table XXII).

TABLE XXII

Stability of the Ferrous Tartrate-Tannin Complex at 545 mμ and slit-width 0.02 mm.

Time (minutes)	Absorption Density	% Increase
2.5	0.495	-
4.0	0.497	0.4
5.0	0.498	0.6
6.0	0.499	0.8
7.0	0.500	1.0
8.0	0.501	1.2
10.0	0.502	1.4
11.5	0.502	1.4
14.5	0.505	2.0
15.0	0.505	2.0
17.0	0.507	2.4
23.0	0.509	2.8
30.0	0.510	3.0

Time was taken from the start of the addition of the ferrous tartrate reagent, and from the above a 3% increase occurs over a 30 minute ageing period. Due to the relatively slow increase in value, a constant result may be obtained only by adopting a standardised procedure.

Selection of the Zero-Point Standard

Due to the instability of the complex, its use in differential analysis is unsatisfactory. The substitution of another solution which is completely stable, giving high absorption at 545 mμ (as in the case of the ultra-violet method), is necessary. For this purpose Thomson's grey solution (358) which has been developed as a standard of optical density, is eminently suitable. This inorganic grey solution contains the following constituents per litre :

$Cr_2(SO_4)_3 \cdot K_2SO_4 \cdot 24\ H_2O$	(Analar)	16.67 grm.
$CuSO_4 \cdot 5\ H_2O$	(Analar)	33.33 grm.
$CoSO_4 \cdot (NH_4)_2\ SO_4 \cdot 6H_2O$	(Recryst.)	39.50 grm.
$K_2Cr_2O_7$	(Analar)	120.0 mgm.

The solution may be used only after an ageing process of 6 weeks, and gives high absorption over a wide wave-band. It is relatively stable on dilution, when treated with organic matter, heat and with time. An already aged solution (4 months) diluted and aged for a further period (1 month) showed the following stability :

TABLE XXIII

Time (days)	Absorption Density
-	0.432
30	0.434

Preparation of Standard Curve

Blank values of the cells were determined as in the ultra-violet method. 1.725 grms. of analysed sample was made to a litre. 10 ml. of this solution was pipetted, and the remainder of the reagents added as rapidly as possible in the succession described. Readings were taken immediately, 2.5 - 3.5 minutes after the addition of the ferrous tartrate reagent, in an instrument standardised against the diluted grey solution at 545 mμ and a slit-width of 0.04 mm. This was repeated with tannin solutions increasing from 1.75 grm. to 2.10 grm/litre in 0.05 grm. amounts (Table XXIV)

TABLE XXIV

Wt. Extract/Litre	Wt. Pure Tannin/Litre	Absorption Density (Against dil. grey soln.)		
1.725	1.139	-.001	.000	
1.75	1.155	.006	.006	
1.80	1.188	.022	.020	.020
1.85	1.221	.034	.033	
1.90	1.254	.047	.046	
1.95	1.287	.062	.060	
2.00	1.320	.075	.075	
2.05	1.353	.085	.0875	.0875
2.10	1.386	.104	.105	

These readings were plotted (Fig. LVIII) giving a linear relationship between absorption density and concentration.

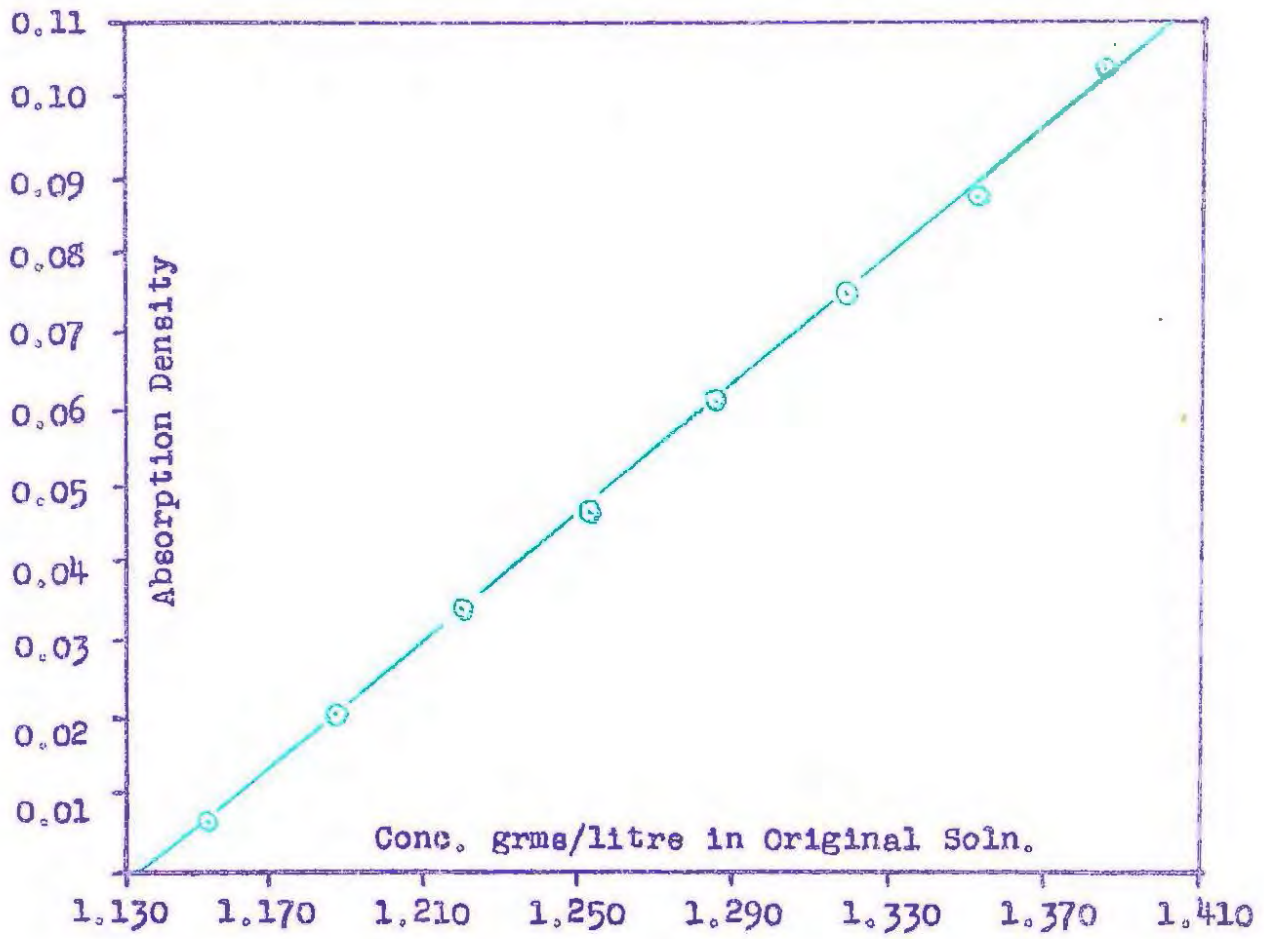


Fig. LVIII. Standard Curve for Colorimetric Method.

Analysis of Analysed Samples

(a) Bark Samples

Separate extractions were performed on the same samples as the solutions previously used for the ultra-violet method had deteriorated due to slight mould growth. The use of fresh solutions was also necessary in order to compare results obtained by

both new methods, and so determine whether deviations shown by the ultra-violet method were reproducible.

The standard procedure for the Procter extractor was used, but successive nearly-complete drainages of the extracting solution were allowed before running in fresh hot distilled water. The extracts were cooled to 20°C., centrifuged as before (3,500 r.p.m. for 4 minutes) and diluted (150 ml. to 500 ml.) before use. The resultant optically clear solution was used to prepare the colorimetric solution. Optical measurement was made 2.5 to 3.5 minutes after the addition of the ferrous tartrate reagent.

TABLE XXV

Code No.	Wt/2L	Readings		% Tannin		
				Colorimetric	U.V.	Hide-Pdr.
W/15	23	0.028	0.027	35.2	33.8	34.9
S/33	23	0.029	0.029	35.4	35.0	35.4
S/34	24	0.012	0.012	32.9	33.2	32.7
D.F.O.	22	0.028	0.027	36.9	36.2	35.2
16/deW	23	0.015	0.015	34.4	33.0	33.3
17/deW	22	0.005	0.005	35.3	35.8	34.3
18/deW	24	0.019	0.017	33.2	33.0	33.2

(b) Tannin Extracts

2.0 grms/litre were weighed, dissolved and made to the mark as before (Table XXVI).

TABLE XXVI

Code No.	Readings		% Tannin Colorimetric	Av.	Hide-Pdr.	Av.
409	0.029	0.027	60.95	60.7	61.8	61.7
410	0.024	0.024	60.5		61.5	
411	0.023	0.023	60.4		61.8	
412	0.028	0.027	61.0		61.8	
413	0.032	0.031	61.4	61.0	61.7	61.2
414	0.030	0.029	61.1		61.1	
415	0.030	0.029	61.1		61.1	
416	0.0225	0.0225	60.3		60.8	
417	0.028	0.027	60.9	60.9	61.9	62.2
418	0.030	0.028	61.1		62.3	
419	0.030	0.029	61.1		62.3	
420	0.024	0.024	60.5		62.3	

(c) Purified Tannin

0.2667 grm. of the lead-salt purified tannin used for the ultra-violet method was made to 200 ml.

Found : % Tannin = 98.0

(Hide-powder method = 94.0%, U.V. method = 95.2%).

Maximum Accuracy Attainable

From theoretical considerations (345) the highest accuracy

may be achieved with the Beckman Model DU Spectrophotometer by working at the maximum sensitivity and maximum concentration possible without causing Beer's Law deviations. Enough light must pass through the zero-point standard to effect the zero setting. This is accomplished on the Beckman by working at much wider slit-widths normally used with water or a reagent blank as zero-point standard. Widening the slit-width, however, increases the band width, and decreases the monochromatic character of the emerging light. Thus with copper sulphate solutions (343) too wide a slit-width gave a curved rather than a straight line, and a compromise, between concentration and monochromaticity was necessary, so that band widths used with wider slit-widths did not stretch into regions of low intensity. Slit-widths used (e.g. for water) are normally wider in the ultra-violet than in the visual range.

Such conditions were observed in the ultra-violet method where a wide slit-width (1.9 mm.) at 280 mμ, corresponding to a band width of 6.17 mμ (from dispersion data supplied with the instrument), and the sensitivity knob as far over to the clockwise side as possible, was used. The choice of the absorption density for the standard solution was arbitrary and only dictated by the above considerations. The slit-widths which may be used in the ultra-violet region vary from instrument to instrument since it is partly dependent on the adjustment and age of the ultra-violet source. (344).

In the colorimetric method the slit-width used (0.04 mm.) corresponded to a band-width of 1.06 mμ at 545 mμ. As the absorp-

tion curve (Fig. LVII) of the black wattle complex shows a wide maximum (> 50 mμ), work at wider slit-widths and higher concentrations is possible. The limiting factor in the accuracy of the method is, however, the relatively unstable complex (Table XXII) and measurement at higher concentrations would bring no added advantage.

Discussion of Results

It is important to note when comparing the above results, that the tannin estimations by the hide powder method of the standardising extract sample, the bark samples and the commercial extracts, were carried out by different individuals or groups of individuals. The Official Method allows a maximum difference of 3% between results obtained by different analysts when using the same sample.

The standardising sample, the purified extract and the barks were analysed and the results calculated in the usual way, but for the commercial extracts (analysed by a commercial firm) a different technique, used for large-scale batch analysis to ensure uniformity of the product, was employed. Here the total solids were determined as usual, but the solubles and non-tannins were estimated as average values per batch of four samples, obtained by mixing equal proportions of the analytical solutions for estimation. The percentage tannin for each sample in the batch was calculated from these averages by making allowance for the different moisture contents of each and using the same averaged

tannin/total solids ratio of the whole batch. With photometric methods the value of each sample could be read directly from the curve, and these results were averaged merely for comparative purposes.

When comparing the accuracy of bark-samples it must be remembered that two factors are involved :- (a) the degree of completeness of extraction and (b) the accuracy of tannin estimation by different analysts. A combination of these two factors probably accounts for some of the variations obtained.

Considering the above factors, both methods offer new, rapid, accurate and direct techniques for large-scale tannin estimation of liquid or solid extracts. The ultra-violet method appears preferable due to its greater simplicity, rapidity and the greater degree of stability of the bisulphite-protected tannin solution used for absorption measurements. Results as accurate, are, however, obtainable with the colorimetric method once some practice in standardising the technique has been obtained. The latter method has the further advantage of being applicable to photometric apparatus of simpler construction. Photoelectric colorimeters are suitable since appropriate filters may easily be selected to cover the broad absorption band exhibited by the complex. The maximum precision attainable with such colorimeters has been discussed (345) and can be assessed. Rough estimations may also be made with Nessler tubes, and may be used for the approximate determination of samples of unknown tannin content as a necessary preliminary to the hide-powder method.

Experimental Summary

To facilitate the practical application of both methods the following summary is presented :

(a) Ultra-violet Method

The following solutions were prepared :

(1) Zero-point standard. 0.8400 grm. benzoic acid per 2 litres diluted 5 times (200 ml/litre). The diluted solution should show an absorption density of 0.434 at 280 mμ and slit-width 1.9 mm. against distilled water.

(2) Tannin solutions for standardisation curve. Five solutions of a previously analysed (hide powder method) well-mixed tannin sample were made, increasing in concentration from 2.1 to 2.5 grms/litre by 0.10 grm. amounts. These were diluted 40 times by accurately pipetting 25 ml. of each into litre flasks, each containing 500 ml. of exactly 0.2% bisulphite solution and diluting to the mark.

Method : The Beckman spectrophotometer was set against the benzoic acid standard at 280 mμ and 1.9 mm. slit-width and the absorption densities of the tannin solutions determined in the usual way. Plotted on accurate graph paper, a straight-line relationship between concentration and absorption-density was obtained.

Unknown samples : Solutions of extracts of unknown tannin content (ca. 60%) were made up as above (2.2 grms/litre diluted 40 times). Bark samples (ca. 35% tannin content) were extracted

in the Procter extractor by the Official Method and the resultant solution centrifuged (3,500 r.p.m. for 4 minutes) and diluted 100 times (10 ml/litre).

The absorption densities of these were determined against the benzoic acid standard as before, and their value in terms of tannin content read directly from the graph.

(b) Colorimetric Method

The following solutions were used :

(1) Ferrous tartrate solution : freshly prepared 1 grm. C.P. ferrous sulphate and 5 grm. Rochelle salt per litre.

(2) Ammonium acetate buffer : 10% solutions filtered.

(3) Zero-point standard : Thomson's grey solution (35%), diluted and suitably aged to give an absorption density of 0.434 at 545 mμ and slit-width 0.02 mm. against water.

(4) Tannin solutions for standardisation curve.

Eight solutions of the previously analysed (hide-powder method) sample were made, increasing in concentration from 1.75 to 2.10 grms/litre by 0.05 grm. amounts.

Method : To exactly 10 ml. of each were added (in sequence) 120 ml. water, 50 ml. ferrous tartrate reagent and 20 ml. ammonium acetate buffer. Absorption density measurement of each was taken 2.5 - 3.5 minutes after the addition of the ferrous tartrate reagent in a Beckman set with the grey solution at 545 mμ and a slit-width of 0.04 mm. Plotted on accurate graph paper a straight-line relationship between concentration and absorption density was

obtained.

Unknown Samples : Solutions of extracts (2 grm/litre) and bark samples (diluted 150/500 ml.) were prepared as in the ultra-violet method.

The absorption densities were determined against the grey solution as above, and their values in terms of tannin content read directly from the graph.

CHAPTER IX

THE "MOLECULAR WEIGHT" OF BLACK WATTLE TANNINS

In the past, "molecular weight" studies on the tannins were complicated by three factors :

(a) The complete elimination of carbohydrate non-tannins could not be achieved without altering the nature of the tannins by oxidation or condensation.

(b) No suitable method was available to cover the molecular range 500 - 2000, and

(c) Suitable solvents had not been found for such determinations.

The latter aspect particularly presents difficulties, as the completely dry amorphous tannins are soluble only in those solvents of small molecular dimension (e.g. water and methanol) which are capable of penetrating the intermolecular spaces; on to which the tannins may hydrogen-bond strongly, and which finally act as dispersing media for the tannins. Strong intermolecular forces between solvent and solute are obviously undesirable.

Hitherto the elimination of sugary non-tannins was accomplished only by prolonged electrodyalysis in aqueous medium. This method necessitated the use of membranes which allowed the penetration of small molecular bodies (e.g. sugars and small phenolic units), and retained the large molecular units (highly condensed tannins) which were then isolated for "molecular weight" studies. As it is now known that gums of large molecular weight

are present in the extract it is obvious that these could have been present amongst the "purified" tannins. Electrodialyses were also run under conditions which permitted the oxidation and possible condensation of the tannins. The average particle size of the products of purification was, therefore, bound to be high, and was not a true index of the average "molecular weight" of the tannins.

Previous work on "mimosa" extracts in this connection may be summarised as in Table XXVII.

"Molecular weight" determinations on the derivatives of black wattle tannins have also been attempted. Rich and Stephen (371) used a cryoscopic method with dioxane as solvent for determining the average molecular size of the methylated and fractionated acetylated tannins. They found :

Substance	Av. Mol. Wt.	Max. Value Found
Methylated Tannins	1700	1950
Acetylated Tannins Fr. I	1900	2200
Acetylated Tannins Fr. II	1300	1450
Acetylated Tannins Fr. III	3000	3500

Due to the hygroscopic nature of the solvent, dioxane, more significance was attached to the maximum values obtained. These were further "corrected" to even higher values (2300, 2600, 1700 and 4500 respectively) as traces of moisture were presumed to

TABLE XXVII

Previous Molecular Weight Determinations of Black Wattle Tannins

Author	Method of Purification	% Tans	% Non-tans	Method	Solvent	"Molecular Weight"
Douglas & Humphreys(118)	Unpurified	70.3	29.7	Cryoscopic	Water	603
"	"	70.3	29.7	"	"	432
"	Electrodialysis	95.4	4.6	"	"	1570
"	"	95.4	4.6	"	"	1704
Corbett (146)	Unpurified	71	29	"	Camphor	600
Rich (173)	Unpurified	71	29	"	Water	525-548
"	Acetone-soluble tannins	-	-	"	"	485-515
"	Electrodialysis	-	-	"	"	1760
"	"	-	-	Ebulliometric	Acetone	1485
"	Acetone-soluble tannins	-	-	"	"	485

be present. The corrected results were then in line with the previously-determined relatively "high" molecular weight of 1760 for the tannin itself.

Williams (157) obtained the values 450, 433, and 470 for the ethyl acetate fresh-bark extracted acetylated tannin. He used acetone as solvent, and the usual ebullioscopic method.

From all the afore-mentioned work it is evident that the proportion of the extract separated by electrodialysis is certainly of high molecular weight (± 1700). It is obvious that this molecular weight of the tannins (95% purity), to which most significance has been attached in the past, is by no means an average value for the tannins, as the crude extract of 70% tannin content has a much lower (average 550) molecular size. A negative value for the 30% non-tannins in the extract would be reflected if both these values were correct. It is also obvious that the origin and treatment of the tannins have to be taken into account before the significance of molecular weight determinations are compared. These facts have not been recognised in the past.

In the present investigation use was made of a derivative of the tannin and a non-polar solvent, to overcome the afore-mentioned difficulties regarding the association between the solvents and the polyphenolic tannins. The cryoscopic method was used with benzene as a solvent. The latter is superior to the hygroscopic dioxane used by Rich and Stephen (371), and their cryoscopic constants are of the same order (benzene = 5.0; dioxane = 5.4).

The tannins were obtained from the commercial extract by the lead-salt "purification" method, which included a methanol extraction to separate the gums. The "purified" product was methylated with dimethyl sulphate and methanolic KOH in the usual way, and finally subjected to chromatography on an alumina column to remove incompletely substituted material. The method was standardised against dilute solutions of crystalline gambier catechin tetramethylether. At higher concentrations the catechin derivative tended to separate from the benzene.

Found :

0.1760 grms. catechintetramethylether in 25.94 grms. "Analar" benzene gave an average depression of 0.1055°C. (six readings).

Whence Molecular Weight = 321 ± 4.3

Theoretical for $C_{15}H_{10}O_2(OCH_3)_4$ = 346

The methylated tannins were dried for 2 hours at 110°C. under vacuum.

0.5262 grms. of methylated tannins in 27.72 grms. "Analar" benzene gave an average depression of 0.072°C. (six readings).

Whence Molecular Weight = 1318 ± 85

Methylated tannins, prepared by the methylation of the acetone extractives of the commercial extract and subsequent chromatography of the product by Kirby's method on an alumina column, were used to confirm the above value using Rast's method and camphor as solvent.

Found :

0.0188 grms. methylated tannins in 0.1981 grms. camphor gave a depression of 3.50°C.

Whence Molecular Weight = 1084

0.0932 grms. methylated tannins in 0.9269 grms. camphor gave a depression of 3.80°C.

Whence Molecular Weight = 1059

Both these samples of methylated tannins were subsequently sent to the National Chemical Laboratories of the C.S.I.R. for investigation by an independent method. A Menzies-Wright ebulliometer, presumably as designed by Kitson et Al (372) and later modified by Ray (373), and benzene as solvent were selected. The methylated tannins were dried for 3 hours at 110°C. Readings on the differential vapour pressure thermometer were made with a cathetometer. The apparatus was standardised against benzil. 0.160 grms. benzil gave a reading of 13.7 mm. From this the constant was calculated.

Found :

A. Methylated Tannins (lead-salt purified)

Wt. (grms.)	Vapour Pressure	Mol. Wt.
.4466	6.6 mm.	1223
.297	4.6 mm.	1170
.249 (additional)	3.8 mm. (additional)	1180

Average Molecular Weight = 1173

B. Methylated Tannins (Acetone extracted)

Wt. (grms.)	Vapour Pressure	Mol. Wt.
0.2366	3.8 mm.	1123
0.2586 (additional)	4.1 mm.	1140

Average Molecular Weight = 1133

Methylated tannins and a non-polar solvent such as benzene are thus more suitable for the molecular weight determination of the tannins, and fairly consistent results are obtained with different methods. The Menzies-Wright method, shown by Ray (373) to be suitable for measurements up to a molecular weight of 5000, seems to be the most suitable technique.

The acetylated tannins which are also soluble in hot benzene, may be used as additional confirmation of the average molecular weight. A comparison of results obtained by methylating portions of the same sample with diazomethane and dimethyl sulphate is desirable before embarking on further work. The diazomethane methylation is gentle and its action should automatically separate traces of carbohydrates (which do not methylate with this reagent and therefore remain soluble in water), from the methylated polyphenolic tannins.

It is thus obvious that care must be exercised in the preparation of tannin samples for molecular weight studies. The influence of the treatment and method of isolation of the tannins before forming a derivative, as well as the method of formation of the derivatives, have still to be determined and compared.

The above molecular weights are roughly half of those obtained by Stephen and Rich on the methylated and acetylated derivatives, and well below those of the electrodialised tannin samples examined by Douglas and Humphreys and Rich. The bulk of the tannins, therefore, falls into a range where many conventional methods are still applicable.

Summary

(1) A critical examination of previous work has shown that undue significance was attached to the high molecular weights (ca. 1700) of a certain fraction of the extract obtained by electrodialysis.

(2) As high-molecular weight gums are now known to be present, it is possible that the previously "purified" tannins were, in fact, still contaminated by gums, due to method of "purification" employed.

(3) Three methods of estimation using the methylated derivatives and non-polar solvents have shown that the average molecular weight of the methylated tannins is of the order of 1150 and, therefore, that of the tannins is of the order of 950.

(4) The method of treatment of the tannins as well as the method of formation of the derivatives may have an appreciable effect on the result obtained. This aspect requires investigation.

(5) Methylations with diazomethane probably represent the

most useful technique of obtaining derivatives without affecting the nature of the tannins. Comparison of the molecular weights of the various derivatives of the tannins is recommended.

(6) The recently-improved Menzies-Wright ebulliometric method possibly represents the most useful and accurate technique for the range required by the derivatives of black wattle tannins

CHAPTER X

BLACK WATTLE NON-TANNINS

All tanning materials contain varying proportions of sugars, uronic acids and sometimes small phenolic bodies which are generally classified as "non-tannins". Few compounds have been identified from this heterogeneous group and therefore little is known of their function in the vegetable tanning process. White (126), for example, has claimed the identification of glucose, arabinose and xylose as carbohydrate non-tannin constituents of quebracho extract. The nature of the non-tannins associated with other condensed tannins is still unknown.

Phillips (374) and Tarboton (375) obtained reactions characteristic of pentoses, hexoses and uronic acids from black wattle non-tannins. Tarboton was able to form glucosazone and obtained increased quantities of reducing sugars from the acid hydrolyses of this fraction. He concluded that the carbohydrates consist mainly of glucose and sucrose and that there was also some evidence of the presence of maltose. He established that the proportion of pentoses present as determined by Phillips was low, due to the reaction of the tannins with the furfural formed during estimation.

The non-tannins examined in this investigation were obtained from the primary fractionation schemes in Chapter IV.

(a) The Gums

A relatively large proportion of true gums is present

in commercial black wattle extract. They are best separated from the tannins and sugars by the addition of a large excess of ethanol, accompanied by vigorous stirring, to a 10% solution of the extract. The gum precipitates as a swollen gelatinous mass from which the mother liquor is removed with difficulty, first by centrifugation, and subsequently by suction in a Buchner funnel. The tannins are completely removed only by repeating the above precipitation 4 or 5 times, and their separation depends on the efficient removal of the mother liquor from the swollen material on each occasion. Large preparations, therefore, require additional precipitations. As gums are known to undergo autohydrolysis on heating in aqueous medium (376)(377), the repeated precipitations andre-solutions of the gums were carried out in the cold during the "purifying" process.

Mainly for this reason, the above method is preferred to the Soxhlet extraction method of the alternative separation scheme, which is otherwise more efficient and less laborious. After a six-hour extraction of the solid extract (10 grms in 5 - 20 mesh particles) with 95% ethanol, a greyish residue remains which occupies the same shape as the original extract chips. The residue constitutes about 6.9% of the commercial extract and thus represents about 8.6% of the total solids present. This method removes the sugars and tannins completely, and the residue obtained, remained stationary on one- and two-dimensional chromatograms (Fig. LIX) where water formed the minor proportion of the developing mixture. The gums correspond apparently to Kirby's

Fig. LIX. Two-Dimensional Chromatogram of Black Wattle Gums from the Commercial Extract.

et Al. "component" N.

The gums were hydrolised quantitatively (see Stephen (377)) and the neutral product investigated by paper chromatography (downward migration) using water-saturated n-butanol as developing mixture. Reference compounds were used and the paper irrigated continuously to accentuate differences in RF value (especially to

differentiate between galactose and glucose which give similar colour-reactions and have RF values in close agreement). Naptho-resorcinol-phosphoric acid (378), aniline hydrogen phthalate (379) and β-naphthylamine (380) spraying reagents were used to locate and identify the sugars. (Fig. LX). Galactose, arabinose and

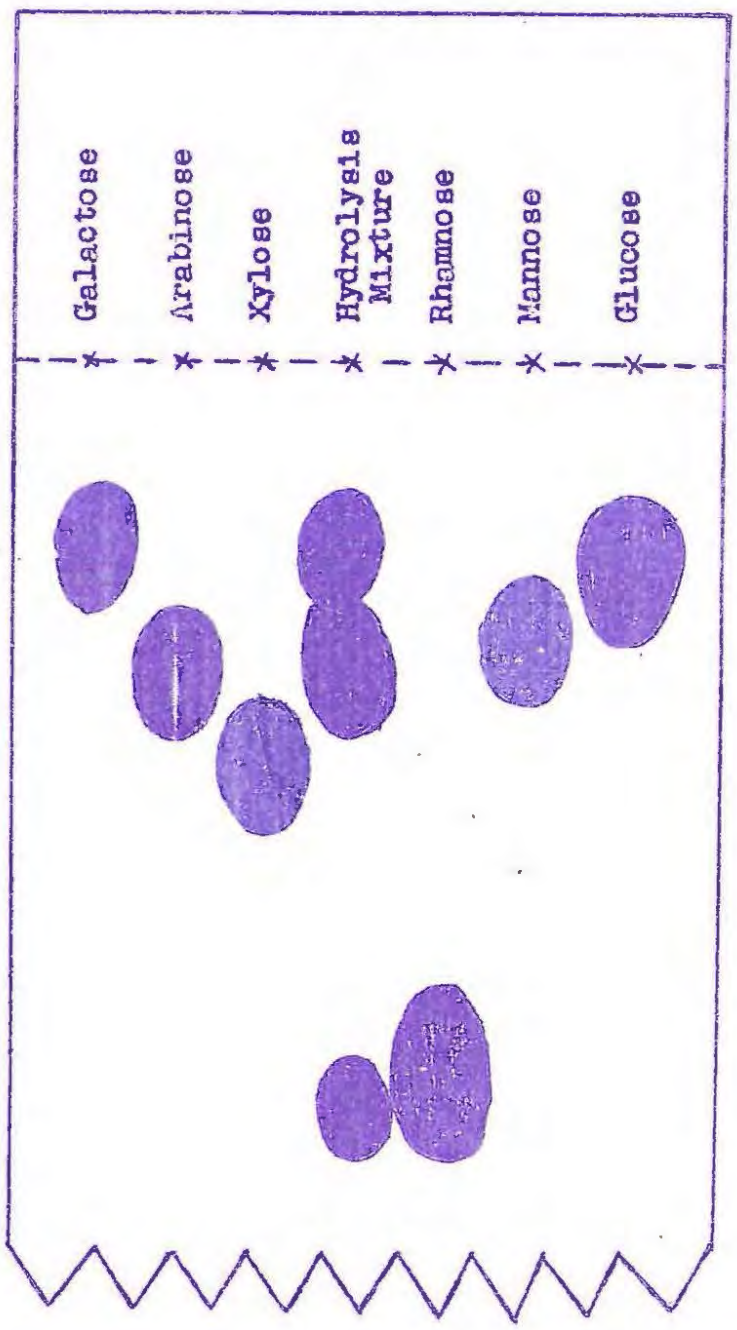


Fig. LX. Chromatogram of the Neutral Hydrolysis Products of the Black Wattle Gums.
(Sprayed with Napthoresorcinol-Phosphoric Acid).

rhamnose residues were, therefore, produced from the gums on acid hydrolysis.

Hydrolysis of the gums (10 grms.) with 2 N sulphuric acid (see Stephen) for six hours gave evidence of the presence of a water-soluble but methanol-insoluble barium salt, probably of a uronic acid residue. The identification of this acid as well as the estimation of the proportions of sugars present by chromatography (381), have yet to be completed.

The equivalent weight of the gums was found to be of the order of 4000, compared with 1880 found by Stephen. Stephen obtained l-arabinose (6), l-rhamnose (1), d-galactose (5), and d-glucuronic acid (1), in the approximate molar ratios indicated, from the acid hydrolysis of black wattle gum. This gum exudes from the bark usually in the form of a clear transparent mass on injury, or as a result of a pathological condition known as "gummosis". The number-average molecular weight of the gums was of the order of 92,000, but autohydrolysis occurs slowly and some fragments of molecular weight below 30,000 are formed as a result.

The gums in the extract have been subjected to a prolonged heat treatment, and although it is surmised that their average molecular weight is below the above, they are still very large molecular particles of remarkable swelling power which retain tannins with avidity due to occlusive and adsorptive effects, and which may seriously retard the tanning process. In concentrated aqueous solution they form a mucilage and their presence probably contributes largely to the water-retaining properties of the ex-

tract. Due to the insolubility of the gums in alcohols, they remain at the point of application on paper chromatograms of the whole extract which are developed with alcohol-water mixtures, and in view of their adsorptive power, their presence could result in the incorrect interpretation of chromatograms of the tannin fraction.

(b) The Sugars

The sugars, separated from the tannins by the lead-salt method, still contained small traces of polyphenolic material. These were removed by shaking with hide-powder. The main solution and the washings from the hide-powder were concentrated under reduced pressure and taken to complete dryness. The sugars were extracted from the excess lead acetate with boiling methanol. The product obtained was weakly reducing (Fehling's solution), and was examined by chromatography. Exactly the same chromatographic methods used for the investigation of the hydrolysis-products of the gums were applied.

One-dimensional chromatograms of the sugar fraction were run in water-saturated n-butanol using reference compounds and naphthoresorcinol-phosphoric acid spraying reagent. The latter reagent was superior to all others for this purpose, and does not affect the paper. Prolonged irrigation (18 - 24 hours) gave excellent separations of sucrose, glucose and fructose from the carbohydrate fraction of the commercial extract (Fig. LXI). The same fraction from the fresh-bark contained only sucrose (Fig. LXII),

which was further identified by hydrolising 5 ml. of the sucrose solution in a sealed tube with 4 ml. 5 N H_2SO_4 for 6 hours (water-bath). The hydrolysis products were neutralised with barium carbonate and the final traces of acid removed by passing the hydrolysate through a column of "Deacidite B", an anion exchange resin. The water was removed under reduced pressure and the concentrate examined by chromatography (Fig. LXIII). Only glucose and fructose were present.

Fig. LXI. Chromatogram of the Carbohydrate Fraction of Commercial Black Wattle Extract

(Napthoresorcinol-Phosphoric Acid Spray)

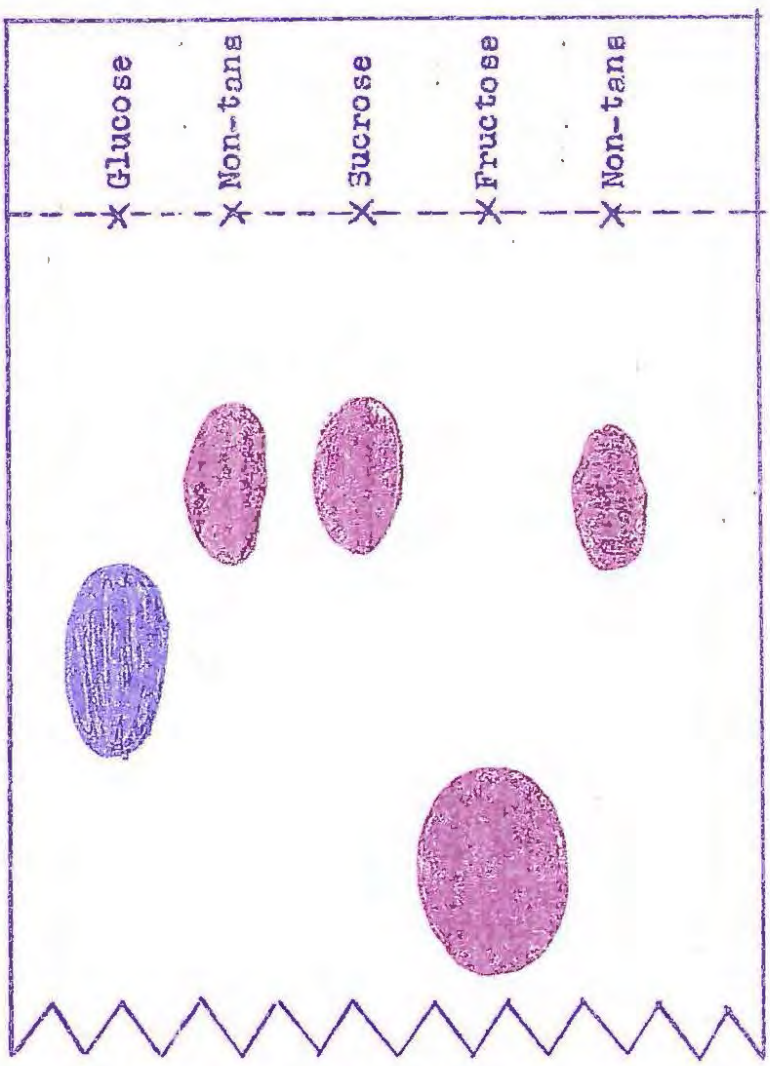


Fig. LXII. Chromatogram of the Carbohydrate Fraction from Fresh Black Wattle Barks

(Napthoresorcinol-Phosphoric Acid Spray)

With the napthoresorcinol-phosphoric reagent the presence of sucrose, glucose and fructose could be established on one-dimensional chromatograms of the commercial extract without the prior separation of the tannins. The exact proportions of sucrose, glucose and fructose in the natural extract still remain to be established. The indications are, however, that this carbohydrate non-tan fraction constitutes about 10 - 13% of the commercial extract.

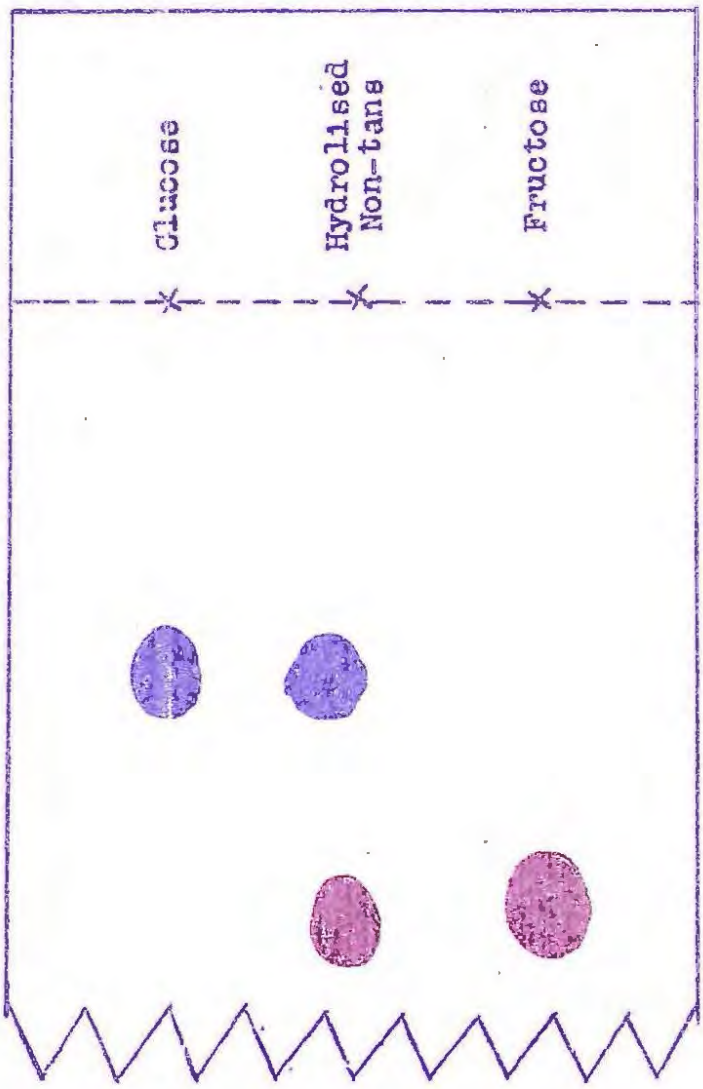


Fig. LXIII. Chromatogram of the Hydrolysis Products of the Sucrose from Fresh Black Wattle Barks

(Napthoresorcinol-Phosphoric Acid Spray)

Discussion

The nature of the non-tannins is of some importance to the tanning industry. Due to lack of efficient separatory methods hitherto, the effect of the small molecular carbohydrates on the tanning process is as yet unknown. The lead-salt method is capable of achieving such separation on a pilot-plant scale, and the author's method has recently been used by Kuntzel and Zissel

(193) for the separation of commercial oak-bark extract into various constituents in order to assess the effect of the presence of the non-tannins on the tanning process and the resultant leather.

Large-molecular particles, such as gums, are semi-colloidal in nature and tend to block the pores of fast filtering paper even in relatively dilute solution. The blocking of the inter-molecular spaces in the collagen structure through which the tannins diffuse during the tanning process, is thus easily envisaged. As black wattle extracts are used primarily for heavy sole-leather manufacture, during which process the slow penetration of the tannins from liquors of high concentration occurs, the retardation-effect of the gum on the penetration rate requires investigation.

Stephen ((149) p. 59) reported a slight increase in acetyl content and a large increase in molecular weight in successive fractions of acetylated acetone-soluble tannins, which were obtained by leaching the same black wattle barks for seven-day periods at 7°C., 35°C., 55°C., and ± 75°C. The increased molecular weight of the hot extract (3200) compared with the cold leach (1760) might be due partly to condensations or oxidations which occur at elevated temperatures. As differences in quality may be observed between skins tanned with cold- and hot-leached wattle barks (Woodhead (362)), this work requires critical repetition using weak bisulphite solutions (see Chapter XI) which minimise oxidation effects. The influence of the extraction-temperature on the solubility of the gums and sugars also requires study.

The presence of the gum in the extract also appears responsible for certain special solubility-phenomena. On dilution of the cold concentrated commercial extracts (prepared at high temperatures), the solutions are known to assume a colloidal appearance at intermediate concentrations. The colloidal suspension usually redissolves on further dilution. In the concentrated aqueous extracts, due to their close association, the less soluble gums are probably held in solution by the tannins through intermolecular forces such as hydrogen bondage. On dilution all these constituents are predominantly hydrogen-bonded on to the solvent and true solubility effects come into operation. The more soluble tannins may displace a proportion of the less soluble gums, resulting in the afore-mentioned phenomenon. At high dilution this displacement effect would obviously disappear.

Summary

(1) Black wattle non-tans consist of gums and sugars predominantly.

(2) The gums are large molecular units of remarkable swelling power which give on hydrolysis galactose, arabinose, rhamnose and a uronic acid residue. They thus appear identical with thenatural exudate of the bark produced on injury or as a result of pathological conditions.

(3) The sugars in the commercial extract are sucrose, glucose and fructose.

(4) The carbohydrate fraction of the fresh-bark extract contains sucrose only. Glucose and fructose thus appear to be formed during the drying or the extraction-process.

(5) The commercial significance and effects of these non-tannins on the tanning process are discussed.

CHAPTER XI

THE EXTRACT OF THE BARK OF ACACIA DECURRENS WILLD.

(Green Wattle Extract)

Of the four species of acacia dealt with in this investigation, the green wattle tree is most closely allied to the black wattle, and until recently they were regarded as varietal forms of the same species (382 - 385). The green wattle has been known in South Africa from the earliest days of the industry, but only in 1929 were trial plantations and later planting on a commercial scale attempted. Bark samples were sent to the Imperial Institute, London, and it was reported that "leather tanned with green wattle barks are not quite so close-grained as those produced with black wattle samples, and were of a decidedly pinker tint" (386). It was chiefly this slightly darker colour of the green wattle tanned leather which decided against its continued use, as the tanner and footwear manufacturer would favour the lighter coloured black wattle tanned leather if both were freely available.

Farmers who had already started planting on a large scale found no ready market as a result, and only the war years with their almost doubled demand for wattle extract, brought temporary relief. During this period, extract manufacturers agreed to accept green wattle bark, and as the proportion was very low compared with black wattle, the colour of the extract

was not unduly impaired. Although the high demand for wattle continued (see p. 7, Chapter I) due to the diminishing reserves and supplies of other vegetable tanning materials, the plantations of green wattle were gradually replaced by black wattle as they reached maturity.

In spite of this colour handicap the green wattle tree has many superior characteristics, when compared with black wattle under identical conditions. Chief of these is its resistance to insect attack. The most damaging of the pests is the wattle bagworm, Acanthopsyche junodi Heyl., a lepidopterous insect which causes intense defoliation of the black wattle tree. This results in retarded and stunted growth; in increased weed and grass growth amongst the trees; and increased difficulty in stripping the trees from the bark. Williams (3) reports that green wattle trees stand out prominently and suffer only slightly in mixed plantations which are heavily infested. Although the green wattle tree is not free from insect attack, it recovers far more rapidly from such attack and seldom suffers serious defoliation. This apparent resistance is probably due to its vigorous growth, especially under adverse conditions.

Other advantages of the green wattle tree may be summarised as follows :

(a) The bark has a tannin content equal to that of black wattle bark.

(b) Green wattle trees continue to strip freely in the dry season when black wattle trees refuse to do so (3).

(c) The green wattle tree grows faster than black wattles on poor sites, and

(d) produces a greater volume of timber under comparable conditions.

(e) The timber from green wattles is straighter than that from black wattles.

(f) Green wattles recover more rapidly than black wattles after hail damage, insect damage or drought.

(g) The seeds ripen more rapidly and have a higher percentage germination than black wattle seeds.

(h) Green wattles produce the same gross weight of bark, but this may be higher than that from black wattle under adverse conditions.

Disadvantages other than colour includes only the slightly lower strength of the wood (6).

Because of the numerous advantageous characteristics listed above, as well as the inherent redness of the bark extract, hybridisation studies of black and green wattles were started by Philip and Sherry (387) and are continued today at the Wattle Research Institute, Pietermaritzburg.

In an attempt to assist such genetic work, green wattle extracts have been studied to

(a) determine the mechanism of colour development and its possible control, and

(b) show up all differences between black and green wattle extracts.

(a) Examination of the Barks

The fresh bark strips, from black and green wattle trees of the same age, were placed in milk cans and covered with paraffin as soon as possible after stripping to exclude oxygen. Under these conditions they retained their fresh appearance for almost a month, and could be transported without change in condition. Barks from trees of equal age growing in close proximity on similar soils were selected, and received after being transported from Natal under the above conditions.

Although Williams (3) reported close agreement in the thickness of black and green wattle barks from 3 - 9 year-old trees, the samples submitted showed a distinct difference. Fresh black wattle bark with an average thickness of 8 mm. was always nearly twice as thick as green wattle bark of the same age and cut at a similar height from the ground from corresponding trees. Williams possibly referred to the dried bark.

In the fresh state, the green wattle bark already shows a pink tinge as opposed to the pale yellow colour of black wattle bark. Both barks darkened fairly rapidly on exposure to air, especially in the moist condition. Maximum darkening takes place on the soft inner side (cambium). When cold aqueous infusions of the fresh bark chips of both barks are made, the green wattle solution is initially already darker. This is almost certainly due to the deeper colour of the tannins present in the bark.

(b) Tannin Analyses of the Extracts

Bark samples from trees 6 years of age, were extracted with cold water and the concentration worked up to 70 - 80° Bk. in a countercurrent process. The aqueous extracts were concentrated under reduced pressure and the resultant extract analysed for tannins by the Official Method. Results were averaged and recalculated to a dry basis :

		Green Wattle	Black Wattle
% Tannins	=	75.02	80.19
% Non-tannins	=	24.06	19.81
% Solids	=	0.92	0.00

A hot leach from the barks of 10 year old trees gave the analysis :

		Green Wattle	Black Wattle
% Tannins	=	73.14, 74.91	77.80, 79.42
% Non-tannins	=	26.86, 25.09	22.20, 20.58

A cold methanol extraction from 7 year old trees contained on a dry basis :

		Green Wattle	Black Wattle
% Tannins	=	81.14	80.00
% Non-tannins	=	18.86	17.60
% Solids	=	0.00	2.40

The above figures show that the tannin/non-tannin ratios are very much the same when identical extraction procedures are applied to barks of the same age. The non-tannins of both extracts are colourless in solution; darken slightly on the water-bath, and yield light brown solids of identical appearance. It is obvious that the green wattle non-tannins are not responsible for the additional redness of green wattle extracts.

(c) Colour Control of the Extracts

(i) Introduction

Commercial black wattle extracts are normally chocolate-brown in colour, although the bark itself is almost colourless. It is also known that black wattle extracts and mimosa-tanned leather darken when exposed to air and light over long periods. Rice (388), Chesire (389), Merry (390) and Jany (391) have all shown that this darkening of tannin liquors is due only to oxidation by atmospheric oxygen. Chesire demonstrated that heat itself is not detrimental to the tannins, but in the presence of oxygen, elevated temperatures caused the acceleration of the oxidation-reaction. The absence of moisture or the addition of small amounts of reducing agents were found to arrest further darkening of the commercial extract. Later (392) he showed that the colour of the fresh-bark extract, which is very light, may be controlled to a major extent by pH adjustment. The effect of pH on the colour development of polyhydroxy compounds has been known since 1921 (393). Jany (391) and chiefly Merry (394)(395)(396)

showed that tannin solutions absorbed oxygen, and that the amounts absorbed increase with increase of pH and produce correspondingly deeper-coloured extracts. Minimum colour values were observed in bark extracts acidified by various acids to pH = 3.

As the green wattle extracts are chemically almost identical (see later work)with black wattle extracts,but appear to redden more easily on account of atmospheric oxidation, the problem of the use of the former resolves itself into one of oxidation control. Green and black wattle tannins were, therefore, first studied from this angle.

(ii) The Effect of Various Compounds on the Oxidation of Green and Black Wattle Tannins

Unless leaching of the fresh bark and subsequent tannage is to be carried out in non-corrosive metal containers, the presence of acids in the extract is not desirable. Small amounts of salts formed on corrosion would combine with tannins to yield dark-coloured complexes. A non-corrosive compound was thus sought, which in low concentration would inhibit colour-development of the fresh bark, but would not interfere with subsequent tanning. Williams attempted to use alum for this purpose (7) and similar trials have been made by Merry (394).

The following classes of compounds were tried out in this investigation :

(a) Complex-forming compounds, e.g. boric acid, phosphoric acid, etc.

(b) Compounds which hydrogen-bond strongly, e.g. methanol, acetone, dioxane etc.

(c) Weak organic acids e.g. oxalic, salicylic, gallic etc. and (d) Reducing agents, e.g. sodium sulphite, bisulphite, nitrite.

30 grm. lots of fresh green wattle bark were placed in contact with 200 ml. of 3 - 5% solutions of the above substances in water, as well as with water only as control. The compounds below are arranged in order of the colour developed in the infusions in which they were present.

1. Colourless Sodium bisulphite, metabisulphite, and sulphur dioxide.

2. Almost Colourless (in order of darkening) Salicylic acid, gallic acid, oxalic acid, sodium silicofluoride, boric acid, benzoic acid.

3. Light Yellow Solutions Phthalic acid, dioxane, formic acid, phosphoric acid.

4. Light Yellow-Amber Aqueous ether, glycerol.

5. Yellow-Amber Water, urea, guaiacol, phenol, acetone, resorcinol, butyl alcohol, o-cresol.

6. Red-Amber Sodium Acetate, sulphite, thiosulphate, pure acetone.

7. Deep Amber Pure dioxane, sodium perborate, sodium nitrate.

The pH effect is again evident as all the acidic solutions are light in colour. All solutions darkened more with time, but

only the sodium bisulphite and sulphur dioxide containing ones remained colourless. Although the latter are also acidic there is an additional effect correctly explained by Cheaire (389).

These fresh bark extracts were protected from discoloration by the presence of a reducing agent, sulphur dioxide. Sulphur dioxide has lower redox potential (-0.200 volts at 25° (397)) than the tannins (+ 0.200 volts at pH 7 at 18°), and is preferentially oxidised in an aqueous mixture of the two. The continued presence of the reducing agent is thus essential to prevent darkening of the tannin. Immediately it is removed or completely oxidised, the tannin solution will commence darkening. The amount of reducing agent necessary will depend on the amount of oxygen dissolved in the water originally, and the amount which will dissolve with time from the atmosphere. Thus a solution which is shaken will require more protective agent than a stationary one, and concentrated tannin solutions a higher percentage than dilute ones, to ensure additional protection in the surface layers of the strong solution.

(iii) Effect of pH on the Natural Oxidation of Black and Green Wattle Extracts

Mention has already been made concerning the effect of pH on colour development. Cheaire (392) oxidised tannin solutions adjusted to varying pH values, by heating for 3 hours at 97°, and then measured the colour developed. From the curve obtained he concluded that pH = 3 was the optimum value for stability. For pH adjustment he used acetic, formic and oxalic acids and in

so doing possibly also introduced effects other than that of pH. This is borne out by his curve with acetic acid, which varies in slope and lies well below that of formic and oxalic. In this investigation, only the sodium acetate-hydrochloric acid buffer, which does not affect the redox potential of the tannins (273) was used. Oxidation was allowed to occur naturally in air, the solutions handled according to a standard procedure, and the colours measured after 34 and 96 hours with a Lovibond tintometer using a cell of 1 cm. internal thickness. From Figs. LXIV and LXV the points of maximum stability at room temperature appear to lie in the region pH = 2.25 for black wattle tannins and pH = 2.45 for green wattle tannins. The value for black wattle is thus about 0.75 pH units below that found by Chesire for the tannins when kept at 97°C.

At higher pH values than those for maximum stability, colour darkening occurs due to oxidation, while at lower values darkening is due to phlobaphene formation which might also be an oxidative reaction. The natural pH for black wattle (50° Bk.) is 4.6 (398) and that for green wattle about 5.0 (cold extraction). Both these values lie above those of maximum stability, and the oxidation of both these natural tannins may thus be inhibited by acidification to the correct pH value. The application of this to the tanning industry has already been suggested by Chesire.

From the above it may be concluded that green and black wattle tannins have tendencies towards phlobaphene formation, maximum stability, and increase of oxidation with increase of pH,

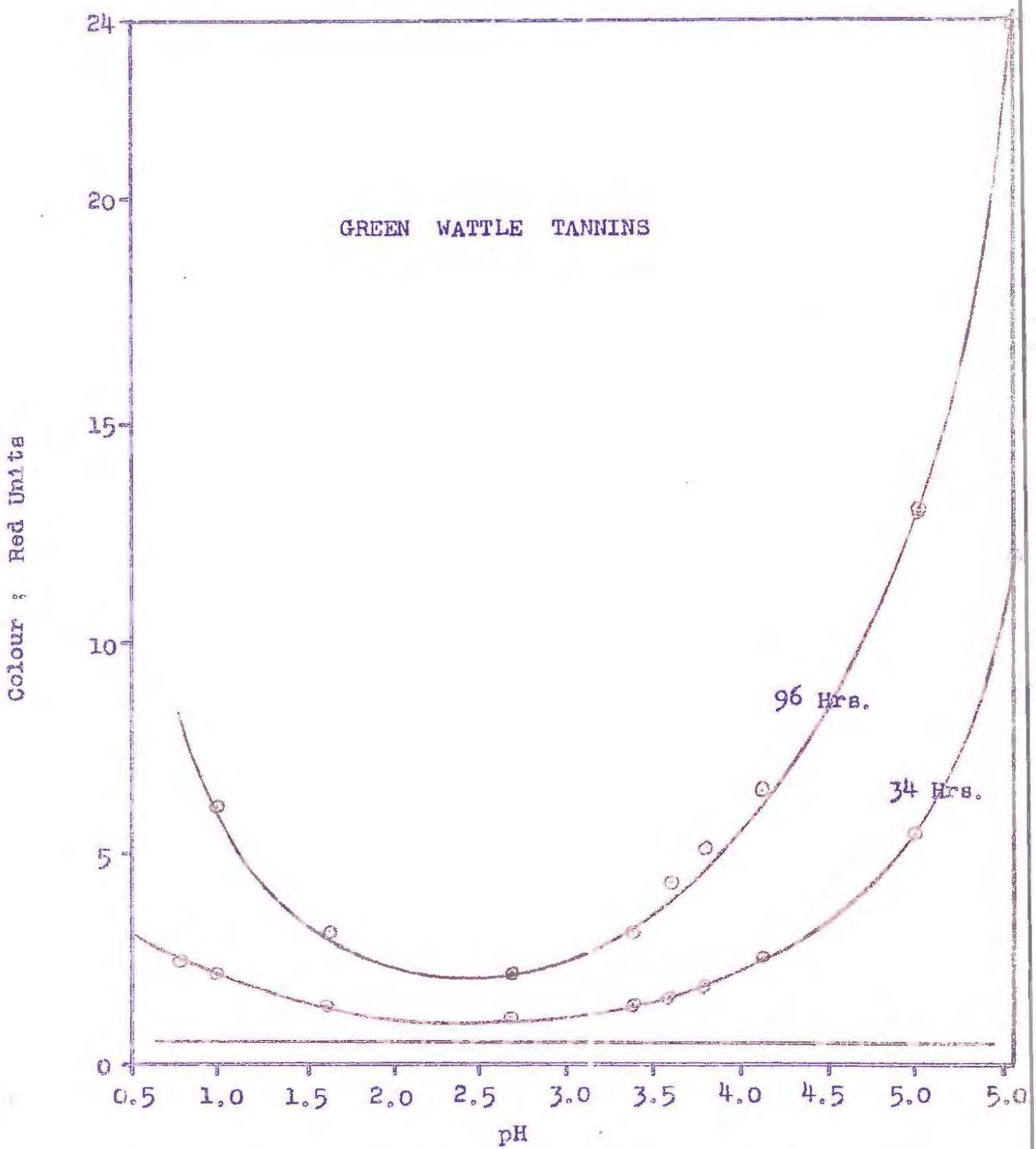


Fig. LXIV. The Atmospheric Oxidation of Green Wattle Tannins at Various pH Values

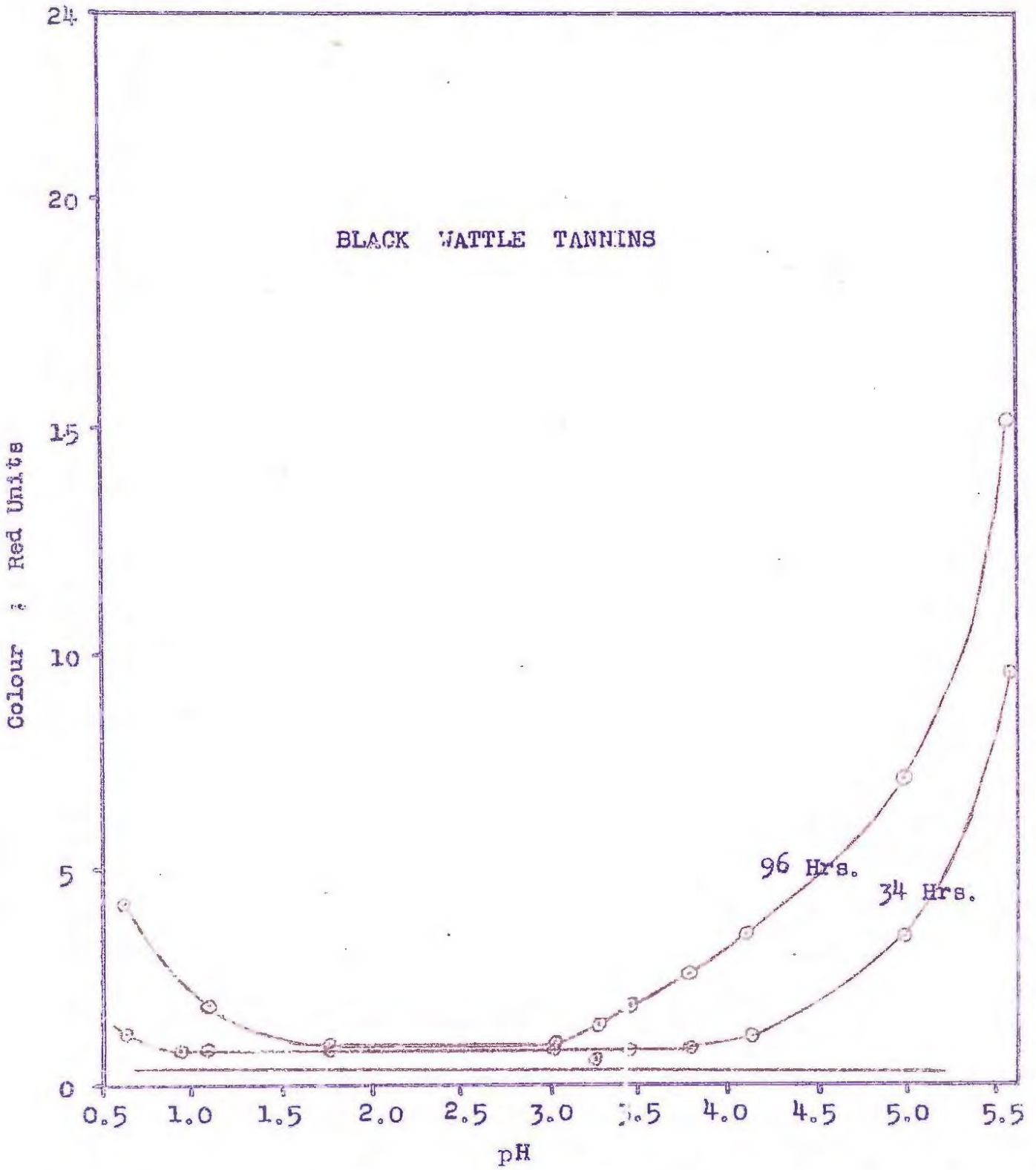


Fig. LXV. The Atmospheric Oxidation of Black Wattle Tannins at Various pH values

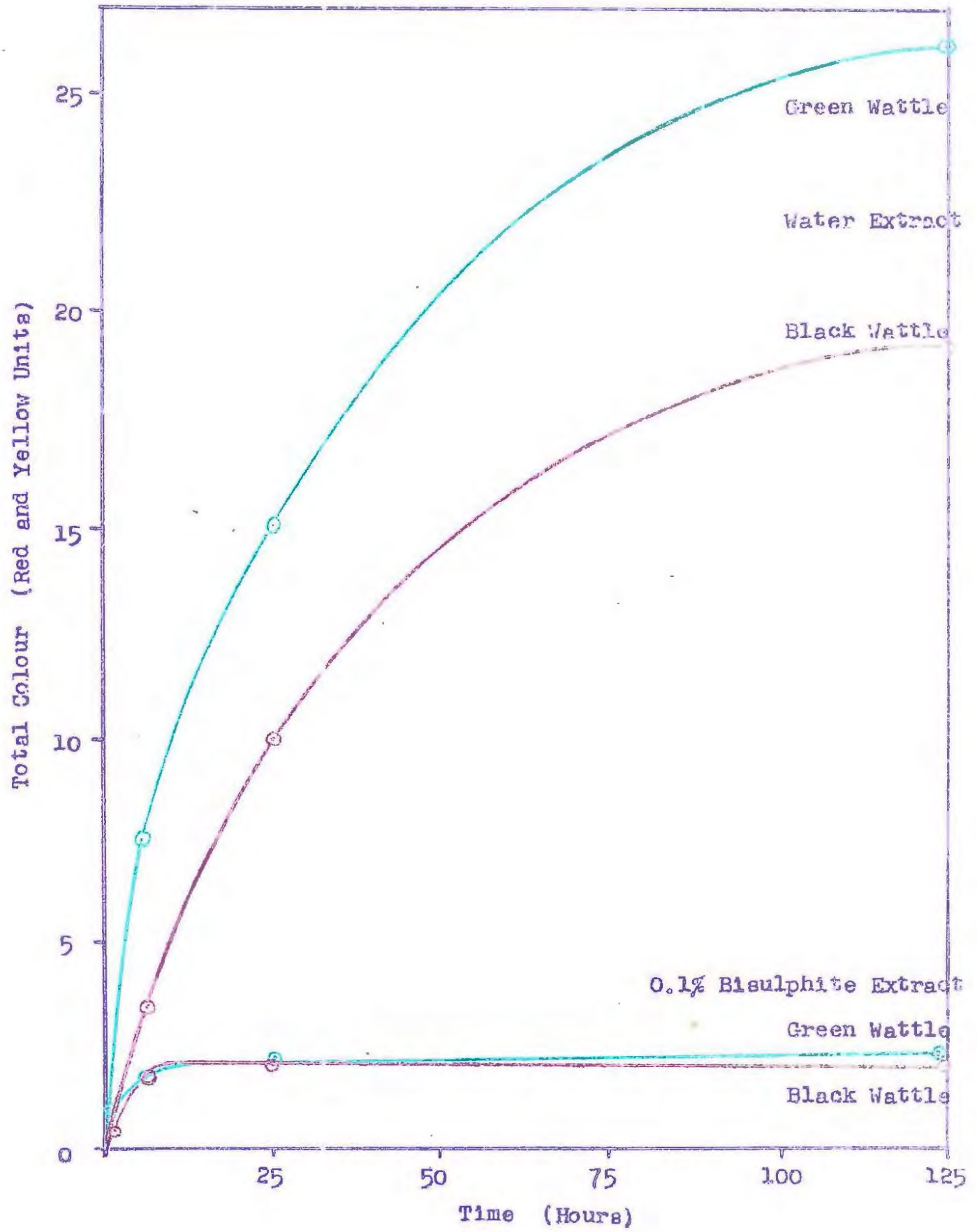
over the same pH ranges. Colour formation, however, develops more rapidly in green than in black wattle tannins over the entire range studied.

(iv) Concentrations of Reducing Agents Required

It was found that with cold leaching very low concentrations of bisulphite were sufficient to give desired protection to both black and green wattle tannins. 30 grms. of fresh black and green wattle bark chips were cut to uniform thickness, and placed in contact with 150 ml. water and 150 ml. 0.1% sodium bisulphite. Commercial bisulphite containing 84.1% $Na_2S_2O_5$ was used in all experiments. The colour, which developed in bisulphite-protected and in unprotected solutions, was measured with a Lovibond tintometer as before and compared (Fig. LXVI). The concentration of these solutions after about 125 hours was 6.7% total extract.

It is well known that when already darkened extracts are autoclaved with comparatively large quantities of bisulphite, light leathers are produced in subsequent tannage. Very low concentrations of the bisulphite, however, when added to the initial leach merely to protect the tannin against atmospheric oxidation, will prevent darkening. Once such darkening has occurred, additions of small quantities of bisulphite will not reduce the colour. The oxidation is apparently permanent and not reversible. Fig. LXVI illustrates the rapidity with which oxidation of the unprotected solution occurs, and shows that when protected against

Fig. LXVI. The Atmospheric Oxidation of Black and Green Wattle Tann
(Room Temperature) in Aqueous and 0.1% Bisulphite Soluti



oxidation there is very little difference in the colour developed in the black and green wattle extracts.

The above protected extracts were evaporated to dryness under reduced pressure, with heating on a waterbath. The extracts were almost identical in appearance and far lighter than the commercial extract marketed. Standard solutions of each were made up and their colour determined (McCandlish (174)).

	Colour Units		
	Red	Yellow	Blue
Green Wattle	0.1	0.5	0.0
Black Wattle	0.1	0.3	0.0
Commercial Black Wattle	1.7	3.3	0.0

Although extraction and concentration were effected here under ideal conditions, it is apparent that during commercial stripping, drying and transport, a large amount of darkening takes place.

Leaching was also carried out with sulphur dioxide solutions (30 grms. bark to 150 ml. solution) of varying concentration over longer periods.

All solutions were finally of 9 or 10 Bk. concentration. The water alone developed mould-growth after two weeks. Sulphur dioxide would be quite effective for cold leaching, but on account of its volatility would pass off on heating.

Concentration of SO_2 in Solution	Colour Units	Time (Days) 1	7	28
0.45%	Red	0.0	0.0	0.0
	Yellow	0.1	0.2	0.4
0.225%	Red	0.0	0.1	0.4
	Yellow	0.2	0.3	0.7
0.1125%	Red	0.0	0.1	0.7
	Yellow	0.2	0.4	1.7
0.0225%	Red	0.1	0.7	2.3
	Yellow	0.4	1.5	5.0
0.00225%	Red	0.4	3.0	6.2
	Yellow	0.9	5.3	24.0
Water	Red	4.0	8.0	30.0
	Yellow	6.0	19.0	30.0

(v) The Effect of Heat on Colour Development in the Presence of Reducing Agents

Bisulphite (0.1%) extracts prepared as above, and while still in contact with bark, were placed on a water-bath at 80° for 3 hours :

	Black Wattle		Green Wattle	
	Red Units	Yellow Units	Red Units	Yellow Units
Colour before heating	0.4	0.9	0.4	1.0
Colour after heating	0.5	1.0	0.5	1.2

Two 50 ml. samples of each of the above solutions were evaporated to dryness on a waterbath in a porcelain basin. One sample of each was taken up again in 50 ml. water and one of each in 50 ml. 0.1% bisulphite solutions. The solutions taken up again in water darkened fairly rapidly, while those redissolved in 0.1% bisulphite darkened only slowly.

	Black Wattle		Green Wattle	
	Red Units	Yellow Units	Red Units	Yellow Units
Original Solution	0.5	1.0	0.5	1.2
After Evaporation and Re-solution in 0.1% Bisulphite	1.2	1.5	1.3	2.6
Same soln. after 24 hrs.	1.0	1.7	1.2	2.9
" " " 48 "	1.2	1.9	1.4	3.3
" " " 120 "	3.5	9.0	4.6	12.0

The above shows that even for drastic conditions, such as evaporation to dryness on a waterbath when exposed to the air, 0.1% bisulphite solutions give good protection to a 7% extract solution. As would be expected, it was found that the bisulphite loses its effectiveness through complete oxidation when taken to dryness with tannin, and on re-solution the protective agent must again be added in order to prevent oxidation of the tannins.

(vi) Minimum Bisulphite Requirements

The minimum quantities of bisulphite necessary to protect the tannins will depend in each case on the method of handling the extracts and on temperature. For each process the amount of oxygen dissolving is a variable factor, and only trial and error experimental work will determine the minimum requirements necessary.

Cold Leaching

Still lower concentrations of bisulphite were used in a countercurrent cold leach and the colour developed measured at daily intervals. 0.16% acetone-bisulphite solution, which has as much total SO_2 as a 0.1% bisulphite solution, was also examined. The acetone-bisulphite was formed as described by Cheaire (389) who found it to give better protection at 92°C., than bisulphite only. This he ascribed to the bigger amount of SO_2 "available" for oxidation, in the former.

The solutions (125 ml.) were added to equal weights of black wattle bark, (30 grm.) and left for 24 hours before addition to the next batch of bark. The concentrations of tannin were checked daily and a standard technique of transference of the solutions adopted (Fig. LXVII). The increases in concentration with each step were remarkably constant and never deviated by more than 2 units from the average Bk. units given.

It is apparent, under the conditions of the above experi-

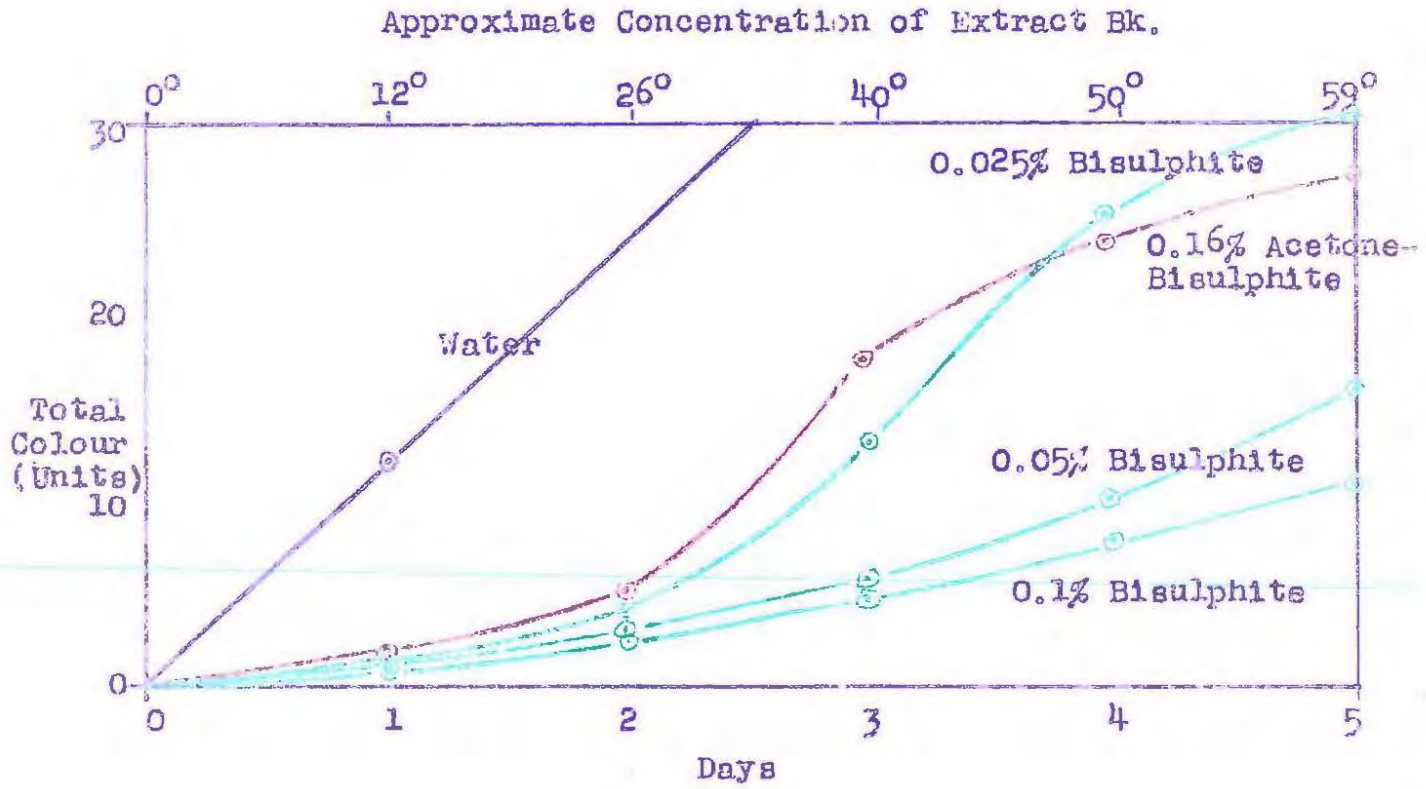


Fig. LXVII. The Countercurrent Cold Leach of Black Wattle Bark Chips with Bisulphite Solutions

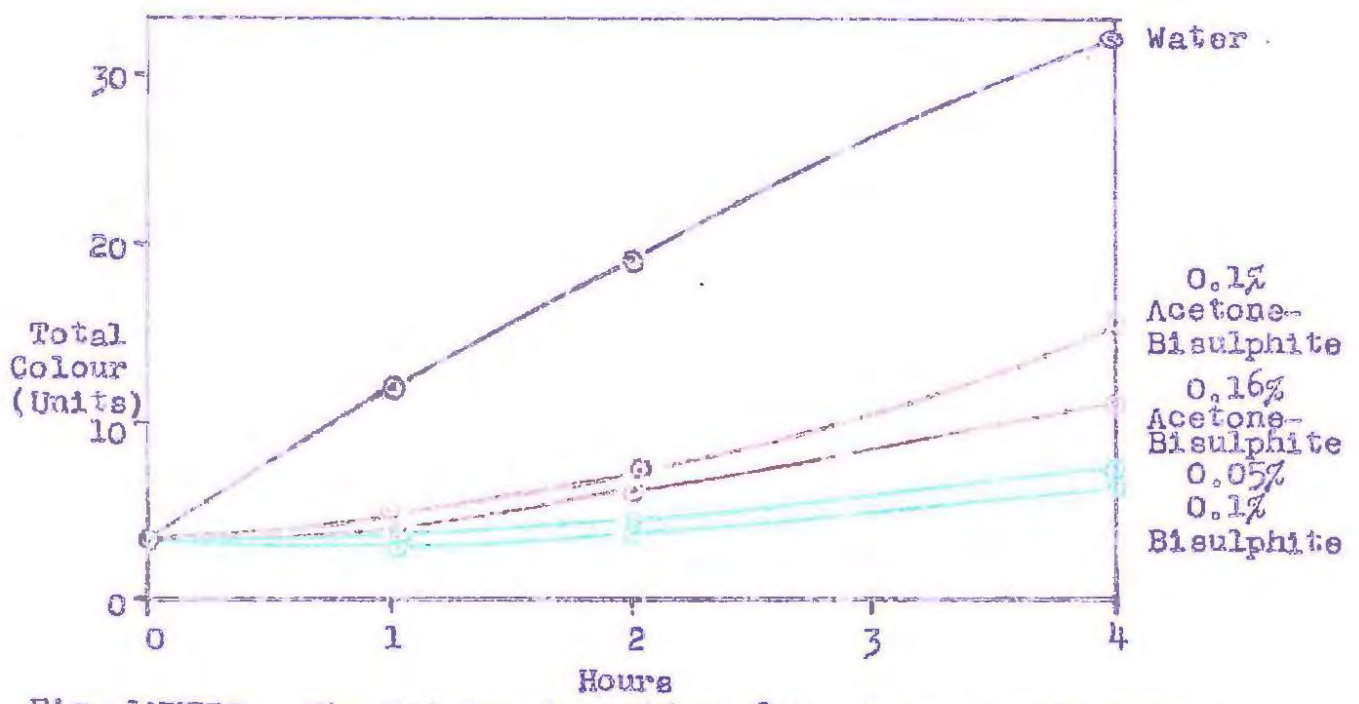


Fig. LXVIII. The Hot Leaching (80-90°C) of Green Wattle Barks with Bisulphite and Acetone-Bisulphite Solutions

ments that :

(a) even very low concentrations of bisulphite are effective in preventing excessive deepening of colour, when used for fresh bark extractions.

(b) the best minimum concentrations are in the region of 0.05% to 0.1%.

(c) for cold leaching, very low concentrations of protectant acetone-bisulphite is less effective than bisulphite only. At such low concentrations probably little or no SO_2 is lost to the atmosphere. As the acetone-bisulphite complex is more stable, and the protective action dependent on concentration of free SO_2 in solution, bisulphite only appears superior.

The solutions were taken to 88° Bk. by further addition of fresh bark, and finally concentrated under reduced pressure. The resulting tannin was very light pink in colour.

Hot Leaching

20 grms. of green wattle chips, cut to uniform thickness, were added to 100 ml. solutions of bisulphite and acetone-bisulphite of varying concentrations. These were heated on the same waterbath (80 - 90°C.) for 4 hours and the colour measured at intervals (Fig. LXVIII). With low concentrations of tannins, and high temperatures, the acetone-bisulphite is apparently still an inferior protectant.

(vii) Control of Colour in Barks

To obtain a lighter leather from both green and black wattle extracts, oxidation has to be minimised during the five processes :

(a) Drying

(b) Transport

(c) Extraction

(d) Concentration

and (e) Tannage

From the colour formulae of standard solutions of fresh bark extracts made in the laboratory, and that offered for sale commercially (page 369), it is obvious that in the aforementioned processes a large amount of oxidation occurs. Much of this takes place in the bark before it reaches the extract manufacturer, and could be prevented. Lightness of colour is one of the desirable qualities in grading of bark (4, 7).

The present practice (4) is to lay the freshly stripped bark, outer side up, at an angle with the ground, in the plantation for partial drying. This takes about three days, and during this period rain, mist, and direct sunlight, cause serious discolouration particularly on the unprotected inner surfaces (7). Rain and mould growth might remove much of the desirable non-tans. Sherry recommends transport of the bark, directly after stripping, to large sheds, where it should be stacked loosely and allowed a good air supply to ensure rapid drying. Once water is removed

the tannins are quite stable, and heating at 120° for 2 hours has little effect on the colour of dry tannin extract. Because of the initial dampness of the bark a fair amount of discolouration is unavoidable, even under ideal conditions.

It was found independently by Watson (399) and also in these laboratories that the fresh bark, retained its original light colour when kept in an atmosphere of 100% relative humidity and low sulphur dioxide content. On subsequent removal and air-drying this bark suffered no immediate discolouration. As was the case with the solutions, once the colour had developed, no improvement could be effected by subsequent treatment with sulphur dioxide gas. The action here is not one of reductive bleaching, nor of preferential oxidation of a reducing agent as in tannin solutions, as a permanently beneficial effect was introduced.

Watson (399) ascribed the cause of most of the darkening in the bark to enzymatic activity. The enzymes amylase and peroxidase are known to occur in other acacias (400). Sulphur dioxide destroys these enzymes and so arrests the initial rapid oxidation caused by them. Subsequent darkening of the bark, if dry, is slow and caused by atmospheric oxygen. Further confirmation of the presence of enzymes was also obtained. He showed that carbon monoxide, cyanic acid gas and heat all eliminated peroxidase activity. Sulphur dioxide, besides being much less toxic, was far more effective than these, and had no detrimental effect on the tannins in the bark. By cutting small uniform sections through the bark with the aid of a microtome, and

measuring the volume of oxygen liberated from each, when the bark is immersed in hydrogen peroxide, Watson determined the location of peroxidase activity. (Fig. LXIX).

The maximum peroxidase concentration lies at the outer surfaces, with low concentration throughout the bark. The first and most rapid darkening of bark kept under paraffin, always takes place at these surfaces. The darkening is particularly noticeable in the thin layer of cambium, which is white immediately after stripping. The peroxidase activity measured, is thus possibly responsible for the darkening produced. The concentration of maximum enzyme activity at the surfaces facilitates their destruction by sulphur dioxide.

Other findings by Watson are :

(a) That addition of tannin to bark has no effect on enzymatic activity.

(b) That exposure to air for a 40 hour period does not affect enzyme activity. The author found, that when stored under paraffin, the enzyme activity of black and green wattle barks diminishes to about 1/8th and 1/15th (respectively) of their original value over 30 days.

(c) That with decreasing pH the enzyme activity diminishes and is very low in strongly acidic solutions.

(d) That small amounts of copper very slightly increase the colour catalytically, although the infusion is protected by sulphur dioxide.

Merry (390)(394) also demonstrated the catalytic effect

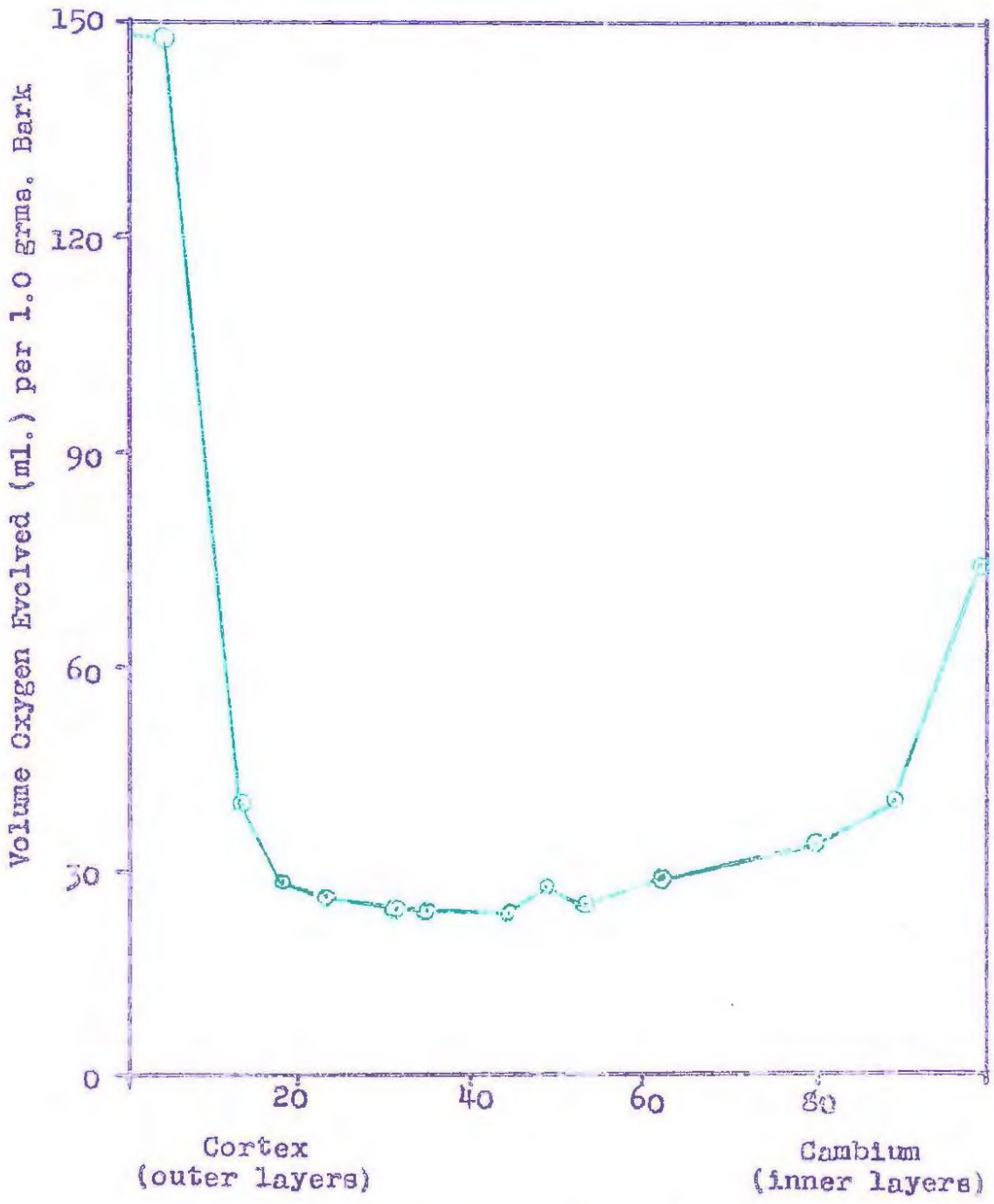


Fig. LXIX. The Location of Peroxidase Activity in "Green" Black Wattle Bark.

of small amounts of metals on the oxidation of chestnut extracts. Such extracts containing traces of ferrous iron, absorbed more than twice the volume of oxygen required to oxidize the ferrous iron present to the ferric state in 17.5 hours. Over 5 days, the absorption rate was twice that of a control solution. Copper had a similar effect.

Rice (388) showed that traces of metals (iron and copper) accelerate the oxidation rate of unprotected black wattle tannin solutions. Szent-Györgyi (401), Laki and Papp (402) showed that metals form complexes with catechol, and cause auto-oxidation of catechol by electron-transfer within the complex. In this process catechol acts as a reduction medium by losing electrons to the ferric ion. Although many hydroxy compounds are able to form complexes, it is only those in which the system

$$\begin{array}{l} R_1 - C - OH \\ \quad\quad\quad\quad \| \\ R_2 - C - OH \end{array}$$

is present, (e.g. dihydroxy maleic acid, ascorbic acid, and its analogues and other ortho-dihydroxy compounds), that electron transfer can take place within the complex (403). Banga (404) obtained similar results with catechol in the presence of copper and iron. As the ortho-dihydroxy structure is present in black and green wattle tannins, such auto-oxidation is possible.

(e) . It was further found by Watson (405) and confirmed in these laboratories, that p-phenylene-diamine in the presence of hydrogen peroxide gave a blue-black stain almost immediately, when

placed in contact with fresh bark. This does not occur once the bark has been autoclaved. The above gives indication of peroxidases (406) which act as enzymes in the presence of hydrogen peroxide. They are said to be iron-porphyrin proteins (407)(408) usually associated with oxidases in plants and their enzymic activity for polyphenols is due to the presence of hydrogen peroxide formed by oxidase action, thus :

O_2 + polyphenol oxidase + polyphenol → Quinones

hydrogen peroxide + peroxidase + polyphenol → Quinones

Elliott (409) showed that peroxidases and hydrogen peroxide oxidise polyphenols.

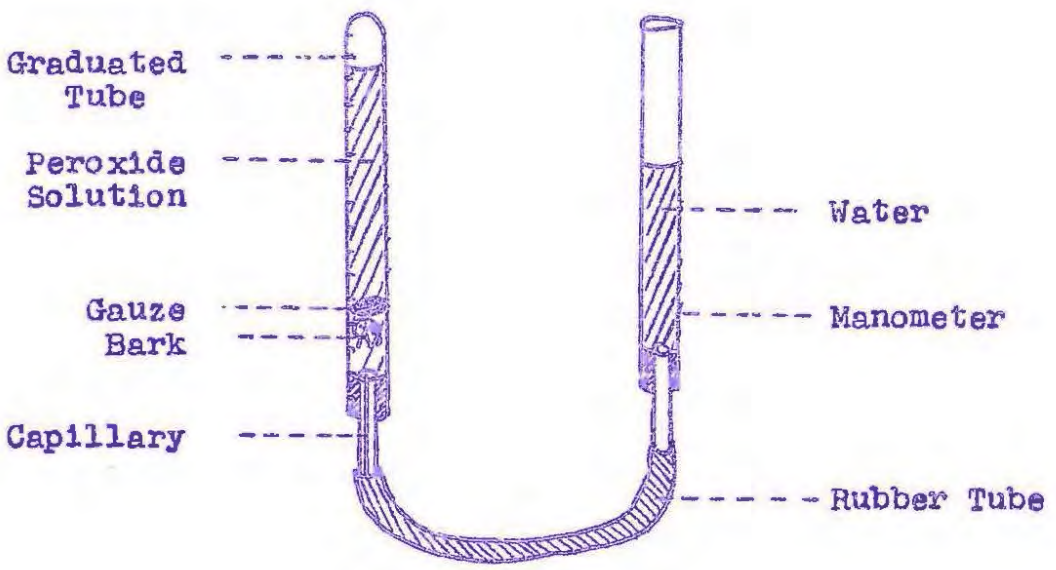
(viii) Comparison of Enzyme Activity of Black and Green Wattle Barks

Watson, in the above experimental work, used the volume of oxygen collected from the reaction of the enzyme in a unit weight of bark with hydrogen peroxide, as an indication of enzyme concentration. This has the disadvantage that even from dilute hydrogen peroxide solutions, once the enzyme is introduced, evolution of oxygen takes place at slowly diminishing rates over very long periods. The instant at which the volume is read is thus entirely arbitrary.

A far more satisfactory method is that of Euler and Josephson (410), modified by Summer (411), for catalase activity. When reaction occurs between catalase and peroxide, the enzyme is gradually destroyed and the velocity constant of the monomolecular

reaction diminishes. By plotting the K value against time and extrapolating, the K_0 value may be obtained. This will give a truer indication of initial enzyme activity.

A narrow tube, 10 cms. long, from a broken burette was sealed at the top, and copper gauze was moved up $\frac{1}{4}$ of the length of the tube so as to remain in position. A rubber stopper fitted with a short length of capillary tube, and a rubber tube, connected the burette tube to a simple water manometer.



The burette tube was inverted and filled to the gauze with a definite volume of hydrogen peroxide of known strength. Bark flakes of known weight, and cut to uniform thickness with a microtome, were placed above the gauze, out of contact of the peroxide. The water manometer was clamped vertically, water added and allowed to run through the rubber tube until all air was dis-

placed. Immediately this took place the cork was joined to the inverted burette tube, the latter righted and shaken to force all bubbles to the top, and the amount of air entrapped measured by levelling with the manometer tube. The gauze prevents the bark pieces from floating to the surface.

Evolution of oxygen was rapid initially, but slowed down and continued almost indefinitely. The volume evolved was measured at 3 minute intervals and the volume read to 0.01 ml. as accurately as possible. The burette tube was well shaken before a reading was taken.

The volume of gas evolved was calculated to N.T.P. and the total concentration of hydrogen peroxide in solution determined by titration with permanganate. The K values were calculated from the equation

$$K = \frac{2.303}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$$

where a = total volume of oxygen which can be evolved from the reacting peroxide solution, and x_1 and x_2 the volumes of gas at N.T.P. evolved at the times t_1 and t_2.

Initial strength of hydrogen peroxide solution = 33.53 vols.
Volume peroxide solution used = 17.00 ml.
Wt. black wattle bark used = 0.394 grm.
Wt. green wattle bark used = 0.397 grm.

The bark used was obtained from 10 year-old trees. These results were further confirmed by using barks of different ages.

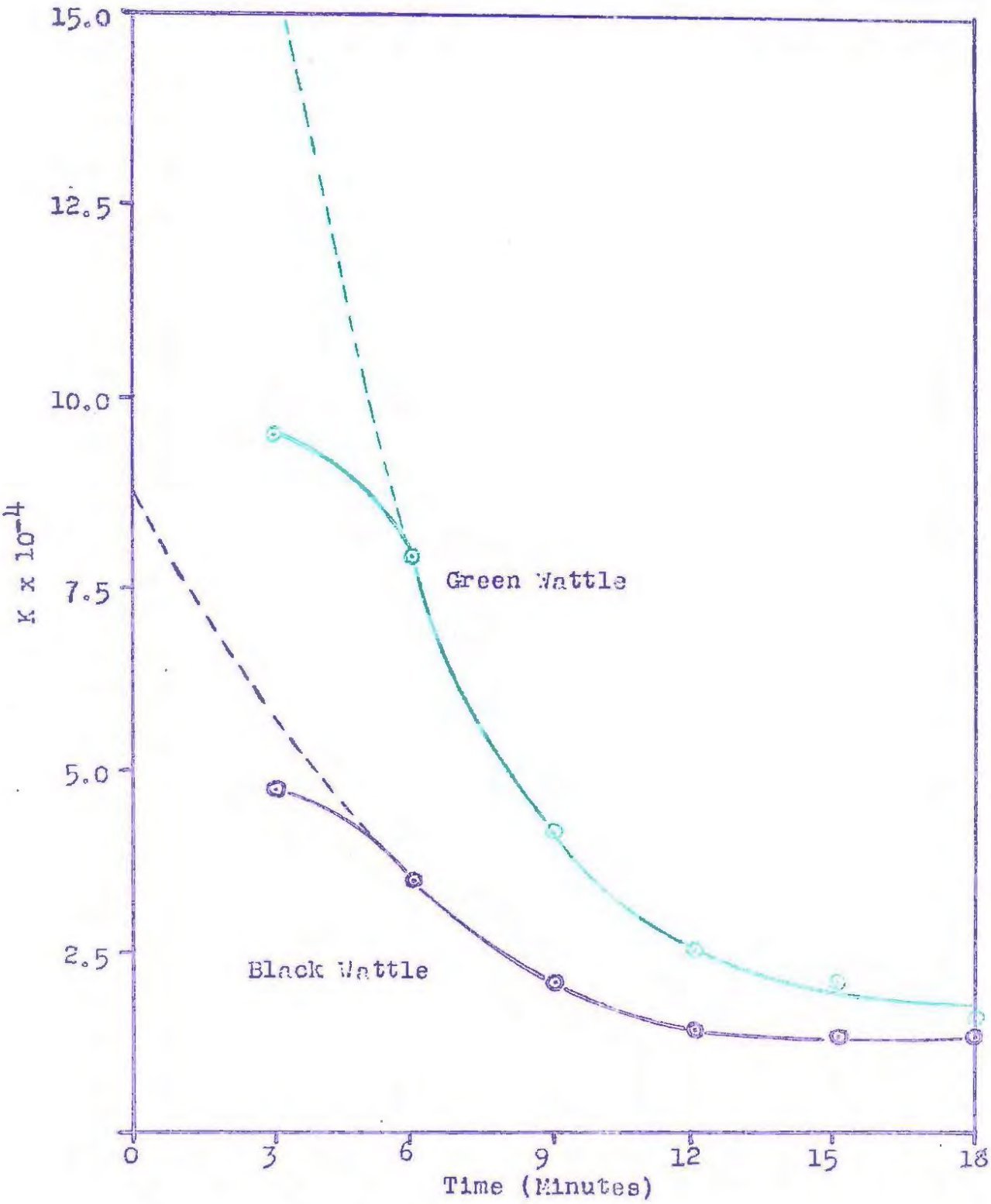


Fig. LXX. The Velocity Constants of the Reaction Between Hydrogen Peroxide and Peroxidases in Black and Green Wattle Barks.

Time (Minutes)	Volume O_2 evolved at 713.5 mm. & 18°C.	Vol. gas at N.T.P.	K x 10^{-4}
3	1.75	1.54	9.06
6	3.20	2.82	7.45
9	4.05	3.57	4.45
12	4.53	3.99	2.38
15	4.91	4.32	2.00
18	5.24	4.61	1.69

Green Wattle

Time (Minutes)	Volume O_2 evolved at 713.5 mm. & 18°C.	Vol. gas at N.T.P.	K x 10^{-4}
3	0.95	0.84	4.91
6	1.62	1.43	3.45
9	2.00	1.76	1.92
12	2.31	2.03	1.65
15	2.58	2.27	1.38
18	2.86	2.52	1.46

Black Wattle

In all measurements the green wattle samples showed higher enzymatic activity than black wattle, and from the K_o values on Fig. LXX, green wattle barks apparently have at least double the peroxidase activity of black wattle barks in the 10 year-old samples. The low K values obtained at the first period (3 minutes) are probably due to the solubility of oxygen in the peroxide solution.

Discussion

Although peroxidases are, therefore, undoubtedly present in black and green wattle barks, and occur in higher concentration in the latter than in the former, there is no proof that this class of enzymes is responsible for rapid oxidation-reactions in the bark after stripping. The fermentative processes of tea leaves, for example, consist essentially of the enzymatic oxidation of the polyphenols present in the leaves. The enzymes responsible for such oxidation were at first considered to be peroxidases (361) also present in the leaves, but later work has shown that no correlation exists between peroxidase concentration and polyphenol oxidation (350). The presence of oxidases were thus postulated. It was shown that the cytochrome system was absent and that the redox potential of the phenols was in any case too high for them to be oxidised by cytochrome oxidases. As a result of later work little doubt remains that polyphenol oxidases of the copper-protein type are responsible for enzymatic oxidation, (360).

Work along similar lines on black and green wattle barks appears to be desirable if not essential, to this problem of the more rapid darkening of green wattle extracts.

(ix) Practical Precautions for Bark

From the above data it is evident that the green wattle tannins in the bark are initially slightly more highly coloured; that the bark has a higher peroxidase activity than black wattle bark, and that a permanently beneficial effect was introduced by treating the fresh bark with SO_2 gas or bisulphite solutions. It appears that green wattle barks could be used commercially provided the enzymatic activity is controlled, and precautions taken against further excessive oxidation.

This could be exercised practically, by stacking the bark in a closed shed, and passing sulphur dioxide gas into the shed from a cylinder. The doors should be kept shut for a short period, and the shed then aired to allow drying of the bark. The volume of the gas and the time required for the above operation will only be found by trial and error methods.

(x) Precautions During Transportation

Once the bark is well dried and kept dry, the tannin is fairly stable and will not darken over short periods. Bark allowed to dampen during transport is known to "sweat" as the result of bacterial activity, and darken excessively. It is best that extraction takes place as soon as possible after harvesting,

although this is not always possible.

(xi) Precautions during Extraction and Concentration Processes

(a) Leaching in Tanneries

Cold or hot leaching processes as carried out in tanneries lend themselves admirably to colour control by the addition of small amounts of bisulphite. 0.1% solutions should give adequate protection.

(b) Commercial Extraction Processes

Extraction and concentration occurs in closed systems and oxidation is likely to occur mainly through oxygen dissolved in the water used for extraction. The proportion of oxygen in relation to the total oxidisable tannins must be very low.

Numerous experiments conducted by Balfe and Phillips showed that heavily bisulphited mimosa and quebracho extracts seriously corrode iron, copper, brass and phosphor bronze in the cold (413). Dilute bisulphite solutions (weak suspender liquors) had similar effects but to a much lesser degree (413). This corrosion was accentuated in the presence of oxygen (413). Nickel-chrome steels however, were completely resistant (at low temperatures) to highly bisulphited mimosa solutions, causing no discolouration and remaining unchanged in appearance (412)(413).

As both the extractors and evaporators are copper-lined, some slight corrosion is likely to occur if bisulphite-solutions

are used to eliminate dissolved oxygen.

(xii) Precautions During Tannage

The solid extracts prepared from 0.1% bisulphite leaches in the laboratory were light in colour, and showed no colour change when stored in bottles in indirect light over long periods. In the absence of moisture, the solid extract, therefore, remains quite stable.

When the solid extracts are redissolved for tannery use, 0.1% bisulphite solutions should give sufficient protection, even where these are heated for the rapid solution of the extract. By the partial or complete application of these precautions, light-coloured green wattle extracts could be used for tanning purposes.

(xiii) Darkening of Mimosa-tanned Leather

The gradual darkening of leather tanned by catechol or condensed tannins is well known. Both green and black wattle tanned leather behave similarly and the former, even if protected against oxidation by weak bisulphite solutions, develops a deeper reddish colour than the latter when exposed to direct or indirect sunlight or ultra-violet light (417). The reason for this accelerated darkening of green wattle extracts is, as yet, unknown.

Mimosa extracts are mainly used for sole-leather tannage and such leathers are invariably "bottom finished" on the completed shoe. These finishes obscure the natural colour of the vegetable-tanned leathers. Provided thus that the leather is not

unduly dark, and as green wattle extract differs only slightly in its tanning qualities from black wattle extracts, there appears to be little disadvantage in the use of green wattle tanned leather. It is essential that the existing prejudice against the slightly reddish leathers be removed, as the green wattle tree has many excellent qualities which recommend its large-scale afforestation.

(d) The Comparative Astringency of Green and Black Wattle Tannins

Page (180) has shown that the composition of gelatin-wattle tannin precipitates are not greatly affected by changes in temperature, salt concentration, initial tannin concentration, the nature of the gelatin or the pH value of the solution as long as this value is below the isoelectric point of the gelatin. He found, furthermore, that the amount of tannin in the gelatin-tannin precipitate decreased with decreasing astringency of the tannin. When black wattle tannins were salted out, for example, the least soluble and most highly-coloured astringent units combined with gelatin in high proportion, while the most soluble colourless units combined in lower proportion. From his data it was clear that there is a close connection between the molecular weight and the composition of the gelatin-tannin precipitate. For ease of comparison Page calculated in each instance the amount of tannin combined with 100 parts of gelatin in the gelatin-tannin precipitate. This figure was termed the "gelatin number" of the tannin. The values obtained were, therefore, an index of the astringency and also the molecular weight of the fractions.

As black and green wattle tannins are closely related, a comparison of the astringency of each was made on a number of samples obtained from different sources. The gelatin-tannin precipitates were made under strictly comparable conditions as described by Page, and the nitrogen in the dried product determined by the Kjeldahl method, using copper sulphate as a catalyst. The same gelatin sample was used for all the estimations. All the extracts were obtained from fresh-barks and concentrated under reduced pressure before analysis :

Extract	Green Wattle		Black Wattle	
	% N	Gelatin No.	% N	Gelatin No.
Aqueous (Cold)	7.21 7.22	146.3	7.88 8.01	123.5
Methanol (Cold) Sample I	7.52 7.45	137.1	7.99 7.87	123.8
Methanol (Cold) Sample II	7.55 7.45	136.8	8.08 7.84	123.1
Methanol (Cold) Sample III	7.24 7.24	144.6	7.58 7.57	134.3
Residual tannins in soln. after ethyl acetate pption from ethanol solution	7.88 7.97	124.3	9.00 8.91	98.37

% N in gelatin = 17.75

All the green wattle samples are more astringent and probably of higher average molecular weight than black wattle

extracts. The differences in gelatin number only vary from 7% to 14%.

(e) The Chemical Nature of Green Wattle Tannins

Methanol-extracted green wattle tannins from fresh bark were methylated with dimethyl sulphate and methanolic KOH. The methylated tannins (found, % $-OCH_3$ = 35.56, 35.62, 35.38) were oxidised with alkaline permanganate in aqueous solution to give a mixture of acids. After recrystallising these from water and decolorising with charcoal, they softened at 129°C., and melted at 137°C. The equivalent weights of the mixtures from different oxidations were 199.8, 201.3, 201.3, 200.8. The acids were separated by Corbett's method (146), and proved to be veratric and O-trimethylgallic acids. These were identified by their equivalent weights and mixed melting-points with authentic specimens. Assuming that only these acids are present, the equivalent weight corresponds to a mixture of 37.3% veratric and 62.7% O-trimethylgallic acids. Green wattle tannins, therefore, closely resemble black wattle tannins in their behaviour on oxidation.

Glueck (41) under the direction of the author, investigated the tannins and non-tannins in greater detail mainly for comparison with black wattle. The extract could be separated into tannins and non-tannins by the lead-salt method used for black wattle extracts. The purified tannins approximated to the same empirical formula ($C_{15}H_{14}O_6$). Fully substituted methoxyl and acetyl derivatives were prepared and analysed. These con-

tained approximately four alkylated and acetylated groups per hypothetical C_{15} unit, with values slightly below those obtained with black wattle tannins. The equivalent of two oxygens per C_{15} unit remain unsubstituted and these may be either ether links or carbonyl groups. Alkaline fusions of the "purified" tannins produced, as in the case of black wattle tannins, β-resorcylic acid and resorcinol, gallic acid and pyrogallol, protocatechuic acid as well as phloroglucinol. From the high yields of resorcinol (6%) and gallic acid (3%), these appear to predominate. A significantly larger proportion of phloroglucinol than was obtained from black wattle tannins was separated from the resorcinol in the meta-hydroxy fraction by fractional sublimation. Green wattle tannins closely resemble black wattle tannins both as regards analyses and degradation-products. Chromatography of the non-tan fraction (methanol extract) showed the presence of sucrose only, as was the case in similar fresh black wattle extracts. One-dimensional chromatograms of the fresh bark indicated the absence of yellow fluorescent compounds (when viewed under ultra-violet light) such as fisetin, which characterise authentic black wattle samples.

These similarities between green and black wattle extracts are not surprising in view of the similar appearance of the trees and their afore-mentioned close relationship. These investigations have shown no differences which could account for the additional rapidity of darkening of green wattle extracts.

(f) Comparison of Two-Dimensional Paper Chromatograms

More recent two-dimensional chromatographic comparisons have shown more differences between black and green wattle tannins and also many similarities. The two-dimensional chromatogram of the methanol-soluble fresh-bark green wattle extract (Fig. LXXI) does not, for example, show the characteristic pattern of five areas of high concentration (Fig. XII. p. 172. A, B, C, D and E) so typical of similar black wattle extracts. Many of the compounds present in green wattle, however, appear to be present in black wattle extracts. On the chromatogram of green wattle tannins

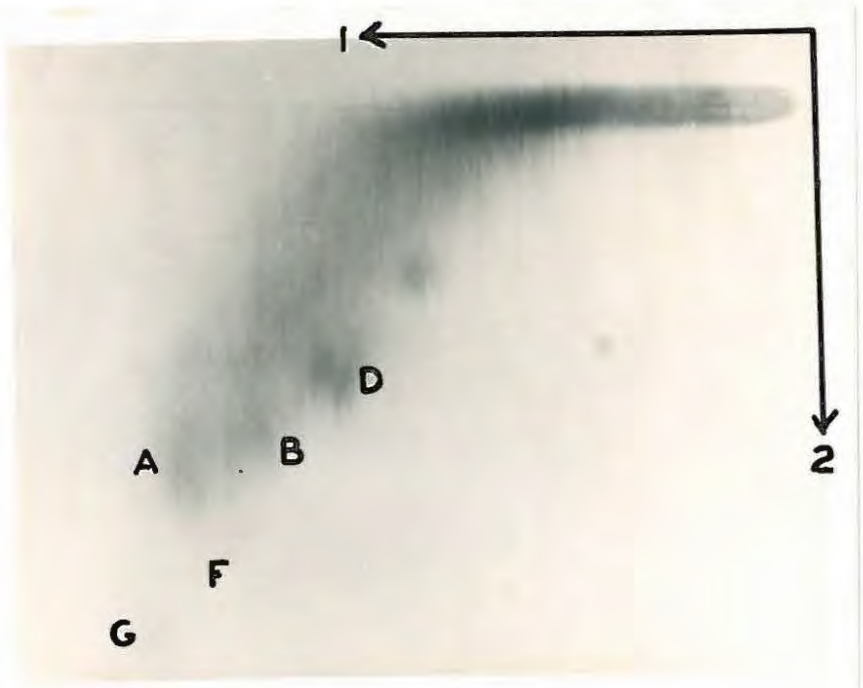


Fig. LXXI. Two-Dimensional Chromatogram of Methanol-extracted Green Wattle Tannins

(Fig. LXXI), the very prominent spot D corresponds in position to constituent D on black wattle chromatograms. Similarly, spots A, B, and G as well as the strongly reducing constituent F, occupy identical positions on both chromatograms. The black wattle constituent C might also be present in green wattle tannins, but is probably obscured by much trailing.

Green wattle chromatograms show the presence of a denser trail in the low RF region, and it is possible that a larger proportion of more highly oxidised or condensed tannins is present here.

This chromatogram was obtained from one sample of authentic bark and its reproducibility requires confirmation. The differences between black and green wattle chromatograms, if characteristic, will be useful when comparing the extracts of hybrids. Different degrees of enzymatic activity in the two barks may be responsible for some of the differences observed.

(g) Summary

(1) The green wattle tree has many advantages over the black wattle, chief of these being its resistance to bagworm attack, and its superior growing qualities under adverse conditions.

(2) The main disadvantage of the tree is the undesirable deeper colour developed by its bark extracts.

(3) The tannins in the fresh bark already have a light pink colour compared with the straw-yellow of black wattle bark.

(4) The additional colour developed by green wattle extracts was shown to be due to the oxidation of the tannin units only. The green wattle tannins develop a reddish coloration more rapidly than black wattle tannins over a wide pH range.

(5) Green wattle barks exhibit much higher peroxidase activity which might account for some of the additional redness observed.

(6) Green wattle tannins are more astringent than black wattle tannins when compared under identical conditions. The increased astringency is considered to be related to a higher molecular weight.

(7) Nietzski's rule states (415)(416) that increases in molecular weight or complexity of organic molecules deepens the colour. It is thus possible that equivalent degrees of oxidation would cause a more pronounced bathochromic effect in the higher-molecular weight green wattle tannins.

(8) Practical colour-control measures may be taken by

(a) destroying the enzymes in the bark by sulphur dioxide treatment soon after stripping;

(b) rapidly transporting the bark under dry conditions;

and (c) extracting the tannin from the bark in tanneries by a hot or cold leach using 0.1% sodium bisulphite solutions. Such extractions do not appear to be applicable to the commercial extraction process, due to possible corrosion of the copper-lined

extractors and evaporators.

(9) In tannin solutions containing a low concentration of sodium bisulphite, the sulphur dioxide set free is preferentially oxidised by dissolved and dissolving oxygen, as the redox potential of sulphur dioxide is below that of the tannins. Probably little, if any, bisulphite enters into chemical combination with the tannin. Once the colour has developed in the tannin solution, small amounts of reducing agents have little or no effect, and drastic reduction is necessary to lighten the colour.

(10) All leathers tanned with condensed tannins are known to redden on exposure to direct or indirect sunlight. Green wattle tanned leathers were found to darken more rapidly than those tanned with black wattle (417). As wattle extracts are used mainly for sole-leather tannage, and the soles are "bottom finished" on the completed shoe, there appears to be little disadvantage, apart from the slightly greater astringency, in the use of green wattle extracts.

(11) Detailed analytical and degradative studies have shown little difference between the tannins from black and green wattle barks. Sucrose constitutes the carbohydrate non-tannin fraction of both. Points of difference which may be of use in genetic studies are :

(a) The green wattle extracts contain no associated yellow fluorescent compounds which characterise the one-dimensional

chromatograms of authentic black wattle extracts, when viewed under ultra-violet light.

(b) The two-dimensional chromatograms of green and black wattles show certain differences, but many similar, if not identical, polyphenols appear present in both extracts.

(c) The difference between the two extracts might well be the presence of a larger proportion of a high molecular fraction in green wattle barks, which results from a greater concentration of active enzymes in the living bark.

CHAPTER XII

THE EXTRACT OF THE BARK OF ACACIA DEALBATA LINK

(Silver Wattle Extract)

The silver wattle tree, also known as "blue wattle" locally on account of its bluish-green foliage, is one of the many species and varietal forms of Acacia used in hybridization studies at the Wattle Research Institute (418). Plantations were originally established in Natal in addition to black wattle as soon as the potentialities of these trees as tannin producers were realised, but afforestation discontinued and the plant eradicated when the bark was found to be inferior. Advantageous characteristics, such as the rapid ripening of its seeds, its vigorous growth under poor conditions and its reputed resistance to frost, are more than outweighed by the low tannin content, its low yield of bark per acre and the reddish colour of its extracts. Thus 8 year-old silver wattle bark (Williams (3)) averaged 16.9% tannin content compared with 34.5% of black wattle bark of the same age, and infusions of the former averaged 8.4 red units compared with 3.9 of the latter.

In an effort to assist the genetic research of the Wattle Research Institute, this short comprehensive examination of silver wattle tannin, aimed at showing up significant differences

between itself and black wattle tannin, was undertaken.

(1) Examination of the Fresh Bark and Tannin Extracts

Freshly-stripped silver wattle bark, kindly supplied by the Wattle Research Institute, was obtained from a tree identified by Mr. S. P. Sherry, and estimated approximately six years old. Oxidation was minimised by placing the strips under paraffin in the usual way. On arrival here the bark appeared much thinner than black wattle barks of corresponding age previously supplied and similarly preserved. The green chlorophyll layer was far more prominent, and on sectioning it displayed the same pink colour as green wattle barks. Sections of fresh black wattle bark by comparison, even when oxidised on the outside, are either light yellow or colourless.

Extraction of the tannin with organic solvents, e.g. acetone and methanol, which appear to inhibit oxidation, gave a light pink tannin on evaporation under reduced pressure. Aqueous extraction, especially at elevated temperatures, produced a pronounced redness in solution and in the solid finally isolated. According to Williams (loc. cit) this redness is even more intense than that produced by green wattle extracts.

(2) Chromatography

Lawrence and Scott-Moncrieff (419) opened up the field of chemical genetics by their work on the petal colours of Dahlia variabilis. They found that five genetic factors influence petal

colour and used tests described by Robinson and Robinson (420) for identification of the various pigments. Bate-Smith (185) demonstrated the usefulness of chromatography for pigment identification in this problem, and its application along similar lines in the hybridisation studies of black, silver, green and golden wattles is considered most important.

(a) One-Dimensional Chromatography of the Fresh Extracts

Solid acetone-extracted tannin from the fresh barks of A. mollissima and A. dealbata was made up to 25% solutions in methanol. 3.6 µl of each solution was spotted on to Whatman's No. 11 paper with a micro-pipette. Small concentrated spots were built up by touching the paper with the pipette, and allowing the spot formed to dry in a current of air before repeating the process. The paper chromatogram was developed with butanol-acetic acid-water (4 : 1 : 5 upper layer) at 22°C. The solvent-front was allowed to advance 10 inches from the starting-line during a 12-hour period and examined under ultra-violet light after air-drying. (Fig. LXXI).

Silver wattle tannin showed a single dull-violet streak extending from the point of application to near the solvent front, but it was most prominent over the region RF = 0.10 to 0.80. Opposed to this, black wattle extract exhibited two clearly yellow fluorescent spots at RF = 0.48 - 0.50 and RF = 0.72 (fisetin); a weak blue fluorescent area at RF = 0.90, and a dull violet streak RF = 0.00 - 0.47. The chromatograms of these two tannins

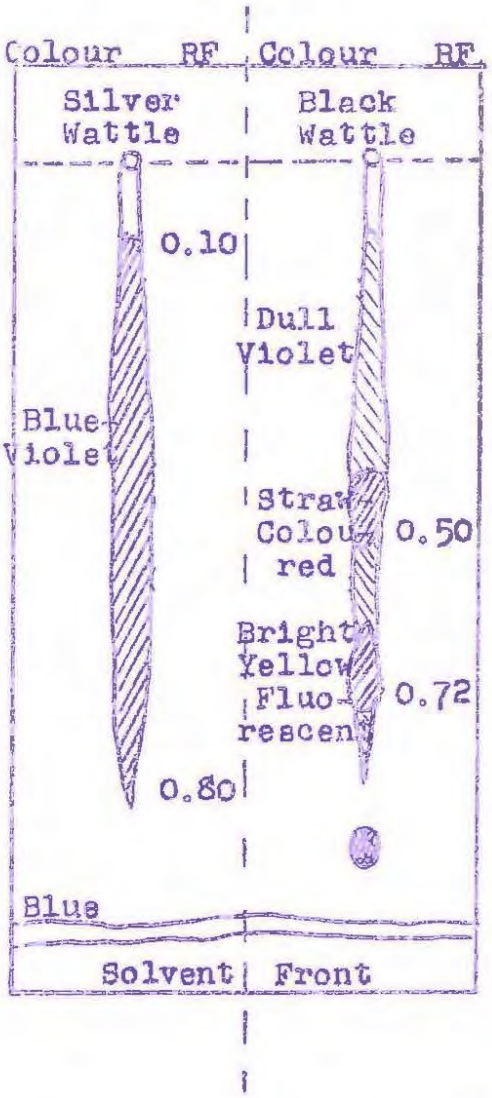


Fig. LXXI. Chromatograms of Black and Silver Wattle Fresh Bark under Ultra-Violet Light.

are thus easily distinguishable.

With 2% $FeCl_3$ spraying reagent the upper regions of the above streaks turned blue, the colour being most concentrated in the area RF = 0.10 - 0.20. The lower region of the silver wattle streak exhibited a far more pronounced and larger green area (RF = 0.50 - 0.70) compared with a similar green-grey area in

black wattle. This appears to indicate a larger proportion of catechol groups in silver wattle tannin.

(b) One-Dimensional Chromatography of the Oxidised Extracts

5 ml. of each of the above methanol solutions of the acetone extracts were pipetted into basins and the solvent removed on a waterbath. 50 ml. water was added and the tannin subjected to atmospheric oxidation for 10 hours at the temperature of the waterbath, the water being replenished from time to time. The dry product, now probably more heavily oxidised than normal commercial extracts, was finally redissolved in 5 ml. methanol and chromatographed as before (Fig. LXXII).

The tannin streaks were easily visible in ordinary light, extending to RF = 0.72 and being most concentrated in the region RF = 0.22. Under ultra-violet light the silver wattle extract had become weakly fluorescent, whereas that of black remained much the same. In both extracts dull brown tannin streaks extended from the point of application to RF = 0.22 while dull fluorescent spots were discernible at RF = 0.48.

This development of fluorescence on oxidation was previously observed by Glueck (414) in green wattle (Acacia decurrens Willd) extracts, and it illustrates the close similarity of the two extracts as evidenced also later from degradative studies.

The differences between the extracts are thus least evident in highly oxidised material and too much importance should not be attached to the presence or absence of yellow weakly fluo-

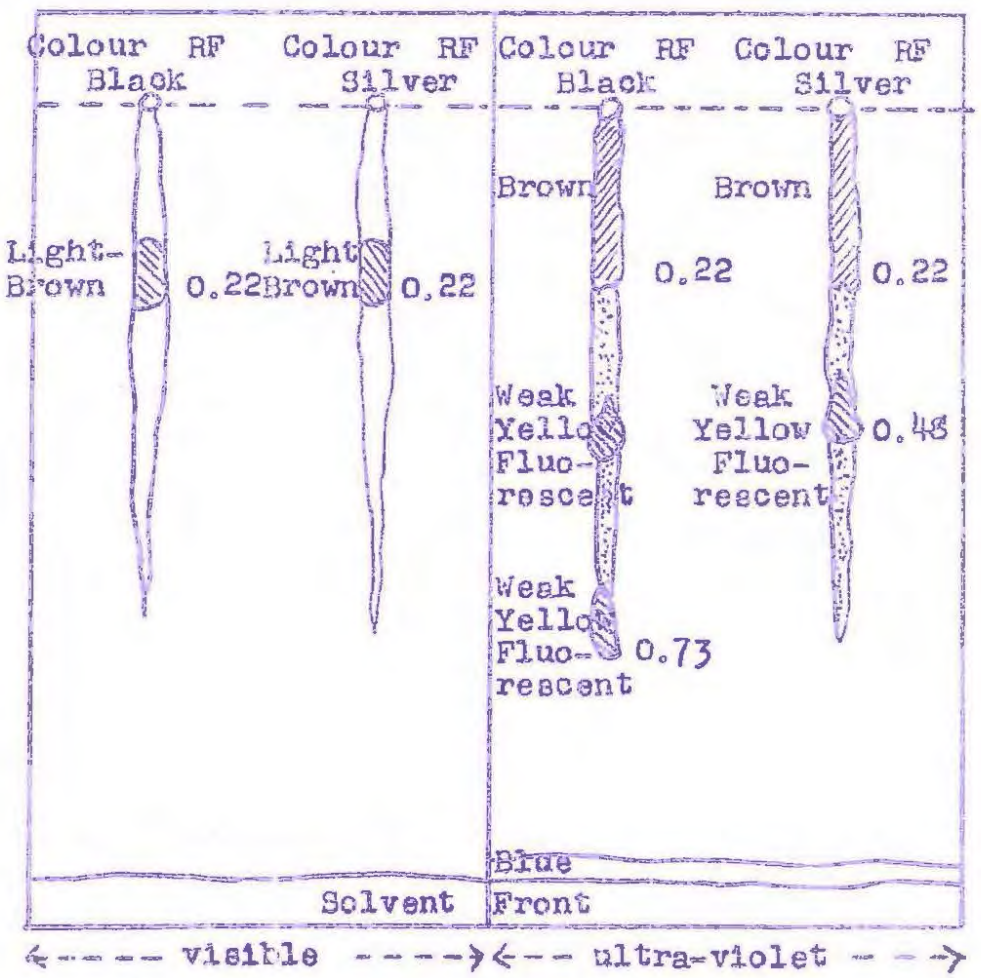


Fig. LXXII. Chromatogram of Oxidised Silver and Black Wattle Extracts in Ordinary and Ultra-Violet Light.

rescent spots in the low RF tannin region on one-dimensional chromatograms.

(c) One-Dimensional Chromatography of the Ethereal Extracts

In previous work it was found that the ethereal extracts of black wattle tannins, when chromatographed, showed two brightly fluorescing spots as well as a third capable of developing brilliant

blue fluorescence in the presence of ammonia vapour when viewed under U.V. light. It was thus considered likely that the tannins would show differences more clearly when their ether extracts were compared.

Fresh bark chips of each Acacia were placed in contact with 150 ml. of pure air-free warm water and the flasks tightly corked. After standing 12 hours, both the above extracts (black wattle 16 Bk, silver wattle 10 Bk), were ether-extracted 6 times with 100 ml. quantities of purified ether. After thorough drying over sodium sulphate, these solutions (600 ml.) were evaporated to dryness, leaving traces of light yellow oily material. 0.5 ml. methanol was added to each and the chromatogram prepared and developed as before (Fig. LXXIII). Whereas the black wattle chromatogram showed the two brilliant yellow fluorescent spots under ultra-violet light previously described, that of silver wattle showed no yellow fluorescence but only a bright blue fluorescent streak RF = 0.45 - 0.81 (Upper part RF = 0.51 blue-white and lower portion RF = 0.81 bright blue) was evident. In the presence of ammonia vapour the whole silver-wattle streak gave an intensified blue fluorescence, while with black wattle, the third blue fluorescent spot RF = 0.87 became evident. Initially the developed chromatograms showed no spots in ordinary light but after 24 hours, presumably due to atmospheric oxidation, a pink-red spot developed in the silver wattle chromatogram, at RF = 0.57 (previously described as blue-white under U.V. light).

Sprayed with 2.0% $FeCl_3$ reagent the silver wattle

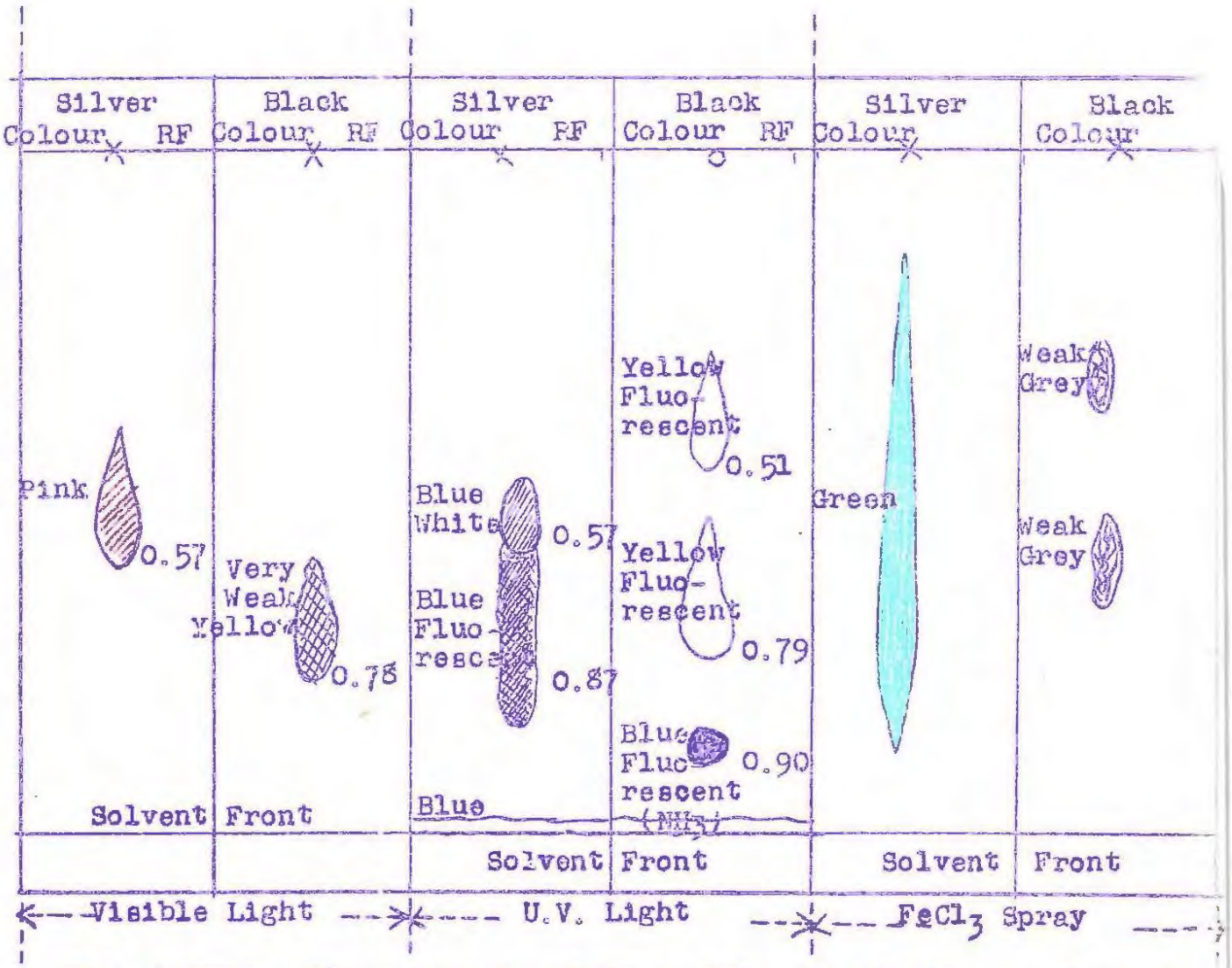


Fig. LXXIII. Chromatograms of Ether Extracts of Silver and Black Wattle Tannins in Ordinary, and U.V. Light and sprayed with $FeCl_3$ (2%). Chromatogram run in butanol-acetic acid-water on Whatman's No. 11 Paper.

chromatogram showed a longitudinal green streak extending from RF = 0.42 to RF = 0.69. This was easily distinguished from that of black wattle which gave very weak, barely discernible, greyish spots.

All these differences are most characteristic and more striking than in the case of original extracts.

(d) Two-Dimensional Chromatography of the Polyphenols in Silver Wattle Extract

The cold methanol extract of fresh-bark chips was examined two-dimensionally on paper chromatograms under the conditions described for black wattle tannins. After spraying with ammoniacal silver nitrate the pattern of reducing spots obtained (Fig. LXXIV) closely resembled that of green wattle extract, but

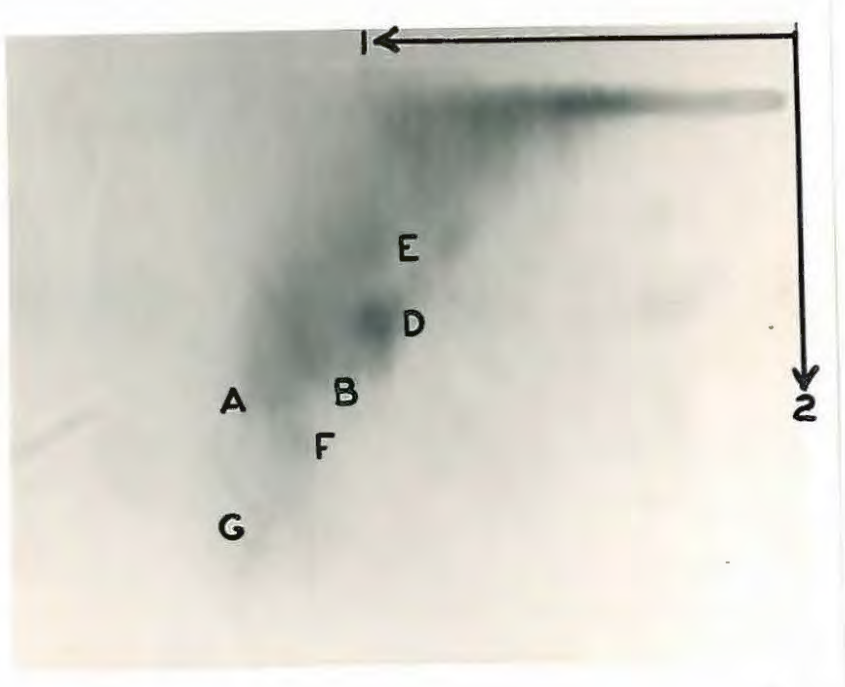


Fig. LXXIV. Two-Dimensional Chromatogram of Silver Wattle Fresh-Bark Extracts.

differed in appearance from typical black wattle chromatograms. The strongly reducing constituent F, also present in black and green wattle chromatograms is very prominent. As in the case of green wattle, constituent D is most prominent and A, B, G and possibly also C and E are present. On chromatograms run under identical conditions, the pattern of five areas A, B, C, D and E of uniformly high concentration do not stand out prominently with silver wattle, as they do in the case of black wattle tannins. Further work is necessary to establish whether any relationship exists between the various spots observed in identical positions on the chromatograms of the two extracts.

The application of chromatography, therefore, particularly to the ethereal extracts, has shown striking differences between black and silver wattle tannins. The development of weak fluorescence in the latter extract on atmospheric oxidation is also of interest.

(3) Colour Reactions of the Tannins

0.01 grm. tannin dissolved in 10 ml. absolute ethanol and treated with one drop 5% aqueous $FeCl_3$ gave a green colour with silver wattle and a deeper blue-green with black wattle.

5 ml. 0.1% lead-salt purified tannins treated with 25 ml. ferrous tartrate reagent (Mitchell (281)) and diluted to 100 ml. gave a green colour with silver wattle and a blue-green with black wattle.

Both the above colour-reactions point to a predominance

of catechol groups in silver wattle tannins, and pyrogallol groups in black wattle tannins.

(4) pH Values of the Natural Extracts

Fresh aquecus extracts (hot leached) of "green" bark previously stored under paraffin, and adjusted to the same concentration (16 Bk.) gave the values :

	pH
Silver Wattle	5.2
Black Wattle	4.6

This difference was also reflected in lead-salt purified extracts.

(5) Degradation of the Tannin

Alkali fusion of lead-salt purified silver wattle tannin at 195°C. for 2 hours was repeated as described for black wattle tannins. The product was subdivided into acidic and phenolic fractions by the bicarbonate technique in the usual way. Paper chromatography showed that the acidic fraction (25% yield) contained protocatechuic, gallic and β-resorcylic acids as with black wattle tannins (Fig. LXXV). Judging by the area of these spots protocatechuic acid appeared present in higher, and gallic acid in lower proportion than in black wattle tannins. Such a comparison must be treated with caution, however, as fusion times and conditions, known to have a pronounced influence on yields of phenolic carboxylic acids, are difficult to reproduce exactly.

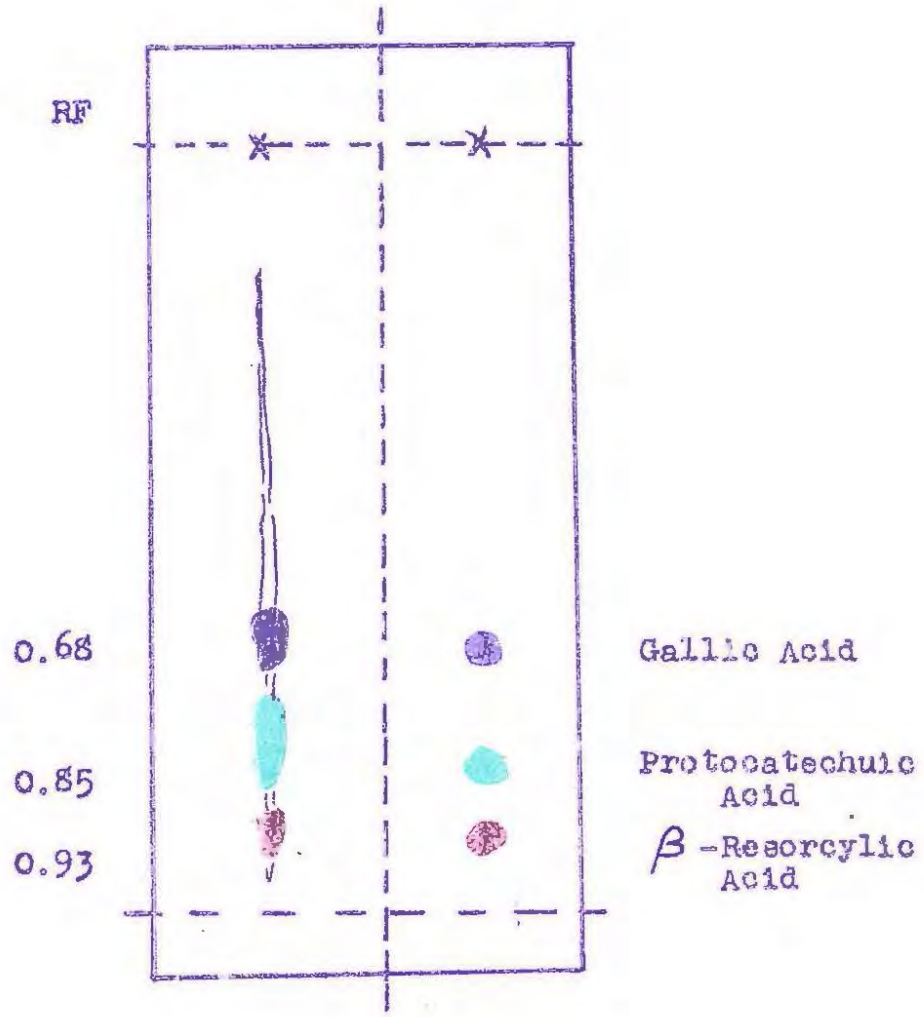


Fig. LXXV. Chromatogram of the Acidic Fraction and β-Resorcylic, Gallic and Protocatechuic Acids (Butanol : Acetic Acid : Water at 22°C. Whatman's No. 11) sprayed with 2% $FeCl_3$ Reagent.

From the phenolic fraction (20% yield) after subdivision into meta- and ortho-hydroxy fractions, the presence of pyrogallol, phloroglucinol and resorcinol was conclusively established by paper chromatography, using a variety of spraying reagents. These were also formed from black wattle tannins although the yield of phloroglucinol from the degradation of silver wattle tannins

appeared greater.

Both the colour reactions of the extract, and the above degradative evidence, point to the presence of larger proportions of catechol groups in silver than in black wattle extracts. A quantitative investigation of ortho-hydroxy groups was thus desirable to confirm these differences.

(6) Quantitative Comparison of Ortho-Hydroxy Groups

From the previous investigation of the formation of ferrous tartrate complexes with phenolic ortho-hydroxy groups, it was evident that catechol groups show lower absorption densities at 545 mμ (absorption maximum), than the equivalent of pyrogallol nuclei, especially at low pH values (5.6 to 7.8). Higher proportions of catechol units as suspected in silver wattle, would thus give a lower total absorption over the above pH range when compared with black wattle.

5 ml. of an exactly 0.1% lead acetate purified tannin solution was treated with 25 ml. fresh ferrous tartrate reagent and diluted to 100 ml. The pH of this solution was usually about 5.6, showing a green colour with silver and a blue-green with black wattle tannin. The absorption density at 545 mμ was measured on a Beckman Model DU Spectrophotometer.

This procedure was now repeated, diluting with water to 80 ml., then adding 1, 3, 5 and 10 ml. quantities of 10% ammonium acetate buffer and finally diluting to 100 ml. The absorption density and pH of each solution was measured. Higher pH values

were obtained by final addition of ammonia before making to 100 ml. (Fig. LXXVI).

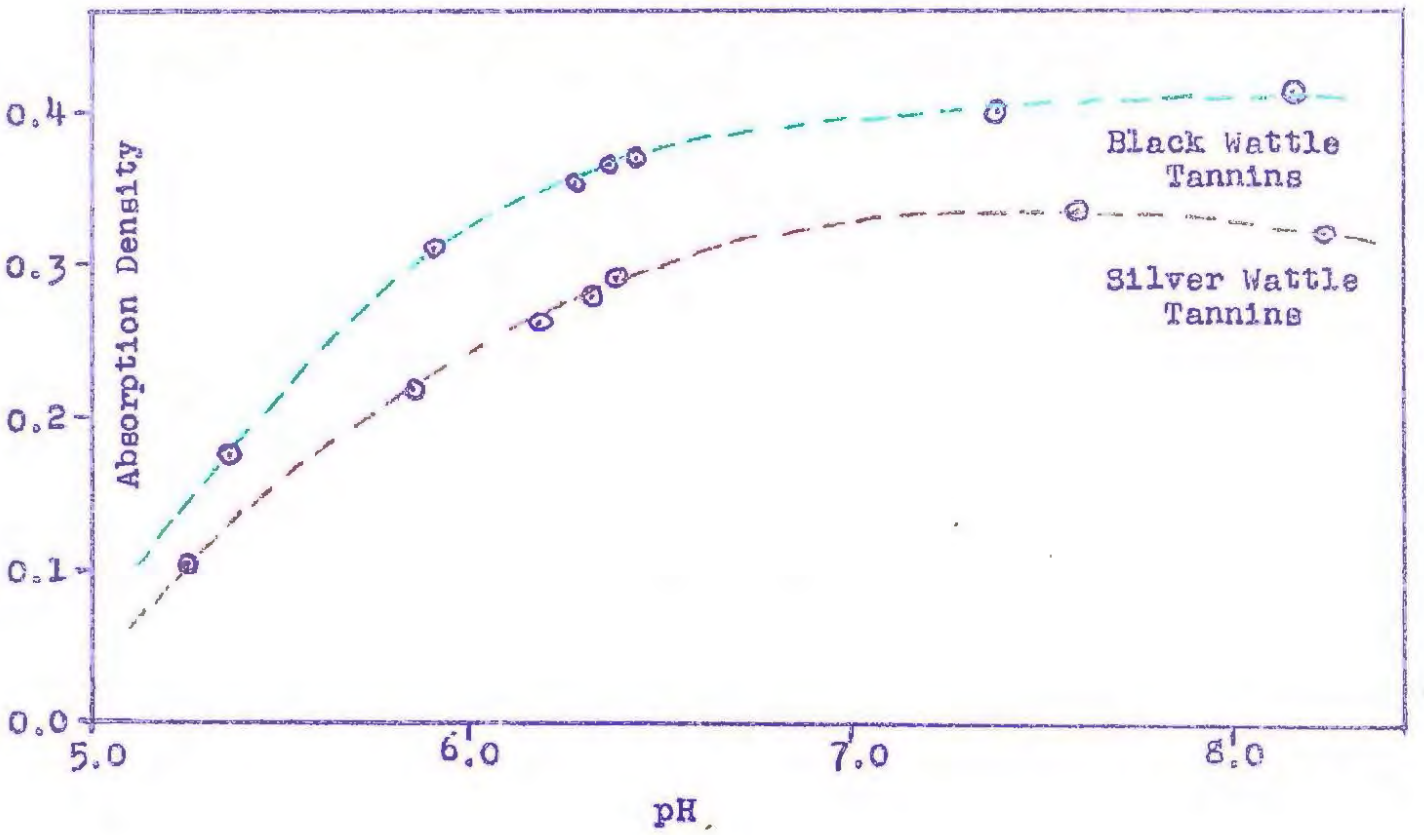


Fig. LXXVI. The Variation of Absorption Density with pH in the Tannin - Ferrous Tartrate Complexes at 545 mμ.

The lower curve obtained with purified silver wattle tannins could be ascribed to three factors :

(a) a lower ratio of "free" ortho-hydroxy-phenolic groups to the remaining tannin residues in silver wattle tannins,

(b) different resonance effects in the constituents of the two tannins which might affect the degree of coordination in the ortho-hydroxy-phenolic complexes,

(c) a higher ratio of catechol to pyrogallol groupings in green wattle tannins than in black wattle tannins.

From an examination of the $FeCl_3$-sprayed one-dimensional chromatograms; qualitative colour tests, and the natural pH of the extracts, the latter is considered to be the most likely. In any event, the spectrophotometric study of silver and black wattle tannins reveals significant differences.

(7) Non-Tannins

These were separated from the predominant tannin fraction by the addition of excess of lead acetate to an aqueous solution of the extract. The lead tannate was centrifuged off and the resulting solution shaken with three lots of 20 grms. each of lightly chromed hide powder. The solution was squeezed through a clean nylon cloth after each shaking, finally stirred with J.S.L.T.C. Kaolin and filtered to clarify as in the Official Method of tannin analysis. This clarified extract was evaporated to dryness under reduced pressure, extracted repeatedly with boiling absolute methanol to remove salts, the methanol extracts centrifuged and finally concentrated. A paper chromatogram of concentrated non-tannins in the presence of standard reference compounds, developed with butanol : acetic acid : water (4 : 1 : 5 upper layer) and allowed to drip off the paper revealed the presence of SUCROSE only with resorcinol-HCl, napthoresorcinol-HCl (369) and napthoresorcinol-phosphoric acid (378) spraying reagents. Napthoresorcinol-phosphoric acid spraying reagent was

also effective for detecting sucrose in the presence of the tannins on chromatograms, i.e., without the above separation. Characteristic colorations were developed when spraying the chromatogram of the original extract as a whole.

Sucrose was also the only detectable carbohydrate non-tan constituent in black and green wattle extracts obtained from the fresh bark.

(8) Summary

The chief differences between silver and black wattle tannins which may be of significance in genetic studies are :

(1) Differences in the thickness of bark.
(2) The lower tannin content of silver wattle bark.
(3) The more pronounced redness of silver wattle extracts.
(4) Marked differences in the one-dimensional chromatograms under U.V. light.
(5) Striking differences in one-dimensional chromatograms of the ethereal extracts viewed in U.V. light, and sprayed with 2% $FeCl_3$.
(6) Differences in the appearance and concentration of reducing areas on two-dimensional chromatograms.
(7) Differences in the proportions of ortho-dihydroxy and ortho-trihydroxy phenolic groups as evidenced by qualitative colour reactions and quantitative spectrophotometric evidence.
(8) Differences in pH values of the natural extracts.

The similarity of the tannins is emphasised by :

(a) The identical degradation products obtained on alkaline fusion (e.g. gallic, β-resorcylic and protocatechuic acids; pyrogallol, resorcinol and phloroglucinol) of the "purified" tannins, although the phloroglucinol was obtained in higher yield.

(b) Developed two-dimensional chromatograms of the tannins show many reducing spots which occupy identical positions on both chromatograms. These may represent either similar or identical tannin constituents.

(c) Sucrose is the predominant carbohydrate non-tannin constituent of both fresh-bark extracts. Gums are also present but have not yet been examined.

CHAPTER XIII

THE EXTRACT OF THE BARK OF ACACIA PYCNANTHA BENTH

(Golden Wattle Extract)

The golden wattle tree is easily distinguished by the lanceolate phyllodes which it bears, compared with the feathery compound leaves of black, green and silver wattle species (see p. 419). The golden wattle is consequently known as the "broad-leaved wattle" in Australia, where it has been used as a tanning material on a fairly large scale (383).

This acacia contains a high proportion of tanning material in the mature bark. Williams (3) records that South African-grown 11 - 14 year-old trees yield barks containing 35 - 41% tannins and 10% non-tannins, while Coombs (384) quotes 39.5% and 41.6% tannin content for Australian commercial golden wattle bark. Acacia pycnantha grows in the interior of Southern Australia in warm areas of medium rainfall, and commercial broad-leaved wattle or "Adelaide" bark, which is supposed to originate from the golden wattle tree only, was considered to be the best bark sold to tanners in Australia. A. pycnantha was also preferred to other acacia species for growth in Morocco, and the barks were shown to contain 42 - 44% tannins and 4.1% glucose, (384). The golden wattle thus succeeds in growing in poorer soils and

under dryer conditions than those required by black wattles.

It would seem that these advantages have not been fully examined in South Africa. Trial plantations were attempted on the Cape Flats, and today some still exist at Eerste River. Small-scale plantings also took place in Natal and the Eastern Cape Province, but commercial afforestation was not attempted. Nevertheless, its different habitat and high tannin content are important, and this acacia should receive more attention in this country with its limited areas of adequate and regular rainfall.

From William's (3) figures, it appears that the extracts are more reddish than black wattle extracts, but lighter than silver wattle extracts, and as dark as green wattle extracts. The sample obtained consisted of dried bark obtained from the government plantations at Eerste River, and appeared more reddish on the inner surfaces than commercial black wattle bark.

No detailed chemical study of the tannins or non-tannins was made but a two-dimensional chromatogram (Fig. LXXVII) of the methanol-extract of the bark was run under the conditions used for the other acacias.

As these tannins originate from the dried bark and were possibly affected by enzymatic oxidation, direct comparison with the fresh-bark methanol-extracts of the other species is not possible. Six spots of relatively high concentration, 3 - 8, are present in the high R^F region and probably correspond to similar reducing areas on the chromatograms of black, green and silver wattle tannins. Constituents 1, 2, 9 and 10 do not appear in the methanol fresh-bark extracts of the other species.

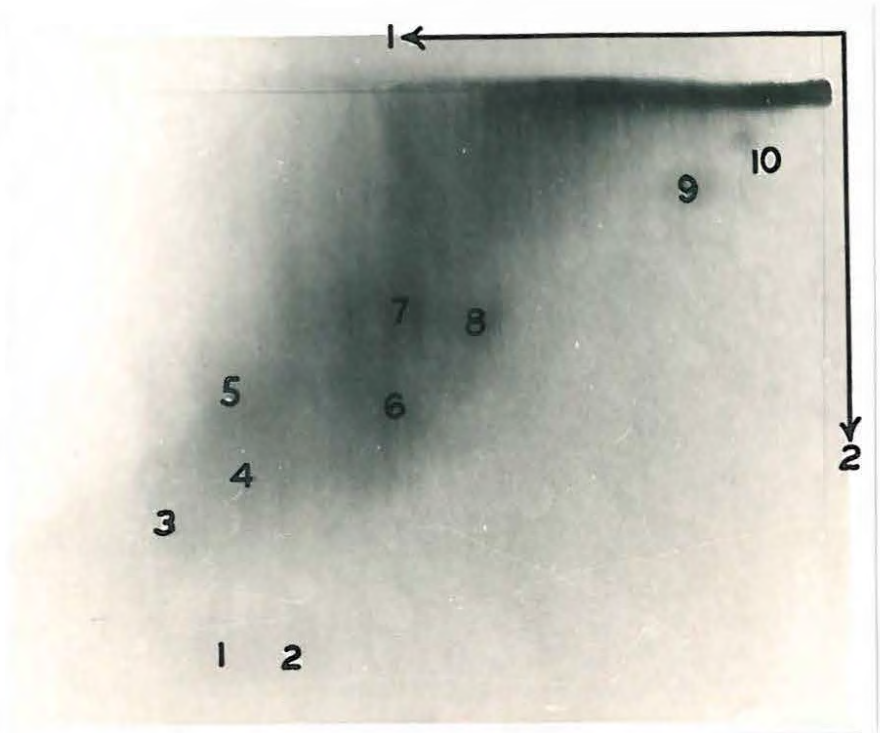


Fig. LXXVII. Two-Dimensional Chromatogram of the Methanol Extract of Dried Golden Wattle Bark.

Summary

(a) Of all the species of acacia used for commercial tannage, the golden wattle bark has the highest tannin content, but its extracts are also reddish in colour.

(b) As it grows successfully in drier areas in Australia,

its possible use in this country should be investigated.

(c) The polyphenolic fraction of the extract consists of a mixture of a number of phenolic tannins which may be related to those present in the other acacia species studied.

Silver Wattle | Black Wattle | Green Wattle

Fig. LXXVIII. The Compound Adult Leaves of Silver, Black and Green Wattle Trees.

Fig. LXXIX. A Twig Showing the Simple Leaves of the Golden Wattle Tree.

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